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Nutritional Quality and In Vitro Rumen Fermentation Characteristics of Silage Prepared with Lucerne, Sweet Maize Stalk, and Their Mixtures

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Abstract: The objective of this work was to evaluate the pH, chemical composition, minerals, vitamins, and in vitro rumen fermentation characteristics of silage prepared with lucerne, sweet maize stalk (MS), and their mixtures. Freshly chopped lucerne and MS were combined in ratios of 100:0 (M0, control), 80:20 (M20), 60:40 (M40), 40:60 (M60), 20:80 (M80), and 0:100 (M100) on a fresh matter basis. Each treatment was prepared in triplicate, and a total of eighteen silos were fermented for 65 days. After 65 days of fermentation, the pH values in M0, M20, M40, M60, M80, and M100 silages were 5.47, 4.84, 4.23, 4.13, 3.79, and 3.61, respectively. As the MS proportion in the mixtures increased, silage K, Ca, P, Na, Fe, and Cu concentrations linearly decreased ($p < 0.001$) and so did vitamins B_5 and K_1 and α-tocopherol. In vitro rumen dry matter and organic matter degradability, pH, ammonia, total volatile fatty acid, and gas production linearly decreased ($p < 0.01$), while neutral detergent fiber concentration linearly increased ($p < 0.001$), with increasing proportion of MS. The in vitro dry matter and organic matter degradability rapidly decreased when the MS percentage was ≥60%. In conclusion, the M40 silage is the most suitable for livestock utilization in local forage production considering the balance of silage pH, nutritional quality, and in vitro ruminal fermentation characteristics.

Keywords: *Medicago sativa*; agricultural by-product; co-ensiling; vitamins; mineral elements; in vitro rumen fermentation

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1. Introduction

With the fast development of animal husbandry, the demand for top-quality green fodder is substantially increasing in China. At present, lucerne (*Medicago sativa* L.) has become an important feed for ruminant animals due to the fact of its high content of crude protein (CP), minerals, and vitamins [1,2]. Lucerne is seasonally harvested and feeding this fresh fodder to ruminants throughout the year is impossible to accomplish. Ensiling is an important method of conserving a moist forage and it can supply livestock with feedstuff throughout the year. However, fresh lucerne is low in water-soluble carbohydrates (WSC) [3,4] and high in buffering capacity (BC) [5], which jointly make it difficult to ensile successfully.

Sweet maize (*Zea mays* L.) is extensively planted in China and many other regions of the world due to the fact of its high sugar content and better flavor [6–9]. China has become the country with the second largest area of growing sweet maize since 2008 [10]. In many provinces of China, sweet maize ear is a popular fruit for human consumption [11]. Sweet maize stalk (MS) remains in the field when maize ear is removed as fruits. Consequently, a

large great deal of MS is accumulated and discarded, which results in bioresource waste. The MS is rich in WSC and low in BC [12–14] and these traits are in favor of MS for ensiling. It is known that lucerne and MS are complementary in terms of CP [12,13]. In addition, one cut of lucerne and the MS harvest periods are usually overlapped in several northern provinces of China such as Hebei [15]. Hence, inclusion of MS to lucerne upon ensiling may improve silage fermentation and be beneficial to lucerne conservation. The aim of making silage is to eventually feed ruminants and carrying out in vitro rumen fermentation trials makes a contribution to preliminarily understanding the nutritive value of silage [16]. Evidence shows that ensiling lucerne with whole-plant maize or sweet sorghum (*Sorghum bicolor* L.) improves the fermentation quality of the mixed silage and that inclusion of lucerne increases silage CP and mineral levels [2,3,14,17]. The vitamin-mineral supplement contributes to ruminant growth and production performance and it is often supplemented to the ruminant diet [2]. Mixing lucerne and MS for making silage may have associative effects in terms of silage pH, nutritional quality, and nutritive value. To date, however, there is little information regarding the pH, chemical composition, minerals, vitamins, and nutritive value of silage prepared with lucerne, MS, and their mixtures. Thus, the objective of this current study was to investigate the influence of combining lucerne with MS in different ratios on silage pH, chemical composition, minerals, vitamins, and in vitro ruminal fermentation parameters. It was hypothesized that the optimal treatment(s) should be attained at the intermediate proportion(s) of MS in terms of silage pH, nutritional quality, and in vitro fermentation profile.

