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ARTICLE

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In vitro protein protection of protein meals using Bioprotect and tannin extract from red grape marc

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Abstract

Protecting crude protein in the rumen may reduce extensive protein degradation and ammonia emission and increase available bypass protein in ruminants. This experiment was conducted to determine the effect of two Bioprotect (15 and 30 mL/kg dry matter (DM)) and two tannin extract (TE) (20 and 40 g/kg DM) inclusion rates on protein protection and in vitro fermentation characteristics of canola and soybean meals incubated for 24 h using an ANKOM *in vitro* gas production system. The treated canola and soybean meals produced lower soluble protein (fraction 'a') and larger slowly degradable protein (fraction 'b') than its untreated counterparts, $p < 0.01$. However, the 20 g/kg DM TE inclusion showed lowest effect on the amount of protein fractions 'a' and 'b' in both meals compared to their other treated counterparts. The increasing concentration of additives reduced the total volatile fatty acids (VFA), $p < 0.001$. The effects of additives differed between the treatments as 15 mL/kg DM Bioprotect and 20 g/kg DM TE did not affect the acetic to propionic acid ratio (A:P) and the time before gas production began. The increase in fraction 'b' and reduction in protein fraction 'a' confirm successful protein protection in this experiment. However, the extensive reduction in ammonia-N and *in vitro* degradable protein after using 30 mL/kg DM Bioprotect suggests possible toxicity to the microbes responsible for protein digestion in higher doses. Therefore, 15 mL/kg DM Bioprotect and 40 g/kg DM TE could be promising protein protection doses for *in vitro* experiments.

Keywords

ammonia-N, gas production, protein degradation, protein fractions, rumen fluid, soybean meal

INTRODUCTION

Excreted nitrogen in the feces and urine of ruminants may reach up to 70% of ingested nitrogen [1, 2]. Nitrogen loss causes environmental pollution as urea, nitrous oxide, and ammonia and economic damage through declining production performance [3–5]. The biological value of protein is also reduced when rumen microorganisms ferment excessive degradable protein [6]. Therefore, protecting crude protein is crucial to

reduce extensive rumen degradation and ammonia emission and helps increase available bypass protein in ruminants [7].

Soybean and canola meals are good sources of digestible protein but ineffective in providing metabolizable protein attributed to their extensive degradation in the rumen [8, 9]. Moreover, lucerne a is highly nutritious legume commonly supplied to ruminants; however, 80% of its protein is degradable in the rumen and leads to reduced nutritional efficiency and increased nitrogen excretion [10]. Thus, several studies have been undertaken to identify potential protein protection options. Protein protection is possible by using feed additives and chemicals, heating, pelleting, feeding protected amino acids, use of tannins, and inhibition of microbial proteolytic activity [11–13]. The applicability of protein protection techniques depends on access, ease, and feasibility. Therefore, the availability of more options could enhance the rate of adoption and benefit the profitability of end users.

The active ingredient in Bioprotect is a stable, nonvolatile organic salt that can react with the primary and secondary amino groups of protein [14]. The ability of Bioprotect to decrease *in vitro* gas production and protect starch from rumen digestion was demonstrated successfully by prior studies. Treating canola meal with 15 mL/kg dry matter (DM), lupin meal with 10%, and wheat grain with 20 mL/kg DM Bioprotect reduced the volume of total gas production and rate of *in vitro* fermentation [14, 15]. Reducing fermentability of grains by 10–40 mL/kg DM inclusion, Bioprotect alleviated heat stress in dairy cows [16] and sheep [17]. Therefore, the inclusion of Bioprotect for protein protection in protein-rich ruminant meals could slow protein degradation and enhance the volume of undegraded protein *in vitro* rumen fermentation.

Condensed tannin binds strongly with protein through phenolic groups of tannins and the carbonyl groups of proteins [18]. The complexes with both Bioprotect and condensed tannin are stable in the rumen pH but dissociate in the low pH of the abomasum and the small intestine, increasing the availability of nutrients for post-ruminal enzymatic digestion and enhancing animal production efficiency [14, 19, 20]. The effects of condensed tannins depend on their chemical composition and structures, which could vary with the source [21, 22]. Therefore, studies on the evaluation of condensed tannins from different tannin sources and optimum inclusion rates in ruminant diets are important [23].

Grape marc is a readily available by-product of the wine industry, consisting of the grape seeds, skin, stalk, and pulp as a residue of the wine-making process. The chemical composition of grape marc ranges from 7.30% to 13.8% MJ/kg DM in metabolizable energy (ME), 3.2%–14.4% DM in CP, 1.30%–17.4% in EE, 19.4%–61.4% DM in neutral detergent fiber (NDF), 16.2%–56.1% DM in acid detergent fiber (ADF), and up to 66.7% DM non-fiber carbohydrates depending on the variety and terroir of the grape [24–26]. Therefore, grape marc is used as an alternative natural nutrient source for different purposes such as antioxidant additives, nutraceuticals, cosmetics, food preservatives, and natural colorants and formulated into functional foods [27]. The studies on the potential of grape marc as a feed supplement demonstrated the possibility of a 20% inclusion level in ruminant diet [28] and variations among grape marc varieties were reported on mitigation of enteric methane emissions [25]. However, the direct supply of grape marc to ruminants as a feed could suppress the digestive system and reduce nutrient utilization resulting from the high concentration of lignified fiber and condensed tannin [29–31]. The condensed tannin content of red grape seed and skin reaches 27% [32]. Thus, the determination of the optimal tannin dosages extracted from grape marc for minimally effective protein protection and anti-nutritional potential in ruminant diets is essential.

There is a paucity of the information on using Bioprotect and tannin extract (TE) from red grape marc for protein protection *in vitro* fermentation. Therefore, this research was conducted to determine the effect of two Bioprotect and 2 TE inclusion rates on protein protection and *in vitro* fermentation characteristics of protein-rich feeds. Mixing limited amounts of Bioprotect and TE from red grape marc with canola or soybean meals was hypothesized to increase the proportion of rumen slowly degradable protein and reduce the amount of ammonia-N, soluble protein, and *in vitro* fermentability.

MATERIALS AND METHOD

All procedures were conducted per the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes [33]. The Department of Jobs, Precincts and Regions Agricultural Research and Extension Animal Ethics Committee approved the preparation and use of cannulated cows from which rumen fluid was sourced for this experiment.

