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The Effect of the Addition of Fermented Natural Lactic Acid Bacterial Liquid and Some Lactic Acid Bacterial Inoculants on Alfalfa Silage Quality, *In Vitro* Digestibility and Gas Production

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ABSTRACT

This study was conducted to evaluate the effect of adding fermented natural lactic acid bacteria (LAB) liquid, also known as pre-fermented juice (PFJ) obtained from different sources and some LAB inoculants to alfalfa (*Medicago sativa* L.) silage on fermentation, *in vitro* organic matter digestibility (IVOMD) and *in vitro* gas production. The silages were prepared in laboratory conditions using 1.5 L glass jars. In the study, the treatments were: the ensiled pure alfalfa plant as a control group; alfalfa plant with the addition of 2% molasses; alfalfa plant +2% molasses, with the addition of PFJ prepared from alfalfa; alfalfa plant +2% molasses, with the PFJ prepared from meadow grass; alfalfa plant +2% molasses, with the PFJ prepared from maize; alfalfa plant +2% molasses with addition of homofermentative LAB inoculant; alfalfa plant +2% molasses with addition of heterofermentative LAB inoculant. Homofermentative and heterofermentative LAB inoculants were added to the alfalfa plant at the level of 10^8 cfu/kg. When the dry matter, ash, acid detergent fiber, neutral detergent fiber, IVOMD, metabolizable energy, and methane values of the prepared silages were examined, the differences between the groups were found to be statistically significant ($p<0.05$). When the fermentation characteristics (pH, NH_3-N, CO_2, LA, AA, BA, mold) of the silages were prepared by adding PFJ and some LAB inoculants to the alfalfa plant, the differences between groups were found to be statistically significant ($p<0.05$). When examined in terms of all parameters, it was determined that the addition of PFJ (3% molasses) prepared from meadow grass +2% molasses to alfalfa plant had positive effects on silage quality, fermentation characteristics and IVOMD.

Keywords: Epiphytic lactic acid bacteria, Fermentation, Legume silage

1. Introduction

Silage is a preferable roughage source in terms of nutrients obtained by fermenting green forage plants and some aqueous industrial residues under anaerobic conditions. Due to the low dry matter and water-soluble carbohydrate (WSC) content in the structure of legume roughages, they are very difficult to ensilage due to their high buffering capacity (McDonald et al. 1991). Plants with high buffering capacity need more WSC and longer fermentation time to ferment. During ensiling, more acid formation is required to lower the pH of the environment. Since the pH values of silages obtained from plants with high buffering capacity are also high, the loss of nutrients is higher in silages made from such plants. During silage fermentation, the use of lactic acid bacteria (LAB) inoculants and easily soluble sources has become widespread in silage material in order to increase the lactic acid content of the silage, decrease the silage pH value, improve aerobic stability and reduce the fermentation losses of the silage (Arriola et al. 2011). The primary purposes of the use of silage additives are to reduce the loss of nutrients, to improve the feed value and quality of the obtained silage, to regulate the fermentation flow, to increase the evaluation levels of the silages by the animals by showing a probiotic effect, and to provide that the silage quality is preserved for a long time after the silage is opened (Gül & Coşkuntuna 2016). The role of LAB in the ensiling process is not only to reduce the pH level by breaking down the WSC but also to prevent the proliferation and activities of microorganisms that compete with the nutrients in the plant but are not desired to be present in the silo environment by forming antifungal and antimicrobial products

such as bacteriocin, hydrogen peroxide, lactate peroxidase or 1.2-propanediol (Davies et al. 1996). As an alternative to commercial LAB inoculants, a new silage additive has recently been developed called fermented LAB liquid. Fermented LAB liquid has important advantages such as being of biological origin as a silage additive, being very easy and economical to prepare and use, being safe, not having any toxic effects, not causing corrosion in the machines used in silage making, not causing environmental pollution and being a natural product (Jin-ling et al. 2013).

This study was conducted to evaluate the effect of adding fermented natural LAB liquid from different sources and some LAB inoculants to alfalfa silage on fermentation, *in vitro* organic matter digestibility (IVOMD), and gas production.

