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In vitro and *in vivo* investigation of Persian manna plant silage as an alternative forage for fattening lambs

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ABSTRACT

The Persian manna plant (*Alhagi maurorum*, PMP) is a species with drought-resistant characteristics and distribution from Asia to America which is widely used in traditional medicine. As a result of recent droughts, many ranchers have become interested in using this plant for feeding their livestock. According to our knowledge, no scientific study has been done regarding the silage quality of PMP with natural-microbial additives. Rumen fluid may also be used as a low-cost and novel-natural microbial additive for improvement in silage quality. Therefore, we planned two *in vitro* and *in vivo* experiments to investigate PMP silage. In the first experiment, we ensiled PMP with molasses (M: molasses, 3%, 6%, and 9% of DM) plus rumen fluid (RF: rumen fluid, 3%, 6%, and 9% of DM) for 60 days with the aim of investigation of nutritional characteristics and its silage potential by *in vitro* methods. In the second experiment, we investigated the nutritional value of the selected superior silage from the *in vitro* section for animal experiments. The growth performance, nutrient digestibility, and ruminal fermentation parameters of forty male lambs were evaluated for substitution of 25%, 50%, 75%, and 100% forage with M6 +RF6 silage in a completely randomized design. Some mineral-chemical composition and digestive-fermentative parameters changed with ensiling PMP. The lowest NDF and ADF were observed in M6 +RF6 ($P < 0.0001$). Higher lactic acid production, total volatile fatty acids, aerobic stability, acid-base buffering capacity, metabolizable energy, Flieg point, and lower butyric acid, ammonia nitrogen, and pH were observed in M6 +RF6 compared to the control. Among the treatments, the highest dry matter intake, average daily gain, final weight, nutrient digestibility, and total volatile fatty acids were observed in lambs fed on the control diet. In total, PMP can be ensiled with molasses and rumen fluid well. As a result of the *in vitro* tests, a better nutritional value was relatively observed in M6 +RF6. The *in vivo* test also showed that M6 +RF6 can be substituted up to about 26% of the dietary DM without adverse effects on growth performance, nutrient digestibility, and ruminal fermentation parameters.

1. Introduction

There are different drought-resistant plants in the world, which can play an essential role in providing a significant part of the nutritional requirements of ruminants. Persian manna plant (*Alhagi maurorum*, PMP) belongs to the *Fabaceae* family and is used extensively in folk medicine as an expectorant, diaphoretic, purgative, laxative, and diuretic agent (Awaad Amani et al., 2006; Naseri and Mard, 2007; Marashdah and Al-Hazimi, 2010). To our knowledge, no nutritional restrictions for the use of PMP in ruminant feeding have not been reported so far. Little data exist about the nutritional characteristics of PMP. For example, Kazemi and Ghasemi Bezdi (2021) reported that PMP at three growth stages can meet the nutrient requirements of ewes at the maintenance level. Also, feeding lactating ewes with PMP had no adverse effect on milk production and composition (Bashtini et al., 2013).

Ensiling is a method mostly used in farms to preserve forages for the off-season. During ensiling, lactic acid bacteria change soluble carbohydrates into lactic acid, thereby reducing the pH to lower values. Creating acidic conditions in the silo environment can prevent the growth of undesirable microorganisms, and these conditions can also cause hydrolyze hemicellulose and lignocellulose bonds (Zheng et al.,

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2017; Shrestha et al., 2017). Molasses is a by-product from sugar factories with a dry matter (DM) content of 70–75% and a soluble carbohydrate content of about 65%, of which sucrose is the main component (McDonald et al., 1991). It is reported that molasses could stimulate microbial fermentation by providing substrates for the growth of lactic acid bacteria (Guo et al., 2014). Rumen fluid may provide a new approach to the development of silage inoculants. The major microorganisms inhabiting the rumen fluid are anaerobic bacteria, archaea, fungi, and protozoa, which participate directly or indirectly in dietary dry matter and organic matter digestibility. The bacteria are the most abundant microorganism with a density of 10^{10-11} /ml of rumen fluid, followed by archaea (10^{8-9} ml; all of them methanogens), ciliate protozoa (10^{6}/ ml), which include up to half of the ruminal microbial biomass due to their large size, and fungi with 10^{6}/ml contributing less than 8% to total biomass (Orpin and Joblin, 1997; Lourenço et al., 2010; Kumar et al., 2013). In a study, the addition of lactic acid-producing bacteria isolated from rumen fluid improved the silage quality [lower pH, ADF, and higher water soluble carbohydrates (WSC) compared to the control] of alfalfa (Guo et al., 2020). No data has been published about the silage prepared from PMP with molasses and rumen liquid as a novel inoculant. Also, every year, a large amount of rumen fluid is produced in livestock slaughterhouses, which are mostly buried unused. We hypothesized a higher ruminal fiber digestibility due to enhanced cleavage of structural carbohydrates by the addition of rumen fluid-+molasess to the PMP silage. Also, we hypothesized that mixed microorganisms in rumen fluid obtained from slaughterhouses as inoculum along with molasses can improve the silage quality of PMP. For this purpose, we tested the nutritional value of prepared silage from PMP *in vitro*. Also, the superior silage selected from the *in vitro* section was nutritionally evaluated in an *in vivo* experiment for its substitution with dietary forage (25%, 50%, 75%, and 100% of DM) by forty Baluchi fattening male lambs in a completely randomized design.

2. Material and methods

2.1. Silage making process and additives

The Persian manna plant (PMP) was harvested from the top 2 cm of the ground surface in the late flowering stage from the Torbaghan rangelands of Kashmar (Latitude of 35°12′N, Longitude of 58°30′E) in August 2020. The fresh samples were chapped into pieces of about 2–3 cm and then were ensiled in four layers of nylon bags for 60 days. The fresh PMP treated with 1] no additive (control), 2] 3% molasses+ 3% rumen fluid (M3 +RF3, DM basis), 3] 6% molasses+ 6% rumen fluid (M6 +RF6, DM basis), and 4] 9% molasses+ 9% rumen fluid (M9 +RF9, DM basis). Six replicates were considered for each treatment. A mixture of rumen fluid was prepared after slaughtering of several sheep in an industrial slaughterhouse located in Kashmar, Khorasan-e Razavi province, Iran. The collected rumen fluid was moved into the vacuum-prewarmed flasks and immediately transferred to the laboratory for silage making. Molasses was purchased from an animal feed supplier in Kashmar.