Experimental materials and design

A solvent-extracted canola meal (*Brassica napus* L.) and soybean meal (*Glycine max* L.) were purchased from a commercial supplier (Peter Gibbs Stock Feeds, Australia) and used as substrates as commonly used protein feeds for ruminants. Bioprotect was supplied by FeedWorks, Australia. Two dosage rates of Bioprotect (15 and 30 mL/kg DM) were incubated by mixing with canola and soybean meals. The Bioprotect was mixed with water in a 1:2 ratio and sprayed on the protein

feeds using a spray bottle slowly and thoroughly inside a 250 mL glass bottle. The untreated canola and soybean meals were used as a control and mixed with a similar volume of water.

Red grape fruit (*Vitis vinifera* L.) was collected from the University of Melbourne's Dookie campus farm, crushed, and pressed to separate the juice and the residue at the Dookie campus winery. The residue was frozen at −20ºC on the same day of wine production and dried in a 60ºC laboratory incubator for 72 h and turned over every 24 h. The grape marc (pomace) contained seeds, skins, pulp, and stalks. The tannin was quantitatively extracted using methanol with a minor modification to the procedure as described by Medini and Fellah [34]. Briefly, the dry grape marc was mixed with 4 mL/g petroleum ether and stirred for 30 min using a magnetic stirrer to remove fats and pigments. The residue was filtered using a Vacuum Pressure Pump (Model No. 400-3912, Barnat company, Illinois, USA) and Whatman filter paper (Grade 4, 20–25 μm, Sigma-Aldrich, Castle Hill, Australia). The residue was mixed with 10 mL/g methanol and water solution (4:1, v/v) as a solvent for 30 min. The supernatant was drained and kept for 24 h at 4ºC. The precipitate from the supernatant was exposed to low temperature drying (30ºC) for 48 h using an incubator (Premium blanket warmer, Thermoline Scientific, Wetherill Park, Australia).

Previous research has indicated that 20–40 g/kg DM of condensed tannin is sufficient for protein protection of protein meals [35]. Therefore, 20 and 40 g/kg DM TE from grape marc was mixed with substrates for in vitro fermentation. Thus, for this experiment, a total of 10 treatments were included, and eight replicates of modules were used in three sequential runs for each treatment during the in vitro fermentation. Six blank modules were also incubated with a rumen fluid as a background in each run. The randomized complete block design was used to compute between two feeds and five doses of additives.

Rumen fluid collection and gas production

Two liters of rumen fluid was collected per run from four mid-lactation Holstein Friesian cannulated dairy cows at Agriculture Victoria (Ellinbank, Victoria) before morning feeding and transported using the procedure described by Tunkala, DiGiacomo [36]. Cows were grazing perennial ryegrass (*Lollium perenne* L.) pasture, and a wheat and barley grain mix (6 kg DM per day per cow) was supplied in the milking parlor. The rumen fluid was transported using an incubator at 39ºC. The rumen fluid was filtered using four layers of cheesecloth after arrival at the *in vitro* fermentation laboratory.

The gas production method of Raab and Cafantaris [37], revised by Karlsson and Hetta [38], was used with minor modifications to collect ammonia-N samples from the ANKOM gas production system [39]. The filtered rumen fluid was mixed with a total of 10 g/L of rapidly soluble carbohydrates (3.33 g of maltose, 3.33 g of starch, and 3.33 g of xylose) as described by Aghajanzadeh-Golshani and Maheri-Sis [40] for preincubation conditioning for 3 hrs which minimizes the background ammonia-N and stimulates microbial activity. Sodium bicarbonate (3.1 g dissolved in 63 mL of McDougall's buffer per L of rumen fluid) was also added to the rumen fluid before preincubation. The rumen fluid was handled under continuous flushing of CO_2 before and during the three-h preincubation.

After 3 h, the preincubated rumen fluid was mixed with McDougall's buffer [41] to obtain a buffered rumen fluid with a 1:2 rumen fluid to buffer ratio. The feed samples were ground into 1 mm particle diameter using a grinder (Breville, The Coffee & Spice Grinder, Stainless Brushed Steel, Myer, Docklands, Australia) and sieved to ensure the size. A 500 mg sample of each substrate was weighed into individual 250 mL ANKOM bottles, mixed with TE or Bioprotect and 90 mL buffered rumen fluid, and incubated for 24 h in a 39ºC water bath (20-L Analogue Water bath, WB20; Ratek Instruments Pty Ltd,).

Measurements and sampling

The chemical composition of substrates were analyzed in a commercial laboratory (FeedTest Laboratory, Agrifood Technology, Werribee, Australia) using near-infrared spectroscopy. The CP of the substrates was quantified by the Kjeldahl system. The post-fermentation pH value of the fermented ruminal fluid was recorded after 24 h fermentation.

Ammonia-N concentration was measured by the colorimetric technique as described by Weatherburn [42] using a multiscan colorimetric plate reader (Thermo Multiskan Spectrum, Thermo Fisher Scientific). 4 mL ammonia-N samples were collected at 4, 8, 12, 16, and 24 h using separate modules for each hour in three replications using the procedure described in Tunkala and DiGiacomo [39]. 4 mL of liquid samples were also collected in three replicates per treatment at the end of 24 h in vitro fermentation for volatile fatty acid (VFA) analysis using gas chromatography fitted with a flame ionization detector with methyl valerate as the internal standard [43, 44]. The ammonia-N and VFA samples were collected in 5 mL capped tubes and stored at −20ºC until analysis.

Dry matter digestibility (DMD) was measured by the pepsin–cellulase method [45, 46]. The organic matter digestibility (OMD) and ME were determined using the equations from SCA [47] and AFFIA [46], respectively.

$$OMD = 6.83 + (0.847 * DMD) \quad (1)$$

$$ME = (0.203 * OMD) - 3.001 \quad (2)$$

The NFC and total digestible nutrient (TDN) were calculated using the following equations from Mertens [48] and Linn and Martin [49], respectively.

$$NFC = 100 - (CP + ash + fat + NDF) \quad (3)$$

$$TDN = 88.9 - (ADF * 0.779) \quad (4)$$

The lag time and the gas production rate were computed using GenStat with the Gompertz model:

$$Y = A + C^{\exp\{-\exp[-B(X-M)]\}} \quad (5)$$

in which A is the y-intercept, B is the rate of gas production (mL/h), C is the maximum gas produced (maximum gas mL/g DM), X is the total time (h) of incubation, and M is the time (h) at which the maximum rate of gas production is reached.