2. Material and Methods

2.1. Study design and silage preparation

In this study, fermented natural LAB liquid formed from alfalfa, meadow grass, and maize plants by adding molasses was prepared using the method reported by Masuko et al. (2002). For this purpose, 2000 mL of distilled water was added to 1000 g of fresh alfalfa, meadow grass, and maize plants and shredded for 2 minutes using a blender. The obtained plant liquid mixtures were filtered using two layers of cheesecloth. The bottles were closed by adding 3% molasses and were incubated at 30 °C for 72 hours. In ensiling, 1 mL of fermented natural LAB liquid was added to 1 kg of alfalfa plants. The alfalfa plant was used as silage raw material in the study. To ensure homogenization in all silage groups prepared in the study, 50 mL/kg of distilled water was added. The total LAB count in fresh silage material was determined by the method reported by Güney and Ertürk (2020) as 3 repetitions 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, the treatments were: the ensiled pure alfalfa plant as a control group; alfalfa plant with the addition of 2% molasses; alfalfa plant +2% molasses, with the addition of pre-fermented juice (PFJ) prepared from alfalfa; alfalfa plant +2% molasses, with the PFJ prepared from meadow grass; alfalfa plant +2% molasses, with the PFJ prepared from maize; alfalfa plant +2% molasses with addition of homofermentative LAB (HoLAB) inoculant; alfalfa plant +2% molasses with addition of heterofermentative LAB (HetLAB) inoculant. Homofermentative and heterofermentative LAB inoculants were added to the alfalfa plant at the level of 10^8 cfu/kg. HoLAB used in the study included the strains such as (*Lactobacillus plantarum* DSM 18112, *Lactobacillus plantarum* DSM 18113, *Lactobacillus plantarum* DSM 18114, *Lactobacillus plantarum* ATCC 55943, *Enterococcus faecium* ATCC 55593, *Enterococcus faecium* ATCC 53519) whereas HetLAB included the strains such as (*Lactobacillus buncheri* ATCC PTA-2494).

Each trial group of silages was compressed into 1.5 liter glass jars with 4 repetitions and ensiled up in an airtight manner. Silages were stored at room temperature for 60 days in a dark environment.

2.2. Fermentation profile analysis

The silages were opened at the end of the 60 day fermentation period. The 3-5 cm part at the top of the jars was discarded, 100 mL of distilled water was added to the homogeneously taken 25 g silage sample and shredded for 2 minutes using a blender, and the pH value of the shredded silage liquid was recorded by measuring rapidly with pH meter measuring device (Polan et al. 1998). The liquid in the blender was filtered and taken into 10 mL tubes; 0.1 mL of 1M HCl was added to the samples to be analyzed for ammonia nitrogen and 25% 0.25 mL of metaphosphoric acid was added to the samples to be analyzed for lactic acid and volatile fatty acid and stored in a deep freezer until analysis. Ammonia nitrogen analyzes of the silage samples were performed according to the method reported by Broderick and Kang (1980), lactic acid and volatile fatty acids (butyric, acetic, and propionic acid) concentrations were determined using a high pressure liquid chromatography device (HPLC) according to the method reported by Suzuki and Lund (1980). For this purpose, it was utilized HPLC device [Shimadzu LC-20 AD HPLC pump, Shimadzu SIL-20 ADHT Autosampler, Shimadzu SPD M20A Detector (DAD), Shimadzu CTO-20ac Colum oven, Icsep Coregel (87H3 colon)]. The silages obtained in the study were subjected to an aerobic stability test (determination of CO_2 production values) for 5 days (Ashbell et al. 1991).

The nutrient contents such as the dry matter, ash, and crude protein analyzes of the silages obtained from alfalfa used as silage material in the study were performed according to AOAC (2005), while ADF and NDF analyzes were performed according to Van Soest et al. (1991). The nutrient analyzes were carried out after the ensiled materials and the obtained silages were dried at room temperature and then ground in a laboratory mill to pass through a 1 mm sieve. The gas production values of the silages and alfalfa herbage were determined through the method described by Menke and Steingass (1988) using four glass syringes as replicate. The rumen fluid used in the analysis was taken with the help of a rumen pump from 2 rams who were given training food (60% forage, 40% concentrate) for

2 weeks. The IVOMD (g/kg OM) and metabolizable energy (ME) (MJ/kg DM) of silages were calculated using equations reported by Menke et al. (1979) as:

