



Co-ensiling pomegranate (*Punica granatum* L.) peels and molasses with berseem (*Trifolium alexandrinum* L.) alters fermentation quality, nutrient composition, ruminal fermentation and methane production in buffalo bulls *in-vitro*

Mariam G. Ahmed^{1,2} · Samir Z. El-Zarkouny² · Adham A. Al-Sagheer³ · Eman A. Elwakeel²

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Abstract

Pomegranate peels are an industrial by-product high in sugar and phytochemical content and pose an environmental concern. Meanwhile, ensiling legume forage such as berseem is difficult due to its lower dry matter content and water-soluble carbohydrate-to-buffering capacity ratio, which leads to a poor fermentation process. To date, no studies have been conducted to investigate the effect of co-ensiling pomegranate peels with berseem. Thus, silage quality was evaluated after co-ensiling of berseem (control) with 50, 100, and 200 g/kg pomegranate peels or 50 g/kg molasses for 0, 15, 30, and 45 days (Experiment 1). Further, rumen nutrient degradation, methane production, and rumen fermentation parameters were evaluated *in vitro* (Experiment 2). Pomegranate peels (200 g/kg) and molasses reduced silage pH compared to control (4.41 or 3.79 vs. 5.02), ammonia-N (2.66 or 3.14 vs. 13.39 g/kg N), and butyric acid (0.05 or 0.1 vs. 0.96 g/kg DM) however, dry matter (323.5 or 283.6 vs. 212.8) and non-fiber carbohydrates (264.8 or 351.8 vs. 136.9 g/kg) were increased, respectively. Pomegranate peels and molasses significantly ($P < 0.05$) increased rumen nutrient degradation and significantly ($P < 0.05$) decreased methane and ammonia-N production (Experiment 2). The chemical composition of silage, *in-vitro* rumen fermentation, and silage quality parameters were significantly ($P < 0.05$) correlated. Pomegranate peels and molasses have potentially improved silage quality and positively influenced rumen fermentation parameters.

Keywords Pomegranate peels · Methane production · Legume forages · Silage · Rumen fermentation

Introduction

Berseem (*Trifolium alexandrinum* L.) is an excellent legume forage extensively utilized in ruminant diets as hay and silage (Ahmed et al. 2023; Yuan et al. 2017). Despite its nutritional advantages, berseem faces challenges related to proteolysis during ensiling and ruminal digestion, leading to an underestimation of its protein value (Barros et al. 2017). This degradation process affects silage quality by increasing buffering capacity, hindering the achievement of low pH levels, and promoting undesirable clostridial fermentation, resulting in poor fermentation quality of silage (Kung et al. 2018). Meanwhile, poor silage quality leads to elevated rumen methane (CH₄) emissions, which subsequently cause a 2–12% decrease in gross energy availability for the host ruminant animal and reduced feed utilization efficiency (Evans 2018). As a result, there is a worldwide need to improve legume forage ensiling techniques to improve silage

✉ Adham A. Al-Sagheer
adham_alsagt@zu.edu.eg; adham_alsagt@hotmail.com

Mariam G. Ahmed
mgmal1984@yahoo.com

Samir Z. El-Zarkouny
selzarkouny@alexu.edu.eg

Eman A. Elwakeel
emankeel@alexu.edu.eg

¹ Animal Production Research Institute, Agricultural Research Center, Nadi El-Said, Giza 11622, Egypt

² Department of Animal and Fish Production, Faculty of Agriculture (El-Shatby), Alexandria University, Alexandria 21545, Egypt

³ Department of Animal Production, Faculty of Agriculture, Zagazig University, Zagazig 44511, Egypt

quality, preserve nutritional value, and reduce environmental impact (Ahmed et al. 2023; Chen et al. 2021). Molasses is a byproduct of sugarcane, sugar beet, starch, citrus, and hemicellulose. It is composed of 79% soluble carbohydrates and 45–50% sucrose. Molasses, a common fermentation stimulant, is employed to promote the growth of lactic acid bacteria in legume forage silage (Gao et al. 2021). However, molasses's high viscosity makes it difficult to handle and does not affect animal performance (Oladosu et al. 2016).

Pomegranate (*Punica granatum* L.) is a member of the Punicaceae family and is extensively grown in different areas including Asia, Africa, and Europe. It is commonly regarded as a functional fruit due to its health-promoting benefits (Ahmed et al. 2022; Karimi et al. 2017). When pomegranates are industrially processed into juice, by-products such as the peel account for 40 to 60% of the weight of the fruit (Natalello et al. 2020). Pomegranate peels are high in botanical compounds such as flavonoids, anthocyanidins, tannins, and readily fermentable soluble sugars (e.g., pectin) (Natalello et al. 2020; Niu et al. 2023). Pomegranate peels have recently been used as natural antibiotic alternatives in ruminant diets to improve animal productivity and control rumen fermentation (Niu et al. 2023). Although ensiling pomegranate peels have demonstrated acceptability and safety in animal feed (Khorsandi et al. 2019), further research is warranted on their utilization to enhance the quality of legume forage fermentation.

To our knowledge, no studies have been conducted to investigate the effect of co-ensiling pomegranate peels with berseem. Given the advantageous properties outlined above, it is hypothesized that the co-ensiling of pomegranate peels with berseem could potentially enhance the chemical composition and fermentation quality of the silage, while also positively manipulating rumen fermentation. Therefore, this study aimed to assess the impact of ensiling dried pomegranate peels or molasses on the fermentation quality and chemical composition of berseem silage across various ensiling durations. Additionally, the study sought to evaluate rumen nutrient degradation, methane production, and in vitro rumen fermentation parameters.

Materials and methods

Experiment 1: evaluation of silage quality

Raw materials and silage preparation

The pomegranate peels were acquired from a food factory in Alexandria's Borg El-Arab industrial zone. Peels were then dried at 50°C in a forced oven for 72 h. Then, the peels were pulverized using (Arthur H. Thomas, Philadelphia, PA, USA) until they could pass through a screen with a 1

mm. Molasses was purchased from Harvest Foods Company (Borg El-Arab, Alexandria). Berseem (*Trifolium alexandrinum* L.) was harvested as a fourth cut from a private farm located in the Abees region of Alexandria city, Egypt. Following a 24-h wilting period, the berseem was chopped by a hand cutter into pieces measuring 15–20 mm in length. Chopped forage was individually ensiled with 0, 50, 100, or 200 g/kg dried pomegranate peels or 50 g/kg molasses (on a fresh matter basis). Then, the ingredient mixture was compressed, packed in a mini plastic silo (9 cm diameter × 16 cm height) with a plastic lid. The silo had a capacity of 1 kg. After 0, 15, 30, and 45 days, 60 silos (4 sampling times × 5 treatments × 3 replicates) were opened and maintained at the ambient temperature (20–22°C).

Evaluation of silage fermentation criteria

The contents of each silo from each treatment were independently evaluated at the end of each period of ensiling to assess the quality of fermentation. Silage samples weighing 20 g were homogenized in 100 mL of distilled water for one minute, allowed to stand at room temperature for one hour, and subsequently filtered using four layers of gauze. The filtered liquid was tested for pH, ethanol, volatile fatty acids (VFA), lactic acid, and ammonia-N (NH₃-N) concentrations. A pH meter (Adwa AD 11 waterproof; Szeged-Hungary Europe, Romania) was used to measure the pH. Lactic acid was measured following Borshchevskaya et al. (2016) procedure. The filtered liquid was prepared in the same manner that Ahmed et al. (2023) carried out to determine the concentrations of VFA, ethanol, and NH₃-N. Fleig's score (FS) was calculated using the following equation, according to Kilic (1986):

$$\text{Fliegss score} = 220 + (2 \times \% \text{DM} - 15) - 40 \times \text{pH} \quad (1)$$

Based on this index, a score of 81–100 signifies very good quality, a score of 61–80 signifies good quality, a score of 41–60 signifies satisfactory quality, a score of 21–40 signifies medium quality and a score of 20 or lower signifies bad quality.

About 100 g of silage was withheld and dried at a temperature of 50°C for 72 h in an oven with forced air circulation for the determination of dry matter (DM). Then samples were pulverized using a screen with a mesh size of 1 mm. Organic matter (OM), ash, ether extract (EE), and crude protein (CP) percentages were determined using the methodology outlined by the AOAC (2006). Acid detergent lignin (ADL), neutral detergent fiber (NDF), and acid detergent fiber (ADF) were determined using an ANKOM 220 fiber analyzer (ANKOM, model A2001, Macedon, NY, USA) as described by Van Soest et al. (1991) without the use of sodium sulfite and heat stable amylase. The calculation of

non-fiber carbohydrates (NFC) involved subtracting the sum of %NDF, %CP, %EE, and ash% from 100%. The condensed tannins (CT) total tannins (TT), and total phenols (TP) in the raw materials were also estimated (Makkar et al. 1993). The aluminum chloride colorimetric method was used to measure total flavonoids (TF), as reported by Zarina and Tan (2013).

