Effects of País grape marc inclusion in high and low forage diets: ruminal fermentation, methane production and volatile fatty acids

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Abstract

The objective of this study was to evaluate the effects of the inclusion of País grape marc (GM) in high (HF) and low forage (LF) ruminant diets on rumen methane (CH₄) production and fermentation parameters in an in vitro (batch) culture. Experimental treatments replaced forage for GM at increasing concentrations (0, 10%, 20% dry matter [DM]) in the incubation substrates. Gas production (GP) and CH₄ production were measured at 6, 12 and 24 h of incubation and in vitro DM disappearance, volatile fatty acids (VFA) and ammonia nitrogen (NH₃-N) were determined at 24 h. The inclusion of 10% and 20% of GM reduced NH₃-N concentrations (p < 0.001) by 48% and 58% respectively, and GM20 inclusion reduced BCVFA concentrations (p = 0.049) by 21% for HF and by 11% LF. Inclusion of 20% GM reduced the percentage of CH₄ in gas (p = 0.038), CH₄ production (p = 0.004) and CH₄ yield (p = 0.027), but leads to a reduction of in vitro dry matter disappearance (IVDMD) (p < 0.001), gas production (p < 0.001) and total VFA (p = 0.001). In conclusion, in batch culture conditions, partial substitution of fibre sources by País GM in high and low fibre diets has the potential to reduce NH₃-N concentrations and CH₄ production and yield with a reduction of IVDMD.

Highlights

- País GM reduces up to 58% in vitro NH₃-N rumen concentrations regardless of forage level in the diet.
- Partial substitution of fibre sources with País GM reduces methane production by up to 15%.
- Partial substitution of forage sources with País grape marc has the potential to improve livestock systems sustainability.

Introduction

Agriculture provides high quality food to humanity but is facing the challenge to feed a growing population whilst reducing its environmental impact (Rojas-Downing et al. 2017). Ruminant livestock production contributes to agriculture’s environmental footprint through the generation of 18% of CH₄ (Xu et al. 2021) and 32% of nitrous oxide (N₂O) emissions (Uwizeye et al. 2020). The use of abundant agriculture by-products as ruminant dietary components has generated increasing interest given its contribution to improve sustainability and promote circular economies (Garcia-Rodriguez et al. 2019). Agro-industrial by-products may also contain functional nutrients such as high concentrations of unsaturated fatty acids which could improve product quality and human health, or secondary plant metabolites such as condensed tannins that potentially reduce enteric CH₄ emissions and improve nitrogen use (Aboagye and Beauchemin 2019). By-products are low cost and from a life cycle assessment perspective, they are free of greenhouse gases, as these are attributed to the primary product (Williams et al. 2014). As such, the inclusion of agro-industrial residues in animal diets are a cost-effective alternative as partial substitute for fodder sources (Halmemies-Beauchet-Filleau et al. 2018). Unconventional feed resources, such as fruit by-products, are increasingly explored as potential feed ingredients.
products, can partially fill feed supply shortage, reduce human-animal competition for food, reduce feed costs and contribute to feed security from locally available resources. Wine industry produces high quantities of organic wastes such as stalks and marc. Grape marc represents 25% of the industry’s solid wastes, and its management presents serious environmental and economic challenges (Spigno et al. 2017). Due to its low cost, fibre and energy contents GM has been used as a substitute for fibrous sources for ruminant feed (Manso et al. 2016). It also contains varying concentrations of lipids (5.2–185 g/kg DM, of which 70% are polyunsaturated fatty acids) and condensed tannins (CT; 6.9–139 g/kg DM), which are considered promising compounds as modulators of ruminal fermentation to reduce CH4 production and nitrogen excretion from livestock production systems (Hixson et al. 2016). Grape seeds are a good source of galloylated condensed tannins and epigallocatechin gallate (a galloylated flavan-3-ol monomer) compounds that are urease inhibitors (Takeuchi et al. 2014), which in turn reduce urinary ammonia emissions and the subsequent formation of N2O (Kingston-Smith et al. 2010), this represents an opportunity to reduce the environmental impact of nitrogen emissions from ruminant.

Variations in lipid and CT concentrations in GM can result from grape variety, cultural practices and winemaking processes (Spanghero et al. 2009), therefore evaluation at local scale is required. Information about the use of GM in dairy ruminants under in vivo conditions is available, although research on this resource under in vitro conditions to assess its effects on rumen fermentation is scarce (Hixson et al. 2016; Russo et al. 2017). Nowadays there are few reports on GM of local varieties (Lopes et al. 2014), but none assessed Pais grape (Vitis vinifera L.), an ancient grape variety cultivated in the regions of Bio Bio and Nuble, central Chile, which has higher amounts of condensed tannins than commercial grape varieties such as Carmenere or Pinot Noir (Villarroel 2009).