$ME(MJ/kgDM)=2.20+0.136\times Gp+0.057\times CP+0.0029\times CP^2$,

$IVOMD(\%)=14.88+0.889\times Gp+0.45\times CP+0.0651\times XA$,

Where; CP is CP in g/100 g DM, crude ash in g/100 g DM and gas production is the net gas production (mL) from 200 mg DM after 24 h of incubation. After recording 24 h gas production values, gas inside the syringe was taken by three-way syringe system and total gas was injected into computer-assisted infrared methane gas meter (Sensor Europe GmbH, Erkrath, Germany) and then methane content was determined as a percentage of 24 h the total amount of gas formed (Goel et al. 2008).The amount of yeast and mold contained in the silages was determined according to the method reported by Filya et al. (2000).

2.3. Statistical analysis

In the study, one way analysis of variance (one-way ANOVA) was used to determine whether the data obtained from the groups were widely different. Duncan's multiple comparison tests were used to control the significance of the difference between the groups, and for this purpose, the SPSS (1991) package program was used.

3. Results and Discussion

The amount of LAB, lactic acid, acetic acid, LA/AA ratio, pH, yeast, and mold values of fermented natural LAB liquid obtained from different sources are given in Table 1. It was determined that the differences were found to be statistically significant between the groups ($p<0.05$). When the LAB value was examined, it was determined that the lowest LAB value was obtained from PFJ prepared from maize, while the highest value was obtained from PFJ prepared from alfalfa. In addition to mineral needs, LAB also need a source of carbon and nitrogen. The higher number of LABs in alfalfa can be explained by the fact that the nitrogen in the structure of alfalfa is higher than that of maize and meadow grass. Total LAB values in fermented natural LAB liquids were higher than the values obtained from the studies of Koç et al. (2017), Aydin and Denek (2019) (5.39 cfu/g), and was found to be similar to the values obtained from the study conducted by Aydın (2019) (10.48 - 11.62 cfu/g).

Table 1- Determined values of naturally fermented LAB liquid prepared from different sources with adding 3% molasses

Plants	*LAB*	*LA*	*AA*	*LA/AA*	*pH*	*Yeast (cfu/g)*	*Mold (cfu/g)*
Alfalfa	10.08^{a}	146.61^{a}	39.22^{a}	3.73^{b}	3.74^{a}	5.58^{c}	3.79^{a}
Meadow grass	9.44^{b}	54.01^{c}	11.16^{c}	4.65^{a}	3.68^{b}	6.96^{a}	0.02^{b}
Maize	8.72^{c}	69.13^{b}	20.61^{b}	3.35^{c}	3.59^{c}	6.72^{b}	0.02^{b}
SEM	0.197	14.340	4.121	0.193	0.022	0.213	0.628
p value	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

$^{a-c}$Values within a same column with different superscripts differ significantly at $p<0.05$; LAB: Lactic acid bacteria, LA: Lactic acid g/kg DM, AA: Acetic acid g/kg DM, LA/AA: Lactic acid/acetic acid ratio

When the LA and AA values were examined, it was seen that the highest was obtained from PFJ prepared from alfalfa, and the lowest from PFJ prepared from meadow grass. The lactic acid production level of LAB differed between species (Evren et al. 2011). The highest LA and AA values in PFJ prepared from alfalfa were due to the high LAB number of PFJs prepared from alfalfa, and it was concluded that microbial species in PFJ prepared from alfalfa better-evaluated molasses. It was determined that although the LAB number of PFJ prepared from maize was lower than the LAB number of PFJ prepared from meadow grass. The fact that the lactic acid and acetic acid values for PFJ prepared from maize were higher than that from meadow grass may be because the LAB species in the maize plant produce higher lactic acid. The LA (154.2 g/kg DM) and AA (36 g/kg DM) values found in the PFJ prepared by Bureenok et al. (2005) by adding 5% molasses were compatible with the results in this study.