Experiment 2: in vitro ruminal fermentation of silage

Experimental design, inoculum donor, and incubation system

In vitro ruminal fermentation of the silage was measured only after 30 days of ensiling, as this period achieved the highest Flieg's score, indicating the best silage quality. Following this period, a total of 200 g from each of the 15 mini-silos were opened, thoroughly mixed, and subjected to drying in an air-forced oven at a temperature of 50°C for a duration of 72 h. Subsequently, the dried contents were finely crushed to a particle size of 1 mm, to be used later as feed substrates. Following the protocol established by Menke and Steingass (1988), 500 mg of dried silage samples were placed into a 120 mL serum bottle for in vitro ruminal batch culture examination.

The rumen fluid used in the study was sourced from two buffalo bulls that were slaughtered at the Agricultural Experimental Station of the Agriculture Faculty (El-Shatby), University of Alexandria, Egypt. The bulls had an average body weight of 500 ± 25 kg. The male buffalo were provided with a primary diet consisting of commercial concentrate (14% CP) and rice straw. Collecting ruminal material from slaughtered animals aligns with animal welfare rules and reduces the stress experienced by live animals requiring a surgical procedure (Mutimura et al. 2013). Rumen contents were individually collected immediately after slaughtering in a tank that had been warmed in advance to a temperature of 39°C. The collected contents were subsequently transported to the laboratory within a time frame of 15 min.

In the laboratory, the ruminal contents from each animal were individually strained through four layers of muslin. The strained contents were then placed on a magnetic stirrer at a temperature of 39°C and flushed with CO₂. Ruminal fluid and McDougall's buffer were blended in a 1:2 v/v ratio and added to each serum bottle containing 50 mL of inoculation medium. This process was done while continuously flushing the bottles with CO₂. Serum bottles were filled with buffered rumen fluid, but no substrate was used as a blank in each inoculum. For each treatment, duplicate fermentations for each of the two inoculums (sources) were inoculated under anaerobic conditions.

Two separate sets of serum bottles containing the inoculum were sealed with butyl rubber stoppers and aluminium crimps. The first set was then incubated at 39°C for 24 h, while the second set was left to incubate for 48 h. The total number of bottles per run was 48 bottles [(blank + 5 treatments) × 4 bottles × 2 time points (24 and 48 h)]. Each parameter was represented by 8 replicates per treatment (4 replicates × 2 runs).

Sample collection and measurements

The same serum bottles ($n = 8$ per treatment) used for gas production measurements were also utilized for estimating the degradability of DM, OM, and NDF and fermentation parameters at each time point (24 and 48 h of incubation). One millimeter of fermentation liquid was collected and centrifuged at 30,000 g for 15 min at 4°C to determine lactate, NH₃-N, and VFA concentrations according to "Evaluation of silage fermentation criteria" section. The remnants in the bottle were strained using pre-weighed crucibles and rinsed with distilled water. To estimate the apparent DM deterioration (ADMD), the crucibles were dried at a temperature of 110°C for 24 h. ADMD was determined by subtracting the weight of filled crucibles from the weight of empty crucibles and adjusting for any residual material present in the blank. The true degradability of DM, OM, and NDF in the dried residue was estimated as described by Ahmed et al. (2023). Using the equation developed by Czerkawski (1986), the amount of microbial nitrogen (MN) was determined as follows;

$$\text{MN (g/kg OMD)} = \text{OMD} \times 19.3 \quad (2)$$

Additionally, methane (CH₄) production was calculated using the formula equation according to Williams et al. (2019):

$$\text{Methane yield (g/kg DM)} = 4.08 \times (\text{acetate/propionate}) + 7.05. \quad (3)$$

Statistical analysis

Data analysis was conducted using the mixed procedure of SAS (version 9.0, SAS Institute Inc., Cary, NC, USA). In Experiment 1. The model incorporated fixed effects treatment (berseem [control], pomegranate peels [50, 100, and 200g/kg] and molasses [50g/kg]), time (0, 15, 30 and 45 days of ensiling) and treatment × time interaction. To assess the treatment trend, orthogonal contrasts were employed to determine the linear, quadratic, and cubic trends. Proper contrasts were also utilized to compare the impact of molasses vs. control treatments. In Experiment 2, the model incorporated fixed effects of treatment (berseem [control], pomegranate peels [50, 100,

and 200g/kg] and molasses [50g/kg]), time (24 and 48 h of incubation) and treatment $\times$ time interaction. Rumen fluid donor or replicate ($n = 8$) as blocking factor (block). Linear, quadratic, and cubic trends of treatments were assessed using orthogonal contrasts, and proper contrasts were also utilized to compare the impact of molasses vs. control treatments similar to Exp. 1. For mean separation of treatments, the Duncan multiple range test was applied, and significance was declared at a threshold of $P < 0.05$. Pearson correlation heatmap was done using GraphPad Prism Software (version 8, San Diego, CA, USA) to determine the relationship between silage quality parameters and chemical compositions of berseem silage.

Results

Experiment 1

Characteristics of raw materials before ensiling

The chemical composition of berseem and dried pomegranate peels is shown in Table 1. Wilted berseem (24 h) and dried pomegranate peels contained 255 and 902.8 g/kg DM, respectively. Dried pomegranate peels contain more EE and NFC than berseem, but less structural carbohydrate and CP. Moreover, the dried pomegranate peels with the highest TP, TT, CT, and TF content were 104.6, 76.9, 1.6, and 3.1 g/kg DM, respectively.

Fermentation quality of dried pomegranate peel silage

The fermentation profile of silages with dried pomegranate peels and molasses addition was significantly influenced by the treatment, ensiling time, and their interaction, as indicated in Table 2 ($P < 0.01$). The pH of the silage decreased linearly ($P < 0.05$) as the proportion of dried pomegranate peels in the mixture increased, and a significant drop in pH was observed at 30 and 45 days of ensiling; pH was less than 4.5 for high mixing proportions of pomegranate peels. Consistent with the declining pH, lactic acid increased linearly ($P < 0.01$), with the highest values occurring at 15 and 30 days of ensiling for all ensiled forages.

At 30 and 45 days of ensiling, increased pomegranate peel content decreased (L,Q,C; $P < 0.01$) acetic acid concentration, with the highest value observed for all ensiled forages after 30 days. After 45 days of ensiling, the amount of propionic acid in mixed silages was also significantly ($P < 0.05$) lower than that of control silages. The response quadratically ($P < 0.05$) of butyric acid and ethanol concentration to increasing pomegranate peel content with a low concentration at an intermediate mixing proportion was observed at 15 and 30 days of ensiling. After 30 days of ensiling, the addition of pomegranate peel to the mixture increased the FS linearly and cubically ($P < 0.01$). The increase in dried pomegranate peel in the mixture decreased cubically in $\text{NH}_3\text{-N}$ concentration ($P < 0.01$) and increased significantly as the ensiling progressed (Fig. 1).

At all ensiling times, the molasses treatments had lower pH values and VFA ($P < 0.01$) but higher lactic acid and

Table 1 Chemical composition (g/kg DM) of raw materials before ensiling

Item	Wilted berseem	Pomegranate peels
Dry matter	255.0	902.8
Organic matter	866.4	960.7
Crude protein	172.2	39.1
Ether extract	17.1	31.9
Neutral detergent fiber (NDF)	592.5	282.2
Acid detergent fiber (ADF)	335.6	172.9
Acid detergent lignin (ADL)	66.7	48.2
Cellulose ^a	268.9	124.7
Hemicellulose ^b	256.9	109.3
Non-fiber carbohydrate ^c	83.6	607.5
Total phenol (eq-g tannic acid/kg DM)	19.0	104.6
Total tannins (eq-g tannic acid/kg DM)	3.0	76.9
Condensed tannins (eq-g leucocyanidin/kg DM)	0.2	1.6
Flavonoids (eq. mg rutin/kg DM)	1.3	3.2

^aHemicellulose was calculated as NDF minus ADF

^bCellulose was calculated as ADF minus ADL

^cNon-fiber carbohydrate calculated by difference [1000 – (NDF + CP + EE + ash)]

Table 2 Effect of berseem ensiled with molasses and pomegranate peels at different levels on pH content, organic acids, ethanol, and Fleig's score after 0, 15, 30, and 45 days of ensiling