In vitro degradable crude protein (IVDP) was calculated from gas production and ammonia-N values using the equation of Raab and Cafantaris [37].

$$IVDP = \frac{\text{Ammonia-N at zero gas production}\left(b^0\text{intercept}\right) - \text{Ammonia-N in blank}}{\text{Total N of incubated feed}} \quad (6)$$

The proportion of protein fractions were estimated by fitting the IVDP to the nonlinear equations of Ørskov and McDonald [50] modified by Karlsson and Hetta [38] for all protected and unprotected feeds using the exponential regression model of GenStat 21st edition.

$$Y = a + b * (1 - e^{-ct}) \quad (7)$$

where Y is the proportion of CP degraded at time t, a is the proportion of CP degraded at time 0 h, b is the proportion of potentially degradable CP, and c is the degradation rate of fraction b.

The effective CP degradation (EPD) value was calculated using the equation of Ørskov and McDonald [50] as follows:

$$EPD = a + \frac{(b * c)}{(k + c)} \quad (8)$$

where a, b, and c are as above, and the passage rate (k) was assumed to be 0.08 h^{-1}.

Statistical analysis

The mean differences of parameters between four inclusion levels in two substrates were computed by two-factor analysis of variance (ANOVA) using GenStat 22nd edition, considering runs as experimental replicates to compare mean differences using the following model.

$$Y_{ij} = \mu + S_i + A_j + SA_{ij} + e_{ij}$$

where Y_{ij} is the general mean of continuous dependent variables, μ is the mean value of substrates and additive combinations examined, and S_i is the fixed effect of each substrate (i = soybean meal and canola meal) on the tested parameter, A_j is the fixed effect of additives (j = Bioprotect and TE), SA_{ij} is the interaction effect between the independent variables, and e_{ij} is the standard error term. The arithmetic mean values were considered statistically significant when the *p*-value is less than 0.05.

The data of IVDP and ammonia-N were analyzed by a 3-factor ANOVA model as described below, following the 2*5*5 factorial design with 2 substrates, 5 doses of additives, and 5 sampling periods.

$$Y_{ijk} = \mu + S_i + A_j + T_k + SAT_{ijk} + e_{ijk}$$

where Y_{ijk}, μ, S_i, and A_j were described above, T_k is the fixed effect of time, STK_{ijk} is the interaction effect between S, A, and T, e_{ijk} is the standard error term for the $(i, j, k)^{th}$ cell. The arithmetic mean values were compared using standard error of differences and considered statistically significant when $p < 0.05$.

The linear regression and correlation coefficient between the volume of additives and EPD values were determined using 2D scatter plot and Pearson's correlation function of Genstat 22nd Edition.

RESULTS

Chemical composition

The canola and soybean meal used in this experiment were varied in chemical composition (Table 1).

Soybean meal was greater in the CP composition, DMD, OMD, ME, NFC, and TDN than canola meal. In contrast, the DM content, ADF, NDF, and fat percentage were greater in canola meal.

In vitro fermentation parameters

The use of Bioprotect and TE from red grape marc reduced the volume, rate of gas production, and total VFA compared to the untreated feeds after 24 h of in vitro fermentation (Table 2). The gas production from untreated canola meal was 158 mL/g and was reduced by 19.0% and 27.9% when treated with 15 and 30 mL/kg DM Bioprotect, respectively, $p < 0.001$. The 20 and 40 g/kg DM TE inclusion decreased the gas production of canola meal by 17.1% and 37.7%, respectively, $p < 0.001$. The untreated soybean meal produced 149 mL/g of gas, and it declined by 18.2%, 24.2%, 25.0%, and 25.5% when treated with 15 and 30 mL/kg DM Bioprotect, and 20 and 40 g/kg DM TE, respectively, $p < 0.001$. However, the gas production curves of treated canola meals followed the same path for 16 h except for 20 g/kg DM TE inclusion and remained overlapped for treated soybean meals all over the 24 h fermentation period (Figure 1). The rate of gas production was lower in treated meals compared to untreated canola meal (6.60 mL/h) and soybean meal (6.20 mL/h), $p = 0.01$. There was no difference between treated and untreated meals in post-fermentation pH of rumen fluid.

The lag time of soybean meal was increased by 30 mL/kg DM Bioprotect and 40 g/kg DM TE from red grape marc, $p < 0.001$. The untreated soybean meal started to produce gas at 0.13 h. The soybean meal mixed with 30 mL/kg DM Bioprotect required 0.40 h and 40 g/kg DM TE needed 0.92 h to start positive gas production. The lag time of canola meal was not affected by mixing it with Bioprotect and TE.

The total VFAs of untreated canola meal (108 mM/L) and soybean meal (102 mM/L) were larger than their

TABLE 1 Chemical composition (g/kg dry matter (DM) or MJ/kg DM) of canola and soybean meals used as a substrate in this experiment.

Substrates	DM, g/kg	CP, g/kg	ADF, g/kg	NDF, g/kg	DMD, g/kg	OMD[a], g/kg	ME[a], MJ/kg	Fat, g/kg	Ash, g/kg	NFC[a], g/kg	TDN[a], g/kg
Canola meal	903	380	202	335	719	712	12.0	5.01	6.81	7.12	68.3
Soybean meal	823	496	122	112	935	920	16.8	1.20	6.30	31.8	77.3

[a]Calculated; ADF, Acid Detergent Fiber; NDF, Neutral Detergent Fiber; DMD, Dry matter digestibility; OMD, Organic matter digestibility; ME, Metabolizable energy; NFC, Non-fiber carbohydrate; RFV, Relative feed value; and TDN, Total digestible nutrient.

TABLE 2 Effect of treating the canola and soybean meals with Bioprotect (15 and 30 mL/kg dry matter (DM)) and tannin extract (TE) (20 and 40 g/kg DM) from red grape marc on in vitro fermentation parameters.

