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Effects of Applying Lactic Acid Bacteria and Molasses on the Fermentation Quality, Protein Fractions and In Vitro Digestibility of Baled Alfalfa Silage

Yixiao Xie, Jinze Bao, Wenqi Li, Zhiqiang Sun, Run Gao, Zhe Wu and Zhu Yu *

College of Grassland Science and Technology, China Agricultural University, Beijing 100193, China; bs20183040396@cau.edu.cn (Y.X.); SY20183040590@cau.edu.cn (J.B.); lwqnd@cau.edu.cn (W.L.); BS20193040405@cau.edu.cn (Z.S.); gaorun@cau.edu.cn (R.G.); wuzhe@cau.edu.cn (Z.W.)
* Correspondence: 02059@cau.edu.cn

Abstract: Alfalfa sometimes cannot be harvested in time due to the rainy season. To improve the fermentation quality, protein quality and digestibility of alfalfa silage harvested late, *Lactobacillus plantarum* (LP) and molasses were applied in an actual production process in this study. Alfalfa harvested at the full bloom stage was ensiled with (1) distilled water (control), (2) 1×10^6 colony-forming units LP/g fresh matter, (3) 15 g molasses/kg fresh matter (M) or (4) LP + M (LPM) for 55 days. Alfalfa ensiled with LP and/or molasses showed significantly lower pH and ammonia nitrogen contents than the control silage ($p < 0.05$). All additive treatments decreased nonprotein nitrogen contents and preserved more true protein ($p < 0.05$). However, molasses increased the acid detergent insoluble nitrogen content in the protein fractions ($p < 0.05$). The LP significantly improved the maximal cumulative gas production and the maximum gas production rate ($p < 0.05$) in the in vitro trial. Finally, both LP and molasses improved the neutral detergent fiber digestibility of the alfalfa silage ($p < 0.05$). The LP and molasses improved fermentation quality and digestibility and preserved more true protein in baled alfalfa silage harvested late in an actual production process. The LP utilized the excessive molasses and partially ameliorated its negative effects of causing higher acid detergent insoluble nitrogen content.

Keywords: baled alfalfa silage; *Lactobacillus plantarum*; molasses; fermentation quality; protein fractions; in vitro digestibility

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1. Introduction

Ningxia is the major alfalfa-producing area in China. However, alfalfa frequently cannot be harvested in time due to the coming local rainy season, which occurs at the same time as the budding stage to the early bloom stage of alfalfa. Delayed cutting leads to a lower protein content and higher fiber content of alfalfa, resulting in a decline in the quality of silage. Improving the quality of alfalfa silage harvested in the full bloom stage is an urgent problem to be solved.

As alfalfa silage is a regular source of protein in the diet of ruminants, it is important to improve its protein quality. Different studies have been conducted with laboratory silos or tubes to investigate the effects of the addition of lactic acid bacteria (LAB) inoculant on protein preservation, but inconsistent and conflicting protein fraction results have been obtained [1–3]. Molasses is a by-product of the sugar beet and sugarcane industries, and its main component is sucrose. Accordingly, molasses can serve as a substrate for the growth of LAB. It is well known that LAB inoculants and molasses can cooperatively promote silage fermentation [2,4]. In an early laboratory silo-scale study, the addition of molasses decreased the pH and increased the content of acetic acid of pre-bloom alfalfa silage. But the molasses showed no effect in reducing the production of nonprotein nitrogen (NPN) [2]. However, to the best of our knowledge, far less is known about the effect of inoculants

and molasses on the protein quality, fermentation quality, and nutritive value of baled full-bloom alfalfa silage on an actual production scale.

Moreover, alfalfa harvested at the full bloom stage usually contains a high fiber proportion. The digestibility of the fiber is the main factor limiting the efficiency of silage use by ruminants. Similar to the protein fraction, many early laboratory silo-scale studies on the effect of *Lactobacillus plantarum* (LP) on digestibility of forages were conducted and the results have often been inconsistent. In these studies, the application of LP unaffected [2,5,6] and improved [7] the digestibility of alfalfa silage. In fact, many factors such as size of the epiphytic population, harvest stage of forage, characteristics and methods of inoculant application may influence the digestibility of the alfalfa silage [7,8]. Again, there have been few studies on the effects of applying LP on the digestibility of full-bloom alfalfa silage, especially on an actual production scale.

