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Research Article

The effect of fermented natural lactic acid bacteria liquid and water-soluble carbohydrate admixture on alfalfa (*Medicago sativa* L.) silage fermentation quality, in vitro digestibility and methane production

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ABSTRACT

This study was carried out to determine the effect of fermented natural lactic acid bacteria liquid and water-soluble carbohydrate (WSC) admixtures on fermentation quality, *in vitro* digestibility, and methane production of alfalfa silage. In the study, analyses (pH, total lactic acid bacteria (LAB), yeast, mold, lactic acid (LA), acetic acid (AA), and LA/AA) of naturally fermented lactic acid bacteria liquid (PFJ) prepared with 3% fructose addition to alfalfa plants were conducted. Treatments included alfalfa (control, C), alfalfa + PFJ (PFJ-C), alfalfa + PFJ + 1.5% molasses (PFJ-CM), alfalfa + PFJ + 1.5% fructose (PFJ-CF), and alfalfa + PFJ + 1.5% sucrose (PFJ-CS). In the study, the differences between the groups were insignificant in the crude ash (CA) and neutral detergent fiber (NDF) values of the silage. On the other hand, the differences between the groups were statistically significant in dry matter (DM), crude protein (CP), acid detergent fiber (ADF), *in vitro* organic matter digestion (IVOMD), metabolizable energy (ME), and methane (CH_4) values. Compared to the control group (57.45%), increases in IVOMD and ME were observed in silage obtained by adding PFJ (58.67%), molasses (59.35%), fructose (63.83%), and sucrose (62.96%). When the fermentation characteristics (pH, ammonia nitrogen (NH_3-N), lactic acid (LA), acetic acid (AA), yeast-mold, and carbon dioxide (CO_2) after aerobic stability) of the silage were analyzed, the differences between the groups were statistically significant. When the CO_2 content and post-aerobic yeast mold values (PAYMV) of the silage were examined, it was observed that there was a decrease in all experimental groups compared to the control group. The LA and AA values of silage increased in all experimental groups compared to the control group. Groups with PFJ had a positive effect on nutritional values, digestibility properties, and fermentation properties compared to the control group. However, considering the groups with PFJ, it can be said that the addition of PFJ-CF is better for silage quality.

INTRODUCTION

Roughage needs to be provided on a regular basis all year round in order for livestock production to be affordable and sustainable, particularly in terms of ruminant nutrition. High-quality, abundant, and cheap roughage sources provide economic profit to the enterprise by minimizing the use of expensive concentrate feeds in animal feeding (Jelan, 2011). Alfalfa plant, which is a source of roughage, is a perennial and polymorphous legume forage plant and is rich in nutrients, especially protein. Since the leaf stalks are thin and weak, significant nutrient losses occur during the period from drying to feeding to animals (Tıknazoğlu, 2009). In regions with high rainfall, nutrient losses occur due to microbial degradation due to incomplete drying. In order to prevent these physical and microbiological losses, ensiling alfalfa plants can be a reasonable solution. Since alfalfa is deficient in water-soluble carbohydrates (WSC) and has a high buffer capacity, additives are necessary during ensiling (Xie et al., 2021). For this purpose, various WSC sources (Gao et al., 2021), and bacterial inoculants (Sun et al., 2021) can be added to the silage to ensure the desired fermentation in alfalfa silage. Lactic acid bacteria (LAB) used in silage fermentation play an essential role in improving silage quality and increasing silage shelf life (Okoye et al., 2023). Lactic acid bacteria are a group of microorganisms that provide the desired fermentation as they cause almost no loss of nutrients during the fermentation process of silage. The main function of LAB in silage is to ferment WSC into organic substances as end products (da Silva et al., 2017). The production of organic acids by LAB depends on the amount of WSC present in the fresh crop and the nature of the bacterial strains used (Kim et al., 2021). The amount and variety of simple sugars in the feed plant, the microbial community in the fresh crop, the LAB strains' resistance to low pH and ability to use particular substrates, and the plant material's capacity, including buffering, all affect how competitive lactic acid bacteria are with other microorganisms in the silo environment (Karademir and Karademir, 2003). Due to the abundance of epiphytic LAB bacterial species, their synergistic action, ease of practical and cost-effective preparation, and numerous advantages, including the ability to use fermented lactic acid liquid (PFJ) as an alternative to commercial LAB inoculants, PFJ has become increasingly popular in recent years (Sun et al., 2021). In this study, it was aimed to evaluate the effect of a PFJ mixture with different WSC sources on the chemical properties and digestibility of alfalfa silage, to determine which WSC source works more effectively with PFJ, and to find out which one is more appropriate to use in future studies.