When the LA/AA ratios were examined, it was seen that the L/A ratios were between (3.35 and 4.5). The lowest LA/AA ratio (3.35) was obtained from PFJ obtained from maize, and the highest was obtained from meadow grass PFJ (4.45). It was reported that homolactic fermentation occurred when the LA/AA ratio was greater than 3.0, while the heterolactic fermentation occurred when the LA/AA ratio was less than 3.0 (Zhang et al. 2015). In this study, the fact that all LA/AA ratios were greater than 3 revealed that homolactic activity was more intense in fermentation. When the pH values of PFJs were examined, it was determined that the lowest value was obtained from PFJs obtained from maize plant and the highest value was obtained from alfalfa PFJ. The higher the buffering capacity of a plant against acidification, the slower the pH decreases in silage. During the ensiling of the alfalfa plant, which has a high buffering capacity, more acid formation is required to decrease the pH of the environment (Atalay 2015). In addition, differences in pH values in PFJs may depend on the type and amount of LAB used, plant species, easily soluble carbohydrate source and amount, and fermented incubation time. It was reported by Can (2010) and Denek et al. (2011) that they found 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 this study. Tao et al. (2017) and Wang et al. (2009) found pH values were in the range of 3.78 and 3.75 in the PFJs prepared by adding glucose to the alfalfa plant. These pH values were compatible with the present study results.

When the yeast value was examined, it was seen that the highest value was obtained from PFJ from meadow grass and the lowest from PFJ from alfalfa. When the mold values were examined, the highest value was determined for PFJ prepared from alfalfa, and the lowest for PFJ prepared from meadow grass and maize. When the total yeast and mold values were examined, the lowest value was obtained for PFJ prepared from maize and meadow grass, and the highest was obtained for PFJ prepared from alfalfa. The amount of acetic acid produced by heterolactic LAB fermentation has an inhibitory effect on the reproduction and activity of yeasts. The report of Ali et al. (2020) supports the report that high acetic acid and the lowest total yeast mold values were observed in PFJs obtained from alfalfa. The results of the study were found to be similar to the yeast and mold values (5.34, 4.18 cfu/g) in the PFJ prepared from the alfalfa plant containing 2% glucose, by Tao et al. (2017).

LAB value ($1.2*10^5$ cfu/mL), buffering capacity value (680 meq kg/DM), yeast value ($4.9x10^5$ cfu/mL), mold value ($8x10^3$ cfu/mL) of fresh alfalfa plant used as silage raw material were determined in the study. The reports on LAB numbers ($1x10^4$, $3x10^5$, and $4.32x10^4$ cfu/mL) in alfalfa plant by Bureenok et al. (2005), Ohshima et al. (1997) and Wang et al. (2009) were found to be compatible with the present study. In the earlier studies, it was stated that the number of LAB infecting the plant before harvesting could vary from $1x10^1$ cfu/mL to $1.0x10^7$ cfu/mL, and there were differences in the number and types of LAB infecting the plants to be ensiled. Among the reasons for these differences, there were reports that many factors such as ultraviolet rays, environment temperature, environmental humidity, and many reasons related to the plant itself were effective and that the decomposition of silage plants increased the number of bacteria on the plant (Jones & Gogerddan 1994; Jones 1995). The buffering capacity of the alfalfa plant in our study with the value of 680 meq/kg DM was lower than the values of the buffering capacities of the alfalfa (720, 728 meq/kg DM) reported by Turan and Önenç (2018) and Çotuk (2016) in their studies and higher than the buffering capacity of alfalfa plant (583, 425), reported by Sun et al. (2021), in their study. Besides, Ohshima et al. (1997) found that the buffering capacity of the alfalfa plant was 683 meq/kg DM and it was found similar to the result of our study.

In the study, alfalfa silage nutrient contents and IVOMD, ME, and *in vitro* CH_4 values of treatments with added fermented natural LAB liquids from different sources, or HoLAB, or HetLAB, and 2% molasses are given in Table 2.