Therefore, the objective of this study was to estimate the effects of LP and molasses on the fermentation quality, protein quality and digestibility of baled alfalfa silage harvested late in an actual production process.

2. Materials and Methods

2.1. Silage Materials and Ensiling

The experiment was conducted in Guyuan, Ningxia, China (106°17′ E, 36°28′ N, elevation 1529 m), on 19 June 2019. The first cutting alfalfa (*Medicago sativa* L. "Longdong") was harvested and wilted at the full bloom stage, leaving a stubble of 5 cm. At the time of harvest, the forage sward has been established for up to 3 years. The wilted alfalfa was chopped into 2–5 cm pieces and baled by a round baler (Comprima, Krone, Germany). Three subsamples of 200 g fresh alfalfa were collected to perform further chemical analysis. Before baling, the alfalfa was treated with (1) distilled water (control), (2) LP, (3) molasses (M) or (4) LP + molasses (LPM). The *Lactobacillus plantarum* strain was isolated from oat silage according to the procedure described by Cai et al. [9]. The identification of ferulic acid easterase (FAE)-producing characteristic of the LP was performed using ethyl ferulate as described by Donaghy et al. [10]. The enzyme activity was measured according to the method of Yue et al. [11] in our previous study. The additives were sprayed and mixed well by the roller mounted on the baler. The application rates of the LP inoculant and the molasses for each bale were 10^6 colony-forming units (cfu)/g and 15 g/kg on a fresh matter (FM) basis, respectively. Each bale (about one cubic meter) contained approximately 800 kg (FM) of alfalfa, and a total of 12 bales (4 treatments × 3 replicates per treatment) were prepared for further analysis. The bales were placed on a flat outdoor ground, and the storage temperature was 16–26 °C. At the time of ensiling, triplicate fresh alfalfa samples were taken for analysis. The bales were unpacked and sampled after ensiling for 55 days.

2.2. Chemical Analysis

At bale opening, the alfalfa silage was mixed well, and two subsamples of 20 g from each bale were homogenized with 180 mL of distilled water for 2 min in a blender jar. The extracts were filtered through four layers of cheesecloth and filter paper. The filtrate was used to determine the pH, ammonia nitrogen (NH_3-N) and organic acid concentration. The pH was measured by a pH meter (PHS-3C, INESA Scientific Instrument Co., Ltd., Shanghai, China), and the NH_3-N content was determined by the sodium hypochlorite and phenol method [12]. The filtrate was further centrifuged at 10,000× *g* at 4 °C for 10 min and processed with a 0.22 μm dialyzer for organic acid analysis. Lactic, acetic, propionic and butyric acids were determined by high-pressure liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) according to our previous report (column: Shodex RS Pak KC-811, Showa Denko KK, Kawasaki, Japan; mobile phase: 3 mmol/L $HClO_4$; flow rate: 1.0 mL/min; column temperature: 50 °C; detection wavelength: 210 nm.) [13]. Two second subsamples of 200 g from each bale were dried by a forced-draft oven at 65 °C for 48 h to determine dry matter (DM) content. The dried samples were ground and passed through a 1.0 mm screen for chemical analysis. Water-soluble carbohydrate (WSC) contents were determined

by the anthrone method [14]. Crude protein (CP) was determined by method 984.13 of the Association of Official Analytical Chemists [15]. The amylase-treated neutral detergent fiber (aNDF) and acid detergent fiber (ADF) were analyzed according to the method of Van Soest et al. [16]. Sodium sulfite and α-amylase were used for the aNDF procedure. Both aNDF and ADF were expressed inclusive of residual ash. The hemicellulose content was estimated as the aNDF value minus the ADF value. The DM, WSC, CP, aNDF and ADF contents of the fresh alfalfa were also measured using the same methods as silage.

2.3. Protein Fraction Analysis

The CP was partitioned into five fractions according to the Cornell Net Carbohydrate and Protein System (CNCPS) [17]. The nonprotein nitrogen, buffer-insoluble protein nitrogen (BIN), neutral detergent insoluble nitrogen (NDIN) and acid detergent insoluble nitrogen (ADIN) were analyzed according to the method of Licitra et al. [18]. Fraction A (PA) and the unavailable fraction (PC) contained NPN and ADIN, respectively. Fraction B (PB) contains the true protein and was further subdivided into rapidly rumen-degradable CP (PB1), intermediate rumen-degradable CP (PB2) and slowly rumen-degradable CP (PB3). The PB fractions were further calculated using CP, NPN, BIN, NDIN and ADIN following the equations described by Licitra et al. [18].