2. Materials and Methods

2.1. Silage Making

Lucerne and maize were grown at the Zhuozhou Experimental Station (N 39°35′25″–39°36′05″, E 115°42′12″–116°14′35″) of the China Agricultural University, Hebei, China. The third-cut lucerne at the late bud stage of maturity was manually harvested from three plots (4 m^2) selected randomly and chopped to approximately 2 cm by paper cutters (MC-440, Beijing Centry Jintu Co., Ltd., Beijing, China) on 25 August 2017. Meanwhile, sweet maize ears at the milk stage of maturity in three plots (8 m^2) were picked as fruits and the remaining stalk was harvested by hand. The collected MS was chopped to approximately 2 cm with a forage chopper (680 type, Qufu Muyuan Machinery Co., Ltd., Qufu, China). Each chopped forage was grouped into eighteen small piles. Lucerne and MS were randomly sampled from respective piles above and combined in ratios of 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100 on a fresh matter (FM) basis, thereby generating treatments of M0 (control), M20, M40, M60, M80, and M100, respectively. Three hundred grams of each of the lucerne-MS mixtures were manually packed into a plastic bag (20 × 30 cm) and vacuumed using a sealer. As to the mixing ratio of 80:20, for instance, 240 g of chopped lucerne was adequately mixed with 60 g of chopped MS by hand in a plastic basin. The mixing procedure of the remaining ratios was similar to the ratio of 80:20. Each treatment was generated in triplicate and a total of eighteen silos were stored for 65 days at room temperature (25–26 °C).

2.2. Silage Quality Analysis

2.2.1. Chemical Parameter Analysis

A 20 g sample was blended with 180 mL of distilled water for 1 min, followed by filtration through double layers of cheesecloth. The supernatant was used for pH determination using an electrode (PHS-3C, INESA, Shanghai, China). Another 200 g sample was dried for 48 h in an air-forced oven at 65 °C to determine dry matter (DM) and ground through a 1 mm sieve with a mill. The ground sample was assayed for CP, ash, and ether extract (EE) according to AOAC (2005) [18]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to Mertens (2002) [19] and Van Soest et al. (1973) [20], respectively, and expressed with residual ash. Hemicellulose (HL) was calculated by the difference between NDF and ADF. Relative feed value (RFV) was estimated in accordance

with Linn and Martin (1989) [21] and RFV = 120 (88.9 − (0.779 × ADF))/(1.29 × NDF). Nitrate was analyzed via the salicylic acid method [22].

2.2.2. Mineral Analysis

Concentrations of K, Ca, Mg, P, Na, Fe, Zn, Mn, Cu, and Ni were determined by simultaneous ICP-OES (7500C, Perkinelmer Inc., Waltham, MA, USA). The silage samples and standard solutions were prepared according to Pacquette and Thompson (2018) [23]. The detection levels for K, Ca, Mg, P, Na, Fe, Zn, Mn, Cu, and Ni were 7, 5, 5, 1, 3, 1, 0.5, 0.1, 0.2, and 0.5 mg/kg DM, respectively.

2.2.3. Vitamin Analysis

The vitamin analysis was performed at the Institute of Resources Environment and Detection Technology, Inner Mongolia Academy of Agricultural and Husbandry Sciences, Hohhot, China. All vitamins, with the exception of vitamin K_3, were analyzed according to AOAC (2016) [24], which contained vitamins B_1, B_2, B_3, B_5, B_6, and K_1 and α-tocopherol. Concentration of vitamin K_3 was determined according to Laffi et al. (1988) [25]. The detection level for vitamins B_2, B_3, and B_5 was 0.02 mg/kg DM and the detection level for vitamins B_1, B_6, C, K_1, K_3, and α-tocopherol was 0.01 mg/kg DM.

2.3. Measurement of In Vitro Fermentation Traits

2.3.1. Preparation of Rumen Fluids and Buffered Inoculums

The rumen fluids and buffered inoculums were prepared according to Menke and Steingass (1988) [26]. Three Simmental × Limousin cross-bred steers with an average body weight of 452 ± 18 kg were fitted with permanent rumen cannula and fed twice daily. The diet was composed of maize silage (456 g/kg DM), maize grain (262 g/kg DM), brewer (112 g/kg DM), bean curd residue (100 g/kg DM), cottonseed meal (30 g/kg DM), and a vitamin-mineral supplement (40 g/kg DM). Prior to morning feeding, rumen fluids were collected from the aforementioned steers, harvested by filtration through double layers of cheesecloth into two pre-heated (39 °C) thermo bottles and transferred to the laboratory within 30 min. The buffered inoculums were produced by combining rumen fluids with the buffer solution in a 1:2 (v/v) ratio under a continuous flux of CO_2.