2.2. Laboratory and analytical methods

Samples of fresh silages were stored at –18 ºC until chemical analyses and *in vitro* protocol. The content of DM in fresh samples was determined by oven-drying at 105 ºC until constant weight. Samples were oven-dried at 65 °C for 48 h, and ground through a 1 mm sieve for chemical-mineral composition analysis. The ash content was measured by a furnace at the temperature of 550 °C for 4 h. The crude protein (CP) content was determined by the Kjeldahl method (AOAC. 2005). The solutions suggested by Van Soest et al. (1991) were employed for neutral detergent fiber (NDF) and acid detergent fiber (ADF) determination by Ankom fiber analyzer (ANKOM, model A2001, New York, USA). Acid detergent lignin (ADL) was determined on ADF using 72% sulphuric acid to cellulose solubilization. The ether extract (EE) was measured using a soxhlet apparatus. The pH of fresh samples was determined using a digital pH meter (Metrohm, model 632, Switzerland) after homogenizing 50 g of the fresh materials with 450 ml of distilled water for 15 min. The extracted fluid was employed for lactic and butyric acids and total volatile fatty acids (TVFA) determination by a KNAUER HPLC system equipped with a UV–VIS detector (Azura, Germany) and with a C18 column (25 cm × 4.6 mm id, 5 µm). The operation was run by a mobile phase of 0.005 mM H_2SO_4 with a flow rate of 0.5 ml/min. The ammonia nitrogen of silage extract was also determined according to the procedure of Broderick and Kang (1980). The content of phosphorus was determined by a spectrophotometer (UV-Vis array Spectrophotometer, Photonix-Ar-2017, Iran) using the molybdovanadate method. The mineral contents, including calcium, sodium, potassium, magnesium, manganese, iron, and zinc were measured using atomic absorption spectrometry (SavantAA, GBC, Australia). The content of non-fiber carbohydrates (NFC) was determined by subtracting CP, NDF, EE, and ash from total DM (Sniffen et al., 1992). The procedure of Jasaitis et al. (1987) was applied for buffering capacity parameters determination. The flieg point was determined using the equation suggested by Kilic (2006). Flieg points = 220 + (2 × %DM–15)–40 × pH. The flieg score with values 81–100 was very good, 61–80 was good, 41–60 was medium, 21–40 was low, and 0–20 was poor. The Aerobic stability was determined according to the protocol suggested by Ke et al. (2015).

2.3. In vitro procedures

The *in vitro* gas production was run according to Menke and Steingass (1988) procedure in two different runs. Three male lambs, weighing on average 40 (± 4 kg) fitted with ruminal fistula, were used in this experiment to gather the ruminal fluid. The lambs were fed at the maintenance levels twice a day. They had free access to clean water and to mineral-vitamin blocks. The samples from rumen fluid were withdrawn three hour after the morning feeding into pre-warmed flasks. Rumen fluid was strained through four layers of cheesecloth and kept at 39 °C under continuous flushing with CO_2 until the start of incubation. Each 100 ml glass syringe was filled with a 200 mg sample (as DM basis), filtered rumen fluid, and artificial saliva (30 ml, 1:2 ratio). The tube connected to each syringe outlet was closed by a plastic clip to avoid gas leakage. Each syringe was then gently shaken and incubated at 39 °C in a water bath for 3, 6, 9, 12, 24, 48, 72, and 96 h (Kazemi et al., 2019; Menke and Steingass, 1988). A medium similar to the gas test was used to determine TVFA, pH, ammonia nitrogen, *in vitro* dry matter digestibility, and *in vitro* organic matter digestibility after 24 h incubation (Kazemi and Ghasemi Bezdi, 2021). The method of sampling for TVFA determination was conducted according to the protocol of Getachew et al. (2004). The method suggested by Barnett and Reid (1957) with the use of the Markham device was employed for *in vitro* TVFA determination (Markham, 1942). The technique described by Komolong et al. (2001) was used for ammonia nitrogen analysis.

2.4. In vivo protocol and methods

This project was conducted in an industrial farm around Kashmar city, Khorasan-e Razavi province, Iran. The M6 +PMP6 to be incorporated into the diet of lambs was arrived at based on *in vitro* study, and it was used because of its higher nutritional value compared to the other experimental silages. Forty Baluchi male lambs (32 ± 2.5 kg BW, 7-month-old) were randomly allocated to five treatments in a completely randomized design followed by a 14-day adaptation period for 90 days (n = 8 per group). The experimental treatments (Table 7) were: 1) control (basal diet, without PMP silage), 2) PMP25% (25% of dietary forages were replaced by M6 +PMP6 silage: 12.9% of dietary DM); 3) PMP50% (50% of dietary forages were replaced by M6 +PMP6 silage: 25.8% of dietary DM); 4) PMP75% (75% of dietary forages were replaced by M6 +PMP6 silage: 38.9% of dietary DM), and 5) PMP100%

(100% of dietary forages were replaced by M6 +PMP6 silage: 51.7% of dietary DM). The ingredients and chemical composition of the experimental diets fed to the fattening lambs are shown in Table 7. The experimental rations were prepared according to NRC (2007), with a forage to concentrate ratio of 52:48. The guidelines suggested by the Iranian Council on Animal Care (1995) were employed for keeping the lambs in the 2 m × 2 m individual pens with a concrete floor. The animals were vaccinated against common contagious diseases and received an internal anti-parasite drug (Albendazole 2.5%, Rooyan, Iran). Before starting the experiment, all pens and equipment were cleaned, washed, and disinfected. The lambs were fed twice a day at 06.30 and 17.30 h with TMR rations *ad libitum* (10% orts) and accessed freely to fresh water.

