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Effect of the addition of pre-fermented juice and soluble carbohydrates to alfalfa silage on silage quality

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ABSTRACT: The purpose of this study was to ascertain how fermented natural lactic acid bacteria (LAB) and different soluble carbohydrate sources affected the silage quality characteristics, *in vitro* organic matter digestibility (IVOMD), metabolizable energy (ME) and *in vitro* methane gas formation of alfalfa (*Medicago sativa* L.) silage. In the study, an analysis (pH, total LAB, yeast-mold, lactic acid (LA), acetic acid (AA), and LA/AA) was conducted on the naturally fermented lactic acid bacteria liquid prepared from alfalfa plants with a 3% molasses addition. While the silage prepared from the pure alfalfa plant in the study constituted the control group (i), the experimental groups; (ii) PFJ + pure alfalfa silage, (iii) PFJ + 1.5% molasses added alfalfa silage, (iv) PFJ + 1.5% fructose added alfalfa silage, (v) PFJ + 1.5% sucrose added alfalfa silage. In the study, the differences between the groups were statistically significant for the dry matter (DM), acid detergent insoluble fiber (ADF), IVOMD, metabolizable energy (ME) and methane (CH₄) values. When the fermentation characteristics (pH, NH₃-N, LA, AA, LA/AA, LA/AA, and yeast-mold after aerobic stability) of the prepared silages were examined, the differences between the groups were found to be statistically significant. When analyzed in terms of all parameters, it was determined that the addition of PFJ + 1.5% sucrose added alfalfa silage had positive effects on silage quality, the fermentation characteristics and *in vitro* organic matter digestibility.

Keywords: Alfalfa (*Medicago sativa* L.); fructose, molasses; pre-fermented juice; silage; sucrose

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INTRODUCTION

Making and using silage on livestock farms is essential for feeding animals and increasing productivity. The purpose of silage making is to can fresh roughage material with the least possible loss of nutrients. In silage making, the choice of silage plant and whether it will be used, the characteristics, and the functions of additives should be well known (Soundharrajan et al., 2021). In legume green forages such as alfalfa, fermentation formation is more difficult due to the plants' high protein and mineral ratio, high buffering capacity, and low amount of water-soluble carbohydrates (Açıkgöz et al., 2011). The low water-soluble carbohydrate content in the plant makes it difficult for lactic acid bacteria (LAB) to become dominant in the silo and limits lactic acid (LA) production in silage fermentation. For this purpose, the use of microbial inoculant bacterial cultures as well as the use of easily soluble carbohydrate sources have recently increased in legume silages to improve silage quality (Bureenok et al., 2005a; Silva et al., 2020; Miralestari et al., 2021).

LAB is the group of microorganisms that provide the desired fermentation since they cause low to no loss of nutrients during the fermentation process of silages. The galactosidase enzyme secreted by LAB produces LA by shredding water soluble carbohydrates (WSC) in the structure of feeds in the silo (Korkut, 2016; Fijałkowska et al., 2020). At the same time, they also inhibit the growth of undesirable bacteria in silages by forming substances with an antimicrobial effect (Gollop et al., 2005; Soundharrajan et al., 2020). If there is not enough substrate for the growth of LAB during fermentation, inoculants may not be effective for the preservation of products in the silo environment (Bureenok et al., 2005b). Sun et al. (2021) reported that the addition of fermented LAB liquid improved the silage fermentation quality even when the inoculant was not effective. Bezerra et al. (2019) also reported that the addition of this liquid to elephant grass silage can improve silage quality, similar to the use of commercial LAB inoculants.

Fermented lactic acid liquid (PFJ) has been widely used in recent years due to its many benefits such as its effectiveness in LA production especially in alfalfa silages with a high moisture content, the presence of many epiphytic LAB bacterial species in its content and the synergistic action of these bacteria, its ease of preparation in a practical and economical way, and its use as an alternative to commercial LAB inoculants

(Sun and Yang, 2021; Boğa and Ayaşan, 2022). This study was carried out to evaluate the effect of the addition of PFJ prepared from alfalfa plants by adding 3% molasses with 1.5% different soluble sources to difficult ensiled alfalfa plants on silage fermentation, *in vitro* organic matter digestibility (IVOMD) and gas production.