Substrates	Canola meal					Soybean meal						
Additives		Bioprotect		Tannin extract			Bioprotect		Tannin extract			
Parameters	Control	15	30	20	40	Control	15	30	20	40	SED[a]	Significance[b]
Gas production, mL/g	158^{a}	128^{c}	114^{e}	131^{c}	98.5^{f}	149^{b}	122^{cd}	113^{e}	112^{e}	111^{e}	7.55	A***; SxA*
Rate of gas prod, mL/h	6.60^{a}	5.35^{b}	4.75^{bc}	5.46^{b}	4.10^{cd}	6.20^{a}	5.08^{b}	4.69^{c}	4.67^{c}	4.62^{c}	0.625	A***; SxA*
Lag time, h	0.18^{c}	0.21^{c}	0.25^{c}	0.16	0.25^{c}	0.13^{c}	0.16^{c}	0.40^{b}	0.18^{c}	0.92^{a}	0.135	S*; A***; SxA**
pH	6.74^{b}	6.76^{ab}	6.76^{ab}	6.76^{ab}	6.74^{b}	6.80^{a}	6.80^{a}	6.78^{ab}	6.79^{a}	6.82^{a}	0.040	S***
TVFA, mM/L	108^{a}	94.3^{d}	64.4^{g}	102^{b}	80.5^{e}	102^{b}	99.1^{c}	51.6^{h}	82.1^{e}	71.3^{f}	1.75	S***; A***; SxA***
Acetic acid, mM/L	41.8^{a}	34.5^{b}	30.3^{d}	31.7^{c}	33.0^{c}	35.5^{b}	31.9^{c}	25.8^{e}	30.0^{d}	31.2^{cd}	1.31	S***; A***; SxA***
Propionic acid, mM/L	9.11^{b}	9.08^{b}	8.13^{cd}	9.10^{b}	8.50^{c}	10.3^{a}	10.1^{a}	7.51^{e}	9.90^{a}	8.63^{c}	0.436	S*; A***; SxA***
Isobutyric, mM/L	1.02^{b}	1.02^{b}	1.00^{b}	0.96^{c}	0.91^{d}	1.10^{a}	0.93^{d}	0.90^{de}	0.91^{d}	0.76	0.024	A***; SxA***
Butyric, mM/L	4.02^{bc}	4.20^{b}	3.88^{c}	3.62^{d}	4.11^{b}	4.25^{b}	5.02^{a}	3.54^{de}	3.78^{d}	4.09^{b}	0.178	S***; A***; SxA***
Isovaleric, mM/L	3.03^{b}	3.10^{b}	2.76^{d}	2.82^{d}	2.84^{d}	3.29^{a}	2.94^{bc}	2.66^{e}	2.81^{d}	2.52^{f}	0.087	S***; A***; SxA***
Valeric, mM/L	59.1^{a}	41.3^{e}	17.2^{i}	53.1^{b}	29.1^{g}	47.4^{c}	45.3^{d}	11.1^{j}	35.1^{f}	23.2^{h}	1.08	S***; A***; SxA***
A:P ratio	3.91^{a}	3.86^{a}	3.78^{b}	3.88^{a}	3.81^{ab}	3.43^{c}	3.44^{c}	3.33^{d}	3.42^{c}	3.35^{d}	0.062	S***; A***; SxA***

Note: Standard error of the difference for Substrate (S) x Additives (A) (Bioprotect and TE). Significance of effects of Substrate (S) x Additives (A) (Bioprotect and TE) and interactions: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; TVFA: total volatile fatty acids; and A:P: acetic to propionic acid ratio. Values in a single row with different superscript letters (a, b, c, d, e, f, g, h, i and j) differ significantly.

treated counterparts; $p < 0.001$. Treating canola meal with 15 and 30 mL/kg DM Bioprotect resulted in a 12.7% and 40.4% reduction of total VFA and a 2.8% and 49.4% reduction of total VFA in soybean meal compared to untreated meals, $p < 0.001$. Increasing the TE concentration from 20 to 40 g/kg DM reduced the total VFA by 19.5% in canola meal and 13.2% in soybean, $p < 0.001$. The acetic acid concentration was reduced in treated meals compared to untreated meals, $p < 0.001$. Moreover, the propionic acid content was also reduced by the inclusion of 30 mL/kg DM Bioprotect and 40 g/kg DM TE in both meals, $p < 0.001$, but no difference between untreated and treated meals using 15 mL/kg DM Bioprotect and 20 g/kg DM TE. Likewise, the acetic to propionic acid ratio (A:P) was also negatively affected by 30 mL/kg DM Bioprotect and 40 g/kg DM TE in both meals, $p < 0.001$, and no effect was observed for using 15 mL/kg DM Bioprotect and 20 g/kg DM TE. Furthermore, the isobutyric and isovaleric proportions were greater in untreated meals than in treated ones and reduced with increasing doses of Bioprotect and TE, $p < 0.001$.

Protein degradation

The ammonia-N concentration was greater for untreated meals than treated meals and reduced over the 24 h fermentation period, $p < 0.001$ (Figure 2). The inclusion of 15 mL/kg DM Bioprotect in canola meal decreased ammonia-N concentration by 74% (0.52 vs. 2.00 μL/L) at 24 h sampling and 33.7% (2.21 vs. 3.28 μL/L) after 4 h of incubation, $p < 0.001$. The use of 15 mL/kg DM Bioprotect in soybean meal decreased ammonia-N concentration by 81.1% (0.40 vs. 2.12 μL/L) at 24 h and 33.4% (2.29 vs. 3.44 μL/L) at 4 h sampling compared to untreated soybean meal, $p < 0.001$. There was no difference between ammonia-N values of fermented meals

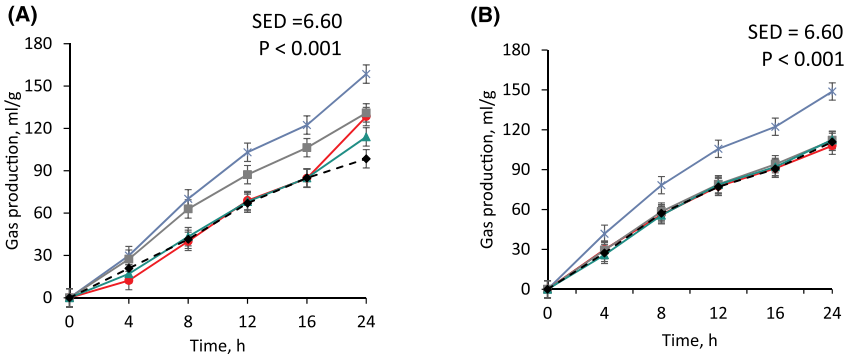

FIGURE 1 Gas production curves of feedstuffs (A): canola meal and (B): soybean meal untreated (—✳—) and treated with 15 mL/kg dry matter (DM) (—●—) and 30 mL/kg DM (—▲—) Bioprotect and 20 g/kg DM (—■—) and 40 g/kg DM (— ◆ -) tannin extracted from red grape marc and incubated at a 39°C water bath for 24 h using fresh rumen fluid. Data are least-square means of gas production after converting gas pressure into gas volumes using Avogadro's law at varying incubation times (4, 8, 12, 16, and 24 h).