2.4. In Vitro Incubation and Degradability Measurements

Rumen fluid was obtained from four Angus steers before morning feeding. These steers were fed 8 kg (DM) of total mixed ration per day, which was composed of 31.25% whole-plant corn silage, 18.75% corn stalks, 27.5% corn grain, 7.5% jujube powder, 7% soybean meal, 4% corn germ oil meal, 0.75% baking soda, 0.75% salt and 2.5% premix. The rumen fluid was strained through four layers of cheesecloth and mixed under CO_2. An artificial buffer solution was prepared and mixed with the rumen fluid at a ratio of 4:1. The buffer consisted of 1600 mL of buffer A (KH_2PO_4, 10.0 g/L; NaCl, 0.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.5 g/L; $CaCl_2 \cdot 2H_2O$, 0.1 g/L and urea, 0.5 g/L) and 340 mL of buffer B ($Na_2S \cdot 9H_2O$, 1.0 g/L and Na_2CO_3, 15.0 g/L). Approximately 1 g of ground sample of silage was added to 125 mL of the mixture under CO_2. In vitro ruminal fermentation was performed in Ankom RFS bottles (310 mL, Ankom Technologies, Macedon, NY, USA). Gas production was recorded using the pressure transducer technique every hour for 48 h. The determinations were carried out in two separate runs, and each run consisted of 27 bottles: 4 treatments × 3 bales × 2 replicates, plus 3 blanks. The cumulative gas production data were fitted to the Gompertz growth equation [19]:

$$V(t) = V(\infty) \times \exp\left[-\exp\left(ke\left(\lambda - t\right)/V(\infty) + 1\right)\right] \quad (1)$$

where $V(t)$ is the cumulative gas production (mL); $V(\infty)$ is the maximal cumulative gas production (mL); k is the maximum gas production rate (mL/h); λ is the lag time (h); t is the time of incubation (h); and e is the base of the natural logarithm (2.718).

Approximately 0.5 g of ground sample of silage was incubated in nylon bags using new rumen fluid mixtures as described by Menke et al. [20]. The residues were weighed after 48 h of incubation to calculate the in vitro dry matter digestibility (DMD). The aNDF contents of the residues were also determined for calculating the in vitro NDFD. Incubation fluid was collected and centrifuged to determine NH_3-N after incubation [12]. An 800-μL volume of supernatant was mixed with 200 μL of internal standard solution (contains 250 g/L of metaphosphoric acid and 2.17 mL/L of 2-ethylbutyric acid). The mixture was further processed with a 0.22 μm dialyzer to subsequent analyze the volatile fatty acids (VFAs) by a gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) according to the procedure of Qiu et al. (column: Rtx-Wax, 0.25 mm ID × 0.25 μm film, Restek, Evry, France; carrier gas: nitrogen; oven program: initial 110 °C for 30 s, rise to 120 °C in 1 min, 120 °C for 4 min, and gradually rise to 150 °C at a rate of 10 °C/min; split ratio: 20:1; flow rate: 2.5 mL/min) [21].

2.5. Statistical Analyses

The data of fermentation characteristics, chemical composition, protein fractions, in vitro degradability, and fermentation parameters of the incubation fluid were subjected to two-way of variance with the fixed effects of the addition of molasses and inoculant, analyzed by SPSS version 19.0 for Windows (SPSS Inc., Chicago, IL, USA). Duncan's multiple range method was used to judge the differences among the means of the treatments when the interaction is significant. Means were considered significantly different at $p < 0.05$. The gas production kinetic parameters V(∞) and k were also estimated by an iterative least square method using a non-linear regression procedure of SPSS.

3. Results

3.1. Chemical Composition of Alfalfa Before Ensiling

Before ensiling, the alfalfa had a DM content of 459.6 g/kg FM, a WSC content of 79.68 g/kg DM, a CP content of 157.09 g/kg DM, an aNDF content of 406.21 g/kg DM, and an ADF content of 291.52 g/kg DM.