MATERIAL AND METHOD

Study design and silage preparation

In this study, fermented natural LA liquid formed from alfalfa by adding molasses was basically prepared using the method reported by Masuko et al. (2002). For this purpose, 1000 ml of distilled water was added to 1000 g of fresh alfalfa plants and shredded for two minutes with the help of a blender. The obtained plant liquid mixtures were filtered using two layers of cheesecloth and 3% molasses was added to the plant liquid mixtures (PFJ) and they were placed in bottles and incubated at 30°C for 72 hours. The study employed the alfalfa plant as its primary source for making silage. In ensiling, 1 ml of fermented natural LAB liquid was added to 1 kg of alfalfa plants. 40 ml/kg of distilled water was added to each silage group prepared for the study in order to guarantee homogeneity. The total LAB count in the fresh silage material was determined using the method reported by Güney and Ertürk (2020) repeated three times for each group according to the tempo automatic bacteria counter test method. The buffering capacity of the fresh alfalfa used in the study was determined according to the method reported by Playne and McDonald (1966). In the study, (Group I) the pure alfalfa plant constituted the control group, while the trial groups were (Group II) the PFJ added alfalfa plant, (Group III) the alfalfa silage with PFJ and 1.5% molasses, (Group IV) alfalfa silage with PFJ and 1.5% fructose, and (Group V) the alfalfa silage with PFJ and 1.5% sucrose. With four repetitions, each silage group was compacted into a 1.5-liter glass jar and ensiled in an airtight fashion. For 60 days, silages were kept in a dark location at room temperature.

Fermentation profile analysis

At the conclusion of the 60-day fermentation period, the silages were opened. The 3-5 cm area at the top of the jars was removed, 25 g of silage collected uniformly was mixed with 100 ml of distilled water, which was then blended for two minutes to shred it, and a pH meter was used to quickly determine the pH of the liquid made from the shred silage (Polan et al.,

1998). After filtering, the blended liquid was transferred into 10 ml tubes, and samples to be examined for ammonia nitrogen and volatile fatty acids each received 25 ml of 25% metaphosphoric acid and 0.1 ml of 1M HCl, respectively. Following that, the samples were stored in a refrigerator at -20°C until analysis. The method described by Broderick and Kang (1980) was used to analyze the silage sample's ammonia nitrogen content, and according to the technique described by Suzuki and Lund (1980), the quantities of LA and volatile fatty acids (acetic, butyric and propionic acid) were measured. High-performance liquid chromatography (HPLC) [Shimadzu LC-20 AD HPLC pump, Shimadzu SPD M20A Detector (DAD), Shimadzu SIL-20 ADHT Autosampler, Isepe Coregel (87H3 colon), Shimadzu CTO-20ac Column oven] was employed for this objective. The silages were subjected to an aerobic stability test (determination of CO₂ production values) for five days (Ashbell et al., 1991).

AOAC (2005) guidelines were used to analyze the dry matter (DM), crude ash (CA), and crude protein (CP) contents of silages. Acid detergent insoluble fiber (ADF) and neutral detergent insoluble fiber (NDF) contents of the silages were analyzed according to procedure of Van Soest et al. (1991). All the analyses were run with four replications in each sample. The studies were completed after the silage material and silages were dried at 20°-22°C and treated in a lab to pass through a 1 mm sieve. The *in vitro* organic matter digestibility (IVOMD), the metabolizable energy (ME), and methane (CH₄) content of the alfalfa and the silages were determined according to the method reported by Menke et al. (1988). The amount of yeast and mold contained in the silages was made according to the procedure reported by Filya et al. (2000).

Statistical method

One Way Analysis of Variance was used to assess the data collected at the conclusion of the study. To compare group means, Duncan multiple comparison tests were applied, and the SPSS (1991) package program was utilized.

RESULTS

In the study, the LAB number, yeast, mold, LA, acetic acid (AA), LA/AA ratio and pH values of the naturally fermented natural LAB liquid obtained from alfalfa plants with 3% molasses were 1.7×10^{10} cfu/g, 4.1×10^5 cfu/g, 6.4×10^3 cfu/g, 147.33 g/kg DM, 40.15 g/kg DM, 3.66, and 3.74, respectively. It was reported that homolactic fermentation occurred when the LA/

AA ratio was greater than 3.0 and heterolactic fermentation occurred when the LA/AA ratio was less than 3.0 (Zhang et al., 2010). In this study, the LA/AA ratio of 3.66 revealed that homolactic activity was more intense in fermentation.