Feed intake and feed refusals were recorded every day for each lamb until the end of the experiment period. The feed samples (offered and refusal) were dried in a forced-air oven at 65 °C for 48 h and saved in plastic bags for further analysis. The lambs were equipped with leather bags for total fecal collection from days 80–90. The lambs were adapted to the leather bags for three consecutive days (days 77–80). The bags were emptied entirely twice a day. The collected feces samples were mixed entirely for each animal and a sub-sample was transferred to a freezer at − 20 °C for further analysis. The nutrient digestibility was measured based on the amount of nutrient consumed and excreted (Kazemi and Ghasemi Bezdi, 2021). After drying the feces samples at 65 °C for 48 h in a forced-air oven until reaching a constant weight, they ground to pass through a 1-mm screen and preserved for further analysis. The analytical procedures followed those described in the laboratory and analytical methods section. At the start of the experiment, lambs were weighed before the morning feeding, and weighing was repeated every 18 days, and the recorded weights were used to calculate final body weight changes. The feed intake and orts were regularly recorded to measure final nutrient digestibility. Feed conversion ratio (FCR) was calculated as the average individual feed intake (Kg) per Kg weight gain.

Rumen fluid (at days 88–90) was gathered 3 h after morning feeding *via* an esophagus tube connected to a vacuum pump and then strained through four layers of cheesecloth. Ruminal pH was measured using a pH meter (Hanna, Model HI 2210–01, USA), and rumen fluid residues were pretreated (Getachew et al., 2004) and preserved at −18 °C for ammonia nitrogen and Volatile fatty acids (VFAs) analysis. The ammonia concentration was measured by the Kjeldahl method (Komolong et al., 2001). The VFAs concentration was determined by gas chromatography equipped (YL6100 GC; Young Lin Instrument, Anyang, South Korea) with a 50 m silica-fused (0.32 mm ID) column (CP-Wax Chrompack Capillary Column, Varian, Palo Alto, CA, USA). The crotonic acid (trans-2-butenoic acid) as an internal standard and helium as carrier gas were used in each run. Detector and injector temperatures were adjusted at 250 °C. The initial temperature of the oven was 55 °C and the final temperature was 195 °C.

2.5. Statistical analysis and calculations

The *in vivo* and *in vitro* data were analyzed in a completely randomized design using the GLM procedure of SAS (SAS., 2002) with initial body weight as a covariate. The data of the *in vitro* and *in vivo* sections were respectively replicated five and eight times. The metabolizable energy (ME) and net energy for lactation (NEl) were determined according to the equation suggested by Menke and Steingass (1988). Means were compared with Tukey's test. A nonlinear equation [$Y = b(1 - e^{-ct})$]proposed by Ørskov and McDonald (1979) was used for *in vitro* gas production parameters measurements. In which Y is the volume of gas produced at time t. c was the fractional rate of gas production (c_{gas}, %/h) and b was the potential gas production (b_{gas}, ml/h).

3. Results

3.1. Chemical and mineral composition

The chemical compositions of PMP silages are shown in Table 1. Except for DM and EE, other chemical compositions changed in different silages of PMP. The lowest concentration of ADF (48.6%) and NDF (56.3%) and the highest content of NFC (22.1%) were observed in PMP+ 6%M+ 6%R ($P < 0.0001$). A different range of CP (10.8–11.4%), ash (9.06–10.2%), and ADL (10.8–12.9%) were observed among the prepared silages.

The mineral compositions of PMP silages are presented in Table 2. Except for calcium, phosphorus, sodium, and zinc concentrations, other minerals including potassium, magnesium, and iron concentrations were changed among the experimental silages. The control silage had the highest magnesium content, while the highest contents of manganese and iron were observed in PMP+ 9%M+ 9%R.

3.2. Silage fermentation pattern

The Fermentation parameters of PMP in the silo environment are exhibited in Table 3. Different fermentation parameters were observed among the experimental silages. A different range of lactic acid (0.8–1.2% of DM), TVFA (2.04–2.45% of DM), aerobic stability (66.5–98 h), and flieg point (9.12–70.7) were observed among the prepared silages. The amount of pH differs from 5.09 for PMP+ 6% M+ 6%R to 6.64 for PMP+ 9%M+ 9%R. The lowest and highest content of butyric acid was observed in PMP+ 6%M+ 6%R and control silages, respectively ($P = 0.0002$). The content of ammonia nitrogen ranged from 0.13% for PMP+ 6%M+ 6%R to 0.32% for PMP+ 9%M+ 9%R.

3.3. The in vitro parameters of the culture medium

The *in vitro* gas production and fermentation parameters of different PMP silages following the incubation in the culture medium are shown in Table 4. The highest amount of b_{gas} was observed in PMP+ 3%M+ 3% R (16 ml/200 mg DM), followed by PMP+ 6%M+ 6%R (15 ml/200 mg DM) ($P = 0.02$). There was no significant difference for c_{gas} and lag time among the experimental silages ($P > 0.05$), however the gas production after 12, 24, 48, and 72 h changed significantly among different silages.

The fermentative-digestive and metabolism parameters measured or estimated for PMP silages incubated in the culture medium are presented in Table 5. The concentration of TVFA in the culture medium was highest in PMP+ 6%M+ 6%R (63 mM), followed by PMP+ 3%M+ 3%R (62.9 mM) and PMP+ 9%M+ 9%R (61.3 mM), respectively. The ammonia nitrogen was unchanged between treatments ($P > 0.05$). The metabolizable energy ranged from 4.06 MJ/kg DM for PMP+ 6% M+ 6%R to 3.50 MJ/kg DM for control silage. The highest pH content of the culture medium was observed for control silage (6.41), followed by PMP+ 9%M+ 9%R (6.37). The amount of DMD ranged from 27.1% for control silage to 32.9% for PMP+ 6%M+ 6%R. Among the prepared silages, the highest amount of OMD was respectively observed in PMP+ 6%M+ 6%R (33.9%) and PMP+ 3%M+ 3%R (32.1%) ($P = 0.002$).