3.2. Fermentation Quality of Baled Alfalfa Silage

The fermentation characteristics and chemical compositions of the baled alfalfa silages are presented in Tables 1 and 2, respectively. The interaction between inoculant and molasses significantly affected ($p < 0.05$) the pH and hemicellulose and NH_3-N levels. The LP, M, and LPM groups showed significantly lower pH and NH_3-N content than the control group ($p < 0.05$). The LP group had lower hemicellulose content than the control group ($p < 0.05$). The addition of molasses decreased the acetic acid content ($p < 0.05$). The LP inoculant decreased the WSC content ($p < 0.05$). Both LP and molasses increased the ratio of lactic acid to acetic acid ($p < 0.05$). No butyric acid was detected in the alfalfa silages.

Table 1. Fermentation characteristics of baled alfalfa silages.

Molasses	0 g/kg		15 g/kg		SEM	Significance		
Inoculant	UN (Control)	IN (LP)	UN (M)	IN (LPM)		I	M	I × M
pH	4.56 ^{a}	4.19 ^{b}	4.15 ^{b}	4.13 ^{b}	0.01	<0.001	<0.001	<0.001
Lactic acid (g/kg DM)	42.47	61.73	53.95	67.61	3.54	0.086	0.186	0.754
Acetic acid (g/kg DM)	4.56	5.55	3.97	2.29	0.38	0.659	0.048	0.267
Propionic acid (g/kg DM)	10.01	11.14	13.07	12.83	0.73	0.938	0.133	0.726
Butyric acid (g/kg DM)	ND	ND	ND	ND	-	-	-	-
Lactic acid: Acetic acid ratio	9.75	12.26	13.9	30.27	1.69	0.036	0.015	0.143
NH_3-N (g/kg TN)	31.06 ^{a}	15.01 ^{b}	16.53 ^{b}	18.12 ^{b}	1.22	0.004	0.013	0.001

DM, dry matter; NH_3-N, ammonia nitrogen; TN, total nitrogen; UN, uninoculated alfalfa silage; IN, inoculated alfalfa silage; LP, *Lactobacillus plantarum* inoculant; M, molasses; LPM, *Lactobacillus plantarum* inoculant + molasses; Means within the same row (a–b) with difference superscripts differ significantly from each other ($p < 0.05$); ND, not detected; SEM, standard error of means, N = 3; I, inoculant; I × M, interaction between inoculant and molasses.

Table 2. Chemical composition of baled alfalfa silages.

Molasses	0 g/kg		15 g/kg		SEM	Significance		
Inoculant	UN (Control)	IN (LP)	UN (M)	IN (LPM)		I	M	I × M
DM (g/kg FM)	446.80	473.63	470.40	446.47	6.92	0.821	0.839	0.071
WSC (g/kg DM)	24.19	18.45	32.32	19.78	1.53	0.026	0.182	0.352
CP (g/kg DM)	145.79	152.77	145.99	149.14	1.25	0.058	0.357	0.669
aNDF (g/kg DM)	423.35	416.43	448.15	425.62	4.79	0.163	0.114	0.439
ADF (g/kg DM)	296.04	306.29	331.58	310.27	4.28	0.537	0.050	0.103
Hemicellulose (g/kg DM)	127.31 ^{a}	110.14 ^{b}	116.57 ^{ab}	115.35 ^{ab}	1.72	0.028	0.444	0.049

DM, dry matter; FM, fresh matter; WSC, water-soluble carbohydrate; CP, crude protein; aNDF, neutral detergent fiber; ADF, acid detergent fiber; UN, uninoculated alfalfa silage; IN, inoculated alfalfa silage; LP, *Lactobacillus plantarum* inoculant; M, molasses; LPM, *Lactobacillus plantarum* inoculant + molasses; Means within the same row (a–b) with difference superscripts differ significantly from each other ($p < 0.05$); SEM, standard error of means, N = 3; I, inoculant; I × M, interaction between inoculant and molasses.

3.3. Protein Fraction of Baled Alfalfa Silage

The protein fractions of baled alfalfa silages are shown in Table 3. Significant interactions between inoculant and molasses were observed for the PA, PB, PB2 and PC levels ($p < 0.05$). The single addition of molasses decreased PA content and increased PB, PB2, and PC content ($p < 0.05$). The LP group had significantly lower PA content and higher PB and PB2 content than the control ($p < 0.05$), but no significant differences in PA and PB content were found between the LP group and LPM group ($p > 0.05$). The LP inoculant increased the PB3 content of the alfalfa silage ($p < 0.05$). Neither LP nor molasses had a significant effect on PB1 content ($p > 0.05$).