Wine production in Chile exceeded one billion L in 2020, generating over 200 thousand tons of marc which are mainly deposited directly in landfills, leading to serious environmental impacts as water pollution, odour generation and phytotoxicity (Beres et al. 2017), that could be reduced if used as animal feed. As the effects of dietary tannins vary according to the forage and concentrate contents of the diet (Vasta et al. 2008; Yogianto et al. 2014), the objective of this study was to evaluate the effects of inclusion of increasing concentrations of Pais GM on rumen fermentation kinetics, CH4, NH3-N and VFA production when used in ruminant diets with different forage-concentrate ratios, using an in vitro gas production technique.

**Materials and methods**

The experimental procedures were conducted in accordance with the ethics and animal welfare committee of the Universidad de Concepcion (CBE-08-2021).

**Grape marc samples**

Pais GM from the Itata valley (Chile), was provided by the vinification laboratory, Universidad de Concepcion (Chillan-Chile). Marc was collected from the grape pile at the winery on the day of production, dried at 60 °C for 48 h using a forced-air laboratory oven, ground through a 2 mm screen (Grain Mill, Breuer, Temuco-Chile) and stored (4 °C) until in vitro incubations.

**Experimental design and treatments**

A batch culture was conducted in a completely randomised design with a 2 × 3 factorial arrangement. The 6 treatments were a combination of two forage-concentrate ratio, high forage (HF, 60:40 forage-concentrate ratio), and low forage (LF, 35:65 forage-concentrate ratio) and three GM concentrations [0, (GM0) 10% (GM10) and 20% (GM20) DM basis]. Forage sources were corn silage and mixed hay, the later included up to 20% DM in each experimental diet, and progressively replaced by GM in experimental diets (Table 1).

All ingredients were ground to 2 mm (Breuer grain mill, Chile), mixed, and thereafter 0.5 g of each substrate was deposited in filter bags (Ankom® F57, ANKOM Technology, Macedon, NY, USA). Each treatment was incubated in three technical replicates and three runs (statistical replicates) in consecutive weeks with three sampling times (6, 12 and 24 h). Thus, 189 bottles [{(2 diets × 3 GM level × 3 technical replicates) + 3 blanks} × 3 sampling times × 3 runs (statistical replicate)] were used in total.

**Animals and in vitro fermentation**

Two hours after morning feeding, rumen fluid was collected from top, bottom, and middle of rumen of two adult non-lactating Aberdeen Angus cows with a permanent rumen cannula. For 14 days before collection, cows were fed mixed hay, corn grain and minerals, balanced to meet the requirements according to their physiological stage and had access to water ad
libitum. The pooled fluid was filtered through 4-layers cheesecloth, transported in preheated thermos (39°C), infused with CO₂ for anaerobiosis and utilised within 15 min of collection. The inoculum was a 1:3 v/v mixture of rumen fluid and buffer solution (Menke et al. 1979).

On the day of incubation, each Ankom® F57 bag with substrate was placed into a 50 mL amber serum bottle. Inoculum (25 mL) was added to the bottles, which were then sealed with rubber stoppers and incubated for 24 h at 39°C (Form Series II 3110 Water-Jacketed CO₂ Incubator, Thermo Fisher Scientific, Waltham, USA) on an orbital shaker set at 90 oscillations/min (Heidolph Unimax, Germany).

**Table 1. Ingredients and chemical composition (% dry matter unless otherwise indicated) of high (HF) and low forage (LF) diets supplemented with Pais grape marc (GM) at concentrations of 0, 10% and 20% (dry matter basis).**