2.3.2. In Vitro Incubations

In vitro incubations were carried out by the method of Menke et al. (1979) [27] and contained two parts, namely, analysis of in vitro rumen DM degradability (IVDMD) and organic matter degradability (IVOMD), rumen pH, ammonia (NH_3), and volatile fatty acid (VFA) and gas production (GP) tests. For the first part, approximately 220 mg of DM was put into a nylon bag, sealed, and pre-heated at 39 °C. A total of 76 bags (four per triplicate sample and another four without sample served as blanks) were used. Each bag was placed into a 100 mL glass syringe filled with 30 mL of buffered inoculum and incubated for 24 h in an incubator with a slow shaking (150 rpm) at 39 °C. For the second part, approximately 220 mg of silage was directly placed into a syringe with 30 mL of buffered inoculum. A total of 38 syringes (two per triplicate sample and another two with only buffered inoculum served as blanks) were incubated for 2, 4, 6, 8, 10, 12, 16, 20, and 24 h at 39 °C. The GP was manually documented at each time point and calibrated by blanks.

2.3.3. In Vitro Rumen Dry Matter and Organic Matter Degradability, Rumen pH, Ammonia, and Volatile Fatty Acid Analysis

After 24 h of incubation, the bags were taken out from the syringes, washed with tap water, dried for 48 h at 65 °C, and weighted for IVDMD determination. The dried residue was used for ash determination and IVOMD calculation. The fermented liquid was measured for pH using an electrode and further centrifugated at 7500× g for 10 min at 4 °C. The NH_3 was tested according to Broderick and Kang (1980) [28]. The VFA profile was detected by GS 3420 gas chromatography according to Erwin et al. (1961) [29]. Total

VFA concentration was counted by summing the area of individual VFA and calibrated by blanks. Each VFA was shown as mol/100 mol of total VFA.

2.4. Calculations and Statistical Analysis

To explore the kinetics of in vitro fermentation, the GP produced was adjusted to the model described by France et al. (2000) [30]:

$$A = b \times (1 - e^{-c(t-l)})$$

where A (mL/g DM) is the volume of GP at time t (h); b (mL/g DM) is the asymptotic GP; c (mL/h) is the rate of GP; l (h) is the lag time prior to GP. The fermentation kinetic parameters (b, c, and l) were estimated through a nonlinear regression model using the SPSS statistical package (version 22.0, SPSS Inc., Chicago, IL, USA) NLIN program. One silo served as an experimental unit and data were analyzed using the SPSS statistical software. Silage pH, chemical composition, mineral profile, vitamins, IVDMD, IVOMD, ruminal pH, NH_3, VFA, GP, and kinetic parameters were tested by analysis of variance using the GLM procedure in SPSS with the treatments (M0, M 20, M 40, M 60, M 80, and M 100) as the fixed effect. The model used was as follows:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where Y_{ij} is the dependent variable; μ is the overall mean; T_i is the impact of treatment; e_{ij} is the error term; i is 1–6; j is the observation number. The Duncan test was used for multiple comparisons of means across treatments. The impact was considered significant at a probability level of $p < 0.05$. Orthogonal polynomial contrasts were employed to test the linear, quadratic, and cubic effects of the MS inclusion in the mixtures on silage pH, nutritional quality, and in vitro rumen parameters. The coefficients of polynomial contrasts were generated in PROC IML of SAS (Version 9.1, SAS Institute Inc., Cary, NC, USA) for the equally spaced MS inclusion. Statistical significance was declared at $p < 0.05$.

3. Results

3.1. Chemical Characteristics of Ensiling Materials

Concentrations of NDF, ADF, EE, ash, and nitrate in fresh lucerne and MS were 416 and 572 g/kg DM, 307 and 298 g/kg DM, 30.7 and 19.9 g/kg DM, 106 and 51 g/kg DM, and 630 and 548 mg/kg DM, respectively. The DM, WSC, CP, and pH values reported previously by Wang et al. (2019) [31] were 187 and 222 g/kg FM, 40.2 and 144.2 g/kg DM, 281 and 112 g/kg DM, and 6.51 and 5.56, respectively.

3.2. Chemical Composition of Silage

Silage pH and chemical composition are displayed in Table 1. Levels of pH, CP, RFV, EE, and ash linearly decreased ($p < 0.001$), whereas DM, NDF, HL, and nitrate concentrations linearly increased ($p < 0.001$), with more MS in the mixtures. There were no significant differences among treatments in the concentration of ADF ($p > 0.05$).

3.3. Minerals of Silage

Table 2 shows the minerals in silage. Concentrations of K, Ca, P, Na, Fe, and Cu linearly decreased ($p < 0.001$), whereas Mg and Mn concentrations linearly increased ($p < 0.001$), as the MS percentage increased from 0% to 100%.