3.4. Buffering capacity parameters

The pH and buffering capacity parameters of different PMP silages are shown in Table 6. The amount of pH extract was differ from 6.18 for PMP+ 3%M+ 3%R to 6.89 for PMP+ 6%M+ 6%R. The highest content of titratable acidity (143 mEq×10^{-3}) was observed in the control silage. The highest amount of acid buffering capacity (58 mEq×10^{-3}), titratable alkalinity (210 mEq×10^{-3}), base-buffering capacity (67.4 mEq×10^{-3}), and acid-base buffering capacity (125 mEq×10^{-3}) were observed in PMP+ 6%M+ 6%R. (Table 7)

Table 1
Chemical compositions (% of DM) of Persian manna plant silages.

Item	DM	CP	NDF	ADF	ADL	EE	Ash	NFC
Control	35.1	11.4^{a}	60.2^{a}	59.6^{a}	12.9^{a}	1.33	10.2^{a}	16.8^{c}
PMP+ 3%M+ 3%R	35.4	11.2^{ab}	59.0^{a}	53.1^{b}	10.9^{b}	1.34	9.10^{b}	19.4^{b}
PMP+ 6%M+ 6%R	34.7	11.1^{ab}	56.3^{b}	48.6^{c}	10.8^{b}	1.49	9.06^{b}	22.1^{a}
PMP+ 9%M+ 9%R	34.8	10.8^{b}	59.8^{a}	53.5^{b}	11.7^{ab}	1.44	10.1^{a}	17.8^{bc}
SEM	0.15	0.08	0.45	1.09	0.25	0.03	0.14	0.56
P-value	0.34	0.05	< 0.0001	< 0.0001	0.0007	0.17	< 0.0001	< 0.0001

Means within columns followed by the same letter are not different Control: Persian manna plant ensiled without additive; PMP+ 3%M+ 3%R: Persian manna plant ensiled with 3% of DM molasses and 3% of DM rumen fluid; PMP+ 6%M+ 6%R: Persian manna plant ensiled with 6% of DM molasses and 6% of DM rumen fluid; PMP+ 9%M+ 9%R: Persian manna plant ensiled with 9% of DM molasses and 9% of DM rumen fluid; DM (% of fresh weight): dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; EE: ether extract; NFC: non-fiber carbohydrate; SEM: standard error of the mean.

Table 2
Mineral compositions of Persian manna plant silages.

Item	Ca	P	Na	K	Mg	Mn	Zn	Fe
Control	13.1	4.39	1.63	11.0^{a}	5.49^{a}	34.7^{ab}	17.9	352^{a}
PMP+ 3%M+ 3%R	12.2	4.07	1.43	8.48^{b}	3.59^{c}	31.1^{c}	17.5	210^{b}
PMP+ 6%M+ 6%R	12.3	4.13	1.63	8.35^{b}	3.71^{bc}	31.9^{bc}	16.0	199^{b}
PMP+ 9%M+ 9%R	12.8	4.16	1.60	8.40^{b}	4.02^{b}	35.8^{a}	15.9	382^{a}
SEM	0.16	0.05	0.03	0.36	0.23	0.66	0.41	27.5
P-value	0.1	0.1	0.1	< 0.0001	< 0.0001	0.004	0.21	0.003

Means within columns followed by the same letter are not different.
Control: Persian manna plant ensiled without additive; PMP+ 3%M+ 3%R: Persian manna plant ensiled with 3% of DM molasses and 3% of DM rumen fluid; PMP+ 6%M+ 6%R: Persian manna plant ensiled with 6% of DM molasses and 6% of DM rumen fluid; PMP+ 9%M+ 9%R: Persian manna plant ensiled with 9% of DM molasses and 9% of DM rumen fluid; Ca (g/kg DM): calcium; P (g/kg DM): phosphorus; Na (g/kg DM): Sodium; K (g/kg DM): potassium; Mg (g/kg DM): magnesium; Mn (mg/kg DM): manganese; Zn (mg/kg DM): Zinc; Fe (mg/kg DM): Iron (mg/kg DM); SEM: standard error of the mean.

Table 3
Fermentation parameters of Persian manna plant in the silo environment.

Item	LA	BA	pH	AN	TVFA	FP	AS
Control	0.80^{b}	0.18^{a}	5.67^{b}	0.24^{ab}	2.25^{ab}	48.3^{b}	81.7^{b}
PMP+ 3%M+ 3%R	1.10^{a}	0.05^{b}	5.33^{c}	0.17^{bc}	2.39^{a}	62.6^{a}	95.5^{a}
PMP+ 6%M+ 6%R	1.20^{a}	0.03^{b}	5.09^{c}	0.13^{c}	2.45^{a}	70.7^{a}	98.0^{a}
PMP+ 9%M+ 9%R	0.92^{b}	0.17^{a}	6.64^{a}	0.32^{a}	2.04^{b}	9.12^{c}	66.5^{c}
SEM	0.04	0.02	0.15	0.02	0.05	6.23	3.27
P-value	< 0.0001	0.0002	< 0.0001	0.0002	0.001	< 0.0001	< 0.0001

Means within columns followed by the same letter are not different Control: Persian manna plant ensiled without additive; PMP+ 3%M+ 3%R: Persian manna plant ensiled with 3% of DM molasses and 3% of DM rumen fluid; PMP+ 6%M+ 6%R: Persian manna plant ensiled with 6% of DM molasses and 6% of DM rumen fluid; PMP+ 9%M+ 9%R: Persian manna plant ensiled with 9% of DM molasses and 9% of DM rumen fluid; LA (% of DM): lactic acid; BA (% of DM): butyric acid; AN (% of total nitrogen): ammonia nitrogen; TVFA (% of DM): total volatile fatty acids; FP: flieg point; AS (hour): aerobic stability; SEM: standard error of the mean.

Table 4
The *in vitro* gas production and fermentation parameters of different Persian manna plant silages following the incubation in the culture medium.