Item	Days	Con	Treatment ^a			SEM ^b		Contrast <i>P</i> -value ^c			
			Pomegranate peels, g/ kg DM								
			50	100	200			Mls	PM-L	PM-Q	PM-C
pH											
	0	5.59	5.05	4.52	4.42	5.84	0.14	<.01	<.01	<.01	<.01
	15	4.95	4.40	4.40	4.30	3.80	0.11	<.01	0.06	0.14	<.01
	30	4.75	4.30	4.05	4.15	3.75	0.10	0.04	0.83	0.33	<.01
	45	5.02	4.74	4.27	4.41	3.79	0.11	<.01	<.01	<.01	<.01
Lactic (g/kg DM)											
	0	3.91	15.1	26.38	47.5	15.40	2.29	<.01	<.01	<.01	<.01
	15	27.98	55.93	39.35	68.15	52.66	2.69	<.01	0.83	<.01	<.01
	30	33.45	62.65	60.88	50.73	66.8	2.97	0.09	0.01	0.42	<.01
	45	21.27	20.13	40.86	37.33	61.19	2.38	<.01	0.47	<.01	<.01
Acetic acid (g/kg DM)											
	0	1.01	1.78	0.47	0.52	3.55	0.23	<.01	<.01	<.01	<.01
	15	16.91	8.76	5.41	1.96	14.75	1.38	0.66	0.23	<.01	0.38
	30	36.07	21.58	8.59	3.15	17.03	2.68	0.31	0.05	<.01	<.01
	45	26.78	14.10	6.69	1.60	8.14	1.95	<.01	<.01	<.01	<.01
Propionic acid (g/kg DM)											
	0	ND	ND	ND	ND	ND	-	-	-	-	-
	15	0.42	0.02	0.01	0.01	0.01	0.04	0.90	0.68	0.63	<.01
	30	1.17	0.22	0.02	0.00	0.01	0.09	0.90	0.79	0.59	<.01
	45	1.32	0.22	0.04	0.01	0.02	0.11	0.01	0.32	0.07	<.01
Butyric acid (g/kg DM)											
	0	0.00	0.15	0.19	0.23	1.22	0.10	<.01	<.01	<.01	<.01
	15	0.02	0.04	0.05	0.10	0.16	0.01	0.18	0.05	<.01	<.01
	30	0.51	0.09	0.07	0.10	0.15	0.04	0.73	0.67	0.84	<.01
	45	0.96	0.06	0.01	0.05	0.10	0.08	0.90	0.45	0.67	<.01
Ethanol (g/kg DM)											
	0	ND	ND	ND	ND	ND	-	-	-	-	-
	15	2.39	9.74	11.51	14.22	10.92	2.31	<.01	0.02	<.01	0.02
	30	5.26	18.58	16.72	28.95	21.56	4.04	0.07	<.01	<.01	0.08
	45	1.56	1.39	6.62	28.33	7.60	2.21	<.01	0.92	<.01	0.08
Fleig's score											
	0	15.40	57.83	85.46	102.71	19.74	7.75	0.01	<.01	<.01	0.09
	15	39.29	85.38	71.65	108.49	110.61	5.89	<.01	0.87	0.01	<.01
	30	46.92	85.39	89.57	110.64	114.46	5.44	0.01	0.97	<.01	<.01
	45	30.75	65.40	76.15	93.31	110.12	6.22	<.01	<.01	<.01	<.01

Effects of treatment, time and treatment x time interaction were <0.01

^aCon: control: 50, 100 and 200 refer to inclusion of pomegranate peels at 50, 100 and 200 g/kg on fresh weight basis^bSEM standard error of means^cPM-L linear effect of pomegranate, PM-Q quadratic effect of pomegranate, PM-C cubic effect of pomegranate^dMls control vs. molasses, ND not detected

ethanol content ($P < 0.01$) than the control (Table 2). From days 15 to 45 of ensiling, the $\text{NH}_3\text{-N}$ content of

molasses treatments was lower ($P < 0.01$) than the control (Fig. 1).

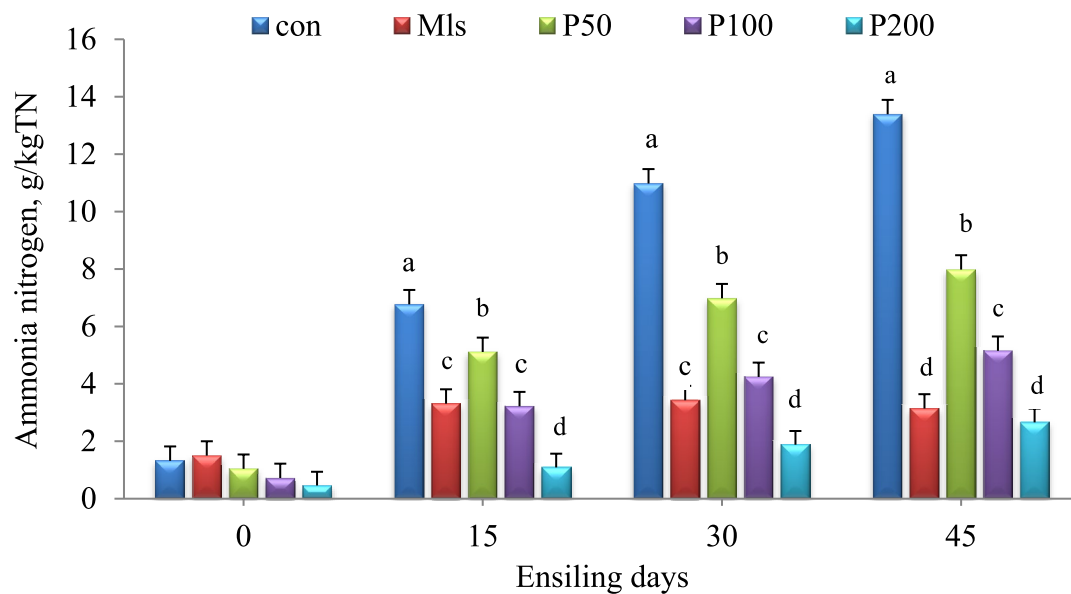


Fig. 1 Ammonia-N (g/kg TN) of berseem ensiled with pomegranate peels and molasses at different levels after 0, 15, 30, and 45 days of ensiling. Con: control; Mls: molasses:50,100 and 200 refer to the

inclusion of pomegranate peels at 50,100 and 200 g/kg on a fresh weight basis. Effects of treatment, time, and treatment $\times$ time interaction are ($P < 0.01$). The standard error of the mean (SEM) is 0.55

Nutrient composition of dried pomegranate peels silage

The chemical composition of berseem silages ensiled with dried pomegranate peels and molasses was significantly influenced by treatment, ensiling time, and their interaction ($P < 0.01$), as indicated in Tables 3 and 4. The content of DM and OM increased ($p < 0.01$; linear, quadratic, and cubic as the proportion of pomegranate peels in the forage mixtures increased. On the contrary, the content of CP in the mixture decreased linearly ($P < 0.01$) with increasing pomegranate peel proportion, with higher values (Q; $P < 0.01$) at intermediate mixing proportions at 45 days of ensiling (Table 3). At 30 days of ensiling, the EE content increased with increasing pomegranate peels in the mixture ($P < 0.01$; linear, quadratic, and cubic). Throughout the ensiling times, the proportion of pomegranate peels in the mixture increased the content of NFC while decreasing the NDF, ADF, hemicelluloses, and cellulose contents of silage ($P < 0.01$; linear, quadratic, and cubic) (Table 4).

The molasses treatment had higher DM, OM, and EE content ($P < 0.01$) but lower CP content ($P < 0.01$) than the control (Table 3). At the ensiling time, the molasses treatment had a higher NFC content ($P < 0.01$) and a lower structural carbohydrate content ($P < 0.01$) than the control. Except for hemicellulose, the silage-treated molasses content increased in NDF, ADF, ADL, and cellulose after 30 and 45 days of ensiling (Table 4).

Experiment 2. Nutrient degradation, methane production and fermentation profiles

Overall, there was no significant interaction between treatment and incubation time for nutrient degradation, CH_4 production, or fermentation patterns. The only interaction between time and treatment on $\text{NH}_3\text{-N}$ concentration was significant ($P < 0.01$) (Fig. 2).

The rumen nutrient degradation, methane production, and microbial protein synthesis of pomegranate peels and molasses ensiled with berseem silage are presented in Table 5. As the proportion of pomegranate peels in the mixture increased, the ADMD increased linearly ($P < 0.001$). The TDMD and TOMD exhibited a quadratic response to the increasing pomegranate peel content, with the highest values observed at intermediate pomegranate levels (Q; $P < 0.05$). At high levels of pomegranate peel in the mixture, CH_4 production decreased cubically ($P < 0.05$). Maximum MN quadratic responses to increasing pomegranate peel content in mixtures ($P < 0.05$). There were no significant differences in lactate, total VFA concentration, or molar proportions of VFA, except for isobutyrate, isovalerate, and Valeric, which decreased (L, Q, C; $P < 0.05$) to increase pomegranate peel content in mixtures while A to P ratio reduced cubically ($P < 0.05$) (Table 6). After 24 h of incubation, the $\text{NH}_3\text{-N}$ concentration was lower ($P < 0.001$) at high levels of dried pomegranate peel (Fig. 2).