3.4. Vitamins of Silage

According to Table 3, readings of Vitamins B_5 and K_1 and α-tocopherol linearly decreased ($p < 0.001$), whereas vitamins B_2, B_6, C, and K_3 contents linearly increased ($p < 0.001$), with more MS included in the forage mixtures.

Table 1. The pH and chemical composition of silage prepared with lucerne, sweet maize stalk, and their mixtures (n = 3).

Item [1]	Silage [2]						SEM [3]	*p*-Value [4]			
	M0	M20	M40	M60	M80	M100		T	L	Q	C
pH	5.47 ^{a}	4.84 ^{b}	4.23 ^{c}	4.13 ^{d}	3.79 ^{e}	3.61 ^{f}	0.153	<0.001	<0.001	<0.001	0.001
DM (g/kg FM)	194 ^{e}	208 ^{d}	219 ^{c}	220 ^{c}	230 ^{b}	238 ^{a}	3.4	<0.001	<0.001	0.115	0.084
CP (g/kg DM)	207 ^{a}	201 ^{a}	179 ^{b}	161 ^{c}	136 ^{d}	105 ^{e}	8.6	<0.001	<0.001	0.001	0.156
NDF (g/kg DM)	395 ^{f}	434 ^{e}	467 ^{d}	514 ^{c}	530 ^{b}	550 ^{a}	13.3	<0.001	<0.001	0.084	0.113
ADF (g/kg DM)	309	305	306	316	310	316	1.8	0.314	0.381	0.754	0.165
RFV	152 ^{a}	139 ^{b}	129 ^{c}	116 ^{d}	113 ^{de}	108 ^{e}	3.8	<0.001	<0.001	0.043	0.253
HL (g/kg DM)	85.8 ^{e}	129.0 ^{d}	161.3 ^{c}	198.2 ^{b}	220.2 ^{a}	220.2 ^{a}	12.03	<0.001	<0.001	0.001	0.592
EE (g/kg DM)	30.3 ^{a}	30.1 ^{a}	29.3 ^{a}	27.3 ^{b}	23.4 ^{c}	19.5 ^{d}	0.97	<0.001	<0.001	<0.001	0.339
Ash (g/kg DM)	107 ^{a}	95 ^{b}	78 ^{c}	71 ^{d}	60 ^{e}	52 ^{f}	4.5	<0.001	<0.001	<0.001	<0.001
Nitrate (mg/kg DM)	66 ^{d}	74 ^{d}	94 ^{c}	108 ^{c}	190 ^{b}	250 ^{a}	16.4	<0.001	<0.001	<0.001	0.006

[1] DM, dry matter; FM, fresh matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; RFV, relative feed value; EE, ether extract; HL, hemicellulose. [2] Lucerne and sweet maize stalk were combined in ratios of 100:0 (M0), 80:20 (M20), 60:40 (M40), 40:60 (M60), 20:80 (M80), and 0:100 (M100) on a fresh matter basis; $^{a–f}$ the same rows with different superscripts are significantly different ($p < 0.05$). [3] SEM, standard error of the mean. [4] T, treatment; L, linear; Q, quadratic; C, cubic.

Table 2. Mineral profile (mg/kg DM) of silage prepared with lucerne, sweet corn maize, and their mixtures (n = 3).

Item	Silage [1]						SEM [2]	*p*-Value [3]			
	M0	M20	M40	M60	M80	M100		T	L	Q	C
K	26,903 ^{a}	22,174 ^{b}	15,423 ^{c}	12,150 ^{d}	7620 ^{e}	4496 ^{f}	1913.5	<0.001	<0.001	0.248	0.780
Ca	16,119 ^{a}	14,753 ^{a}	10,172 ^{b}	9323 ^{b}	6990 ^{c}	5512 ^{c}	947.9	<0.001	<0.001	0.412	0.352
Mg	4162 ^{c}	5005 ^{b}	4820 ^{bc}	5846 ^{a}	5835 ^{a}	6480 ^{a}	206.3	<0.001	<0.001	0.613	0.990
P	4144 ^{a}	3296 ^{b}	2873 ^{c}	2572 ^{c}	2237 ^{d}	1955 ^{d}	178.5	<0.001	<0.001	<0.001	0.035
Na	1585 ^{a}	1235 ^{b}	897 ^{c}	526 ^{d}	450 ^{d}	87 ^{e}	122.9	<0.001	<0.001	0.010	0.066
Fe	180 ^{a}	158 ^{b}	139 ^{c}	130 ^{d}	123 ^{de}	116 ^{e}	5.4	<0.001	<0.001	0.002	0.981
Zn	35.2 ^{bc}	35.2 ^{bc}	34.4 ^{bc}	39.1 ^{a}	36.4 ^{ab}	33.2 ^{c}	0.54	0.01	0.064	0.967	0.056
Mn	20.7 ^{c}	28.4 ^{ab}	26.2 ^{b}	30.0 ^{a}	28.7 ^{ab}	30.5 ^{a}	0.87	<0.001	<0.001	0.013	0.193
Cu	13.2 ^{ab}	14.7 ^{a}	12.3 ^{b}	12.4 ^{b}	10.3 ^{c}	8.5 ^{d}	0.52	<0.001	<0.001	0.027	0.275
Ni	5.3 ^{a}	4.0 ^{b}	4.9 ^{ab}	4.9 ^{ab}	4.4 ^{ab}	4.3 ^{ab}	0.15	0.045	0.334	0.577	0.009