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MATERIALS and METHODS

Study design and silage preparation

Using the technique described by Masuko et al. (2002), fermented natural LAB liquid was created in this work by adding fructose to the alfalfa plant. The 1000 ml of distilled water and 1000 g of fresh alfalfa plant were blended for two minutes to shred the mixture. The obtained plant liquid mixtures were filtered using two layers of cheesecloth, and 3% fructose was added to the plant liquid mixtures (PFJ), which were then placed in bottles and incubated at 30°C for 72 hours. The alfalfa plant was used as silage raw material in the study. Fresh alfalfa was harvested and wilted for 24 hours during the early flowering period (terrestrial climate, 37.8°N, 38.46°E, 518 m altitude) in Şanlıurfa province. The wilted material (DM 266 g/kg FW) was chopped into 1-2 cm lengths. 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 guarantee homogeneity in all the silage groups created for the study. According to the Tempo automatic bacteria counter test method, the total LAB count in the fresh silage material was calculated using the procedure described by Güney and Ertürk (2020), and it was repeated three times for each group. 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, experimental groups were formed from non-additive alfalfa (control, C), alfalfa with added PFJ (PFJ-C), alfalfa with added PFJ and 1.5% molasses (PFJ-CM), alfalfa with added PFJ and 1.5% fructose (PFJ-CF), and alfalfa with added PFJ and 1.5% sucrose (PFJ-CS). With four repetitions, each silage group was compressed into 1.5-liter glass jars, which were then tightly sealed. Silages were stored at room temperature for 60 days in a dark environment.

Fermentation profile analysis

After the silage were opened, the silage subsamples (25 g) were thoroughly shaken with 100 ml of sterile deionized water to measure the pH using a glass electrode and pH meter (Hanna Instruments, Analog pH/ORP meter, Romania) (Polan et al., 1998), and then they were filtered using qualitative filter paper before being stored at -20°C for additional ammonia-nitrogen (NH_3-N) and organic acid analyses. The silage samples underwent NH_3-N analysis using the procedure described by Broderick and Kang (1980), and using a high-pressure liquid chromatography device (HPLC) (Shimadzu SPD M20A Detector (DAD), Shimadzu LC-20 AD HPLC pump, Shimadzu cto-20ac Colum oven, Shimadzu SIL-20 ADHT Autosampler, Icsep Coregel (87H3 colon)), the organic acid contents (known as lactic, butyric, acetic, and propionic acid) were determined in accordance with the technique described by Suzuki and Lund (1980). The silages obtained in the study were subjected to an aerobic stability test (determination of CO_2 production values) for five days (Ashbell et al., 1991).

While the acid detergent insoluble fiber and neutral detergent insoluble fiber analyses of the silage obtained from the alfalfa plants used as silage material in the study were carried out in accordance with Van Soest et al. (1991), the raw nutrient content, such as dry matter, crude ash, and crude protein analyses, were carried out in accordance with AOAC (2005). After the silage ingredients and the produced silages were dried at room temperature and ground in a laboratory mill to pass through a 1 mm sieve, the raw nutrient analyses were conducted. The IVOMD of the silages and the metabolizable energy (ME) and methane (CH_4) content of the forages obtained in the study were determined according to the method reported by Menke et al. (1988). The method described by Filya et al. (2000) was used in the study to determine the yeast and mold content of silage groups.

Statistical method

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 $p<0.05$ was considered significant. For this purpose, the IBM SPSS 20 (1991) software program was used.

RESULTS

In the present study, the LAB count, yeast, mold, lactic acid, acetic acid, LA/AA ratio, and pH values of fermented natural LAB liquid (PFJ) obtained from alfalfa plants with 3% fructose supplementation were determined as 3.2×10^{11} cfu/ml, 3.8×10^5 cfu/ml, 2.6×10^5 cfu/ml, 160.38 g/kg DM, 38.12 g/kg DM, 4.2, 3.60, respectively, as indicated in Table 1. The LA/AA ratio of 4.2 in the PFJ employed in the present study reveals that homolactic activity is more intense in fermentation, according to Zhang et al. (2010).

Table 1. Analysis values of naturally fermented lactic acid bacteria liquid prepared from alfalfa plant by adding 3% fructose.