Table 1 contains an examination of the raw alfalfa plants utilized as silage in the study. When Table 1 was examined, the LAB number of the fresh alfalfa plants was determined as 1.3×10^5 cfu/g. The buffering capacity of the alfalfa plant was 675 meq/kg DM. The total yeast number of alfalfa plants was 4.85×10^5 cfu/g and the mold number was 8.2×10^3 cfu/g.

The nutrient content, IVOMD, ME, and *in vitro* CH₄ values of the silages prepared by adding naturally fermented LAB liquid and easily soluble carbohydrate sources to the alfalfa plants used as silage material are given in Table 2.

As it was seen in Table 2, the differences between the groups were statistically insignificant ($p > 0.05$) in the crude ash (CA), crude protein (CP), and NDF values of the silages, while the differences between the groups were statistically significant ($p < 0.05$) in the DM, ADF, IVOMD, ME and CH₄ values. When the DM contents of the silages obtained by adding PFJ and easily soluble sources such as molasses, fructose and sucrose at the rate of 1.5% were examined, increases were observed in the DM values in the groups where 1.5% molasses and 1.5% sugar were added compared to the other groups ($p < 0.05$). When the silages' ADF and NDF values were examined, the group with the 1.5% sugar addition was found to be lower than the other silage groups. When the IVOMD and ME values of alfalfa silages obtained by adding PFJ and 1.5% easily soluble sources such as molasses, fructose, and sucrose are examined, increases were observed in all trial groups compared to the control group ($p < 0.05$). Methane (CH₄) values of the silages increased in the 1.5% fructose and sucrose added group compared to the other groups ($p < 0.05$).

The fermentation characteristics of the silages prepared by adding naturally fermented LAB liquid and easily soluble carbohydrate sources to the alfalfa plants and their correlation in the analysis (pH, NH₃-N, LA, AA, CO₂, yeast-mold, IVOMD, ME, CH₄) are given in Tables 3 and 4.

The fermentation characteristics (pH, NH₃-N, LA, AA, LA/AA, and aerobic stability after yeast-mold) of the silages prepared by adding naturally fermented

Table 1. Analysis of the fresh alfalfa plant

	DM	CA	CP	ADF	NDF	IVOMD	ME	CH4	LAB	Yeast	Mold	BC
Fresh Alfalfa Plant	30.34	11.19	20.17	34.62	43.26	58.96	8.92	14.36	1.3*10 ⁵	4.85*10 ⁵	8.2.10 ³	675

DM: Dry matter, %; CA: Crude ash DM%; CP: Crude protein, DM%; ADF: Acid detergent fiber, %DM; NDF: Neutral detergent fiber, %DM; IVOMD: *In vitro* organic matter digestibility, ME: Metabolizable energy, CH4: *In vitro* methane gas (%), LAB: Lactic acid bacteria cfu/g, BC: Buffering capacity (meq kg/DM)

Table 2. Nutrient content and IVOMD, ME, and CH4 values of alfalfa silages prepared by adding naturally fermented lactic acid bacteria liquid and readily soluble carbohydrate source

Groups	DM	CA	CP	ADF	NDF	IVOMD	ME	CH4
Group I	29.42 ^b	10.96	19.86	32.94 ^{ab}	41.52	57.44 ^b	8.50 ^c	14.18 ^b
Group II	26.81 ^c	12.13	20.26	34.27 ^a	41.47	63.00 ^a	9.55 ^a	15.18 ^a
Group III	31.77 ^a	10.99	20.4	30.41 ^{bc}	41.49	60.56 ^a	8.97 ^{bc}	15.69 ^a
Group IV	29.53 ^b	10.53	19.16	31.00 ^{bc}	41.48	61.78 ^a	9.08 ^{ab}	14.49 ^b
Group V	31.76 ^a	10.2	20.04	30.27 ^c	40.83	61.67 ^a	9.07 ^{ab}	14.31 ^b
SEM	0.459	0.234	0.298	0.485	0.102	0.567	0.102	0.152
p	0.000	0.059	0.759	0.013	0.134	0.008	0.013	0.000

a,b,c Values with different letters in the same column were found to be different (P<0.05); DM: Dry matter, %; CA: Crude ash DM%; CP: Crude protein, DM%; ADF: Acid detergent fiber, %DM; NDF: Neutral detergent fiber, %DM; IVOMD: *In vitro* organic matter digestibility, ME: Metabolizable energy, CH4: *In vitro* methane gas (%), SEM: Standard Error Mean