[1] Lucerne and sweet maize stalk were combined in ratios of 100:0 (M0), 80:20 (M20), 60:40 (M40), 40:60 (M60), 20:80 (M80), and 0:100 (M100) on a fresh matter basis; $^{a–f}$ the same rows with different superscripts are significantly different ($p < 0.05$). [2] SEM, standard error of the mean. [3] T, treatment; L, linear; Q, quadratic; C, cubic.

Table 3. Vitamin profile (mg/kg DM) of silage prepared with lucerne, sweet maize stalk, and their mixtures (n = 3).

Item	Silage [1]						SEM [2]	*p*-Value [3]			
	M0	M20	M40	M60	M80	M100		T	L	Q	C
Water-soluble vitamins											
Vitamin B_1	3.62 ^{a}	0.85 ^{d}	1.09 ^{cd}	1.29 ^{bcd}	1.70 ^{b}	1.46 ^{bc}	0.237	<0.001	<0.001	<0.001	<0.001
Vitamin B_2	14.1 ^{d}	17.9 ^{ab}	16.2 ^{c}	16.8 ^{bc}	17.0 ^{bc}	18.9 ^{a}	0.39	<0.001	0.004	0.009	0.002
Vitamin B_3	8.86 ^{b}	11.23 ^{a}	9.75 ^{ab}	10.92 ^{a}	9.35 ^{ab}	7.97 ^{b}	0.342	0.018	0.734	0.052	0.593
Vitamin B_5	103.3 ^{a}	83.5 ^{b}	45.2 ^{c}	40.6 ^{c}	33.5 ^{d}	26.7 ^{e}	6.82	<0.001	<0.001	<0.001	0.019
Vitamin B_6	1.55 ^{b}	1.59 ^{b}	2.98 ^{a}	1.63 ^{b}	2.07 ^{b}	1.73 ^{b}	0.134	<0.001	0.049	0.006	0.388
Vitamin C	3.53 ^{f}	7.32 ^{e}	21.36 ^{d}	25.74 ^{c}	46.17 ^{b}	50.42 ^{a}	4.301	<0.001	<0.001	<0.001	0.003
Fat-soluble vitamins											
α-tocopherol	807.1 ^{a}	745.0 ^{a}	645.3 ^{b}	505.7 ^{c}	418.8 ^{d}	370.7 ^{d}	40.03	<0.001	<0.001	0.058	0.029
Vitamin K_1	31.4 ^{a}	26.8 ^{b}	21.9 ^{c}	17.3 ^{d}	15.2 ^{d}	11.1 ^{e}	1.73	<0.001	<0.001	0.082	0.285
Vitamin K_3	1.47 ^{e}	2.09 ^{d}	2.57 ^{c}	3.36 ^{b}	4.01 ^{a}	4.35 ^{a}	0.252	< 0.001	< 0.001	0.046	1.000

[1] Lucerne and sweet maize stalk were combined in ratios of 100:0 (M0), 80:20 (M20), 60:40 (M40), 40:60 (M60), 20:80 (M80), and 0:100 (M100) on a fresh matter basis; $^{a–f}$ the same rows with different superscripts are significantly different ($p < 0.05$). [2] SEM, standard error of the mean. [3] T, treatment; L, linear; Q, quadratic; C, cubic.

3.5. In Vitro Rumen Degradability, Rumen Fermentation Parameters, and Gas Production of Silage

As shown in Table 4, readings of IVDMD, IVOMD, rumen pH, NH_3, total VFA, propionate, iso-butyrate, valerate, and iso-valerate linearly decreased ($p < 0.01$) and the acetate to propionate and butyrate values increased ($p < 0.05$), with a greater proportion of MS. No significant differences among treatments in the concentration of acetate were observed ($p > 0.05$). Over the whole in vitro incubation, the silage GP amount deceased ($p < 0.01$) with an increasing ratio of MS (Table 5).