| Ingredients, % of dry matter (DM) | GM0  | GM10 | GM20 | GM0  | GM10 | GM20 |
|----------------------------------|------|------|------|------|------|------|
| Mixed hay                        | –    | –    | 20   | 10   | 0    | 20   |
| Pais grape marc                  | –    | –    | 0    | 10   | 20   | 0    |
| Corn silage                      | –    | –    | 38   | 38   | 38   | 16   |
| Corn grain                       | –    | –    | 10   | 13   | 13   | 12   |
| High-moisture corn               | –    | –    | 10   | 10   | 11   | 22   |
| Soybean meal                     | –    | –    | 8    | 8    | 8    | 12   |
| Malasses                         | –    | –    | 3    | 3    | 3    | 5    |
| Sugar beet pulp                  | –    | –    | 10   | 7    | 6    | 12   |
| Vitamin premix                   | –    | –    | 1    | 1    | 1    | 1    |
| **Chemical composition, %**      |      |      |      |      |      |      |
| Ash                              | 10.2 | 9.5  | 4.8  | 6.20 | 6.1  | 5.90 |
| Crude protein                    | 9.8  | 14.4 | 5.7  | 13.50| 13.3 | 13.10|
| Ether extract                    | 10.0 | 2.0  | 5.2  | 1.53 | 2.0  | 2.45 |
| Neutral detergent fibre          | 42.6 | 50   | 54.8 | 35.10| 33.5 | 33.10|
| Acid detergent fibre             | 41.2 | 37.6 | 33.3 | 21.80| 19.9 | 18.40|
| Non-fibrous carbohydrates*       | 32.6 | 25.5 | 29.5 | 43.70| 45.1 | 45.45|
| Total polyphenol content         | 18.8 | –    | –    | 0.06 | 0.1  | 0.13 |
| Condensed tannins                | 6.8  | –    | –    | 0.00 | 0.5  | 0.90 |

*Non-fibrous carbohydrates (NFC) = 100 – CP – EE – NDF – Ash.

Concentrations of CH₄ were presented as percentage in gas, production (mg/g DM) and yield (mg/g DM digested).

At the end of the incubation, pH of the culture medium was measured using a pH metre (Thermo Scientific, Orion Star A121 pH Portable Metre, USA) and the Ankom®-F57 filter bags were washed with distilled water and oven dried at 55°C for 48 h. Percent weight loss was calculated and presented as in vitro DM disappearance (IVDMD).

**Determination of ammonia nitrogen and volatile fatty acids**

Two individual samples (1.5 mL) of inoculum were collected at the beginning of the incubation (0h) and from each bottle, after pH measurement. Samples were transferred to 2 mL microcentrifuge vials. A sample for NH₃-N determination was mixed with 150 µL of trichloroacetic acid (0.65 w/v), centrifuged at 14,000 × g for 10 min at 4°C and stored at −20°C until analysis. Concentrations of NH₃-N were measured with UV-Vis spectrophotometer (Spectroquant Pharo 300, Merck KGaA, Germany) at 625 nm, by the Berthelot method modified according to Rhine et al. (1998). A sample to determine VFA concentrations was mixed with 300 µL of metaphosphoric acid (0.25 w/v), centrifuged, filtered (Clarinert™, 22 µm, Agela Technologies, China) and stored at −20°C until analysis. The VFA concentrations were analysed by GC (Agilent 7890B, Agilent Technologies, Inc., Santa Clara, USA). The GC was fitted with a flame ionisation detector and a capillary column (30 m × 0.250 mm ×
Chemical analysis

The GM and substrates were analysed by the following AOAC (1995) methods: dry matter (DM Method 934.01), crude protein (CP Method 954.01), ash (Method 942.05), ether extract (EE Method 920.39) and acid detergent fibre (ADF Method 973.18). Neutral detergent fibre (aNDF) content was tested according to Mertens (2002) using heat stable alpha amylase. Total polyphenol contents (TPC) of substrates were determined by the Folin-Ciocalteu method, according to the methodology reported by Trujillo-Mayol et al. (2019). Condensed tannin contents were determined by the DMac method (Payne et al. 2010) using catechins as calibration standard.

Calculations

Kinetic parameters for IVDMD were determined by fitting the results to the nonlinear Gompertz equation (Lavrenčič et al. 1997):

\[ y = B \exp \left[ -C \exp \left( -At \right) \right], \]

where \( y \) is the rate of IVDMD at a time \( t \), \( B \) is the potentially degradable DM fraction, \( C \) is the relative rate of degradation, and \( A \) is a constant factor of the microbial efficiency.

Kinetic parameters for GP and CH\(_4\) were determined by fitting the results obtained to the nonlinear Gompertz equation (Schofield et al. 1994):

\[ y = b \exp \left( -\exp \left[ 1 - c \left( t - L \right) \right] \right), \]

where \( y \) is GP (mL/gDM incubated [DMi]) or CH\(_4\) (mg/gDM incubated [DMi]) after time \( t \), \( b \) is the value of the component (total potential mL of GP or mg of CH\(_4\)), \( c \) is the specific rate of fermentation and \( L \) is the lag time (time axis intercept).

The efficiency of fermentation was determined by the partitioning factor (PF) at 24 h according to Blümmel et al. (1997). Gas and CH\(_4\) yields were estimated as the net gas (mL) or CH\(_4\) (mg) at each sampling time, divided by the corresponding g of degraded DM (DMd).