Table 3 Nutrient composition (g/kg DM) of berseem ensiled with pomegranate peels and molasses and at different levels after 0, 15, 30, and 45 days of ensiling

Item	Days	Con	Treatment ^a			Mls	SEM ^b	Contrast <i>P</i> -value ^c			
			Pomegranate peels g/ kg DM					PM-L	PM-Q	PM-C	Mls ^d
			50	100	200						
Dry matter											
	0	250.0	273.2	306.3	372.6	240.7	1.26	< .01	< .01	< .01	0.45
	15	241.5	281.9	293.3	377.5	288.1	1.17	< .01	< .01	< .01	< .01
	30	239.6	262.0	312.9	358.2	297.3	1.03	< .01	< .01	< .01	< .01
	45	212.8	250.0	288.7	323.5	283.6	0.95	< .01	< .01	< .01	< .01
Organic matter											
	0	866.4	871.1	875.8	885.3	845.9	0.37	< .01	< .01	< .01	0.10
	15	854.7	874.0	884.8	900.8	898.1	0.40	< .01	< .01	< .01	< .01
	30	851.3	869.8	888.2	903.6	891.1	0.43	< .01	< .01	< .01	< .01
	45	858.5	860.1	875.2	895.0	887.7	0.38	< .01	< .01	< .01	< .01
Crude protein											
	0	172.2	165.6	158.9	144.3	164.6	0.26	< .01	< .01	< .01	< .01
	15	154.7	165	145.2	114.1	136.2	0.48	< .01	0.01	< .01	< .01
	30	164.3	146.9	132.2	126	131.4	0.34	< .01	0.02	0.09	< .01
	45	172.1	139.3	154.7	140.1	140.1	0.34	0.02	0.01	0.22	< .01
Ether extract											
	0	17.1	18.9	20.0	23.1	18.4	0.05	0.44	0.51	0.52	0.69
	15	18.2	21.8	22.3	22.7	18.8	0.29	0.33	0.08	0.82	0.06
	30	19.2	21.9	21.7	25.4	19.7	0.29	0.02	< .01	0.03	< .01
	45	19.6	22.0	22.6	24.8	19.7	0.20	0.02	< .01	0.10	< .01

Effects of treatment, time, and treatment × time interaction were ($P < 0.01$)

^aCon: control; 50, 100, and 200 refer to the inclusion of pomegranate peels at 50, 100, and 200 g/kg on a fresh weight basis

^bSEM standard error of means

^cPM-L linear effect of pomegranate, PM-Q quadratic effect of pomegranate, PM-C cubic effect of pomegranate

^dMls control vs. molasses

The molasses treatment had higher ($P < 0.01$) ADMD, TDMD, TOMD, and MN, but lower ($P < 0.01$) CH₄ production (Table 5). Propionate, butyrate, and isobutyrate levels increased ($P < 0.05$), but the acetate/propionate ratio decreased significantly in the molasses treatment compared to the control (Table 6). With time incubation, the NH₃-N concentration in molasses was lower than the control (Fig. 2).

Correlation analysis

In EXP 1, the silage pH exhibited positive correlations with CP content (0.712) during the ensiling process, whereas FS and lactate demonstrated negative correlations (−0.779 and −0.665, respectively) with CP content ($P < 0.01$; Fig. 3). A negative correlation was observed between the high NFC concentration and pH (−0.867), while it showed a positive correlation with FS (0.859) and lactate levels (0.761). On the other hand, NDF, ADF, and cellulose exhibited positive correlations

with pH (0.862, 0.814, and 0.814, respectively) but negative correlations with FS (−0.816, −0.842, and −0.848, respectively) and lactate (−0.751, −0.640, and −0.659, respectively; $P < 0.01$). In Exp 2, the ADMD, TDMD, and TOMD were negatively associated with nonstructural carbohydrates and positively associated with NFC content in silage after 24 and 48 h of incubation ($P < 0.01$; Figs. 4 and 5). The contents of CP were found to be positively correlated with isobutyrate (0.698) and isovalerate (0.704) while negatively correlated with MN (−0.685), ADMD (−0.586), TDMD (−0.636), and TOMD (−0.685) after 24 h ($P < 0.01$; Fig. 4).

Discussion

Experiment 1

In the current study, the DM content of wilted berseem was significantly lower than the recommended range

Table 4 Non-fiber carbohydrates and structural carbohydrates compositions (g/kg DM) of berseem ensiled with molasses and pomegranate peels at different levels and ensiled for 0, 15, 30, and 45 days

Item ^a	Days	Con	Treatment ^b				SEM ^c	Contrast <i>P</i> -value ^d			
			Pomegranate peels, g/kg DM					PM-L	PM-Q	PM-C	Mls ^e
			50	100	200	Mls					
NFC											
	0	83.8	109.9	134.8	187.4	95.3	1.07	< .01	< .01	< .01	< .01
	15	123.9	203.4	241.3	332.5	377.7	2.20	< .01	< .01	< .01	< .01
	30	123.8	205.7	267.8	321.2	359.5	1.88	< .01	< .01	< .01	< .01
	45	136.9	208.1	220.8	264.8	351.7	1.73	< .01	< .01	< .01	< .01
NDF											
	0	593.2	577.7	562.2	530.5	592.5	0.67	< .01	< .01	< .01	0.38
	15	557.9	483.8	475.9	431.5	365.5	1.56	< .01	< .01	< .01	< .01
	30	544	495.3	466.4	430.9	380.5	1.40	< .01	< .01	< .01	< .01
	45	529.9	490.7	477.2	465.4	376.2	1.09	< .01	< .01	0.06	< .01
ADF											
	0	335.7	327.5	319.7	303.1	335.3	0.33	< .01	< .01	< .01	0.33
	15	365.2	304.0	298.9	255.6	229.7	1.17	< .01	< .01	< .01	< .01
	30	363.8	314.2	302.4	264.3	246.8	1.03	< .01	< .01	< .01	< .01
	45	367.6	328.4	308.5	246.5	243.4	1.05	< .01	< .01	< .01	< .01
ADL											
	0	66.7	65.7	64.9	63.0	66.6	0.05	< .01	< .01	< .01	0.22
	15	74.5	64.8	63.1	51.4	46.1	0.29	< .01	< .01	< .01	< .01
	30	70.6	70.9	65.6	56.0	50.3	0.25	< .01	< .01	< .01	< .01
	45	69.6	59.2	69.5	56.9	46.4	0.22	< .01	< .01	< .01	< .01
Cellulose											
	0	269.0	261.8	254.7	240.1	268.7	0.29	< .01	< .01	< .01	0.43
	15	290.7	239.2	235.8	204.3	183.6	0.90	< .01	< .01	< .01	< .01
	30	293.2	243.4	236.8	208.4	196.6	0.83	< .01	< .01	< .01	< .01
	45	298.1	269.2	239.0	189.6	197.0	0.90	< .01	< .01	< .01	< .01
Hemicelluloses											
	0	257.7	250.2	242.5	227.4	257.3	0.34	< .01	< .01	< .01	0.56
	15	192.8	179.8	177.1	175.9	135.8	0.46	< .01	< .01	0.13	< .01
	30	180.2	181.1	164.0	166.7	133.7	0.45	< .01	< .01	< .01	< .01
	45	162.3	162.2	168.7	218.9	132.9	0.57	< .01	< .01	< .01	< .01

Effects of treatment, time, and treatment × time interaction were < 0.01

^aNFC non-fiber carbohydrates, NDF Neutral detergent fiber, ADF Acid detergent fiber, ADL Acid detergent fiber^bCon = control: 50, 100, and 200 refer to the inclusion of pomegranate peels at 50, 100, and 200 g/kg on fresh^cSEM standard error of means^dPM-L linear effect of pomegranate, PM-Q quadratic effect of pomegranate, PM-C cubic effect of pomegranate^eMls control vs. molasses

(300–350 g/kg) for optimal silage quality (Kung et al. 2018). To address this issue, common techniques include the incorporation of dried materials or the addition of additives, aiming to reduce moisture content (Borreani et al. 2018). As expected, the inclusion of dried pomegranate peels in this study may help reduce moisture levels of berseem from 750 to 630 g/kg. In comparison to berseem, dried pomegranate peels would make a good ferment

substrate for ensiling due to their high NFC content (607 vs 83.6 g/kg DM), which could support sufficient acid production during the ensiling process (Rooke and Hatfield 2003). The TP, TT, and CT values of dried pomegranate peel were lower than those reported by Natalello et al. (2020) and Niu et al. (2023). These differences may be explained by analytical methods or growth conditions that affect polyphenol concentrations, as reviewed by (Aboagye

Fig. 2 Ammonia -N (mg/100 ml rumen buffered liquid) of berseem ensiled with pomegranate peels and molasses at different levels after 30 days of ensiling. Con: control; MIs: molasses; The p-value for the effect treatment ($P=0.6$), time ($P<0.01$), treatment $\times$ time ($P<0.001$). The standard error of the mean (SEM) is 1.08