Table 2- Nutrient contents and IVOMD, ME, *in vitro* CH_4 values of silage groups

Groups	*DM*	*CA*	*CP*	*ADF*	*NDF*	*IVOMD*	*ME*	*CH_4*
Control	26.40^{e}	10.97^{bc}	18.04^{c}	32.54^{a}	46.20^{a}	55.90^{b}	8.35^{bc}	14.34^{a}
2% molasses added to group	30.41^{bc}	10.88^{c}	18.91^{bc}	32.05^{ab}	45.86^{a}	56.75^{b}	8.35^{bc}	13.96^{a}
Alfalfa PFJ +2% molasses	31.14^{ab}	10.96^{bc}	19.60^{ab}	30.13^{c}	44.82^{a}	55.68^{b}	8.21^{c}	12.81^{b}
Meadow grass PFJ +2% molasses	31.94^{a}	10.88^{c}	19.55^{ab}	30.37^{bc}	44.45^{a}	60.80^{a}	9.00^{a}	14.07^{a}
Maize PFJ +2% molasses	30.44^{bc}	10.89^{c}	19.20^{ab}	30.21^{c}	45.44^{a}	60.43^{a}	8.94^{a}	13.91^{a}
HoLAB +2% molasses	30.17^{cd}	11.31^{ab}	19.45^{ab}	30.60^{bc}	43.19^{b}	59.92^{a}	8.86^{ab}	13.75^{a}
HetLAB +2% molasses	29.35^{d}	11.44^{a}	20.15^{a}	31.25^{abc}	40.59^{b}	57.87^{ab}	8.55^{abc}	14.15^{a}
SEM	0.331	0.058	0.168	0.463	0.468^{a}	0.526	0.079	0.132
p value	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

$^{a-e}$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 insoluble fiber, DM %; NDF: Neutral detergent insoluble fiber, %DM; IVOMD: *In vitro* organic matter digestibility g/kg OM, ME: Metabolizable energy MJ/kg DM, CH_4: *In vitro* methane (%)

The fermentation characteristics of the silages obtained with using the fermented natural LAB liquids from different sources, or some LAB inoculants are given in Table 3 and the correlations of the values are given in Table 4.

Table 3- Fermentation characteristics of the experimental silages

Groups	*pH*	*NH_3N*	*CO_2*	*LA*	*AA*	*BA*	*Mold*	*Yeast*
Control	5.58^{a}	15.26^{a}	6.77^{b}	9.56d	13.70^{ab}	3.44^{a}	1.70^{a}	<10
2% molasses added to group	4.53^{bcd}	8.44^{cd}	4.52^{c}	26.82^{bc}	16.67^{a}	0.00^{b}	1.00^{d}	<10
Alfalfa PFJ +2% molasses	4.71^{b}	8.84^{cd}	1.66^{d}	11.40^{d}	6.23^{c}	0.00^{b}	1.01^{d}	<10
Meadow grass PFJ +2% molasses	4.35^{d}	7.31^{d}	4.49^{c}	38.91^{a}	15.65^{a}	0.00^{b}	1.60^{b}	<10
Maize PFJ +2% molasses	4.45^{cd}	9.88^{bc}	1.74^{d}	31.29^{ab}	16.58^{a}	0.00^{b}	1.30^{c}	<10
HoLAB +2% molasses	4.50^{bcd}	9.01^{bcd}	9.31^{a}	15.90^{cd}	7.85^{bc}	0.00^{b}	1.30^{c}	<10
HetLAB +2% molasses	4.67^{bc}	11.10^{b}	3.54^{c}	7.82d	19.88^{a}	0.00^{b}	0.00^{e}	<10
SEM	0.078	0.519	0.520	2.490	1.182	0.242	0.100	<10
p value	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<10

$^{a-e}$Values with different letters in the same column were found to be different (p<0.05); NH_3-N/TN: Ammonia nitrogen; CO_2: Carbon dioxide g/kg KM; LA: Lactic acid g/kg DM; AA: Acetic acid g/kg DM; BA: Butyric acid g/kg DM