Statistical analyses

For statistical analyses, the model included GM level, diet type and their interaction as fixed effects. For all evaluated parameters, the three replicates were averaged before statistical analysis and those averages were the statistical unit. An ANOVA test was carried, using the model:

\[ Y_{ijk} = \mu + \alpha_i + \beta_j + \delta_k + (\alpha\beta)_{ij} + \epsilon_{ijk}, \]

where \( Y_{ijk} \) is the experimental data; \( \mu \) is the overall mean of observations; \( \alpha_i \) is the fixed effect of GM inclusion; \( \beta_j \) is the fixed effect of diet effect (fixed effect); \( \delta_k \) is the incubation (random effect); \( (\alpha\beta)_{ij} \) is the interaction between diet \( \times \) GM and \( \epsilon_{ijk} \) is error. Means were compared by Tukey test and Pearson correlation coefficients were calculated to establish the relationship between diet components and gas and CH\(_4\) production, NH\(_3\)-N and VFA concentrations. Differences were considered significant when \( p \leq 0.05 \). All analyses were performed using Stata Ver. 15.1 (StataCorp LP, College Station, Texas, USA).

Results

In vitro dry matter disappearance, gas and methane production kinetics

A significant \( (p = 0.039) \) diet \( \times \) GM interaction (Figure 1(A)) resulted in a 4% reduction in asymptotic gas production (b) by GM\(_{20}\) inclusion in the HF diet as compared to the GM\(_{0}\) diet, whereas in the LF diet, inclusion of GM\(_{10}\) and GM\(_{20}\) resulted in 8 and 11% reductions, respectively. The potentially degradable fraction (b) \( (p < 0.001) \), relative degradation rate (C; \( p = 0.004 \)) of IVDMD and Lag phase (L) of GP \( (p < 0.001) \) as well as asymptotic CH\(_4\) \( (p = 0.018) \) were lower in the HF as compared to LF diet (Table 2). Grape marc inclusion slightly reduced (4%) the potentially degradable fraction (b) of IVDMD \( (p = 0.006) \) regardless of its inclusion level, and GM\(_{20}\) inclusion resulted in a 10% reduction of asymptotic CH\(_4\) production \( (p = 0.003) \).

24 h fermentation parameters

With the exception of NH\(_3\)-N production \( (p = 0.108) \), diet affected all fermentation parameters, resulting in increased IVDMD \( (p < 0.001) \), PF \( (p = 0.004) \), total \( (p = 0.001) \) and branched chain VFA (BCVFA; \( p = 0.015 \))
and decreased pH \((p < 0.001)\), NDFd \((p < 0.001)\), Acetate \((p = 0.029)\) and A:P ratio \((p = 0.023)\) in LF diets as compared to HF diets (Table 3). Likewise, with the exception of NDF disappearance (NDFd; \(p = 0.934\)) and PF \((p = 0.678)\), GM inclusion affected all fermentation parameters and resulted in increased pH \((p = 0.011)\), butyrate \((p < 0.001)\), and reduced IVDMD \((p < 0.001)\), total VFA \((p = 0.001)\) and acetate proportions \((p = 0.029)\). Diet × GM interactions were recorded for propionate proportions \((p = 0.027)\), BCVFA proportions \((p < 0.001)\) and for acetate-propionate (A:P) ratio \((p = 0.045); \) Figure 2). In HF diets, GM20 inclusion reduced by 13% propionate proportion as compared to HF-GM0, whereas for LF, GM10 and GM20 inclusion reduced propionate proportions by 5% and 8%, respectively. The inclusion of GM20 reduced BCVFA molar ratio by 21% in HF, and by 11% as compared to their controls. Inclusion of GM20 in HF diet resulted in a 13% increase of A:P ratio compared to its control diet. Total VFA were 8.5% higher for LF than HF diet \((p < 0.001)\), GM20 inclusion reduced by 7% total VFA concentrations, although there were no effects by GM10. Inclusion of GM20 reduced the acetate molar proportions \((p = 0.029)\) by 6%, but increased butyrate molar ratio \((p < 0.001)\) according to the inclusion level, by 5% with GM10 and by 8% with GM20 as compared to GM0.

**Gas and methane production**

There was a significant diet × GM \((p = 0.019)\) interaction on the total GP (mL/DM; Figure 1(B)) which was unaffected by GM10 for HF diet. Inclusion of GM10 reduced by 8% total GP for LF diet, whereas inclusion of GM20 reduced total GP by 4% for HF and by 11% for LF, when compared to their corresponding controls.