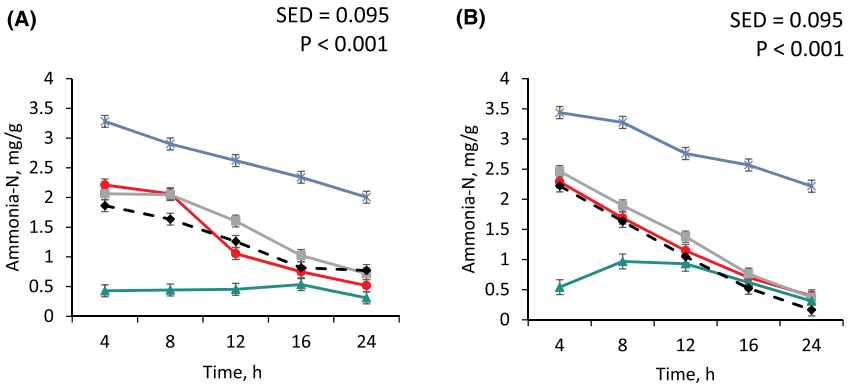

FIGURE 2 The ammonia-N concentration produced from (A): canola meal and (B): soybean meal untreated (—✳—) and treated with 15 mL/kg dry matter (DM) (—●—) and 30 mL/kg DM (—▲—) Bioprotect and 20 g/kg DM (—■—) and 40 g/kg DM (— ◆ -) tannin extracted from red grape marc and incubated at a 39°C water bath for 24 h using fresh rumen fluid. Data are least-square means of ammonia-N values measured at varying incubation times (4, 8, 12, 16 and 24 h).

treated with 20 and 40 g/kg DM TE at 4 h sampling period. Moreover, the ammonia-N concentration was decreased with increasing incubation time for all treated and untreated meals, $p < 0.001$, but not the meals treated with 30 mL/kg DM Bioprotect. The ammonia-N concentration measured from meals treated with 30 mL/kg DM Bioprotect was lowest compared to other treatments at 24 h sampling with 86.9% and 84.2% reduction in canola and soybean meals.

The IVDP of untreated canola meal increased from 14% at 4h to 74% during 24 h of in vitro fermentation, $p < 0.05$ (Table 3). The 15 and 30 mL/kg DM inclusion of Bioprotect in canola meal reduced the IVDP by 33.0% and 46.0% compared to untreated canola meal after 24 h of incubation, $p < 0.05$, whereas the canola meal treated with 20 and 40 g/kg DM TE showed a reduction of 8.0% and 31.0% IVDP, respectively, $p < 0.05$. Likewise, the IVDP of soybean meal treated with 15 and 30 mL/kg DM Bioprotect decreased by 26.6% and 53.2% compared to untreated soybean meal, $p < 0.05$. The 20 and 40 g/kg DM TE inclusion in soybean meal reduced the IVDP by 12.8% and 23.4% after 24 h of *in vitro* fermentation, $p < 0.05$.

The protein fraction 'a' of untreated canola meal (212 g/kg CP) was greater than that of treated meals, $p < 0.01$ (Table 4). The inclusion of 15 and 30 mL/kg DM Bioprotect in canola meal declined the proportion of fraction 'a' by 83.9% and 86.0%, $p < 0.01$. Increasing the dose of TE in canola meal from 20 to 40 g/kg DM reduced the proportion of fraction 'a' from 88.5 to 31.2 g/kg CP, $p < 0.01$. The protein fraction 'b' of untreated canola meal (709 g/kg CP) was lower than canola meal treated with Bioprotect and TE, $p < 0.01$. There was no difference between canola meals treated with 40 g/kg DM TE and 15 and 30 mL/kg DM Bioprotect in fraction 'b'. The inclusion of 15 and 30 mL/kg DM Bioprotect increased the fraction 'b' of canola meal by 15.2% and 16.6% compared to untreated canola meal; $p < 0.01$. The 20 and 40 g/kg DM inclusion of TE in canola meal increased the fraction 'b' by 10.5% and 16.3% compared to untreated canola meal; $p < 0.01$. There was no difference between fraction 'b' content of

TABLE 3 The in vitro degradable crude protein (IVDP) of canola and soybean meals treated with Bioprotect (15 and 30 mL/kg dry matter (DM)) and tannin extract (TE) (20 and 40 g/kg DM) from red grape marc calculated using the intercept of gas production and ammonia-N values at 4, 8, 12, 16, and 24 h of in vitro fermentation.

Substrates	Canola meal					Soybean meal						
Additives		Bioprotect		Tannin extract			Bioprotect		Tannin extract			
Time, h	Control	15	30	20	40	Control	15	30	20	40	SED[a]	Significance[b]
4	0.14^{b}	0.06^{bc}	0.03^{bc}	0.11^{b}	0.09^{b}	0.34^{a}	0.15^{b}	0.13^{b}	0.31^{a}	0.29^{a}	0.085	S*, T*
8	0.21^{c}	0.11^{cd}	0.09^{cd}	0.18^{c}	0.14^{c}	0.62^{a}	0.33^{b}	0.19^{c}	0.38^{b}	0.11^{cd}		
12	0.36^{c}	0.18^{d}	0.17^{d}	0.25^{cd}	0.23^{cd}	0.77^{a}	0.47^{b}	0.28^{c}	0.52^{b}	0.53^{b}		
16	0.54^{b}	0.30^{d}	0.35^{d}	0.44^{c}	0.32^{d}	0.85^{a}	0.54^{b}	0.36^{cd}	0.62^{b}	0.61^{b}		
24	0.74^{b}	0.41^{d}	0.28^{e}	0.66^{bc}	0.43^{d}	0.94^{a}	0.69^{bc}	0.44^{d}	0.82^{b}	0.72^{bc}		

Note: Standard error of the difference for Substrate (S) x Additives (A) (Bioprotect and TE). Significance of effects of Substrate (S) x Additives (A) (Bioprotect and TE) and interactions: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; TVFA: total volatile fatty acids; and A:P: acetic to propionic acid ratio. Values in a single row with different superscripts (a, b, c, d and e) differ significantly ($p = 0.05$).

TABLE 4 Protein fractions (a, b, undegraded, g/kg CP) and degradation rate (c, %/h) of canola meal and soybean meal treated with Bioprotect (15 and 30 mL/kg dry matter (DM)) and tannin extract (TE) (20 and 40 g/kg DM) from red grape marc after 24 h of in vitro rumen fermentation.