Table 3. Protein fractions of baled alfalfa silages.

Molasses	0 g/kg		15 g/kg		SEM	Significance		
Inoculant	UN (Control)	IN (LP)	UN (M)	IN (LPM)		I	M	I × M
PA (% CP)	71.50 ^{a}	66.05 ^{bc}	66.94 ^{b}	65.26 ^{c}	0.18	<0.001	<0.001	0.003
PB (% CP)	24.24 ^{c}	29.64 ^{a}	27.25 ^{b}	29.94 ^{a}	0.18	<0.001	0.013	0.038
PB1 (% CP)	2.24	2.57	3.38	3.04	0.23	0.742	0.210	0.724
PB2 (% CP)	17.33 ^{d}	21.48 ^{a}	18.76 ^{c}	20.26 ^{b}	0.20	<0.001	0.966	0.020
PB3 (% CP)	4.66	5.59	5.11	6.64	0.27	0.048	0.307	0.456
PC (% CP)	4.26 ^{b}	4.32 ^{b}	5.81 ^{a}	4.8 ^{b}	0.09	0.013	0.001	0.009

PA, fraction containing non-protein nitrogen; CP, crude protein; PB, fraction containing true protein; PB1, rapidly rumen-degradable fraction of true protein; PB2, intermediate rumen-degradable fraction of true protein; PB3, slowly rumen-degradable fraction of true protein; PC, fraction containing acid-detergent insoluble nitrogen; UN, uninoculated alfalfa silage; IN, inoculated alfalfa silage; LP, *Lactobacillus plantarum* inoculant; M, molasses; LPM, *Lactobacillus plantarum* inoculant + molasses; Means within the same row (a–d) with difference superscripts differ significantly from each other ($p < 0.05$); SEM, standard error of means, N = 3; I, inoculant; I × M, interaction between inoculant and molasses.

3.4. In Vitro Incubation of Baled Alfalfa Silages

The in vitro net gas production profiles and in vitro parameters of baled alfalfa silages are presented in Figure 1 and Table 4. Since the fermentation curves got plateau in 48 h, the gas production kinetic parameters were also calculated. Although there was no significant difference in DMD among the treatments ($p > 0.05$), both LP and molasses increased the NDFD of the silages ($p < 0.05$). Furthermore, the LP inoculant increased the 24-h cumulative gas production, maximal cumulative gas production and maximum gas production rate of baled alfalfa silage ($p < 0.05$).

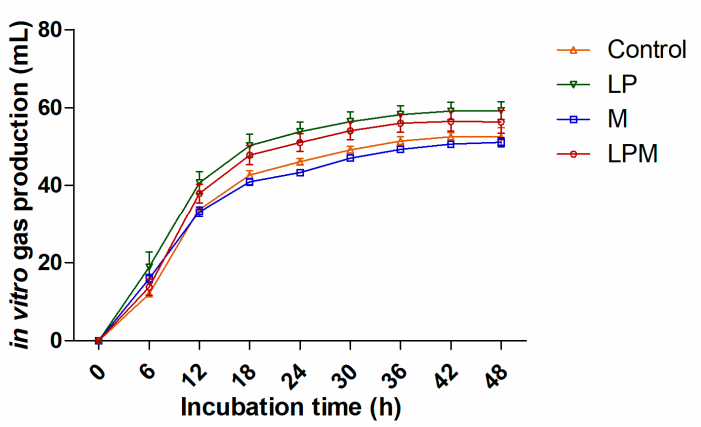

Figure 1. Gas production profiles from in vitro fermentation of the baled alfalfa silages for 48 h (N = 3, bars indicate standard error of the mean). LP, *Lactobacillus plantarum* inoculant; M, molasses; LPM, *Lactobacillus plantarum* inoculant + molasses.

Table 4. In vitro degradability of baled alfalfa silages.