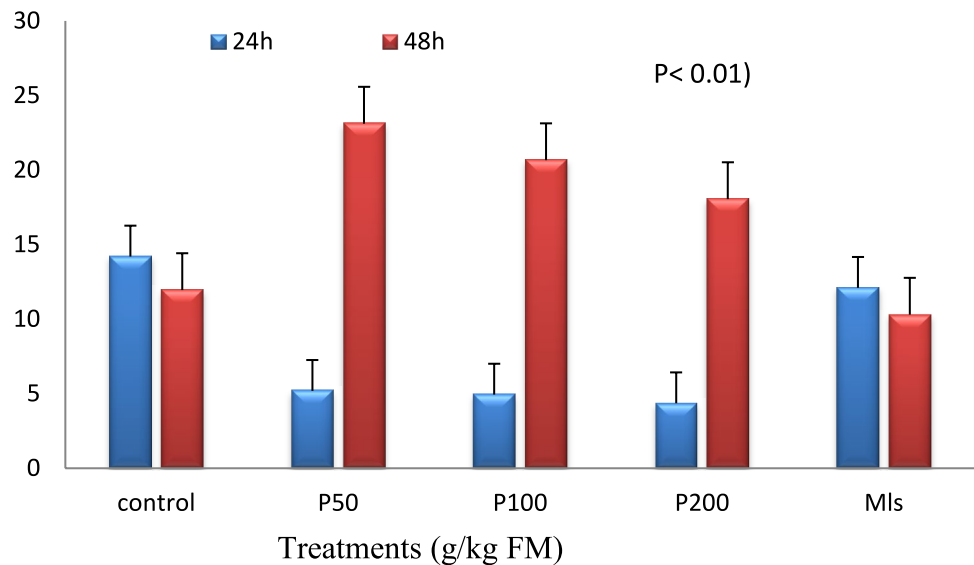


Table 5 Effects of berseem ensiled with molasses and pomegranate peels (ensiled for 30 days) on apparent and true dry matter (ADDM and TDDM), true organic matter (TDOM), neutral detergent fiber disappearance (NDFD), methane (CH_4) and microbial protein (MP) in vitro

Item	Treatment ^a					SEM ^b	Contrast <i>P</i> -value ^c			
	Pomegranate peels, g/kg DM						PM-L	PM-Q	PM-C	MIs ^d
	Con	50	100	200	MIs					
ADDM (%)	54.94	60.87	63.05	52.79	65.33	1.217	<.001	0.32	0.08	<.01
TDDM (%)	65.75	68.58	70.33	62.44	74.08	0.871	<.001	0.06	0.16	<.01
TOMD (%)	62.15	66.11	68.4	58.05	72.21	1.037	<.001	0.04	0.08	<.01
NDFD (%)	33.12	35.47	36.48	34.35	34.92	1.176	0.52	0.96	0.31	0.89
CH ₄ (g/kg DM)	24.26	24.29	24.72	24.11	21.89	0.164	0.10	0.03	0.02	<.01
MN (g/kg of OMD)	118.09	125.61	129.97	110.29	137.2	1.970	<.01	0.02	0.09	<.01

^aValues are average concentrations at 24 and 48 h of incubation. No treatment $\times$ time interaction was observed

^bAverage of SEM among treatments

^cPM-L linear effect of pomegranate, PM-Q quadratic effect of pomegranate, PM-C cubic effect of pomegranate

^dMIs control vs. molasses

and Beauchemin 2019). Thus, it is hypothesized that adding dried pomegranate peel to ensiled berseem would accelerate fermentation, reducing extensive proteolysis, preventing the negative effects of undesirable bacteria growth, and reducing rumen protein degradation and CH_4 production in ruminants fed such silage.

In the current study, the pH values of silages found in this study ranged from 4.30 to 5.00, which are considered suitable for legume silage (Kung et al. 2018). The values of lactic acid ranged from 20 to 68 g/kg, which is recommended for legume silages with a low DM concentration (300–350 g/kg), according to Kung et al. (2018). The high tannin content of dried pomegranate peels (Table 1) may explain the low pH of silage and significant lactic acid production by improving silage antioxidative potential and decreasing berseem silage buffering capacity,

promoting the growth of lactic acid bacteria and inhibiting undesirable bacteria (He et al. 2020; Zou et al. 2021). Furthermore, the high content of dried pomegranate peels of NFC, specifically pectin (data not estimated), which acts as a substrate for the growth of lactic acid-producing bacteria (e.g., *Leuconostoc*), results in higher organic acids and lower silage pH (Gao et al. 2021; Wang et al. 2020a).

The greatest decrease in pH values for all ensiled forages was observed within the first 30 days of ensiling, which agrees with previously reported data for various materials (Ahmed et al. 2023; Gao et al. 2021). Lactic acid bacteria, particularly heterofermentative bacteria, grew quickly and reduced pH during the initial fermentation ensiling times, and then their growth decreased with ensiling time due to decreased WSC or low pH sensitivity, which could explain this (Wang et al. 2020b).

Table 6 Effects of berseem ensiled with molasses and pomegranate peels (ensiled for 30 days) on concentrations of short-chain fatty acids (acetic, propionic, isobutyric, butyric, isovaleric, valeric), total VFA, acetate to propionate (A to P) ratio, and lactate in vitro (Exp. 2)

Item	Treatment ^a					Contrast <i>P</i> -value ^c				
	Pomegranate peels, g/kg DM					SEM ^b	PM-L	PM-Q	PM-C	MIs ^d
	Con	50	100	200	MIs					
Lactate (mM)	29.31	29.65	28.16	30.22	21.21	1.38	0.94	0.79	0.69	0.16
Total VFA (mM)	115.07	113.54	111.04	112.01	124.68	2.084	0.97	0.93	0.32	0.07
Acetic acid (mM)	77.44	76.36	74.73	74.79	79.58	1.218	0.84	0.80	0.25	0.27
Propionic acid (mM)	18.37	18.08	17.25	17.94	21.85	0.382	0.78	0.70	0.10	0.01
Butyric acid (mM)	13.44	13.57	14.02	12.73	17.11	0.447	0.44	0.49	0.80	0.02
Isobutyric acid (mM)	2.38	2.28	2.09	2.79	2.52	0.089	<0.001	<0.001	0.24	0.05
Isovaleric acid (mM)	1.94	1.85	1.64	2.21	2.03	0.091	<0.001	<0.001	0.02	0.08
Valeric acid (mM)	1.48	1.41	1.32	1.56	1.6	0.034	<0.001	0.03	0.04	0.64
Acetic acid/ Propionic acid	4.22	4.23	4.33	4.18	3.64	0.040	0.10	0.03	0.02	<0.01

^aValues are average concentrations at 24 and 48 h of incubation. No treatment × time interaction was observed

^bAverage of SEM among treatments

^c*PM-L* linear effect of pomegranate, *PM-Q* quadratic effect of pomegranate, *PM-C* cubic effect of pomegranate

^d*MIs* control vs. molasses

Acetic acid levels in the mixture were found to be lower than the optimal range (10–30 g/kg DM; (Kung et al. 2018)). The decrease in acetic acid concentrations could be explained by the presence of tannins that inhibit bacteria producing acetic acid such as enterobacteria (Zou et al. 2021). The energy cost of ensiling dried pomegranate peel with berseem silage was expressed as the production of highly undesirable butyric acid and ethanol. This is consistent with the results of Eliyahu et al. (2015) in pomegranate pulp silage. However, the values of butyric acid and ethanol were less than 5 and 30 g/kg DM, respectively, and were within the recommended ranges (Kung et al. 2018; Muck 2010). The massive fermentation of carbohydrates by yeast, heterofermentative lactic acid bacteria, and *Clostridium tyrobutyricum* during the ensiling process may explain the high levels of lactate, butyrate, and ethanol production in dried pomegranate peel (Eliyahu et al. 2015). The NH₃-N concentration was less than 5 g/kg TN of dried pomegranate peel, which was significantly lower than the recommended ranges for legume silage (Zhang et al. 2016). This could be explained by tannin-protein complex formation and inhibition of plant protease activity during ensilage (Chen et al. 2021).

Molasses additives improved berseem silage quality, which is consistent with the findings of Gao et al. (2021). The high molasses content of soluble substrates may promote homolactic fermentation, raise lactate concentration, and lower pH, resulting in lower butyric acid and NH₃-N production as well as high ethanol (Desta et al. 2016; Gao et al. 2021).

The higher DM and NFC content observed with higher ratios of dried pomegranate peels in the mixture can be linked to the chemical composition of dried pomegranate peels. However, the decrease in DM content observed in ensiled forages over time is most likely due to microbial fermentation of silage DM, which results in the decomposition of nutrients into liquids, gases, and VFA (Desta et al. 2016). The fluctuations in CP levels in ensiled forages can be ascribed to the breakdown of diverse protein compositions in the ensiled forages, caused by the interaction between pomegranate peel and berseem. This interaction may affect the speed and degree of fermentation (Rooke and Hatfield 2003).

The higher DM content in molasses silage could be due to the higher DM content of the molasses used, which is consistent with the findings of Desta et al. (2016). Furthermore, the high NFC content in molasses silage with ensiling time may be linked to the acid hydrolysis of structural carbohydrates, which liberates WSC and sugars, or the inhibition of silage microbial growth by reducing pH, which prevents the absorption of fermentable substrates (Desta et al. 2016). Further, the reduction in hemicellulose content of silage-treated molasses could be attributed to organic acid accumulation during forage ensiling (Desta et al. 2016).