Substrates	Canola meal					Soybean meal						
Additives		Bioprotect		Tannin extract			Bioprotect		Tannin extract			
Fractions and rate	Control	15	30	20	40	Control	15	30	20	40	SED[a]	Significance[b]
a, g/kg CP	212^{b}	34.2^{f}	29.7^{f}	88.5^{d}	31.2^{f}	221^{a}	85.1^{d}	78.1^{de}	134^{c}	83.0^{d}	5.91	S**; A**
b, g/kg CP	709^{d}	836^{a}	850^{a}	792^{b}	847^{a}	758^{c}	854^{a}	863^{a}	806^{ab}	857^{a}	28.2	S**; A**
Undegraded, g/kg CP	80.0^{b}	130^{a}	121^{a}	120^{a}	122^{a}	21.1^{d}	60.7^{c}	59.8^{c}	60.2^{c}	60.5^{c}	11.04	S**; A**
c, %/h[a]	7.17^{b}	3.48^{g}	3.54^{f}	3.30^{i}	3.57^{e}	9.25^{a}	3.55^{f}	3.60^{d}	3.32^{h}	3.66^{c}	0.171	S**; A**

Note: Standard error of the difference for Substrate (S) x Additives (A) (Bioprotect and TE). Significance of effects of Substrate (S) x Additives (A) (Bioprotect and TE) and interactions: *, $p < 0.05$; **, $p < 0.01$; ***, and $p < 0.001$. Values in a single row with different superscripts (a, b, c, d, e, f, g, h and i) differ significantly ($p = 0.01$).

canola meal treated with 40 g/kg DM TE and 15 and 30 mL/kg DM.

The untreated soybean meal had a greater fraction 'a' and lower fraction 'b' than its treated counterparts, $p < 0.01$. Mixing the soybean meal with 15 and 30 mL/kg DM Bioprotect reduced the fraction 'a' by 61.5% and 64.7% compared to the untreated soybean meal, whereas the fraction 'b' increased by 10.1% and 12.2%, respectively, $p < 0.01$. The fraction 'b' of soybean meal treated with 20 and 40 g/kg DM TE was greater than that of untreated soybean meal by 6.00% and 11.6% CP, $p < 0.01$. There was no difference between treated soybean meals in the proportion of fraction 'b', except for 20 g/kg DM TE inclusion. Moreover, the use of additives increased the amount of undegraded protein fractions in both meals compared to their untreated counterparts.

The degradation rate of protein fraction 'b' was significantly lower in treated diets compared to untreated meals, $p < 0.01$. The degradation rate of fraction 'b' was reduced from 7.17%/h to 3.48% and 3.54%/h after 15 and 30 mL/kg DM Bioprotect inclusions in canola meal, respectively, $p < 0.01$. Treating canola meal with 20 and 40 g/kg DM TE resulted in a 3.30% and 3.57%/h degradation rate of protein fraction 'b'; $p < 0.01$. The degradation rate for fraction 'b' of untreated soybean meal was 9.25%/h and reduced to 3.55 and 3.60 when mixed with 15 and 30 mL/kg DM Bioprotect, $p < 0.01$. The inclusion of 20 and 40 g/kg DM TE in soybean meal reduced the degradation rate of fraction b to 3.32% and 3.66%/h, $p < 0.01$.

The EPD values were linearly reduced by the increasing doses of Bioprotect ($R^2 = 0.90$ in canola meal and 0.71 in soybean meal, $p < 0.001$) and TE ($R^2 = 0.78$ in canola meal and 0.91 in soybean meal, $p < 0.001$) (Figures 3 and 4). However, there was no difference between EPD values of soybean meal treated with 15 and 30 mL/kg DM Bioprotect, indicating a slight variation between meals in response to these two additives.

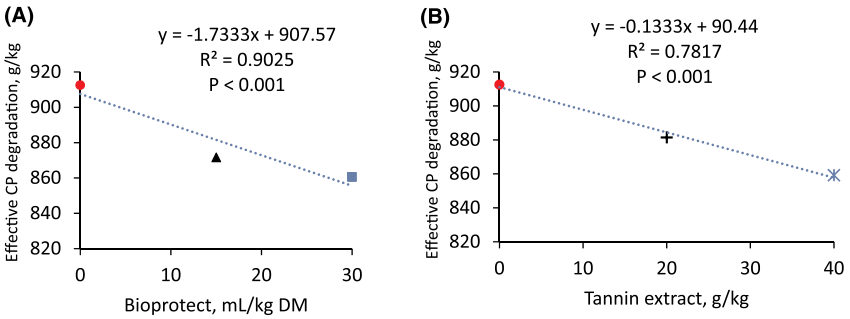

FIGURE 3 The correlation curves, coefficients (R^2), and linear regression equations between the volume of Bioprotect (mL/kg dry matter (DM)) (A) and tannin extract (TE) (g/kg DM) (B) in the *X* axis and the effective crude protein degradation (g/kg CP) in the *Y* axis for **canola meal** treated with 0 (●), 15 (▲), and 30 mL/kg DM (■) Bioprotect and 20 (+) and 40 g/kg DM (✳) TE from red grape marc and in vitro fermented using rumen fluid for 24 h. The *p*-values for the correlation between the additives and the effective crude protein degradation were $p < 0.001$.

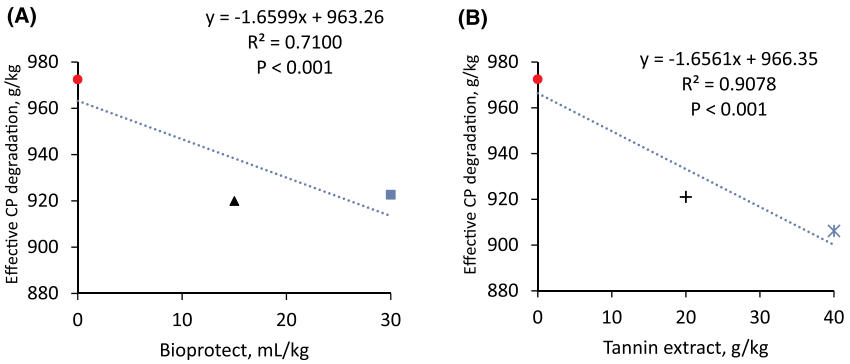

FIGURE 4 The correlation curves, coefficients (R^2), and linear regression equations between the volume of Bioprotect (mL/kg dry matter (DM)) (A) and tannin extract (TE) (g/kg DM) (B) in the *X* axis and the effective crude protein degradation (g/kg CP) in the *Y* axis for **soybean meal** treated with 0 (●), 15 (▲), and 30 mL/kg DM (■) Bioprotect and 20 (+) and 40 g/kg DM (✳) TE from red grape marc and *in vitro* fermented using rumen fluid for 24 h. The *p*-values for the correlation between the additives and the effective crude protein degradation were $p < 0.001$.