### Table 2.

*In vitro* dry matter disappearance (IVDMD), gas and CH4 production kinetics of high (HF) and low forage (LF) diets with inclusion of *Pa/C19*ıs grape marc at 0, 10% and 20% (dry matter basis) at 24 h of incubation.

| Itemsa | Diet | SEMb diet | Grape marc | SEM GM | D | GM | D × GM |
|--------|------|-----------|------------|--------|---|----|--------|
| IVDMD  |      |           |            |        |   |    |        |
| B      | 58.53 | 63.59     | 0.283      | 0.291  | 62.38A | 60.85B | 59.93B | 0.347 | <0.001 | 0.006 | 0.086 |
| C      | 1.22  | 1.35      | 0.250      | 0.250  | 1.27 | 1.29 | 1.29 | 0.030 | 0.004 | 0.899 | 0.241 |
| A      | 0.33  | 0.36      | 0.170      | 0.170  | 0.34 | 0.34 | 0.35 | 0.248 | 0.387 | 0.997 | 0.619 |
| Gas production | | | | | | | | | | | |
| b      | 128.78 | 138.54   | 1.416      | 1.416  | 139.55A | 131.27B | 130.17B | 1.693 | 0.003 | 0.027 | 0.039 |
| c      | 0.17  | 0.16      | 0.005      | 0.005  | 0.17 | 0.16 | 0.16 | 0.006 | 0.542 | 0.066 | 0.373 |
| L      | 1.67  | 2.39      | 0.943      | 0.943  | 2.01 | 2.05 | 2.05 | 0.105 | <0.001 | 0.966 | 0.855 |
| CH4 production | | | | | | | | | | | |
| b      | 22.87 | 19.06     | 0.432      | 0.432  | 21.45A | 20.75B | 19.19B | 0.056 | 0.018 | 0.003 | 0.460 |
| c      | 0.15  | 0.16      | 0.006      | 0.006  | 0.14 | 0.14 | 0.14 | 2.853 | 0.452 | 0.760 | 0.066 |
| L      | 5.91  | 6.16      | 0.267      | 0.267  | 6.09 | 6.01 | 5.97 | 0.286 | 0.286 | 0.996 | 0.078 |

### Footnotes

- A:B: Means within the same line with a different superscript differ.
- B: potentially degradable fraction (%); C: relative degradation rate (/h); A: microbial efficiency factor; b: asymptotic production (mL or mg/DM); c: rate of production (/h); L: initial delay before production begins (/h).
- D: diet; GM: grape marc; D × GM: diet × grape marc interaction.
Methane yield was unaffected by diet \( (p = 0.163) \) (Table 4). However, LF diets resulted in reduced gas yield \( (p = 0.003) \), \( \text{CH}_4 \)% in gas \( (p < 0.001) \), and methane production \( (p = 0.047) \) but increased \( \text{CH}_4 \) per g NDF disappeared \( (p < 0.001) \) as compared to HF diets. Grape marc (20% DM) reduced gas yield \( (p = 0.027) \), \% \( \text{CH}_4 \) in gas by 9\% \( (p = 0.038) \), \( \text{CH}_4 \) production by 15\% \( (p = 0.004) \) and \( \text{CH}_4 \) yield by 13\% \( (p = 0.027) \), whereas \( \text{CH}_4 \) production expressed per gram of NDF disappeared was unaffected \( (p = 0.507) \).

**Table 3. In vitro ruminal fermentation parameters of high (HF) and low forage (LF) diets with inclusion of Paas grape marc at 0, 10% and 20% (dry matter basis) at 24 h of incubation.**