Experiment 2

The higher in vitro degradation of dried pomegranate peels in comparison to berseem silage could be ascribed to the lower content of their respective fiber fractions, a relationship

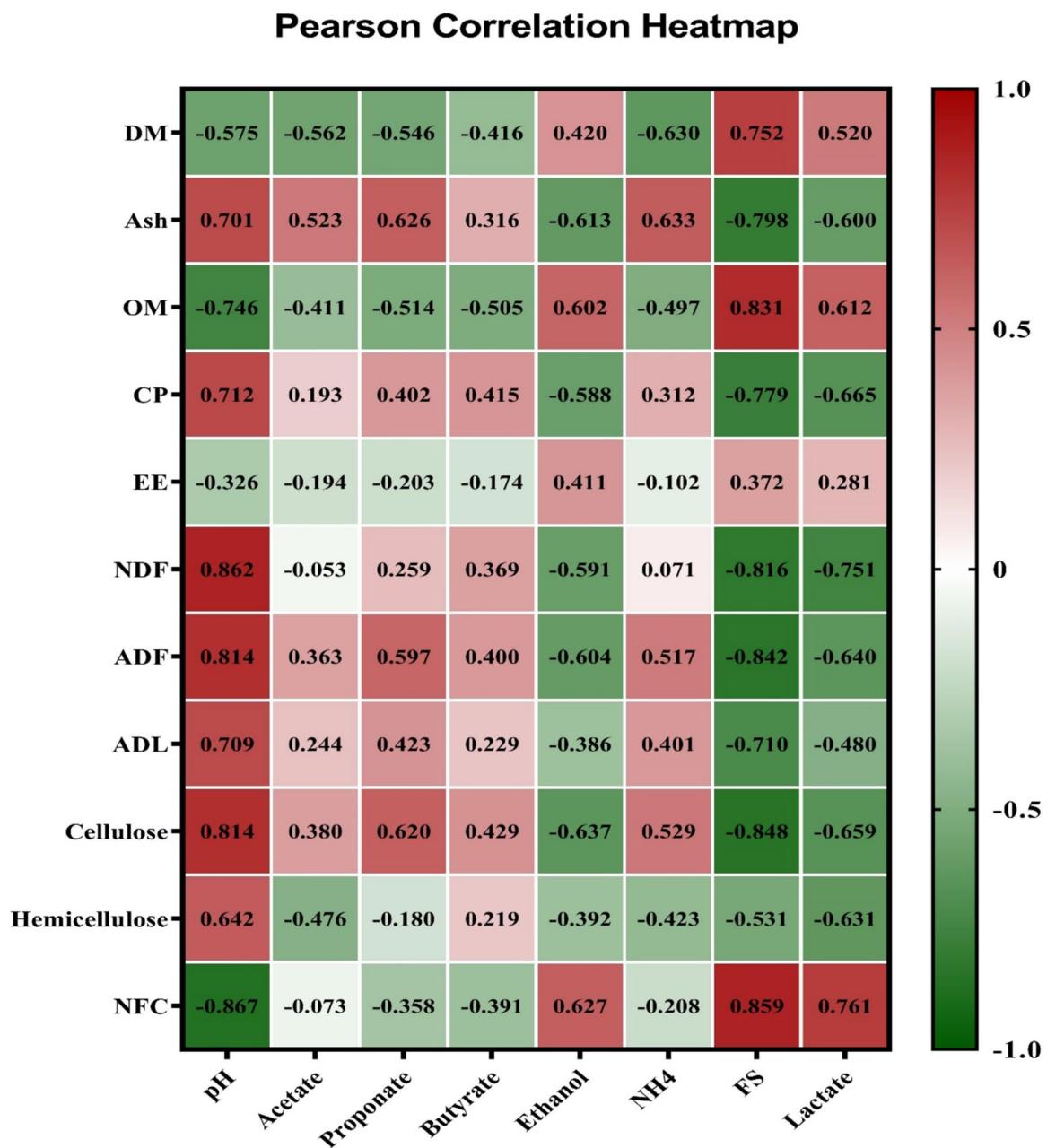


Fig. 3 Pearson correlations between the silage quality parameters and chemical composition of berseem silages throughout the ensiling period. DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; NDF: neutral detergent fiber; ADL: acid detergent lignin; NFCM: non-fibrous carbohydrates; FS: Fleig' score. The num-

ber in each square represents the correlation extent; the color represents a significant correlation ($p < 0.05$), the deeper the color of the square is the more significant the correlation. the red color means a positive correlation, and the green color means a negative correlation

supported by the personal correlation of this study. The study revealed a significant negative correlation ($P < 0.01$) between ADMD, TDMD, and TOMD with the silage content of NDF (-0.681 , -0.724 , and -0.772 , respectively), ADF (-0.635 , -0.690 , and -0.741 , respectively), and ADL (-0.682 , -0.697 , and -0.744 , respectively). Pomegranate peel silage has a high content of substrates potentially degradable (e.g., pectin) (data not estimated) by the ruminal microbiota and

low-lignin, which has antimicrobial efficacy (Medjekal et al. 2017). The associative impact of combining berseem and pomegranate peel forages may account for the variations in nutrient degradation observed in different forage mixes. The diverse characteristics and chemical components of the forage blend lead to a lack of synchronization in the release of nutrients, which in turn leads to variations in the growth of microbial biomass (Chen et al. 2022).

Pearson Correlation Heatmap

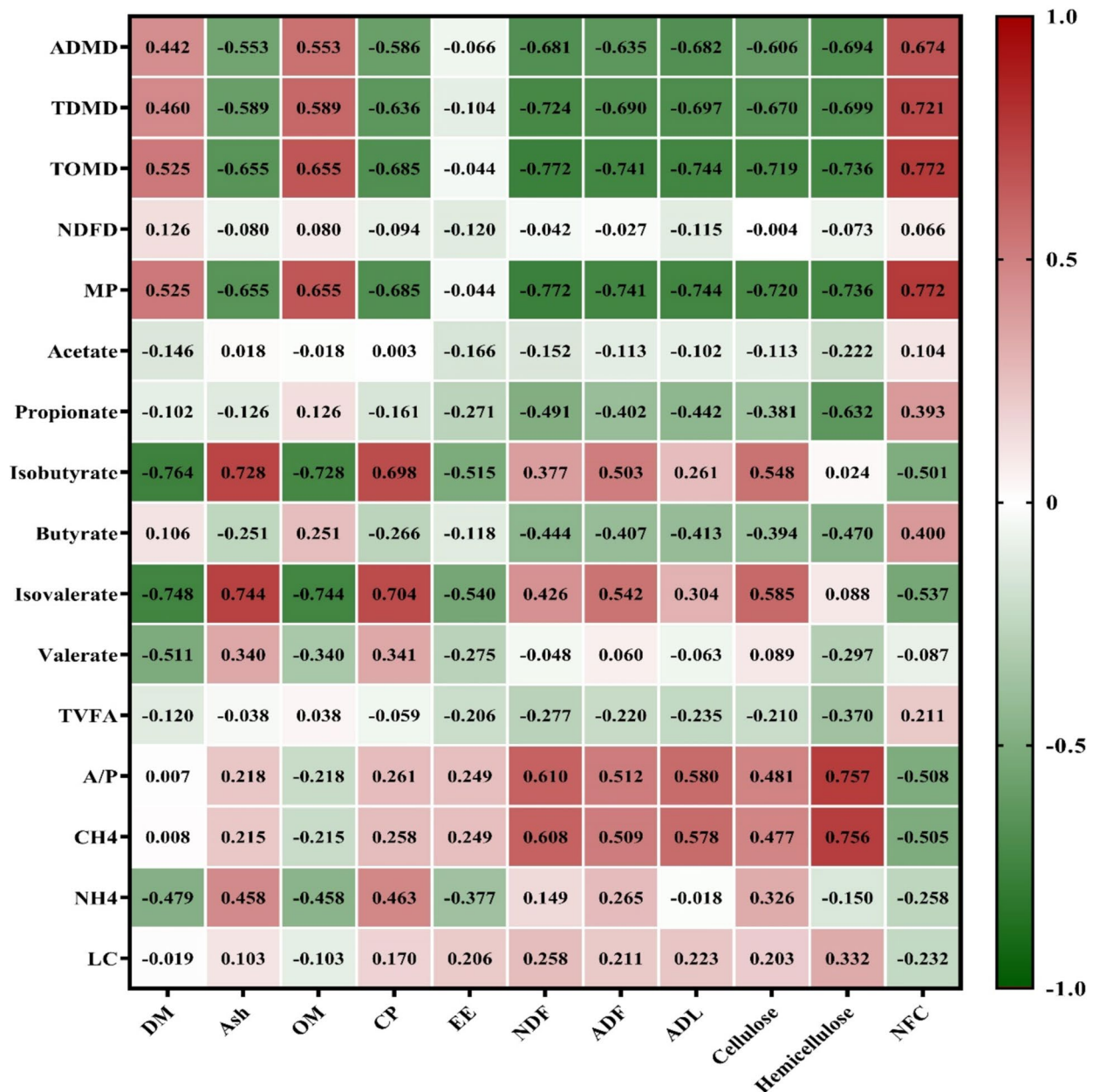


Fig. 4 Pearson correlations between the in vitro fermentation parameters at 24 h of incubation and chemical composition of berseem silages. ADMD: apparent dry matter degradability; TDMD: truly dry matter degradability; TDOM: truly degraded organic matter; NDFD: neutral detergent fiber degradability; MP: microbial protein; TVFA: total volatile fatty acid; A/P: acetate to propionate ratio; CH₄: methane. DM: dry matter; OM: organic matter; CP: crude protein; EE:

ether extract; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; NFC: non-fibrous carbohydrates. The number in each square represents the correlation extent; the color represents a significant correlation ($p < 0.05$), the deeper the color of the square the more significant the correlation. the red color means a positive correlation, and the green color means a negative correlation