DISCUSSION

The addition of Bioprotect and TE from red grape marc to soybean and canola meals inhibited in vitro fermentation characteristics and protein solubility as measured by the gas production, total VFA, ammonia-N, IVDP, fraction 'a' and the degradation rate of fraction 'b'. The reduction in gas production and soluble protein and the increase in lag time, slowly degradable protein, and EPD following the application of additives could be attributed to the formation of complexes from the bonding of active compounds with feed molecules, which reduce enzymatic and microbial activities. However, no prior reports on the impact of Bioprotect and TE from red grape marc determined protein fractions 'a', 'b', undegraded protein, and EPD during in vitro studies.

Protein degradation

Ammonia-N is the end product of the protein degradation and deamination of peptides/amino acids in the rumen [51]. The inclusion of Bioprotect and TE reduced ammonia-N concentration in this experiment. This is consistent with the study by Alipour and Rouzbehan [52], who demonstrated that the application of 15, 30, 45, and 60 g/kg DM TE on DM basis from grape marc decreased the ammonia-N of soybean meal by 6%, 13.4%, 19.4%, and 32.8%, respectively, in 24 h of in vitro fermentation using rumen fluid from cannulated sheep. Moreover, Sinz and Marquardt [53] have reported that mixing of 50 g/kg DM TE on DM basis from grape seeds with ryegrass hay reduced 12% ammonia-N formation measured after 24 h of in vitro fermentation in the Hohenheim gas test using rumen fluid from lactating cows. Reduction in ammonia-N after applying Bioprotect and TE confirms successful protein protection in this experiment. However, the inclusion of 30 mL/kg DM Bioprotect resulted in an extensive reduction of ammonia-N concentration, possibly caused by toxicity and a detrimental effect on the microbial activities responsible for ammonia-N production in the rumen fluid.

The IVDP is the estimation of crude protein that could potentially be degraded during the in vitro fermentation. The IVDP values were lower in treated meals. However, there is no information on additives used for protein protection *in vitro* experiments. The lower IVDP is related with decreased ammonia-N, fraction 'a', increased volume, and reduced degradation rate of fraction 'b', attributed to reduced protein solubility and formation of protein–tannin complexes during the in vitro rumen fermentation process.

Fraction 'a' represents the fraction of protein that is readily soluble and rapidly degradable in the rumen while fraction 'b' refers to the slowly degradable protein portion. The fraction 'a' was reduced and 'b' was increased after mixing the meals with both doses of Bioprotect and the highest dose of TE in this experiment. Alipour and Rouzbehan [52] have demonstrated that 15, 30, 45, and 60 g/kg DM inclusion of TE from grape marc reduced the fraction 'a' of soybean meal by 13.3%, 53.3%, 73.3%, and 73.3% using *in sacco* bag technique in rumen cannulated rams. Moreover, treating soybean meal with 15 and 30 g/kg DM TE from *Cistus ladanifer* L. showed a decrease of fraction 'a' by 26.7% and an increase in fraction 'b' by 10.3% in an in situ experiment using rams [54]. Tannins promote the formation of protein–tannin complexes [55] or induce structural changes in proteins and enzymes, leading to their decreased availability to proteolytic activities and solubility [56, 57]. The changes in protein fractions 'a' and 'b' in this experiment suggest that the inclusion of Bioprotect and TE can reduce the availability of soluble proteins for microbial degradation and increase protein proportion that is less susceptible to rapid breakdown. This can positively affect the nutritional value of Bioprotect and TE for protein protection in ruminants. However, the effect of 20 g/kg DM TE inclusion in fractions 'a' and 'b' was lowest compared to other additives.

Bioprotect and TE applications delayed the degradation rate of fraction 'b' by making it more resistant to breakdown, resulting in a more sustained availability of fraction 'b' over time. Alipour and Rouzbehan [29] showed that the degradation rate of untreated soybean meal was 0.09%/h *in situ* study using cannulated rams and reduced to 0.06%/h with 30 and 45 g/kg DM grape marc TE inclusion. The reduction of the fraction 'b' degradation rate implies a decreased proteolysis caused by the inclusion of Bioprotect and TE during the 24 h in vitro fermentation period.

The Bioprotect and TE treatments increased the undegraded protein fraction in this experiment, assisted by the protein protection properties of these additives against proteolytic microorganisms and enzymes in the rumen fluid. This is consistent with Ramaiyulis and Zain [58], who showed an average of 42.4% increase in rumen undegraded protein from *in vitro* fermented feed supplement containing soybean meal and treated with 6.80, 11.7, and 16.0 g/kg DM TE from gambier leaf. Tabacco and Borreani [59] also reported 40 and 60 g/kg DM inclusion of TE from chestnut significantly increased the proportion of undegraded protein in alfalfa silage fermented *in situ* using cannulated dry cows. The formation of complex bonds between tannin and protein molecules could justify the increased volume of undegraded protein and prove the increase in bypass protein for post-rumen digestion.

The EPD is the total amount of protein degraded during the *in vitro* rumen fermentation [38, 60]. The application of Bioprotect and TE decreased the EPD values in both meals. This is consistent with different *in situ* studies. Dentinho and Moreira [61] demonstrated a

linear reduction of EPD in soybean meal treated with 12.5, 25, 50, 100, and 150 g/kg DM of TE from *Cistus ladanifer* L. fermented *in situ* using cannulated rams. Likewise, Alipour and Rouzbehan [52] concluded that increasing the dose of TE from grape marc resulted in a linear reduction of *in situ* rumen protein degradability of soybean meal incubated in cannulated sheep. Tabacco and Borreani [59] found that adding 40 and 60 g/kg DM commercial chestnut tannin reduced *in situ* protein degradability of alfalfa silage by 5%–10% in cannulated dairy cows. The reduction of EPD was attributed to the increased proportion of the undegraded protein fraction, which escaped the *in vitro* rumen degradation available for intestinal digestion. Thus, a limited amount of TE could reduce protein degradation in the rumen and enhance ruminant production efficiency by increasing the amount of utilizable protein in the small intestine [62, 63].

In vitro fermentation parameters

The volume and the rate of gas production from untreated meals were greater than meals treated with Bioprotect and TE from red grape marc. This is consistent with Alipour and Rouzbehan [52] who demonstrated that linear reduction of *in vitro* gas production from soybean meal with increasing doses of TE from grape marc. Moreover, Lavrenčič and Pirman [64] treated soybean meal with 60 g/kg DM TE from chestnut wood and found a 9% reduction in gas production. Likewise, treating a canola meal with 15 mL/kg DM and lupin meal with 100 mL/kg DM Bioprotect depressed the volume of total gas production by 16.7% and 45%, respectively [15]. Prathap and Chauhan [17] showed incorporation of a 40 mL/kg DM Bioprotect *in vitro* fermentation decreased gas production of wheat grain by 21.8%. The formation of complexes from the bonding of these additives with feed molecules could decrease the availability and inhibit the degradation of feed in the rumen fluid, resulting in a reduced total gas production [14, 55, 56].