Table 4- Correlation of the analysis in the silages

Fermentation parameters		*pH*	*NH_3N*	*LA*	*AA*	*BA*	*CO_2*	*Mold*	*IVOMD*	*ME*	*Methane*
pH	**PK**	1	0.854**	-0.527**	-0.091	0.936**	0.249	0.211	-0.496**	-0.458*	-0.003
	P		0.000	0.004	0.644	0.000	0.201	0.282	0.007	0.014	0.987
NH_3N	**PK**		1	-0.374*	0.240	0.796**	0.235	0.065	-0.341	-0.295	0.129
	P			0.050	0.219	0.000	0.228	0.742	0.076	0.127	0.513
LA	**PK**			1	0.443*	-0.333	-0.107	0.384*	0.530**	0.523**	0.114
	P				0.018	0.084	0.589	0.043	0.004	0.004	0.564
AA	**PK**				1	-0.034	-0.189	-0.271	0.265	0.278	0.501**
	P					0.863	0.336	0.163	0.173	0.152	0.007
BA	**PK**					1	0.333	0.426*	-0.332	-0.295	0.145
	P						0.083	0.024	0.085	0.128	0.463
CO_2	**PK**						1	0.311	0.064	0.079	0.195
	P							0.107	0.746	0.690	0.321
MOLD	**PK**							1	0.143	0.187	0.060
	P								0.467	0.340	0.760
IVOMD	**PK**								1	0.987**	0.243
	P									0.000	0.213
ME	**PK**									1	0.321
	P										0.095

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

The differences between the groups were found to be statistically significant ($p<0.05$) when the DM, CA, ADF, NDF, IVOMD, ME, and CH_4 values of the silages were examined. When the DM values were examined, increases were observed in all trial groups compared to the control group. The highest DM value (31.93) was obtained from the group with PFJ +2% molasses added to the meadow grass. Henderson et al. (1982) determined the reason for the increase in silage DM level due to the inhibition of butyric acid bacteria and many types of microorganisms according to the decrease in pH level in the silo. Also, the increase in DM value in the group with the addition of HoLAB supports the report that DM loss decreases due to the increase in the amount of lactic acid in the environment and conversion of sugars such as glucose and fructose into lactic acid by homofermentative LAB in the silo (Kung 2018). When the CA values of the silages were examined, increases were observed in the HetLAB +2% molasses added group. When the CP values were examined, increases were observed in all trial groups compared to the control group. The reason is that readily fermentable soluble carbohydrate sources have a positive effect on silage fermentation and reduce proteolysis (Okuyucu 2018; Bingöl et al. 2009; Görü & Seydoşoğlu 2021). When the ADF values of the silages were examined, a decrease was observed in all trial groups compared to the control group, and a decrease was observed in the NDF value in the HetLAB +2% molasses added group ($p<0.05$). In the studies carried out by Nsereko et al. (2008) and Ding et al. (2019), it was reported that as it was known that LAB cannot degrade the polysaccharides that form the cell wall, in recent researches it was found that some special HetLAB strains such as *L. buchneri*, *L. reuteri*, *L. crispatus,* and *L. brevis* produced ferulate esterase, which could reduce the cell wall coverage, and this result was compatible with the result of our study that the lowest NDF content was in the HetLAB +2% molasses added group. Drouin et al. (2019), in their study of alfalfa plant with *Pediococcus pentosaceus* and *Lactobacillus plantarum* inoculant, reported that they increased the breakdown of hemicellulose polysaccharides, one of the plant cell wall elements and this result supported the current study.

In the study, when the IVOMD and ME values of the silage groups were examined, increases were observed compared to the control group, but the highest was obtained in the group with meadow grass PFJ +2% molasses ($p<0.05$). The high *in vitro* digestibility of organic matter of supplemented silages can be explained by the lowest content of NDF and ADF relativeto control group. In addition, this increase supports the report of Okuyucu (2018) that the main fermentation product in silages is LA and that LA is fermented in the rumen and evaluated by ruminants, and accordingly, it increases the IVOMD and ME values. A positive correlation was observed between LA and IVOMD (R=0.530), and between LA and ME (R=0.523). When the CH_4% value of the silages was examined, a decrease was observed in the PFJ +2% molasses added group compared to the other groups ($p<0.05$). The reason for this may be the decrease in methane production parallel with the decrease in *in vitro* organic matter digestibility.

In the study, when the fermentation characteristics (pH, NH_3-N, CO_2, LA, AA, BA, mold) of alfalfa silage groups were examined, the differences between the groups were found to be statistically significant ($p<0.05$). When yeast values were examined in all silages, no yeast was detected in all trial groups. The pH values of the silage groups were determined in the range of (4.35-5.58). While a decrease was observed in all trial groups compared to the control group, the lowest was determined in the group with meadow grass PFJ +2% molasses. It was concluded that this decrease in the additive groups was due to higher lactic acid content in these groups than in the control group. The pH values in the whole additive group were found to be by the statement of Kung and Shaver (2001) that the pH value for quality legume silages should be in the range of 4.3-4.7. The pH values of silages were affected by many factors such as the type of LAB used as the inoculant source, the buffering capacity of the plant, the content of WSC, the structure of the microbial flora present in the plant, and the process applied in the preparation of the silage. When Table 4 was examined, a negative correlation (R=-0.527) was observed between pH and LA. Luo et al. (2021) reported that pH values were in the range (of 4.48-4.84) in their study by adding 1-3 percent molasses to the alfalfa plant, and this report was similar to the current study.