	LAB	LA	AA	LA/AA	pH	Yeast (cfu/ml)	Mold (cfu/ml)
PFJ	$3.2*10^{11}$	160.38	38.12	4.2	3.60	$3.8*10^5$	$2.6*10^5$

LAB: Lactic acid bacteria cfu/ml, LA: Lactic acid g/kg DM, AA: Acetic acid g/kg DM

Table 2. Analysis of fresh alfalfa plant used as silage raw material.

	LAB Log10	Yeast Log10	Mold Log10	BC
Fresh alfalfa	4.38	5.66	4.04	660

LAB: Lactic acid bacteria, BC: Buffering capacity (meq/kg DM)

The analysis of the fresh alfalfa plant used as silage raw material in the study is presented in Table 2.

The nutrient contents, IVOMD, ME, and *in vitro* CH_4 values of the silage groups are provided in Table 3. According to Table 3, while the differences between the groups were found to be statistically insignificant ($p>0.05$) in the CA and NDF values of the silage, the differences between the groups were found to be statistically significant ($p<0.05$) in the DM, CP, ADF, IVOMD, ME, and CH_4 values.

Table 3. Nutrient contents, IVOMD, ME and CH_4 values of silages

	DM	CA	CP	ADF	NDF	IVOMD	ME	CH_4
Control	29.47^{c}	10.63	20.02^{b}	32.41^{ab}	40.78	57.45^{b}	8.50^{c}	14.05^{ab}
PFJ-C	30.57^{b}	10.69	20.38^{ab}	33.83^{a}	42.52	58.67^{b}	8.69^{bc}	13.86^{ab}
PFJ-CM	30.95^{b}	11.23	20.41^{ab}	32.92^{a}	42.77	59.35^{b}	8.77^{bc}	14.55^{a}
PFJ-CF	31.98^{a}	10.96	20.24^{b}	30.93^{b}	42.37	63.83^{a}	9.46^{a}	12.67^{c}
PFJ-CS	31.24^{b}	10.87	20.74^{a}	33.19^{a}	41.42	62.96^{a}	9.09^{ab}	13.49^{bc}
SEM	0.212	0.101	0.080	0.307	0.290	0.668	0.097	0.190
P	0.000	0.366	0.047	0.017	0.138	0.000	0.003	0.009

$^{a-c}$: 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 insoluble fiber, %DM; NDF: Neutral detergent insoluble fiber, %DM; IVOMD: *In vitro* organic matter digestion, ME: Metabolizable energy, CH_4: *In Vitro* methane gas (%), SEM: Standart Error Mean

The fermentation characteristics of the silage groups are presented in Table 4. When the fermentation characteristics (pH, NH_3-N, LA, AA yeast mold after aerobic stability) and CO_2 of the silage prepared in the study were analyzed, the differences between the groups were determined to be statistically significant ($p<0.05$).

Table 4. Fermentation characteristics of silages

	pH	NH_3-N/TN	PAYMV	LA	AA	CO_2
Control	5.09^{a}	11.95^{a}	2.48^{a}	12.07^{e}	7.62^{e}	5.81^{a}
PFJ-C	4.83^{b}	9.81^{ab}	1.12^{b}	13.48^{d}	9.68^{d}	2.24^{b}
PFJ-CM	4.67^{c}	8.05^{bc}	0.00^{c}	16.34^{c}	20.16^{a}	1.52^{c}
PFJ-CF	4.31^{d}	7.02^{c}	0.00^{c}	23.35^{a}	12.26^{c}	1.22^{d}
PFJ-CS	4.34^{d}	6.40^{c}	0.00^{c}	17.08^{b}	14.37^{b}	1.04^{e}
SEM	0.070	0.546	0.261	1.042	1.155	0.473
P	0.000	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 ($P<0.05$); NH_3-N/TN: Ammonia nitrogen, CO_2: Carbondioxide g/kg DM, LA: Lactic acid g/kg DM, AA: Acetic acid g/kg DM, PAYMV: Post aerobic yeast-mold values.

DISCUSSION

Total lactic acid bacteria (LAB) values (3.2×10^{11} cfu/ml) of PFJ prepared in this study were higher than the values obtained from the study of Aydın and Denek (2019) and similar to the values obtained from the study of Aydın and Denek (2022). The yeast and mold values in the natural LAB liquids used in the study had higher yeast and mold values (5.78 cfu/ml and 5.41 cfu/ml, respectively) than the fermented liquid made from the alfalfa plant by Tao et al. (2017), which had values of 5.34 cfu/ml and 4.18 cfu/ml, respectively. The pH value of PFJ prepared from alfalfa plants was calculated at 3.60. The LA and AA values in PFJ were 160.38 and 38.12 g/kg DM, respectively. The LA (154.2 g/kg DM) and AA (36 g/kg DM) values found in the PFJ prepared by Bureenok et al. (2005) agree with the present study. In the studies conducted so far, molasses, sucrose, and glucose additives were used as nutrient sources in the preparation of fermented lactic acid bacteria liquid, while no study with fructose addition was encountered. The possible reason for the microbiological and chemical differences between the fermented natural lactic acid bacteria liquids may be due to the type of plants used in the studies, the source and amount of WSC used, and the incubation time.