Table 3. Effect of PFJ and soluble carbohydrates addition on fermentation characteristics of alfalfa silage groups

Groups	pH	NH3-N/TN	YMAAS	LA	AA	LA/AA	CO2
Group I	5.11 ^a	12.32 ^a	2.59 ^a	11.59 ^c	9.15 ^c	1.29 ^{bc}	5.64 ^a
Group II	4.99 ^a	12.37 ^a	1.02 ^e	22.44 ^b	23.48 ^a	0.94 ^c	4.53 ^b
Group III	4.67 ^b	8.02 ^b	2.02 ^b	16.76 ^{bc}	11.48 ^{bc}	1.46 ^b	2.66 ^c
Group IV	4.61 ^b	9.05 ^b	1.62 ^c	20.07 ^b	13.65 ^b	1.52 ^b	2.67 ^c
Group V	4.22 ^c	6.47 ^b	1.31 ^d	54.45 ^a	21.68 ^a	2.52 ^a	2.05 ^d
SEM	0.074	0.647	0.126	3.568	1.382	0.132	0.3104
P	0.000	0.001	0.000	0.000	0.000	0.000	0.000

a-c Values with different letters in the same column were found to be different (P<0.05); NH3-N/TN: Ammonia nitrogen, CO2: Carbon dioxide g/kg DM, LA: Lactic acid g/kg DM, AA: Acetic acid g/kg DM, YMAAS: Yeast-Mold after aerobic stability

Table 4. Correlation of the analysis in the silages.

		pH	NH3-N	LA	AA	CO2	Yeast-Mold	IVOMD	ME	CH4
pH	PC	1	,842 ^{**}	-,788 ^{**}	-,335	,905 ^{**}	,403	-,321	-,135	,077
	P		,000	,000	,149	,000	,078	,168	,572	,747
NH3-N	PC		1	-,491 [*]	-,057	,789 ^{**}	,168	-,098	-,018	-,019
	P			,028	,811	,000	,480	,680	,941	,935
LA	PC			1	,670 ^{**}	-,600 ^{**}	-,557 [*]	,403	,276	-,215
	P				,001	,005	,011	,078	,240	,363
AA	PC				1	-,235	-,881 ^{**}	,658 ^{**}	,680 ^{**}	,110
	P					,319	,000	,002	,001	,644
CO ₂	PC					1	,426	-,407	-,219	-,158
	P						,061	,075	,354	,506
Yeast-Mold	PC						1	-,726 ^{**}	-,689 ^{**}	-,166
	P							,000	,001	,483
IVOMS	PC							1	,903 ^{**}	,263
	P								,000	,263
ME	PC								1	,372
	P									,106
CH ₄	PC									1
	P									

PK: Pearson correlation; *: Correlation is significant at 0.05 level; **: Correlation is significant at 0.01 level; P: Significance degree

LAB liquid and easily soluble carbohydrate sources to the alfalfa plants were shown in Table 4, the differences between the groups were statistically significant ($p < 0.05$). The pH values of alfalfa silages with PFJ and 1.5% molasses, fructose and sucrose added were lower than the control group and alfalfa silage with only PFJ. The highest pH value (5.11) was identified in the control (group I) and the lowest pH value (4.22) was identified in group V. The Ammonia-N ($\text{NH}_3\text{-N}$) values of the Group I, II, III, IV and V were found to be 12.32, 12.37, 8.02, 9.05 and 6.47, respectively; the LA and AA values were 11.59, 22.44, 16.76, 20.07, 54.45 g/kg DM and 9.15, 23.48, 11.48, 13.65, 21.68 g/kg DM, respectively; the LA/AA values of each group were 1.29, 0.94, 1.46, 1.52, and 2.52 respectively, and the CO_2 values were 5.64, 4.53, 2.66, 2.67, and 2.05, respectively.