| Items \( ^a \) | HF | LF | SEM \( ^b \) | GM \( _0 \) | GM \( _1 \) | GM \( _2 \) | SEM GM | D | GM | D × GM | p-Value \( ^c \) |
|----------------|----|----|-------------|----------|----------|----------|--------|----|----|--------|----------|
| pH             | 5.63 | 6.45 | 0.184      | 6.47\( ^a \) | 6.49\( ^{AB} \) | 6.51\( ^{A} \) | 0.013  | <0.001 | 0.011 | 0.511  |
| IVDMD (%)      | 57.33 | 62.67 | 0.431      | 58.57\( ^{A} \) | 57.51\( ^{A} \) | 55.90\( ^{B} \) | 0.503  | <0.001 | <0.001 | 0.825  |
| NDFd (%)       | 28.97 | 21.34 | 0.803      | 29.70     | 29.53     | 29.31     | 1.292  | <0.001 | 0.934 | 0.753  |
| NH\(_3\)-N (mg/dL) | 2.99 | 2.74 | 0.211      | 4.20\( ^{A} \) | 2.18\( ^{B} \) | 1.76\( ^{B} \) | 0.450  | 0.108  | <0.001 | 0.807  |
| PF (mg DM/d/mL) | 4.65 | 4.90 | 0.036      | 4.70      | 4.65      | 4.60      | 0.066  | 0.004  | 0.678 | 0.072  |
| VFA \( ^d \) | Total (mM) | 154.95 | 168.10 | 2.729 | 167.11\( ^{A} \) | 159.91\( ^{AB} \) | 156.12\( ^{B} \) | 2.543  | 0.001  | 0.001 | 0.502  |
| Acetate (%)    | 45.31 | 43.95 | 0.443      | 47.01\( ^{A} \) | 44.81\( ^{AB} \) | 44.23\( ^{B} \) | 0.626  | 0.007  | 0.029 | 0.648  |
| Propionate (%) | 27.56 | 27.73 | 0.412      | 28.76\( ^{A} \) | 28.10\( ^{AB} \) | 26.06\( ^{B} \) | 0.504  | 0.187  | <0.001 | 0.027  |
| Butyrate (%)   | 25.37 | 25.71 | 0.023      | 1.24\( ^{A} \) | 1.03\( ^{AB} \) | 0.87\( ^{B} \) | 0.065  | 0.026  | <0.001 | 0.119  |
| BCVFA \( ^e \) (%) | 1.07 | 1.18 | 0.023      | 1.24\( ^{A} \) | 1.03\( ^{AB} \) | 0.87\( ^{B} \) | 0.065  | 0.015  | 0.049 | <0.001 |
| A:P ratio      | 1.80 | 1.73 | 0.221      | 1.71\( ^{B} \) | 1.73\( ^{B} \) | 1.85\( ^{A} \) | 0.048  | 0.023  | 0.002 | 0.045  |

\( ^{A} \)Least significant difference within the same line without a common superscript differ.

\( ^{a} \)IVDMD: in vitro dry matter disappearance; NDFd: neutral detergent fibre disappearance; NH\(_3\)-N: ammonia nitrogen; PF: partitioning factor; VFA: volatile fatty acids; BCVFA: Branched-chain volatile fatty acids; A:P: Acetate-propionate ratio.

\( ^{b} \)Standard Error of Mean.

\( ^{c} \)D: diet; GM: grape marc; D × GM: diet × grape marc interaction.

\( ^{d} \)BCVFA: iso-valerate + iso-butyrate.

**Correlations between diets chemical composition and fermentation parameters**

Among all the chemical composition factors of the treatments, CT \( (r = -0.49, p < 0.05) \) and TPC \( (r = -0.40, p < 0.05) \) concentrations had the greatest correlation to NH\(_3\)-N (Table 5). The IVDMD was negatively correlated with NDF content \( (r = -0.49, p < 0.001) \) and TPC \( (r = -0.34, p < 0.05) \). Gas production negatively correlated with TPC \( (r = -0.42, p < 0.001) \) and CT \( (r = -0.3, p < 0.05) \). Production and yield of \( \text{CH}_4 \) had no correlations with any of the components of the diet. Total VFA positively correlated with CP \( (r = 0.28, p < 0.05) \) but negatively correlated with TPC \( (r = -0.37, p < 0.05) \) and CT \( (r = -0.31, p < 0.05) \), whereas acetate was negatively correlated with CP \( (r = -0.32, p < 0.05) \) and positively correlated with NDF \( (r = 0.32, p < 0.05) \). Butyrate was correlated with ash \( (r = 0.50, p < 0.001) \) and CT \( (r = 0.43, p < 0.001) \).

**Discussion**

The interactions for diet × GM for the asymptotic GP and total GP indicate that the effects of substitution of a fibrous source as mixed hay by GM on rumen fermentation vary according to the forage-concentrate ratio of the diet. Inclusion of GM up to 10\% DM had no negative effects on GP parameters of the HF diet, although it did for the LF diet. The reduction of total GP is compatible with the reduction of IVDMD and the total VFA concentrations when GM\(_{20}\) was included. These results concur with previous reports in which fibrous sources were substituted for GM for forage diets (Vinyard et al. 2018; Caetano et al. 2019; Zhang et al. 2022) and for high concentrate diets (Ishida et al. 2015). The IVDMD reduction is of low magnitude as compared to an 11\% reduction for DM digestibility reported by Caetano et al. (2019) for a forage diet and a 35\% reported by Vinyard et al. (2021) for a LF diet. Contrastingly, no effects were reported on gas production or IVDMD when GM was included up to 50\% DM in hay for an *in vitro* fermentation (Giller et al. 2021). Reduction in IVDMD is probably related to an increase in fibrous carbohydrates and lignin with the inclusion of GM to the diet, although it is also possible that CT of GM may reduce the digestion of nutrients by inhibiting the growth and activity of ruminal microorganisms, thus limiting the activity of microbial enzymes, and through complexes with nutrients that bind carbohydrates and proteins (Vasta et al. 2019). Condensed tannins have a high affinity for cell wall polysaccharides and starch to form complexes (Watrelot and Norton 2020). The addition of GM\(_{20}\) to diets substantially increased the CT content, so it is possible that CT caused the decrease in IVDMD by action of CT on rumen fibrolytic microbes and cell wall degradation.
inhibiting rumen gas and CH$_4$ production (Vasta et al. 2019) which could explain the reduction in asymptotic GP and total GP.