Table 4. In vitro dry matter and organic matter degradability, rumen pH, ammonia, and volatile fatty acid of rumen-incubated silage prepared with lucerne, sweet maize stalk, and their mixtures after 24 h of incubation ($n = 3$).

Item [1]	Silage [2]						SEM [3]	*p*-Value [4]			
	M0	M20	M40	M60	M80	M100		T	L	Q	C
IVDMD (g/kg DM)	510 ^{a}	493 ^{b}	482 ^{c}	434 ^{d}	428 ^{d}	413 ^{e}	6.2	<0.001	<0.001	0.138	0.001
IVOMD (g/kg DM)	503 ^{a}	460 ^{c}	479 ^{b}	422 ^{de}	430 ^{d}	407 ^{e}	8.2	<0.001	<0.001	0.209	0.903
pH	6.84 ^{a}	6.80 ^{ab}	6.75 ^{bc}	6.72 ^{c}	6.69 ^{cd}	6.63 ^{d}	0.022	<0.001	0.001	0.746	0.831
NH_3 (mg/dL)	27.4 ^{a}	25.4 ^{b}	20.2 ^{c}	18.2 ^{d}	17.1 ^{de}	15.7 ^{e}	1.01	<0.001	<0.001	0.003	0.132
Total VFA (mM)	26.8 ^{a}	25.2 ^{ab}	24.0 ^{bc}	23.6 ^{bc}	21.9 ^{cd}	19.9 ^{d}	0.61	0.002	0.002	0.878	0.589
Acetate	63.6	64.4	66.0	66.0	65.5	65.2	0.45	0.667	0.215	0.402	0.769
Propionate	23.5 ^{a}	21.4 ^{ab}	21.4 ^{ab}	20.4 ^{bc}	19.8 ^{bc}	18.9 ^{c}	0.42	0.01	0.004	0.445	0.441
Acetate to propionate	2.72 ^{b}	3.02 ^{ab}	3.08 ^{ab}	3.24 ^{a}	3.30 ^{a}	3.45 ^{a}	0.074	0.048	0.015	0.491	0.768
Butyrate	4.44 ^{e}	5.82 ^{d}	7.70 ^{c}	8.59 ^{c}	10.14 ^{b}	12.43 ^{a}	0.655	<0.001	<0.001	0.579	0.874
iso-Butyrate	3.15 ^{a}	3.14 ^{a}	1.34 ^{b}	1.53 ^{b}	1.24 ^{b}	0.85 ^{b}	0.254	0.002	0.002	0.357	0.314
Valerate	0.51 ^{a}	0.42 ^{ab}	0.26 ^{bc}	0.21 ^{cd}	0.15 ^{cd}	0.07 ^{d}	0.041	0.001	<0.001	0.374	0.666
iso-Valerate	4.70 ^{a}	4.71 ^{a}	3.19 ^{b}	3.15 ^{b}	3.01 ^{b}	2.49 ^{b}	0.244	0.003	0.002	0.432	0.275

[1] IVDMD, in vitro DM degradability; IVOMD, in vitro organic matter degradability; DM, dry matter; NH_3, ammonia; VFA, volatile fatty acid, and each VFA is shown as mol/100 mol. [2] Lucerne and sweet maize stalk were combined in ratios of 100:0 (M0), 80:20 (M20), 60:40 (M40), 40:60 (M60), 20:80 (M80), and 0:100 (M100) on a fresh matter basis; $^{a–e}$ the same rows with different superscripts are significantly different ($p < 0.05$). [3] SEM, standard error of the mean. [4] T, treatment; L, linear; Q, quadratic; C, cubic.

Table 5. In vitro gas production (mL/g DM) dynamics and estimated parameters of silage prepared with lucerne, sweet maize stalk, and their mixtures ($n = 3$).