Molasses	0 g/kg		15 g/kg		SEM	Significance		
Inoculant	UN (Control)	IN (LP)	UN (M)	IN (LPM)		I	M	I × M
DMD (g/kg DM)	534.43	588.33	551.77	572.2	14.80	0.137	0.675	0.698
NDFD (g/kg DM)	194.74	291.86	213.43	331.95	5.15	<0.001	0.046	0.358
V_{24h} (mL)	46.02	53.73	43.28	50.96	1.14	0.015	0.273	0.940
V_{48h} (mL)	52.47	59.21	51.06	56.27	1.23	0.062	0.484	0.999
V(∞) (mL)	51.07	57.91	49.56	55.27	1.12	0.037	0.456	0.981
k (mL/h)	3.51	4.06	2.9	4.19	0.18	0.028	0.369	0.622

DMD, dry matter digestibility; DM, dry matter; NDFD, neutral detergent fiber digestibility; V_{24h}, 24-h cumulative gas production; V_{48h}, 48-h cumulative gas production; V(∞), maximal cumulative gas production; *k*, maximum gas production rate; UN, uninoculated alfalfa silage; IN, inoculated alfalfa silage; LP, *Lactobacillus plantarum* inoculant; M, molasses; LPM, *Lactobacillus plantarum* inoculant + molasses; SEM, standard error of means, N = 3; I, inoculant; I × M, interaction between inoculant and molasses.

The fermentation parameters of the incubation fluid of the baled alfalfa silages are presented in Table 5. The interaction between molasses and inoculant significantly affected branched-chain VFA content ($p < 0.05$). The fluids from the M group, LP group, and LPM group had lower branched-chain VFA content than the fluid from the control group ($p < 0.05$), but no significant difference in branched-chain VFA content was found among the treated groups ($p > 0.05$). The addition of molasses significantly decreased the isobutyrate and isovalerate content in the incubation fluid ($p < 0.05$).

Table 5. Fermentation parameters of the incubation fluid of baled alfalfa silages.

Molasses	0 g/kg		15 g/kg		SEM	Significance		
Inoculant	UN (Control)	IN (LP)	UN (M)	IN (LPM)		I	M	I × M
NH_3-N (mg/100 mL)	28.03	25.71	26.00	23.92	1.14	0.258	0.602	0.879
Acetate (mmol/L)	22.74	17.16	26.02	17.33	1.90	0.069	0.460	0.484
Propionate (mmol/L)	10.94	9.89	8.85	8.85	0.39	0.345	0.144	0.746
Isobutyrate (mmol/L)	0.91	0.75	0.64	0.69	0.02	0.216	0.015	0.104
Butyrate (mmol/L)	4.54	3.46	4.63	3.44	0.37	0.128	0.796	0.777
Isovalerate (mmol/L)	1.89	1.38	1.33	1.27	0.06	0.053	0.042	0.146
Valerate (mmol/L)	1.01	0.79	0.84	0.79	0.05	0.150	0.451	0.431
Acetate: Propionate	2.07	1.75	2.93	1.93	0.16	0.068	0.126	0.256
branched-chain VFAs (mmol/L)	2.80 ^{a}	2.13 ^{b}	1.98 ^{b}	1.95 ^{b}	0.07	0.029	0.005	0.039
Total VFAs (mmol/L)	42.04	33.43	42.33	32.36	2.17	0.065	0.930	0.880

NH_3-N, ammonia nitrogen; VFAs, volatile fatty acids; UN, uninoculated alfalfa silage; IN, inoculated alfalfa silage; LP, *Lactobacillus plantarum* inoculant; M, molasses; LPM, *Lactobacillus plantarum* inoculant + molasses; Means within the same row (a–b) with difference superscripts differ significantly from each other ($p < 0.05$); SEM, standard error of means, N = 3; I, inoculant; I*M, interaction between inoculant and molasses.

4. Discussion

All baled alfalfa silages were well preserved after 55 days of ensiling, as indicated by the low pH, dominant lactic acid content, low NH_3-N content, and undetectable butyric acid content. Surprisingly, the pH of the control group also reached 4.56. This result may be due to the high DM of the alfalfa before ensiling. Wilting could decrease the availability of inorganic ions to form a buffer system with weak organic acids in the silage [22].