The reduction in NH$_3$-N concentrations by GM inclusion in this study is of greater magnitude than previously reported results for GM trials under in vitro or in vivo conditions. Tayengwa et al. (2021) reported a 12% reduction in NH$_3$-N concentrations by inclusion of 15% DM of GM in a fibrous diet, whereas Ream et al. (2021) reported reductions of 15% in NH$_3$-N concentrations by GM 15% DM inclusion in a silage-based diet, and Hixson et al. (2018) reported reductions of 30% in NH$_3$-N concentrations by a 30% DM inclusion of red GM in a low-fibre diet. However, results from this study are consistent with those obtained using polyphenolic plant extracts with higher doses of tannins than those used in this study, such as those reported by Hassanat and Benchaar (2013), with NH$_3$-N reductions of 62% and 66% in diets containing increasing levels of acacia and quebracho tannins up to 20% DM; and Vera et al. (2018) who reported NH$_3$-N reductions of 50% with a pine bark extract (2% DM) on a forage diet. This probably could be due to the higher CT contents of País grapes (and its marc) as compared to the commercial varieties commonly reported in literature.

The diet × GM interaction on BCVFA suggests a differential effect of GM on protein metabolism according to the type of diet in which it is included. There was no effect on BCVFA proportion by the inclusion of GM$_{10}$. The GM$_{20}$ inclusion reduced BCVFA proportion in both diets, but the reduction was nearly two-fold greater for HF than LF. Branched-chain VFA are products of amino acid fermentation, thus a reduction in BCVFA proportion would be expected with a reduction in NH$_3$-N production. As such, the effects on NH$_3$-N may be a result of the TC present in GM. A

**Figure 2.** Interaction diet × grape marc (GM) sliced by High forage (HF) and Low Forage (LF) diets for (A) propionate molar proportion (%), (B) A:P ratio, and (C) branched-chain volatile fatty acids (BCFA) molar proportion (%), at 24 h. Means values within substrate without common letters differ (p < 0.05).

**Table 4.** In vitro gas and methane production of high (HF) and low forage (LF) diets with inclusion of País grape marc of grape marc at 0, 10% and 20% (DM basis) at 24 h of incubation.

| Items | Diet | SEM$^b$ diet | Grape marc | SEM GM | p-Value$^c$ |
|-------|------|-------------|------------|--------|-------------|
|       | HF   | LF          | GM$_0$     | GM$_{10}$ | GM$_{20}$   | D  | GM | D × GM |
| Gas   | mL/g DM | 126.46 | 131.05 | 2.17 | 133.94$^a$ | 128.44$^a$ | 123.88$^a$ | 1.417 | 0.009 | <0.001 | 0.019 |
|       | mL/g DMd | 213.78 | 207.81 | 1.39 | 214.48$^a$ | 209.91$^{ab}$ | 207.99$^{a}$ | 1.704 | 0.003 | 0.027 | 0.126 |
| CH$_4$ | % net gas | 9.80 | 7.67 | 0.348 | 8.85$^a$ | 8.77$^a$ | 8.06$^a$ | 0.426 | <0.001 | 0.038 | 0.898 |
|       | mg/g DM | 17.25 | 16.10 | 0.56 | 17.79$^a$ | 16.84$^{ab}$ | 14.99$^{b}$ | 0.482 | 0.047 | 0.004 | 0.393 |
|       | mg/g DMd | 27.82 | 26.59 | 0.94 | 28.47$^a$ | 27.59$^{ab}$ | 24.77$^{b}$ | 0.749 | 0.163 | 0.027 | 0.199 |
|       | mg/g NDFd | 39.03 | 56.60 | 2.37 | 49.59 | 48.03 | 44.88 | 1.43 | <0.001 | 0.507 | 0.705 |

$^a$Means within the same line without a common superscript differ.
$^b$Standard error of mean.
$^c$D: diet; GM: grape marc; D × GM: diet × grape marc interaction.
The differentiated effect for tannins incubated on different substrates was reported by Menci et al. (2021), who found higher depression of NH$_3$-N production and BCVFA proportions in a hay than a high protein pasture by tannins addition. The authors attributed this effect to the high concentrations of crude protein in the pasture which may require higher tannin concentrations to produce effects.