DISCUSSION

In this study, the total LAB values (1.7×10^{10} cfu/ml) in the fermented natural LAB liquids prepared with 3% molasses addition were higher than the values obtained from the studies of previous research (Aydın and Denek, 2019; Güney et al., 2018; Koç et al., 2017) and similar to the values obtained from the study of Aydın and Denek (2022). Yıldırım et al. (2022) prepared PFJ with sucrose in their study. The LAB number of PFJ prepared with sucrose is similar to the results of the present study. The yeast and mold values in the natural LAB liquids used in the study (4.1×10^5 cfu/ml and 6.4×10^3 cfu/ml) were similar to the yeast and mold values in the PFJ prepared from alfalfa plants by Tao et al. (2017). The pH value of the fermented natural LAB liquid obtained from the alfalfa plants at 3% molasses levels was 3.74. Denek et al. (2011) reported that the pH values in PFJ prepared by adding molasses to barley, wheat and meadow grass were in the range of 3.75-3.84, similar to the present study. The LA and AA values in PFJ were 147.33 ml/L and 40.15 ml/L, respectively. In the study conducted by Bureenok et al. (2005a), the LA (154.2 ml/L) and AA (36 ml/L) values found in the PFJ prepared by adding 5% molasses were in agreement with the present study. The differences between the PFJ analyses in the present and previous studies may be due to the type of plant used, the sources and amount of easily soluble carbohydrates used, and the incubation period.

The value of the LAB number of the fresh alfalfa plants (1.3×10^5 cfu/g) was higher than the value reported by Sun and Yang (2021), lower than the value

reported by Silva et al. (2020), and similar to the value reported by Zhang et al. (2020). In these studies, it was reported that the number of LAB contaminating the plant before harvest can vary from 1×10^1 cfu/g to 1.0×10^7 cfu/g and there were differences in the number and types of LAB contaminating the plants to be silaged. Among the reasons for these differences, there were reports that ultraviolet rays, environmental temperature, environmental humidity, and many reasons related to the plant itself were affected and the breakdown of silage plants increased the number of bacteria carried by the plant (Turan, 2015). The buffering capacity of the alfalfa plants (675 meq/kg DM) was lower than the values reported by Turan and Önenç (2018) and Çotuk (2016) (720 and 728 meq/kg DM, respectively), higher than the values reported by Sun et al. (2021) (583 and 425 meq/kg DM), and Liu et al. (2017) (226 mEq/kg DM), and similar to the value reported by Ohshima et al. (1997) at 683 meq/kg DM. The total yeast number (4.85×10^5 cfu/g) and the mold number (8.2×10^3 cfu/g) of the alfalfa plants were similar to the values reported by Silva et al. (2020). The number and types of natural microorganisms in the plants vary according to environmental conditions, location of the silo, time (season), degree of contamination, plant species, plant variety and DM content (Kızıllımsık et al., 2016a).

According to Zhang et al. (2022), molasses added to silages increased the amount of LAB, a type of anaerobic bacteria, and decreased the amount of ADF and NDF in the silages. Drouin et al. (2019) reported that *Pedococcus pentosaceus* and *Lactobacillus plantarum* inoculants increased the breakdown of hemicelluloses polysaccharides, one of the plant cell wall elements, in alfalfa plants. This statement can be explained by the fact that the highest homofermentative activity in the current study was in the sugar-added group, which had low ADF and NDF content.

Aydın (2022) reported that the silages produced by adding various amounts of molasses to a barley and vetch combination had a significantly higher digestibility compared to the control silage. In a study conducted by Şen et al. (2022) adding molasses to ryegrass-Hungarian vetch mixtures, it was reported that molasses addition increased the digestibility values of silage. They claimed that adding molasses to silage raised the IVOMD and ME values and that molasses increased the digestibility values by speeding up the breakdown of ADF and NDF. The increase observed in *in vitro* %CH₄ values may have increased in paral-

lel with the increase in IVOMD.