When the data was analyzed, the LAB count of the fresh alfalfa plant was determined as Log10 4.38 cfu/g. This value was higher than the value reported by Sun and Yang (2021), lower than the value reported by Silva et al. (2020), and Wang et al. (2023), and similar to the value reported by Si et al. (2023). A meta-analysis by Oliveira et al. (2017) reported that LAB counts above 10^5 cfu/g could ensure efficient fermentation, while values below 10^4 cfu/g might decrease DM recovery and

increase ammonia nitrogen concentration. In the study, the buffering capacity of the alfalfa plant was found to be 660 meq/kg DM. This value was lower than the values reported by Turan and Önenç (2018) (720 meq/kg DM), and higher than Sun et al. (2021) (583, 425). In our study, the total yeast count of the alfalfa plant was determined as Log10 5.66 cfu/g and the mold count as Log10 4.04 cfu/g. These yeast values are higher than the values reported by Wang et al. (2023) and Si et al. (2023) in their studies, while the mold value of Wang et al. (2023) is lower than our study. The yeast and mold count of Silva et al. (2020) in alfalfa plants are similar to our study. The number and types of natural microorganisms in plants vary according to environmental conditions, location of the silo, time (season), degree of contamination, plant species, plant variety, and DM content (Kılıç, 1986).

When the DM contents of the silages obtained by adding WSC such as molasses, fructose, and sucrose at a rate of 1.5% in addition to PFJs were examined compared to the control group, increases in DM values were observed in all groups, while the highest value (PFJ+CF) was detected.

When the ADF values of the silages were analyzed, the (PFJ+CF) addition group was found to be lower than the other silage groups. The decrease in ADF value is similar to Wang et al. (2023) and Si et al. (2023). It was found to be insignificant in terms of NDF values. The report that LAB had little or no degrading effect on the cellulose value was consistent with our study (Muck, 1996).

In the study, when the IVOMD and ME values of the silages obtained by combining WSC such as molasses, fructose, and sucrose at a rate of 1.5% in addition to PFJs were examined, increases were observed in the PFJ+CF and PFJ+CS groups compared to the control group. This increase supports the report of Okuyucu (2018) that LA is the main fermentation product in silages, and LA is fermented in the rumen and evaluated by ruminants, accordingly, increasing the IVOMD and ME values.

When the percentage CH_4 values of silages were analyzed, the lowest value was observed in the PFJ+CF group. Carbohydrate sources are converted into volatile fatty acids (VFA), H_2, and CO_2 in the rumen. H_2 and CO_2 released as a result of rumen fermentation cause methane (CH_4) gas formation (methanogenesis) by methanogenic microorganisms (bacteria, archaea, and protozoa) (Hegarty and Klieve, 1999). It is stated that when bacteria utilizing lactic acid convert this lactic acid to propionic acid, the production of hydrogen and formic acid, which are precursors of methane, decreases, resulting in a decrease in methane production (Sarıpınar and Sulu, 2005). The PFJ+CF-supplemented group had the highest level of lactic acid, and it is believed that methane emission is inhibited by bacteriocins produced by lactic acid bacteria species that use fructose in silage more effectively (Hegarty and Klieve, 1999).

The pH values obtained from all supplemented silage groups were lower than the control group values. The highest pH value (5.09) was obtained from the control group silage, and the lowest pH values 4.31 and 4.34 were determined in the PFJ+CF and PFJ+CS groups, respectively. It is expected that the addition of WSC to alfalfa plants will decrease the pH value of silage. The pH values in the supplemented groups in our study are in accordance with the report of Kung and Shaver (2001) that the pH value should be in the range of 4.3–4.7 for quality legume silages.