Item [1]	Silage [2]						SEM [3]	*p*-Value [4]			
	M0	M20	M40	M60	M80	M100		T	L	Q	C
2 h	29.4 ^{a}	26.7 ^{b}	25.8 ^{b}	27.4 ^{ab}	20.7 ^{c}	19.6 ^{c}	0.67	<0.001	<0.001	0.077	0.001
4 h	53.9 ^{a}	50.2 ^{b}	49.5 ^{b}	48.4 ^{b}	41.0 ^{c}	39.8 ^{c}	0.95	<0.001	<0.001	0.069	0.014
6 h	69.2 ^{a}	69.0 ^{a}	70.6 ^{a}	63.9 ^{b}	63.2 ^{bc}	60.0 ^{c}	0.77	<0.001	<0.001	0.211	0.003
8 h	90.4 ^{a}	86.0 ^{b}	85.3 ^{b}	84.4 ^{b}	82.5 ^{b}	71.6 ^{c}	1.08	<0.001	0.012	0.342	<0.001
10 h	105.9 ^{a}	103.0 ^{ab}	100.7 ^{b}	101.4 ^{b}	96.6 ^{c}	84.1 ^{d}	1.31	<0.001	0.124	0.904	<0.001
12 h	118.2 ^{a}	115.2 ^{ab}	115.8 ^{ab}	112.7 ^{bc}	109.2 ^{c}	97.4 ^{d}	1.29	<0.001	0.513	0.714	<0.001
16 h	130.2 ^{a}	130.7 ^{a}	130.7 ^{a}	126.1 ^{a}	127.3 ^{a}	120.6 ^{b}	0.86	0.001	0.006	0.710	0.003
20 h	146.4 ^{a}	145.4 ^{a}	146.0 ^{a}	138.9 ^{b}	136.9 ^{b}	133.6 ^{b}	1.09	<0.001	<0.001	0.189	0.559
24 h	159.3 ^{a}	156.2 ^{a}	155.1 ^{a}	153.7 ^{a}	144.3 ^{b}	137.2 ^{c}	1.53	<0.001	<0.001	0.138	0.179
Estimated parameters											
b (mL/g DM)	183.4	180.7	181.2	178.1	163.3	174.1	2.85	0.365	0.081	0.332	0.533
c (mL/h)	0.08 ^{b}	0.08 ^{b}	0.09 ^{ab}	0.08 ^{b}	0.10 ^{a}	0.08 ^{b}	0.011	0.042	0.220	0.257	0.367
l (h)	–0.14 ^{c}	0.08 ^{bc}	0.14 ^{bc}	0.00 ^{bc}	0.75 ^{a}	0.39 ^{ab}	0.074	0.001	0.001	0.129	0.029

[1] DM, dry matter; b, the asymptotic gas production; c, the rate of gas production; l, the lag time prior to gas production. [2] Lucerne and sweet maize stalk were combined in ratios of 100:0 (M0), 80:20 (M20), 60:40 (M40), 40:60 (M60), 20:80 (M80), and 0:100 (M100) on a fresh matter basis; $^{a–d}$ the same rows with different superscripts are significantly different ($p < 0.05$). [3] SEM, standard error of the mean. [4] T, treatment; L, linear; Q, quadratic; C, cubic.

4. Discussion

4.1. Silage pH and Nutritional Quality

It is well acknowledged that a low pH in the range of 3.6–4.5 is a basic indicator of well-fermented silages [32]. The pH of the M40, M60, M80, and M100 silages ranged from 4.23 to 3.61, falling into the range of 3.6–4.5 and indicating sufficient lactic acid production, whereas the pH values in M0 and M20 silages ranged from 5.47 to 4.84, which indicated unsatisfactory fermentation [5]. The higher pH in these silages was possibly attributed to a low WSC concentration and a high BC value, which jointly hindered silage pH decline during fermentation [5,33]. Regretfully, both WSC and BC in silage were not determined in the present work in addition to organic acids, ammonia nitrogen, and ethanol. The NDF concentration was numerically lower in M0 or M100 silage after 65 days of fermentation compared to respective fresh herbage. A possible explanation is that the acid environment produced in the silage hydrolyzed part of cell wall fraction [34]. Likewise, a great deal of nitrate in ensiling materials was degraded after 65 days of fermentation in the present work and this finding was in accordance with Wang et al. (2018) [35], who reported that nitrate concentration substantially decreased from 383 mg/kg DM in fresh maize to 61–91 mg/kg DM in maize silage after 55 days of ensiling. All or a part of the nitrate present in a forage crop is broken down by plant enzymes and microbes, and nitrite and nitric oxide are temporarily accumulated during the early stage of fermentation [36]. Degradation is complete in poorly fermented silage and high pH and ammonia nitrogen values correlate with nitrate reduction [37,38].

The greater concentrations of K, Ca, P, Na, and Fe in the M0 silage compared to the M100 silage was observed in the present work, which confirms the results of Zhang et al. (2015) [2]. Nonetheless, Fe concentration (117–180 mg/kg DM) in the present work was lower than the data documented by the authors above. Differences in mineral content across forages depend on many factors such as plant species, growth environment, and fertilizer application [2]. In addition, Miller et al. (2015) investigated the impact of long-term manure application on the mineral composition of irrigated barley silage and found that with a greater application rate, concentrations of P, K, Na, and K to Ca + Mg ratio increased, but Ca and Mg concentrations decreased at higher rates compared to a low rate [39]. More studies on minerals are needed to increase our understanding of their changes during ensiling.