The addition of molasses, fructose, and sugar to alfalfa plants is expected to decrease the pH value of silage. This is because the readily fermentable carbohydrate content creates the ideal acidic environment required for a good silage (Seydoşoğlu, 2019). In the study, the low pH values in the added groups compared to the control group could be influenced by the low amount of water-soluble carbohydrates in the alfalfa plants and the high amount of LA in the added groups. LA is reported to be a stronger silo acid than AA. When Table 4 was examined, a negative correlation ($R: -0.788$) was observed between the pH and LA values. This correlation was consistent with this study's data in the group with 1.5% sucrose and PFJ added alfalfa silage, in which the LA content (54 g/kg DM) was the highest and the pH (4.22) value was the lowest. The pH values in the added groups in the present study were similar to the report of Kung and Shaver (2001) which stated that the pH value should be in the range of 4.3–4.7 for quality legume silages. Luo et al. (2021) reported that molasses-added silage groups decreased the pH values (4.48–4.84) compared to the control group in their silage study by adding 1–2–3% molasses to alfalfa plants, which supports the current study. The pH values of silages are affected by several factors such as the LAB species used as an inoculant source, the buffering capacity of the plant, the SCC content, the structure of the microbial flora present in the plant and the process applied in the preparation of the silage (Çayıroğlu et al., 2016; Kızıllımşek et al., 2016b; Seydoşoğlu, 2019).

In the study, when the silage $\text{NH}_3\text{-N}$ values were compared with the control group, a decrease was observed in the silages prepared with molasses, fructose and sugar additions ($p < 0.05$). It is thought that easily soluble carbohydrate sources have a favorable impact on the fermentation of silage and reduce proteolysis (Bingöl et al., 2009). Aydın et al. (2022) reported that the silage $\text{NH}_3\text{-N/TN}$ value should be lower than 11% for silages to be evaluated in a good quality silage class. The reports that the addition of molasses and fructose (Gao et al., 2021), molasses (Luo et al., 2021), and molasses and sucrose (Bureenok et al., 2005b) to alfalfa silages decreased the ammonia nitrogen value were in agreement with this study.

After looking at the silages' LA and AA evaluated, increases occurred in all trial groups compared to the control group ($p < 0.05$). When Table 3 was examined, the highest LA content and the lowest pH value were

observed in the silage group with the PFJ and 1.5% sucrose. When Table 4 was examined, a negative correlation ($R: -0.788$) was observed between the LA and pH values. Gao et al. (2021) reported an increase in LA values due to the addition of molasses and fructose to alfalfa silages, which was in agreement with the present study.

LA/AA ratios in silages varied between 0.94 and 1.52. Homolactic fermentation occurred in silage when the LA/AA ratio exceeded 3.0, and heterolactic fermentation occurred when it was below 3.0 (Zhang et al., 2010). In all groups, when the LA/AA ratio was less than 3.0 heterolytic fermentation was observed, and the highest heterolytic activity occurred in the PFJ-added group, while the lowest heterolytic activity occurred in the sugar-added group. In a quality silage, the LA level should be 65–70% of the total silage acids (Kung, 2010). In this study, the amount of LA was found below the specified rate in the control group, while the highest rate was found in the PFJ and silage group with 1.5% sugar added.

When Table 3 was examined, a decrease was observed in the amount of CO_2 and yeast mold values of silages in which all additives were added compared to the silage of the control group, and an increase in AA content was observed. The amount of AA produced by heterolactic LAB fermentation in the silages of the additive groups had an inhibitory effect against microorganisms that caused silage degradation, halted the development and activity of yeasts, and reduced the generation of CO_2 , which in other words, improved the aerobic stability values (Ali et al., 2020). When Table 4 was examined, a negative correlation ($R: -0.881$) was observed between AA and yeast and mold, and a negative correlation ($R: -0.600$) was observed between LA and yeast and mold.

CONCLUSION

The purpose of this study was to ascertain the impact of naturally fermented LAB liquid on the alfalfa silage quality traits, IVOMD, ME, and *in vitro* methane gas formation. All trial groups' silages had lower pH, CO_2 , yeast, and mold values than the control group. By inhibiting the growth and activity of yeasts and molds and providing an inhibitory effect against microorganisms that cause silage deterioration, the amount of AA produced by heterolactic LAB fermentation in the silages of the addition groups improved the aerobic stability values. The amount and composition of pH, $\text{NH}_3\text{-N}$, and organic acids (AA, butyric,

and LA) that occur during silage fermentation determine the quality of fermentation. Specifically, silages with low pH and NH₃-N values and high LA/AA ratios can be considered as well-fermented silages. The

results showed that silage fermentation and *in vitro* organic matter digestibility were both improved by the addition of PFJ and 1.5% sugar to alfalfa silage.

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