The increase of Bioprotect from 15 to 30 mL/kg DM and TE from 20 to 40 g/kg DM did not affect the gas production in soybean meal. Moreover, the gas production curves of treated canola meal followed an overlapping path until 16 h, except for canola meal treated with 20 g/kg DM TE. The study by Salawu and Acamovic [65] showed that gas production curves of *in vitro* fermented *Calliandra* stems treated with 50 g/kg DM Quebracho TE mixed with 1 or 10 g/kg DM Browse plus additives were not separated until after 32 h of fermentation. This suggests exceeding the optimal volume of additives may not change the fermentation pattern. In contrast, the lower dose of TE produced a greater gas volume than other treated canola meals, indicating the occurrence of insufficient bonding with feed molecules. Moreover, the inhibition effect and bonding of the additives with feed molecules could be decreased over time in some feeds, as observed in gas production curves for the inclusion of 15 mL/kg DM Bioprotect in canola meal after 16 h. This implies the gas production values from the inclusion of 30 mL/kg DM Bioprotect and 40 g/kg DM TE could be recommended for consistent in vitro fermentation characteristics than lower doses.

Lag time is the time required by microbes to colonize the feed, multiply, and grow before positive gas production begins [66, 67]. The lag time was increased by 30 mL/kg DM Bioprotect and 40 g/kg DM TE applications in both meals but not for treatments with a lower volume of additives. This finding is consistent with Alipour and Rouzbehan [52] who demonstrated that the application of 30, 45, and 60 g/kg DM TE from grape marc increased the lag time of soybean meal fermentation from 0.07 h to 0.09, 0.12, and 0.36 h, respectively. In addition, Salawu and Acamovic [65] fermented *Calliundra calothyrsus* leaf and stem *in vitro* with 50 g/kg DM Quebracho TE, which resulted in a delay of gas production for 1.6 h in leaves and 2.1 h in stems. The extended lag time confirms that higher doses of Bioprotect and TE from red grape marc have the potential to slow the microbial activities responsible for gas production, leading to delayed onset of gas production.

The total VFA was reduced with the increasing volume of additives as VFA concentration is directly correlated with the amount and rate of gas production [68, 69]. Likewise, the isobutyric and isovaleric acid concentrations were greater in untreated meals and reduced with increasing doses of Bioprotect and TE. The isobutyric and isovaleric acids are by-products of microbial proteolytic activities in the rumen [70]. Therefore, the binding of tannins with proteins might reduce protein degradation and proportions of iso-acids *in vitro* fermentation [71]. However, the effects differed between the treatments as 15 mL/kg DM Bioprotect and 20 g/kg DM TE did not affect the A:P ratio. Therefore, the impact of additives on VFA production depends on the volume of additives, as higher concentration promotes a strong effect on the enzymes and microorganisms responsible for VFA generation [72, 73].

The effect of TE from grape marc on *in vitro* feed fermentability and protein protection could vary based on different factors. The variety of the grapes, the ratio of seed, skin, and pulp in the grape marc, and inclusion doses could change the tannin concentration and its effect [24, 25, 32]. Moreover, feed fermentation responses could vary between substrates as they differ in nutritional characteristics, fermentation process, and interactions with the additives [72, 74, 75]. Therefore, these factors need to be considered before application in the ruminant's diet.

The quantification of feed digestibility plays a pivotal role in optimizing feed resource utilization and

mitigating excessive nutrient excretion to the environment. However, the controlled environment *in vitro* experiments may not fully represent the dynamic and diverse environment within the ruminants. Moreover, variability in methodologies and lack of standardized protocols among different *in vitro* techniques can lead to inconsistent results between laboratories. This makes it challenging to compare data across studies or to establish universal standards [76, 77]. Conversely, *in vivo* feed digestibility experiments are encumbered by time, expense, labor intensiveness, and substantial feed quantity requirements. Such methodologies are impractical for the expeditious and routine evaluations demanded by laboratories catering to the needs of livestock producers and feed manufacturers [78]. Consequently, *in vitro* digestibility techniques have emerged as a preferred alternative due to their rapidity, cost-efficiency, and precision in predicting digestibility in ruminants, presenting a favorable contrast to the protracted and resource-demanding nature of *in vivo* approaches [79].

In summary, *in vitro* fermentation characteristics and protein fractions were impacted using Bioprotect and TE in a dose-dependent manner but not for the post-fermentation pH. The responses differed between the treatments as 15 mL/kg DM Bioprotect and 20 g/kg DM TE did not affect the lag time and the A:P ratio. Moreover, the 20 g/kg DM TE inclusion produced greater gas production in canola meal and showed lowest effect on the amount of protein fractions 'a' and 'b' in both meals compared to their other treated counterparts. On the other hand, the extensive reduction of ammonia-N and IVDP from the application of 30 mL/kg DM Bioprotect suggests possible toxicity to microbes responsible for protein digestion in higher doses. Therefore, 15 mL/kg DM Bioprotect and 40 g/kg DM TE could be promising protein protection doses for *in vitro* protein protection, and these additives are required to be evaluated for the *in vivo* rate.

CONCLUSION

The *in vitro* fermentability and protein fractions of canola and soybean meals were affected by the addition of Bioprotect and TE from red grape marc. The increase in fraction 'b' and reduction in protein fraction 'a', ammonia-N, IVDP, and EPD confirms successful protein protection in this experiment. However, the effect from TE in a lower dose could be insufficient, and a higher dose of Bioprotect could have detrimental effects. Therefore, treating canola and soybean meals with 15 mL/kg DM Bioprotect and 40 g/kg DM TE could be promising protein protection methods. Further research is required to evaluate these additives for *in vitro* intestinal digestibility and *in vivo* inclusion rates.

AUTHOR CONTRIBUTIONS

Bereket Zeleke Tunkala: Conceptualization; methodology, investigation; data curation; formal analysis; writing—original draft preparation and project administration. **Kristy DiGiacomo**: Resources; funding acquisition; supervision, and writing—review and editing. **Pablo S. Alvarez Hess**: Resources; supervision and writing—review and editing. **Frank R. Dunshea**: Methodology; resources; supervision; and writing—review and editing. **Brian J. Leury**: Conceptualization; funding acquisition; supervision, and writing—review and editing. All authors have read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The Department of Jobs, Precincts and Regions Agricultural Research and Extension Animal Ethics Committee approved the preparation and use of cannulated cows from which rumen fluid was sourced for this experiment.

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