Item	b_{gas}	c_{gas}	lag time	gas 12 h	gas 24 h	gas 48 h	gas 72 h
Control	8.02^{b}	0.029	5.30	0.54^{b}	4.54^{b}	5.71^{c}	6.03^{c}
PMP+ 3%M+ 3%R	16.0^{a}	0.032	5.21	1.50^{ab}	7.87^{ab}	11.1^{ab}	13.2^{a}
PMP+ 6%M+ 6%R	15.0^{a}	0.035	4.97	1.67^{a}	8.60^{a}	11.6^{a}	13.2^{a}
PMP+ 9%M+ 9%R	13.5^{ab}	0.024	5.23	0.67^{ab}	5.0^{ab}	7.70^{bc}	8.87^{b}
SEM	1.12	0.003	0.17	0.18	0.64	0.82	0.93
P-value	0.02	0.63	0.94	0.03	0.02	0.004	< 0.0001

Means within columns followed by the same letter are not different.
Control: Persian manna plant ensiled without additive; PMP+ 3%M+ 3%R: Persian manna plant ensiled with 3% of DM molasses and 3% of DM rumen fluid; PMP+ 6%M+ 6%R: Persian manna plant ensiled with 6% of DM molasses and 6% of DM rumen fluid; PMP+ 9%M+ 9%R: Persian manna plant ensiled with 9% of DM molasses and 9% of DM rumen fluid; bgas (ml/200 mg of DM): potential gas production; cgas (%/h): fractional rate of gas production; gas 12, 24, 48, and 72 h (ml/200 mg of DM): the gas produced at times 12, 24, 48, and 72 h incubation; SEM: standard error of the mean.

3.5. *In vivo trial*

3.5.1. *Feed intake and nutrient digestibility*

The effect of the substitution of dietary forages with PMP silage on nutrient digestibility and growth performance of fattening lambs are presented in Table 8. The highest dry matter intake ($P < 0.0001$), final body weight ($P = 0.007$), and average daily gain ($P = 0.002$), and lower FCR ($P = 0.05$) were observed in the control followed by PMP25% and PMP50%, respectively. The lowest amount for the above parameters was observed in PMP100%. The highest digestibility for DM ($P = 0.0003$), OM ($P = 0.0005$), CP ($P < 0.0001$), and NDF ($P < 0.0001$) was also observed in the control followed by PMP25% and PMP50%, respectively. The lowest nutrient digestibility was observed in PMP100%.

3.5.2. *Ruminal fermentation*

The effects of the substitution of dietary forages with PMP silage on ruminal fermentation characteristics of fattening lambs are exhibited in Table 9. The amounts of pH and ammonia nitrogen were not affected by

Table 5
The fermentative-digestive and metabolism parameters measured or estimated for Persian manna plant silages incubated in the culture medium.

Item	TVFA	NH_3-N	pH 24 h	OMD	ME	NEl	DMD
Control	59.3^{b}	14.0	6.41^{a}	29.0^{b}	3.50^{b}	1.43^{b}	27.1^{b}
PMP+ 3%M+ 3%R	62.9^{a}	13.4	6.33^{b}	32.1^{a}	3.96^{ab}	1.75^{ab}	31.5^{a}
PMP+ 6%M+ 6%R	63.0^{a}	13.3	6.35^{b}	33.9^{a}	4.06^{a}	1.82^{a}	32.9^{a}
PMP+ 9%M+ 9%R	61.3^{a}	13.0	6.37^{ab}	29.4^{b}	3.56^{ab}	1.47^{ab}	28.7^{b}
SEM	0.44	0.17	0.009	0.78	0.09	0.06	0.86
P-value	0.0002	0.22	0.002	0.002	0.02	0.02	0.0009

Means within columns followed by the same letter are not different.
OMD and DMD were determined after 24 h incubation.
Control: Persian manna plant ensiled without additive; PMP+ 3%M+ 3%R: Persian manna plant ensiled with 3% of DM molasses and 3% of DM rumen fluid; PMP+ 6%M+ 6%R: Persian manna plant ensiled with 6% of DM molasses and 6% of DM rumen fluid; PMP+ 9%M+ 9%R: Persian manna plant ensiled with 9% of DM molasses and 9% of DM rumen fluid; TVFA (mM): total volatile fatty acids; NH3-N (mg/dL): ammonia nitrogen; pH 24 h: the pH measured in the culture medium after 24 h incubation; OMD (% of DM): organic matter digestibility; ME (MJ/kg DM): metabolizable energy; NEl (MJ/kg DM): net energy for lactation; DMD (% of DM): dry matter digestibility; SEM: standard error of the mean.

Table 6
The pH and buffering capacity (mEq×10^{-3}) parameters of different Persian manna plant silages.

Item	pH	Titratable acidity	Acid-buffering capacity	Titratable alkalinity	Base-buffering capacity	Acid-base buffering capacity
Control	6.75^{a}	143^{a}	55.7^{a}	84.3^{d}	37.5^{c}	91.9^{c}
PMP+ 3%M+ 3%R	6.18^{c}	104^{c}	49.0^{b}	188^{b}	66.6^{a}	115^{ab}
PMP+ 6%M+ 6%R	6.89^{d}	110^{c}	58.0^{a}	210^{a}	67.4^{a}	125^{a}
PMP+ 9%M+ 9%R	6.43^{b}	124^{b}	49.0^{b}	148^{c}	57.6^{b}	107^{b}
SEM	0.1	4.62	1.29	14.5	3.70	3.83
P-value	< 0.0001	< 0.0001	0.0005	< 0.0001	< 0.0001	0.0003

Means within columns followed by the same letter are not different.
Control: Persian manna plant ensiled without additive; PMP+ 3%M+ 3%R: Persian manna plant ensiled with 3% of DM molasses and 3% of DM rumen fluid; PMP+ 6%M+ 6%R: Persian manna plant ensiled with 6% of DM molasses and 6% of DM rumen fluid; PMP+ 9%M+ 9%R: Persian manna plant ensiled with 9% of DM molasses and 9% of DM rumen fluid; SEM: standard error of the mean.

different treatments ($P > 0.05$). The content of TVFA was highest in PMP25% (70.7 mM), followed by control (70.3 mM) and PMP50% (69.2 mM), respectively ($P = 0.005$). The concentrations of propionate, valerate, and isovalerate were not affected by the experimental diets ($P > 0.05$). Animals fed on PMP100% exhibited the lowest ruminal acetate ($P = 0.0001$) and butyrate ($P = 0.01$) concentrations compared to the control group.