The dried pomegranate peel was effective in reducing CH₄ production at higher levels due to tannins, which have the potential to reduce CH₄ production, according to Chen et al. (2021). Furthermore, increased microbial protein synthesis plays a significant role in mitigating methanogenesis by

creating a competitive environment for hydrogen ions (H⁺), which are required for CH₄ formation. This competition can successfully reduce CH₄ emissions (Czerkawski 1986). Pomegranate peel was effective in improving the efficiency of rumen microbial N synthesis, which could be attributed to

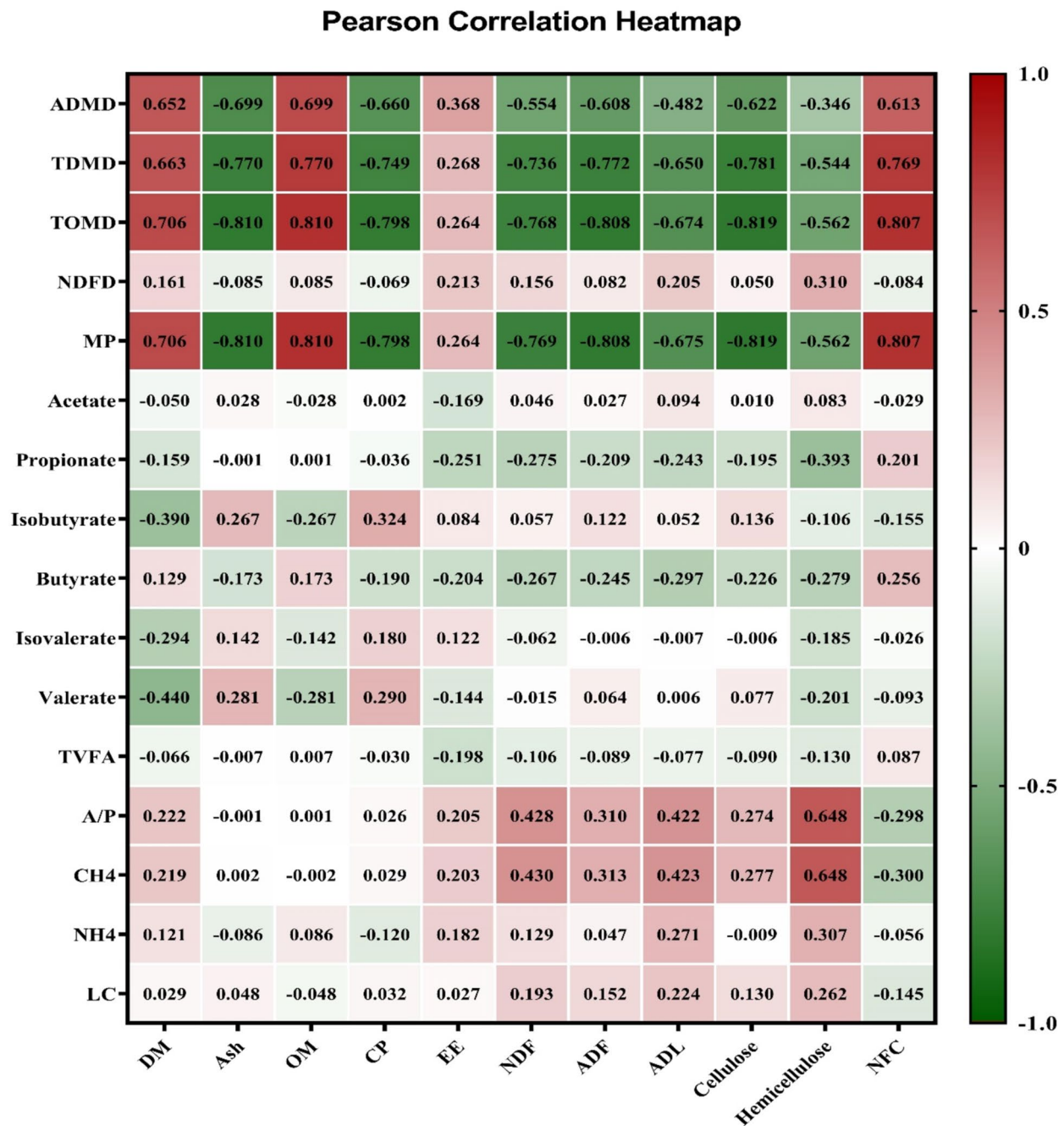


Fig. 5 Pearson correlation between the invitro fermentation parameters at 48 h of incubation and chemical composition of berseem silages. ADMD: apparent dry matter degradability; TDMD: truly dry matter degradability; TDOM: truly degraded organic matter; NDFD: neutral detergent fiber degradability; MP: microbial protein; TVFA: total volatile fatty acid; A/P: acetate to propionate ratio; CH₄: methane. DM: dry matter; OM: organic matter; CP: crude protein; EE:

ether extract; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; NFC: non-fibrous carbohydrates. The number in each square represents the correlation extent; the color represents a significant correlation ($p < 0.05$), the deeper the color of the square the more significant the correlation. The red color means a positive correlation and the green color means a negative correlation

tannin having antiprotozoal properties (data not estimated) that lead to ammonia reduction, which could be due to decreased bacterial lysosome activity or an increase in NH₃-N uptake for microbial protein biomass synthesis (Abarghuei and

Salem 2021). The substantial amounts of dried pomegranate peel shift VFA molar proportions like monensin (i.e., decrease the acetate and propionate ratio), which agrees with Khorsandi et al. (2019). The decrease in BCVFA levels

can be attributed to the high tannin content of pomegranate peels, which is consistent with the findings of Chen et al. (2021) on alfalfa treated with tannic acid. Additionally, the diminished accumulation of BCVFA is associated with a lower concentration of $\text{NH}_3\text{-N}$ (Fig. 2). This could be explained by the formation of a tannin and protein complex via hydrogen bonding, as well as the tannins' inhibitory effects on rumen proteolytic bacteria (Makkar et al. 1993; Ahmed et al. 2024).

In our study, molasses was used as a positive control when adding pomegranate peels to the silage. For ensiling, molasses is a well-known ingredient that works especially well with forages like Berseem that have WSC content. Molasses additions are used as stimulants to increase the availability of fermentable carbohydrates and promote the growth of lactic acid bacteria to improve the quality of fermentation (Li et al. 2010; Gao et al. 2021). Molasses added to silage modifies rumen fermentation profiles, which is consistent with the findings of Chen et al. (2016). This could be due to sucrose's high molasses content, which serves as an important substrate for microbial fermentation, improving rumen microbial protein synthesis, nutrient degradation, and mitigation of CH_4 (Chen et al. 2016; Palmonari et al. 2023). The low $\text{NH}_3\text{-N}$ concentration of molasses-treated silage could be attributed to the silage's reduced protein fraction, which should result in a lower protein fraction (Pámanes-Carrasco et al. 2023).

Conclusions

Both dried pomegranate peel and molasses with berseem as carbohydrate sources were found to be excellent cleaners for the environment and animal nutrition after 30 days of ensiling, with higher fermentation quality and a suitable nutritional composition. In addition, adding dried pomegranate peel and molasses to silage improved nutrient degradation and microbial protein synthesis, reduced ruminal protein degradation, and supported the reduction of CH_4 . As a result, these additives are thought to be safe for both animals and humans when used as antibiotic alternatives in ruminant diets. More in-vivo research is required to corroborate our in-vitro findings.

Authors' contributions M.G.A.: Conceptualization, Investigation, Data curation, Methodology, Writing – original draft preparation, and Visualisation. S.Z.E.-Z.: Conceptualisation, Writing – review & editing, Formal analysis. A.A.A.-S.: Conceptualization, Investigation, Writing– review & editing. E.A.E.: Conceptualization, Investigation, Data curation, Methodology, Writing – original draft preparation, and Visualisation. All authors have read and agreed to the published version of the manuscript.

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

Declarations

Ethical approval The animal study protocol was approved by the Institutional Animal Care and Use Committee of Alexandria University.

Consent to participate Not applicable.

Consent to publish Not applicable.

Conflicts of interest The authors declare no conflict of interest.