In the study, when the silage NH_3-N values were compared with the control group, a decrease was observed in all trial groups ($p<0.05$). In the study, the lowest ammonia nitrogen value was determined in the group with PFJ +2% molasses. This is because the protease enzymes in the plant break down the proteins in the plant into peptides, amides, and ammonia during the proteolysis event. Due to the decrease in the efficiency of proteolytic enzymes as a result of the lactic acid production rate and rapid pH decrease in the environment, proteolysis decreases, so the degradation of proteins also decreases (Reich & Kung Jr 2010). Carpintero et al. (1979) reported that the proportion of NH_3-N should be lower than 11% of silage total N to be evaluated in the good-quality silage class. The statement reported by Bureenok et al. (2011) that the addition of molasses to alfalfa silage reduced the ammonia nitrogen value was compatible with the result of this study.

The LA value of the silages increased in all trial groups compared to the control group ($p<0.05$). When the LA value of the silages (Table 3) was examined, the highest lactic acid content and the lowest pH value were observed in the meadow grass PFJ +2% molasses added silage group. There was determined, a negative correlation (R=-0.527) between pH and LA, and a positive correlation (R=0.443) between LA and AA. In quality silage, the lactic acid level should be 65-70% of the total silage acids (Kung & Shaver 2001). In this study, the amount of lactic acid remained below the specified ratio in the control group, while the highest ratio was determined in the silage group with meadow grass PFJ -2% molasses. Gao et al. (2021) reported that LA values increased due to the addition of molasses to alfalfa silage and this statement was compatible with the present study.

When the AA values of the silages were examined, the highest acetic acid value (19.88) was determined in the heterofermentative inoculant +2% molasses added group. When BA values were examined, BA was detected in the control group, but not in the additive groups. In this study, it is thought that the high CP ratio and low DM and WSC content of the alfalfa plant used as silage material cause insufficiency in the production of lactic acid, which is necessary to inhibit the growth of clostridial bacteria (Weinberg et al. 1988). As a result of this state, saccharolytic clostridia transform the WSC and organic acids in the plant into butyric acid (Ohshima 1997). The low LA and high pH explains the presence of butyric acid in the control group.

When the CO_2 and mold values of the silages were examined, a decrease was observed in all experimental groups compared to the control group ($p<0.05$). In the study, the highest CO_2 value of the silages was determined in the homofermentative inoculant +2% molasses added to the group, while the lowest value was found in the alfalfa PFJ +2% molasses added group. When compared with all groups, there were obtained the highest acetic acid, the lowest yeast, and mold content and the lowest CO_2 ratio in the heterofermentative inoculant +2% molasses added to the group. These were supported by the results reported by Ali et al. (2020) that the amount of acetic acid produced by heterolactic LAB fermentation in silages has an inhibitory effect against microorganisms that cause silage deterioration, prevents the reproduction and activity of yeasts, reduces CO_2 production, in other words, and improves aerobic stability values.

4. Conclusions

This study was conducted to evaluate the effect of adding fermented natural LAB liquid from different sources and some LAB inoculants to alfalfa silage on fermentation, IVOMD, and gas production. It was seen that the pH, CO_2, yeast, and mold values of the silages decreased in all trial groups compared to the control group. In terms of all parameters, it was concluded that adding meadow grass PFJ +2% molasses had positive effects on silage quality, fermentation characteristics and *in vitro* organic matter digestion, and this fermented natural LAB liquid can be efficiently used as silage additive.

Ethical Statement: In this study, it was reported by the Harran University Animal Experiments Local Ethics Committee that there is no need for an ethics committee document. (decision no: 2022/006/08, date: 14.09.2022).

Data availability: Data are available on request due to privacy or other restrictions.

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