4. Discussion

4.1. Chemical and mineral composition

The present work investigated the impact of the addition of molasses and rumen fluid as two low-cost additives on the silage quality of PMP. Recently, Kazemi and Valizadeh (2023) reported that PMP can be ensiled with molasses and *saccharomyces cerevisiae* well. Also, it is reported that PMP can meet the nutrient requirements of milking ewes at the maintenance levels (Kazemi and Ghasemi Bezdi, 2021). The DM, CP, ash, EE, and ADL contents of prepared PMP silages are similar to those reported by Kazemi and Ghasemi Bezdi (2021). Persian manna plant silage treated with 6% molasses+ 6% rumen fluid showed lower NDF and ADF compared to other silages. In line with our results, a direct inoculation with ruminal fluid has improved the chemical composition of different silages (Hartinger et al., 2022). The decrease in NDF and ADF concentrations of 6% molasses+ 6% rumen fluid treated PMP may be related to the acidic hydrolysis of hemicelluloses during proper fermentation in the silo environment. Thus, our hypothesis was confirmed, and it can be assumed that the microorganisms present in the rumen fluid precleaved lignocellulosic complexes in the silages, thus allowing a higher fiber degradability (Hartinger et al., 2022). A higher reduction in CP content of PMP+ 9%M+ 9%R compared to the control can be attributed to enhancing the proteolysis degradation of rumen fluid added to the silo.

Most of the mineral requirements of ruminants are provided through the release of minerals available in the diet. In this research, in addition to trying to measure some essential minerals in the plant after ensiling, the effects of rumen liquid and molasses on the mineral composition of PMP were also investigated. Some mineral compositions of PMP were within the contents (phosphorus: 2.8–5.4 g/kg DM; sodium: 1.58–3.61 g/kg DM; potassium: 7.43–9.14 g/kg DM; magnesium: 2.60–5.83 g/kg DM) reported by Kazemi and Valizadeh (2023).

4.2. Silage fermentation pattern

So far, rumen fluid has not been used as a silage additive except in limited research works, but it can be a diverse source of microorganisms, especially *lactobacilli* and fibrolytic microorganisms with their enzymes, which may be helpful in ensiling (Hartinger et al., 2022). A higher lactic acid and lower pH in PMP treated with 6% molasses and 6% rumen fluid can be attributed to the increase in fiber degradability due to the addition of molasses and rumen liquid, which has provided more substrate for lactic acid-producing bacteria. Also it was routinely suggested that lactic acid production is only related to non-spore bacteria, commonly referred to lactic acid bacteria, because of their fermentative lifestyle, mostly converting a variety of carbohydrates into lactic acid (Okoye et al., 2022). Generally, in well-preserved silage, at least 65–70% of the total acid (or 4–7% of silage DM) will be lactic acid (Kung and Shaver, 2001). The pH value is one of the primary indicators to evaluate silage quality. Most of the harmful microorganisms are not survive in acidic conditions, and in fact, a pH less than 4.2 is considered an indicator of high-quality silage (Zi et al., 2021). In this experiment, although the pH values of the experimental silages were all above 4.2, they had the standards of desirable silage. Likewise, the rumen fluid along with molasses (PMP+3%M+3%R or PMP+6%M+6%R) decreased the butyric acid concentration in PMP silages, affecting the ammonia nitrogen concentration, indicating a shift towards a homolactic-dominated fermentation. Interestingly, decrease in ammonia nitrogen content of PMP treated with rumen fluid+molasses (3 or 6%), indicating less protein degradation. However, it has to be noted that ammonia proportion still accounted for less than 10% of total

Table 7
Ingredients and chemical composition of the experimental diets fed to the fattening lambs.

Item	Experimental diets				
	Control	PMP25%	PMP50%	PMP75%	PMP100%
Ingredients (% of DM)					
Alfalfa hay	23	17.2	11.5	5.7	0
Corn silage	23	17.2	11.5	5.7	0
Wheat straw	5.7	4.4	2.8	1.4	0
Persian manna plant silage	0	12.9	25.8	38.9	51.7
Sugar beet pulp	2.6	2.4	3.3	5.2	2.4
Corn grain, ground	14.3	15.3	15	14.5	16
Barley grain, ground	14.3	15.3	15	14.5	16
Soybean meal 44% CP	5.6	5.5	5	5	5.5
Cottonseed meal	3.4	3.1	2.5	2.2	1.8
Wheat bran	5.7	4.3	5.2	4.5	4.2
Vitamin-mineral premix[1]	1.1	1.1	1.1	1.1	1.1
Salt	0.7	0.7	0.7	0.7	0.7
Sodium bicarbonate	0.6	0.6	0.6	0.6	0.6
Chemical composition (% of DM)					
DM (% of fresh weight)	56.3	55.45	54.7	53.8	52.3
CP	14	14	14	13.9	13.9
NDF	42	41	41	40.8	40.4
EE	2.5	2.5	2.4	2.4	2.3
Ash	9.3	9.3	9	9	9.1
ME (Mcal/kg DM)	2.19	2.18	2.17	2.16	2.15
Calcium	0.6	0.6	0.6	0.7	0.7
Phosphorus	0.3	0.3	0.3	0.4	0.4

PMP: Persian manna plant; Control = basal diet without PMP; PMP25% = 25% of dietary forages were replaced by PMP silage (or 12.9% of dietary DM replaced with PMP); PMP50% = 50% of dietary forages were replaced by PMP silage (or 25.8% of dietary DM replaced with PMP); PMP75% = 75% of dietary forages were replaced by PMP silage (or 38.9% of dietary DM replaced with PMP); PMP100% = 100% of dietary forages were replaced by PMP silage (or 51.7% of dietary DM replaced with PMP); DM = dry matter; CP = crude protein; NDF = neutral detergent fiber; NFC = non-fiber carbohydrates; EE = ether extract; ME = metabolizable energy.
[1]each kg of premix contained 100 mg vitamin E, 11 mg vitamin B_1, 25 mg vitamin B_2, 400000 IU vitamin A, 100000 IU vitamin D, 3.2% Ca, 1.3% P, 3.8% Na, 10 g Mg, 1000 mg Cu, 50 mg I, 50 mg Co, 2000 mg Mn, 2000 mg Zn, and 4000 mg Fe.

nitrogen in all silages, which is considered a sufficient true protein conservation (Kung et al., 2018). Also, a decrease in ammonia nitrogen concentration in rumen fluid/molasses-treated (3 or 6%) silages compared to the control indicated the positive effect of these additives on the silage quality of PMP. It has been reported that different microbial additives can have different effects on the fermentation characteristics of silage, such as pH, lactic acid level, digestibility and *etc.*, so that these effects are different in different studies (Kazemi et al., 2022; Kazemi and Valizadeh, 2023). In terms of quality, silage is considered good if it has a high fermentation rate, less butyric acid production, and more remarkable recovery of energy and DM (Kung et al., 2003). We found PMP+ 6%M+ 6%R had better fermentation rate than other prepared silages.