The WSC in the M group was consumed by the epiphytic LAB, but the consumption was lower than that in the LPM group, as indicated by the latter's higher remaining WSC content. Molasses promoted the growth of the LAB and decreased the acetic acid content in the silage; similar results were reported by Umaña et al. [23]. The LPM group exhibited the highest lactic acid content and the lowest acetic acid content, indicating that the inoculant made good use of the hexoses in molasses for homofermentation. However, lactic acid also is substrate for yeasts and molds, and the higher ratio of lactic acid to acetic acid in the treated groups may make the silage aerobically more unstable [24]. It should be noted that silage with such a high ratio of lactic acid to acetic acid should be used shortly after bales are unpacked.

In general, combining molasses with LAB inoculant decreased the NH_3-N content [2,23], while LP alone contributed to a fairly low NH_3-N content in the present study. Although molasses also decreased the NH_3-N content, it did not seem to aid the inoculant and sometimes even had opposing effects. It is difficult to explain the tiny increase in NH_3-N content in the LPM group. A similar result was reported by Kwak et al. [25], who found that the combination of LAB and molasses resulted in higher NH_3-N content in mushroom substrate silage than the use of molasses alone. We tentatively suggest that molasses also promotes the growth of epiphytic LAB, which have a weaker ability to inhibit protein breakdown than the inoculant. Further study is needed to confirm this speculation.

Though NPN can be utilized by the rumen, it is still important to decrease the proteolysis of true protein in alfalfa silage [26]. In the first few days of ensiling, extensive protein breakdown occurred, and a large amount of NPN was produced [27]. The addition of molasses and LP inoculant inhibited this process and preserved more true protein, similar to the results of Wang et al. [1]. Both LP and molasses inhibited the conversion of the intermediate rumen-degradable fraction of true protein to NPN. Furthermore, the conversion of the slowly rumen-degradable fraction of true protein to NPN was inhibited when the LP was applied. The ADIN contains proteins associated with lignin or tannin and Maillard products, and it cannot be degraded by ruminal bacteria and does not contribute to absorbable amino acids [17]. The addition of molasses increased the ADIN content of

alfalfa silage, similar to the effects reported by Hashemzadeh-Cigari et al. [2]. This proved that the additional true protein provided by molasses in the rumen is partially unavailable to ruminants. This result may be attributed to the higher WSC contents of the groups treated with molasses, which accelerated the Maillard reaction [2]. This reaction usually occurs in alfalfa silage with high protein, carbohydrate and DM contents and usually contributes to increases in fiber constituents [28–30]. The addition of LP consumed most of the molasses (carbohydrate), and an early study showed that some *Lactobacillus* species degrade Maillard products, which is the component of ADIN [31]; thus, the ADIN content was lower in the LPM group than in the M group. The higher content of ADIN might also increase the nitrogen content of the ADF in the M group [32].

Interestingly, though the molasses provided more readily fermentable carbohydrates, the M group did not show higher gas production or a higher maximum gas production rate. This may be partially due to the Maillard products resisting the rumen fermentation [33,34]. The inoculant increased gas production and the gas production rate, in contrast to the report of Contreras-Govea et al. [35], who found that LP had no effect on the in vitro ruminal asymptotic gas production of alfalfa silage. Furthermore, the LP also improved the NDFD of the baled alfalfa silage. The LP applied in this study was identified to have a FAE activity of 69.8 mU and 134.4 mU at 20 °C and 30 °C respectively. The FAE could disrupt the ester bonds and improve the accessibility of the cell wall to depolymerase, making the fiber more convenient for the rumen to degrade [36]. However, since this study could not separate the effect of FAE and other effects from the LP on the parameters we measured, we could not conclude that the increase in digestibility is caused by FAE. Actually, the other inherent characteristics of LP and epiphytic LAB might also have the potential to improve digestibility under certain fermentation conditions. Jin et al. found that a non-FAE-producing inoculant showed a comparable effect to FAE-producing inoculants in improving NDFD in barley silage and mixed small-grain silage [37,38]. Further research is needed to isolate the effect of FAE from the FAE-producing inoculants alone, which may provide further insight into the relationship between the inoculants and the digestibility of silage.

The main end products of structural carbohydrates and nonstructural carbohydrates of rumen fermentation are acetate and propionate, respectively [21]. The M group tended to preserve a higher proportion of carbohydrate structure than the other groups. Branched-chain VFAs, which are the end products of amino acid deamination, have been used as an indicator of ruminal protein degradation [21,39]. The lower isoacid contents in treatment groups than in the control group in the present study indicated that the additives protected the proteins from degradation, which is consistent with the results for the protein components.

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