The diet × GM interactions for propionate and for A:P ratio agree with Foiklang et al. (2016), who identified interaction between GM inclusion level and dietary fibre source. Changes in total and individual VFA molar proportion recorded in this study are in line with those obtained in other GM studies (Moate et al. 2020; Tayengwa et al. 2021; Vinyard et al. 2021). Individual VFA concentrations in this study are similar to those reported by Oskoueian et al. (2013) who found that myricetin and kaemferol, two majors grape flavonols, reduced total VFA concentrations, propionate concentrations, and increased butyrate, as well as A:P ratio, under in vitro conditions. Reduction of total and individual VFA and IVDMD are of small magnitude, as compared to the reductions reported for these parameters these authors.

Reductions in CH$_4$ percentage, production and yield, are consistent with previous reports. Foiklang et al. (2016) reported a reduction in calculated CH$_4$ production for GM at 20% DM basis with forage sources under in vitro conditions. Additionally, a 20% reduction in CH$_4$ emissions by substituting 36% alfalfa hay with GM in dairy cows, without affecting production was previously reported (Moate et al. 2014). However, Moate et al. (2020) reported a 15% reduction in CH$_4$ emissions and a 10% reduction in milk production when replacing fresh perennial ryegrass by GM. These authors attributed these reductions to the GM contents of lipids, lignin and CT. Lipids are not fermented in the rumen, and cover the surface of feed particles preventing fermentation by microorganisms, and may have toxic effects on methanogens and protozoa, thereby contributing to reduced CH$_4$ production (Williams et al. 2020). Lignin is not fermented in the rumen, so it does not contribute to ruminal CH$_4$ production, but it can bind carbohydrates, inhibiting their enzymatic degradation, thus reducing their fermentation and CH$_4$ production. Pellikaan et al. (2011) demonstrated under in vitro conditions the antimethanogenic effects of CTs present in grape seed, however, recently Moate et al. (2020) indicated that GM CTs are not particularly potent antimethanogenic molecules. Similarly, Hixson et al. (2018) suggested a synergistic effect of CTs, fatty acids and lignin. Indeed, high-fat GM varieties affect GP and VFA concentrations, thus reducing CH$_4$ production and GM varieties with higher concentrations of extractable tannins of smaller molecular size have higher antimethanogenic potential.

Correlations registered in this study between the main parameters of ruminal fermentation (IVDMD, GP and yield) and CP and NDF are in accordance with normal parameters of ruminal fermentation. Total polyphenols and CT were negatively correlated with in vitro fermentation products (NH$_3$-N and VFA), but not with CH$_4$ production or yield. It is possible that the lack of correlation for CH$_4$ production parameters, support the suggestion by Hixson et al. (2018) of an additive effect between CT and lipids on the antimethanogenic effect of GM. Future research is warranted to determine whether the CH$_4$ effects of GM are due to polyphenols, lipids or the combination of both.

The results suggest that Pais GM has the potential to improve nitrogen utilisation by ruminants, and reduce CH$_4$ emissions, although these results are dependent on the level of inclusion. Inclusion of Pais GM at 10% DM did not generate important effects on most of the assessed parameters. However, GM inclusion at 20% DM basis generated greater impacts, which appear to be dependent on forage-concentrate ratio of the diet. Further evaluations are necessary to confirm these findings and to evaluate the pertinency of the use of Pais GM as a feed compound for ruminants.

**Conclusions**

Partial substitution of mixed hay by Pais GM up to 10% DM in high and low fibre diets under *in vitro*
conditions reduces NH₃-N concentrations without negative effects on rumen fermentation parameters. Inclusion of GM 20% DM reduces NH₃-N and CH₄ production and yield, as a result of reduced gas production and CH4 in gas and decreases IVDMD and total VFA. The mechanisms of action of GM on nitrogen partitioning are not clear, further studies are warranted to understand these mechanisms and to examine if these positive effects are extended to in vivo conditions. These results suggest that Pais GM has the potential to partially substitute fibrous sources in ruminant feed thus adding value to a highly available agro-industrial residue and contribute to livestock systems sustainability.

Ethical approval

The experimental procedures were conducted in accordance with the ethics and animal welfare committee of the Universidad de Concepcion (CBE-08-2021).

Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

Data supporting the findings of this study are available from the corresponding author, JA-S, upon reasonable request.

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