The α-tocopherol in forage is an antioxidant and plays an important role in the immune system of ruminants [40]. A great α-tocopherol concentration (807 mg/kg DM) was detected in the M0 silage. Similarly, Wang and Yu (2020) recorded that the α-tocopherol concentration in lucerne silage with different DM levels reached up to 1217 mg/kg DM after 30 days of ensiling [41]. However, Jia et al. (2019) reported that its concentration was 142 mg/kg DM [1]. Moreover, it is reported that ensiling can decrease silage α-tocopherol concentration [1,42]. Kalač and Kyzlink (1979) found that poor fermentation quality led to greater α-tocopherol loss [43]. Zong et al. (2021) stated that the application of *Lactobacillus* inoculation decreased silage α-tocopherol loss via altering the relative abundance of cocci lactic acid bacteria and *Citrobacter* [42]. However, Silage fermentation does not always decrease α-tocopherol. Lindqvist et al. found that the α-tocopherol concentration of birdsfoot trefoil (*Lotus corniculatus* L.) and timothy (*Phleum pratense* L.) mixtures remained unchanged after 100 days of fermentation [44]. These variations can be explained by plant species, plant maturity stage, silage quality, ensiling time, etc. [1,41,43,44].

4.2. In Vitro Rumen Degradability, Rumen Fermentation Parameters, and Gas Production of Silage

Digestibility largely affects forage intake of ruminant animals and lucerne often has a relatively high DM digestibility, and IVDMD and IVOMD are positively related to total VFA production [2]. The reduced IVDMD in silage with more MS was associated with the NDF increase and CP decrease, leading to a drop in total degradable DM. This result was consistent with Zhang et al. (2015) [2] and Chen et al. (2019) [45]. Silage

IVDMD and IVOMD decreased significantly from 482 and 479 g/kg DM to 434 and 422 g/kg DM, respectively, when MS percentage increased from 40% to 60%. In addition, no significant differences between the M60 silage and M80 silage were recorded for IVDMD and IVOMD. This indicates that if a high DM or organic matter degradability is needed to be obtained, the MS percentage in the mixtures should be below 60%. The rumen pH in the present work ranged from 6.63 to 6.84, which fell into the normal range of 5.5–7.0 [45]. The greater NH_3 concentration in the M0 silage reflected a higher protein decomposition occurring during in vitro fermentation than the M100 silage. This finding was in accordance with Zhang et al. (2015) [2]. In addition, Dhiman and Satter (1997) stated that the higher rumen NH_3 concentration is related to the higher CP content of lucerne silage compared to maize silage [46]. The decreased propionate concentration and increased butyrate concentration of fermented liquid with more MS might be attributed to ruminal microbial community. Fermentation products of *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* consist mainly of succinate, which can be employed to generate propionate in the rumen [47]. Likewise, the *F. succinogenes* and *R. flavefaciens* populations in silage increased, accompanied by increased propionate concentration, as the sweet sorghum (*Sorghum bicolor* L.) proportion in the mixtures increased from 25% to 100% [45]. Branched-chain VFA could arise from the fermentation of branched-chain amino acids [48]. Therefore, the higher iso-butyrate and iso-valerate concentrations in the M0 silage compared to the M100 silage in the present work could be because of a higher CP concentration and its extensive degradation.

In vitro GP is highly reliant on the availability of soluble fraction and reflects silage DM degradation extent [49]. A higher GP was detected in the M0 silage relative to the M100 silage during 24 h of in vitro fermentation. A possible explanation is that the M0 silage had a lower NDF content compared to the M100 silage, as a negative relationship between NDF and GP was reported by Chen et al. (2019) [45]. A higher structural carbohydrate concentration can hinder GP by depressing microbial fermentation or enzymatic hydrolysis of forage polysaccharides [50].

5. Conclusions

Ensiling fresh lucerne alone produced a silage of higher pH compared to the M100 silage. Meanwhile, ensiling MS alone resulted in a lower IVDMD and IVOMD value compared to the M0 silage. The overall quality of the lucerne-MS mixture silage was higher than the silage gained from ensiling lucerne or MS alone. It can be concluded that the M40 treatment is the most suitable for making high-quality silage in local forage production considering the balance of silage pH, nutritional quality, and in vitro ruminal DM and organic matter degradability.

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