Molasses addition is effective in compensating for the WSC loss caused by initial undesirable microorganisms (yeast, mold, and aerobic bacteria) and ensure that a sufficient amount of WSC remains at vigorous stages of lactic acid bacteria (LAB) growth to produce enough lactic acid and maintain low pH. Because of the low water-soluble carbohydrates in PMP, adding molasses to the silage was considered. The aerobic stability of silage is an essential factor to ensure that well-prepared silage can retain nutrients for animals with lower amounts of mold spores and toxins (Yuan et al., 2015). When the silage is opened and exposed to air, fermented acids, and other substrates are oxidized by aerobic bacteria, yeasts, and mold, which increases the temperature of the silage and causes nutrient deterioration (Wilkinson and Davies, 2013). Therefore, improving fermentation in silage through monitoring the microbiological and chemical status of silage is necessary to evaluate aerobic stability. In the present study, PMP+ 3%M+ 3%R and PMP+ 6%M+ 6%R had a better aerobic stability compared to other silages. It is believed that silages with good aerobic stability will provide a TMR diet with good aerobic stability (Kung, 2010). Two prepared silages including PMP+ 6%M+ 6%R and PMP+ 3%M+ 3%R with flieg point scores of 70.7 and 62.6, were grouped in a range of good silages.

4.3. The in vitro parameters of the culture medium

Molasses is one of the by-products of sugar factories, which is known for its high fermentability. The beneficial effects of this additive have been confirmed during the preparation of different silages (Wanapat et al., 2013; Limón-Hernández et al., 2019; Kazemi et al., 2022; Kazemi and Valizadeh, 2023; Gül, 2023). It can also be used as sucrose supply for LAB growth and lactic acid (LA) production. In this study, we used molasses with the aim of providing a suitable fermentable source for the growth of lactic acid-producing bacteria and subsequently producing more lactic acid. The *in vitro* gas test was primarily used to predict ruminal digestibility and the ME content of feedstuffs (Menke and

Table 8
The effect of the substitution of dietary forages with Persian manna plant silage on nutrient digestibility and growth performance of fattening lambs.

Item	Treatments					SEM	*P*-value
	Control	PMP25%	PMP50%	PMP75%	PMP100%		
DMI (kg/day)	1.27^{a}	1.25^{ab}	1.23^{ab}	1.18^{bc}	1.16^{c}	0.01	< 0.0001
Final BW (kg)	52.9^{a}	51.1^{ab}	50.0^{ab}	49.0^{b}	48.1^{b}	0.47	0.007
ADG (Kg/day)	0.222^{a}	0.196^{ab}	0.188^{ab}	0.170^{b}	0.159^{b}	0.006	0.002
FCR	5.8b	6.4ab	6.7ab	7.0a	7.3a	0.17	0.05
Nutrient digestibility (% of DM)							
DM	64.2^{a}	63.1^{ab}	60.9^{abc}	60^{bc}	57.8^{c}	0.57	0.0003
OM	67.6^{a}	66.2^{ab}	64.3^{abc}	62.5^{bc}	61.1^{c}	0.60	0.0005
CP	69.1^{a}	67.2^{a}	65.0^{ab}	61.0^{bc}	57.9^{c}	0.89	< 0.0001
NDF	41.9^{a}	40.6^{a}	38.5^{ab}	36.2^{b}	35.9^{b}	0.55	< 0.0001

Means within a row with different subscripts differ significantly.
PMP: Persian manna plant; Control = basal diet without PMP; PMP25% = 25% of dietary forages were replaced by PMP silage (or 12.9% of dietary DM replaced with PMP); PMP50% = 50% of dietary forages were replaced by PMP silage (or 25.8% of dietary DM replaced with PMP); PMP75% = 75% of dietary forages were replaced by PMP silage (or 38.9% of dietary DM replaced with PMP); PMP100% = 100% of dietary forages were replaced by PMP silage (or 51.7% of dietary DM replaced with PMP); DMI = dry matter intake; BW = body weight; ADG = average daily gain; FCR (kg DMI/kg ADG): feed conversion ratio; DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber; SEM = standard error of the mean.

Table 9
The effect of the substitution of dietary forages with Persian manna plant silage on ruminal fermentation characteristics of fattening lambs.

Item	Treatments					SEM	*P*-value
	Control	PMP25%	PMP50%	PMP75%	PMP100%		
pH	6.54	6.50	6.54	6.47	6.46	0.01	0.11
NH_3-N (mg/dL)	18.2	18.4	18.1	17.3	17.5	0.20	0.31
Total VFA (mM)	70.3^{ab}	70.7^{a}	69.2^{abc}	68.1^{bc}	67.6^{c}	0.34	0.005
Individual VFA (mol/100 mol)							
Acetate	65.5^{a}	64.0^{a}	64.7^{a}	63.9^{a}	60.8^{b}	0.39	0.0001
Propionate	18.7	19.8	19.8	20.0	20.3	0.2	0.1
Butyrate	14.0^{a}	13.0^{ab}	13.2^{ab}	13.6^{ab}	12.7^{b}	0.13	0.01
Valerate	1.30	1.35	1.40	1.37	1.30	0.01	0.12
Isovalerate	0.42	0.41	0.43	0.35	0.46	0.02	0.45
Acetate/Propionate	3.50^{a}	3.24^{ab}	3.27^{ab}	3.19^{ab}	3.0b	0.05	0.007

Means within a row with different subscripts differ significantly.
PMP: Persian manna plant; Control = basal diet without PMP; PMP25% = 25% of dietary forages were replaced by PMP silage (or 12.9% of dietary DM replaced with PMP); PMP50% = 50% of dietary forages were replaced by PMP silage (or 25.8% of dietary DM replaced with PMP); PMP75% = 75% of dietary forages were replaced by PMP silage (or 38.9% of dietary DM replaced with PMP); PMP100% = 100% of dietary forages were replaced by PMP silage (or 51.7% of dietary DM replaced with PMP); VFA: volatile fatty acids; SEM = standard error of the mean.