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References

- Abarghuei MJ, Salem AZM (2021) Sustainable impact of pulp and leaves of *Glycyrrhiza glabra* to enhance ruminal biofermentability, protozoa population, and biogas production in sheep. *Environ Sci Pollut Res* 28:33371–33381
- Aboagye IA, Beauchemin KA (2019) Potential of molecular weight and structure of tannins to reduce methane emissions from ruminants: a review. *Animals* 9:856
- Ahmed MG, Al-Sagheer AA, El-Zarkouny SZ, Elwakeel EA (2022) Potential of selected plant extracts to control severe subacute ruminal acidosis in vitro as compared with monensin. *BMC Vet Res* 18(1):356
- Ahmed MG, Al-Sagheer AA, El-Waziry AM, El-Zarkouny SZ, Elwakeel EA (2023) Ensiling characteristics, in vitro rumen fermentation patterns, feed degradability, and methane and ammonia production of berseem (*Trifolium alexandrinum* L.) co-ensiled with artichoke bracts (*Cynara cardunculus* L.). *Animals* 13:1543
- Ahmed MG, Elwakeel EA, El-Zarkouny SZ, Al-Sagheer AA (2024) Environmental impact of phytobiotic additives on greenhouse gas emission reduction, rumen fermentation manipulation, and performance in ruminants: an updated review. *Environ Sci Pollut Res* 31:37943–37962 <https://doi.org/10.1007/s11356-024-33664-5>
- AOAC (2006) Official methods of analysis, 18th ed. Washington, DC
- Barros T, Powell JM, Danes MAC, Aguerre MJ, Wattiaux MA (2017) Relative partitioning of N from alfalfa silage, corn silage, corn grain and soybean meal into milk, urine, and feces, using stable ^{15}N isotope. *Anim Feed Sci Technol* 229:91–96
- Borreani G, Tabacco E, Schmidt RJ, Holmes BJ, Muck RE (2018) Silage review: factors affecting dry matter and quality losses in silages. *J Dairy Sci* 101:3952–3979

- Borshchevskaya LN, Gordeeva TL, Kalinina AN, Sineokii SP (2016) Spectrophotometric determination of lactic acid. *J Anal Chem* 71:755–758
- Chen L, Guo G, Yuan X, Zhang J, Li J, Shao T (2016) Effects of applying molasses, lactic acid bacteria and propionic acid on fermentation quality, aerobic stability and in vitro gas production of total mixed ration silage prepared with oat–common vetch intercrop on the Tibetan Plateau. *J Sci Food Agric* 96:1678–1685
- Chen L, Bao X, Guo G, Huo W, Xu Q, Wang C, Liu Q (2021) Treatment of alfalfa silage with tannin acid at different levels modulates ensiling characteristics, methane mitigation, ruminal fermentation patterns and microbiota. *Anim Feed Sci Technol* 278:114997
- Chen Y-H, Chen C-Y, Wang H-T (2022) The effect of forage source and concentrated liquid feedstuff supplementation on improving the synchronization of ruminant dietary energy and nitrogen release in vitro. *Fermentation* 8:443
- Czerkawski JW (1986) An introduction to rumen studies. Elsevier. <https://doi.org/10.1016/C2009-0-00551-0>
- Desta ST, Yuan X, Li J, Shao T (2016) Ensiling characteristics, structural and nonstructural carbohydrate composition and enzymatic digestibility of Napier grass ensiled with additives. *Bioresour Technol* 221:447–454
- Eliyahu D, Yosef E, Weinberg ZG, Hen Y, Nikbachat M, Solomon R, Mabjeesh SJ, Miron J (2015) Composition, preservation and digestibility by sheep of wet by-products from the food industry. *Anim Feed Sci Technol* 207:1–9
- Evans B (2018) The role ensiled forage has on methane production in the rumen. *Anim Husb Dairy Vet Sci* 2:1–4
- Gao R, Wang B, Jia T, Luo Y, Yu Z (2021) Effects of different carbohydrate sources on alfalfa silage quality at different ensiling days. *Agriculture* 11:58
- He L, Lv H, Chen N, Wang C, Zhou W, Chen X, Zhang Q (2020) Improving fermentation, protein preservation and antioxidant activity of *Moringa oleifera* leaves silage with gallic acid and tannin acid. *Biores Technol* 297:122390
- Karimi M, Sadeghi R, Kokini J (2017) Pomegranate as a promising opportunity in medicine and nanotechnology. *Trends Food Sci Technol* 69:59–73
- Khorsandi S, Riasi A, Khorvash M, Hashemzadeh F (2019) Nutrients digestibility, metabolic parameters and milk production in postpartum Holstein cows fed pomegranate (*Punica granatum* L.) by-products silage under heat stress condition. *Anim Feed Sci Technol* 255:114213
- Kilic A (1986) Silo feed (instruction, education and application proposals). Bilgehan Press, p 327
- Kung L Jr, Shaver R, Grant R, Schmidt R (2018) Silage review: interpretation of chemical, microbial, and organoleptic components of silages. *J Dairy Sci* 101:4020–4033
- Li J, Shen Y, Cai Y (2010) Improvement of fermentation quality of rice straw silage by application of a bacterial inoculant and glucose. *Asian Australas J Anim Sci* 23:901–906
- Makkar HPS, Blümmel M, Borowy NK, Becker K (1993) Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. *J Sci Food Agric* 61:161–165
- Medjekal S, Bodas R, Bousseboua H, López S (2017) Evaluation of three medicinal plants for methane production potential, fiber digestion and rumen fermentation in vitro. *Energy Procedia* 119:632–641
- Menke KH, Steingass H (1988) Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Anim Res Dev* 28:7–55
- Muck RE (2010) Silage microbiology and its control through additives. *Rev Bras Zootec* 39:183–191
- Mutimura M, Myambi C, Gahunga P, Mgheni D, Laswai G, Mtenga L, Gahakwa D, Kimambo A, Ebong C (2013) Rumen liquor from slaughtered cattle as a source of inoculum for in vitro gas production technique in forage evaluation. *Agric J* 8:173–180
- Natalello A, Hervás G, Toral PG, Luciano G, Valenti B, Mendoza AG, Pauselli M, Priolo A, Frutos P (2020) Bioactive compounds from pomegranate by-products increase the in vitro ruminal accumulation of potentially health promoting fatty acids. *Anim Feed Sci Technol* 259:114355
- Niu P, Kreuzer M, Liesegang A, Kunz C, Schwarm A, Giller K (2023) Effects of graded levels of dietary pomegranate peel on methane and nitrogen losses, and metabolic and health indicators in dairy cows. *J Dairy Sci* 106:8627–8641
- Oladosu Y, Rafii MY, Abdullah N, Magaji U, Hussin G, Ramli A, Miah G (2016) Fermentation quality and additives: a case of rice straw silage. *Biomed Res Int* 2016:1–14
- Palmonari A, Federiconi A, Cavallini D, Sniffen C, Mammi L, Turroni S, D'Amico F, Holder P, Formigoni A (2023) Impact of molasses on ruminal volatile fatty acid production and microbiota composition in vitro. *Animals* 13:728
- Pámanes-Carrasco G, García-Piña E, Araiza-Rosales E, Herrera-Torres E (2023) Inclusion of molasses to garlic foliage silage and its effect on in vitro ruminal fermentation and gas production. *J Anim Feed Sci* 32:316–323
- Rooke JA, Hatfield RD (2003) Biochemistry of ensiling. *Silage Sci Technol* 42:95–139
- Van Soest PJ, Robertson JB, Lewis BA (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci* 74:3583–3597
- Wang B, Sun Z, Yu Z (2020a) Pectin degradation is an important determinant for alfalfa silage fermentation through the rescheduling of the bacterial community. *Microorganisms* 8:488
- Wang Y, Zhou W, Wang C, Yang F, Chen X, Zhang Q (2020b) Effect on the ensiling performance and microbial community of adding *Neolamarckia cadamba* leaves to corn stalks. *Microb Biotechnol* 13:1502–1514
- Williams SRO, Hannah MC, Jacobs JL, Wales WJ, Moate PJ (2019) Volatile fatty acids in ruminal fluid can be used to predict methane yield of dairy cows. *Animals* 9:1006
- Yuan X, Wen A, Desta ST, Wang J, Shao T (2017) Effects of sodium diacetate on the fermentation profile, chemical composition and aerobic stability of alfalfa silage. *Asian Australas J Anim Sci* 30:804–810
- Zarina Z, Tan S (2013) Determination of flavonoids in *Citrus grandis* (Pomelo) peels and their inhibition activity on lipid peroxidation in fish tissue. *Int Food Res J* 20:313
- Zhang Y, Yang J, Wang J, Zheng N, Li S, Zhao S, Wen F, Wang J (2016) Progress assessment of chemical indicators of silage. *Chinese J Anim Husbandry* 52:37–42
- Zou X, Chen D, Lv H, Zhang Q, Zheng P (2021) Effect of ellagic acid on fermentation quality and bacterial community of stylo silage. *Fermentation* 7:256

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