Ni et al. (2017) reported that adding sucrose to silages boosted the growth of Lactobacillus and Pseudomonas while preventing the formation of unwanted Enterobacter. In addition, Zi et al. (2022) reported that the addition of WSC altered the bacterial assemblage in silage, increasing the number of acids producing *Megamonas, Bacteroides, Megasphaera, Faecalibacterium, Stenotrophomonas,* and *Bifidobacteriums* and decreasing the number of *Weissella* and *Enterobacteriums*. The strongest of the acids formed during silage fermentation is lactic acid, and with the addition of an effective LAB inoculant, a large volume of lactic acid production and subsequently a low pH value are obtained in silage (Muck, 1996). When the pH and LA values of silage were evaluated together, a negative relationship was observed. This relationship was consistent with our data in the PFJ+CF group, where lactic acid content was 23.45 g/kg, DM was the highest, and pH 4.31 was the lowest.

Silage groups prepared with the addition of molasses, fructose, and sucrose showed a decrease in NH_3-N values when compared to the control group in the study. Gao et al. (2021) reported that the addition of molasses and fructose to alfalfa silage reduces the nitrogen content of ammonia. With the use of inoculants and WSC, the pH of the silage decreased quickly. This was likely caused by the inhibition of *Enterobacter, Clostridium*, and other microorganisms that consume the crude protein and inhibit the plant's protease activity, preserving some true proteins and lowering the concentration of NH_3-N in the silage (Muck, 1996).

Interpretation of the results of the LA and AA values of silages shows that there were increases in all experimental groups compared to the control group. Table 3 indicates that the highest lactic acid content and the lowest pH value were observed in the PFJ+CF group. Gao et al. (2021) reported an increase in LA values due to the addition of molasses and fructose to alfalfa silage, which is in agreement with the present study. In the groups with WSC additives, the highest AA value was observed in the molasses-added group, while the lowest AA value was observed in the fructose-added group. This could be because molasses contains nitrogenous chemicals that microbes can use in addition to sucrose (Otero et al., 1993).

The silage groups to which all additives were added showed higher levels of acetic acid content and lower levels of CO_2 and yeast mold values when the data was analyzed in comparison to the silage in the control group. Yeast and mold contamination reduces silage quality (Blajman and Vinderola, 2020). It is recognized that yeasts in the silage environment during the aerobic period produce CO_2 intensively. In this study, the CO_2 values of silages prepared by adding various additives were found to be low. The amount of acetic acid produced by heterolactic LAB fermentation in the silages in the additive groups supports the report that it has an inhibitory effect against microorganisms that lead to the deterioration of silage, prevents the growth and activity of yeasts, and reduces

CO_2 production, i.e., improves aerobic stability values (Ali et al., 2020).

Lactic acid bacteria are characterized by degrading different carbohydrates at different levels. The higher the molecular weight of the carbohydrate type, the lower the level of fermentation. More complex carbohydrates, such as sucrose and polysaccharides, are more difficult to break down than monosaccharides. In addition, microbial and plant enzymes play an important role in this breakdown process (Kılıç, 1986). When all parameters were analyzed, the higher silage quality in the fructose-supplemented groups compared to the other groups can be attributed to the better utilization of fructose, which is a monosaccharide, by LAB species in the PFJ and in the silage.

CONCLUSION

The purpose of this research was to ascertain how alfalfa silage fermentation quality, *in vitro* digestibility (IVOMD), and methane generation were affected by the addition of WSC and fermented natural lactic acid bacteria. The pH, CO_2, yeast, and mold values of silages decreased in all experimental groups compared to the control group silage. By preventing the development and activity of yeasts and molds as well as the microorganisms responsible for silage spoiling, the quantity of acetic acid generated by heterolactic LAB fermentation in the silages of the addition groups increased the aerobic stability values. The amount and composition of pH, NH_3-N, and organic acids (acetic acid, butyric acid, and lactic acid) formed during silage fermentation determine the quality of fermentation. Especially silage groups with low pH and NH_3-N can be considered well-fermented silages. In terms of all parameters, it was concluded that the addition of 1.5% fructose to PFJs prepared by adding 3% fructose had positive effects on silage fermentation, *in vitro* organic matter digestion, metabolizable energy (ME), and *in vitro* methane gas formation.

DECLARATIONS

Ethics Approval

Harran University Local Ethics Committee, the letter no: 2022/006/06

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Conflict of Interest

Not applicable.

Consent for Publication

Not applicable.

Author contribution

Idea, concept and design: S.S.A, N.D., M.A.

Data collection and analysis: N.K., Ş.T.

Drafting of the manuscript: S.S.A, N.D., M.A., N.K., Ş.T.

Critical review: S.S.A, N.D., M.A., N.K., Ş.T.

Data Availability

The data used to prepare this manuscript are available from the corresponding author when requested.

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