Steingass, 1988). A lot of satisfaction has been created among animal science researchers for the use of gas production system due to their low cost and rapid implementation (Kazemi et al., 2021; Kazemi and Valizadeh, 2023). In this study, the lag time observed for PMP silages could be due to their fibrous nature, which made it more difficult to degrade by ruminal microorganisms *in vitro*. The increase in gas production of PMP treated with 6% rumen fluid and 6% molasses after 12, 24, 48, 72, and 96 h can be attributed to the positive effect of two additives on increasing the digestibility of PMP fiber. This result is difficult to explain but probably due to the molasses or urea-treated PMP silage altered ruminal TVFA production.

In line with our results, the addition of molasses increased the ruminal DM degradability of king grass silage (Li et al., 2014). A negative correlation between the amount of fiber and *in vitro* DM digestibility has been reported (Kazemi, 2019; Kazemi and Valizadeh, 2019). Some of the increase in *in vitro* digestibility of DM or OM in rumen fluid/molasses-treated PMP can be attributed to the reduction of the cellulose wall of the plant by two additives, which has facilitated the conditions for an ideal digestion in the culture medium. On the other hand, molasses has provided a suitable environment for the activation of microorganisms in the rumen liquid added to the PMP silage. Also, a negative correlation between *in vitro* ruminal TVFA and pH was reported in the study of Kazemi (2019) which can explain the lower pH in PMP+ 3%M+ 3%R and PMP+ 6%M+ 6%R of the present study. In line with our results (as seen in Tables 4 and 5), Blümmel et al. (1997) stated that ruminal VFAs produced by microorganisms are positively related to *in vitro* gas production.

4.4. Buffering capacity parameters

The buffering capacity of each of the feedstuffs ingredients plays a crucial role in equilibrating ruminal pH levels. The use of feeds with a suitable buffering capacity can improve the digestibility of the feed in the digestive system, guarantee the digestive system's health, and improves animal performance. In this study, although the additives caused a change in the buffering capacity of experimental silages, PMP+ 6% M+ 6% had the highest acid-base buffering capacity. Determining the buffer capacity of feedstuffs can play an essential role in our decision for timing to use buffer additives. In line with our results, a buffering capacity higher than 85 mEq$\times 10^{-3}$ for some protein feeds, and leguminous forages has been reported by Montanez-Valdez et al. 2013. Kazemi and Valizadeh (2023) reported that the initial pH and titratable acidity are the most critical determinants of ruminal pH. Regarding our results, the highest titratable acidity was observed in the control (143 mEq$\times 10^{-3}$), indicating high resistance to acidification. All silages had a pH near the neutral zone, so their consumption couldn't lead to a severe decrease in ruminal pH.

4.5. In vivo trial

4.5.1. Feed intake and nutrient digestibility

In line with the present study, dietary supplementation with ensiled PMP up to 210 g/kg DM had no deleterious effects on animal performance (Karamshahi Amjazi et al., 2017). In the present study, when 75% of the dietary forages was replaced with PMP silage, dry matter intake decreased compared to the control treatment. Some of the decreases in DMI in diets with high levels of PMP silage can be attributed to its lignocellulose nature, which is consistent with the results reported by Kazemi and Ghasemi Bezdi (2021). Also it is suggested, to some extent, this decreasing trends might be due to lower palatability of high PMP diets because of 6% rumen fluid. Recently, Mokhtarpour and Jahantigh (2023) reported that camelthorn (or PMP) can be substituted with alfalfa and wheat straw up to 30% of diet DM in the fattening lambs' ration without any significant adverse effects on animal performance. A lower body weight and ADG of animals fed on PMP75% and PMP100% can be attributed to lower nutrient digestibility of them compared to the control group. Lower nutrient digestibility (DM, OM, CP, and NDF) in animals fed on PMP75% and PMP100% can be related to the higher fibrous nature of PMP silage compared to the primary forages (corn silage and alfalfa) in the control diet.

4.5.2. Ruminal fermentation

The most of energy requirements of ruminants meet by ruminal VFAs (Kazemi and Ghasemi Bezdi, 2021). A positive correlation between OMD and TVFA was reported by Kazemi and Valizadeh (2019). Some of the decreases in ruminal TVFA of animals fed on PMP100% compared to control can be attributed to lower OMD, as seen in Table 8. The amount of VFAs produced in the rumen depends on DMI and nutrient digestibility and finally, the degraded materials will supply the original substrate for ruminal fermentation (Firkins et al. (1986); Robinson et al., 1986). In this study, the concentrations of ruminal VFAs for acetate and butyrate in animals fed on PMP100% were lower than control. As stated by Huo et al. (2021), this was mainly due to the lower NDF digestibility of the diet containing PMP100% (Table 8) than the control. Also, it is reported that the main products of fiber degradation are acetic and butyric acids (Dijkstra et al., 1993). Furthermore, PMP100% diets decreased acetate concentrations without effect on the propionate proportion, thereby resulting in a significant reduction in the acetate to propionate ratio compared to control.

5. Conclusion

Rumen fluid as a cheap and natural inoculant can be used as a silage additive. Also, PMP had the potential for ensiling with molasses and rumen fluid well. We found a better nutritional reply from PMP+ 6% M+ 6%R rather than other prepared silage, *in vitro*. As seen in the *in vivo* section, PMP as a cheap and available plant, can be replaced up to about 26% of dietary DM without any harmful effects on growth performance, nutrient digestibility, and ruminal fermentation of fattening male lambs.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Compliance with ethical standards

Animal handling and experimental procedures were performed according to the guidelines approved by Iranian Council of Animal Care (1995).

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