TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2021) 45: 726-734

doi:10.3906/vet-2104-15

The effects of the *L. plantarum* strain RC1.4 starter culture with or without sucrose addition on fermentation efficacy, microbial content, and aerobic stability indicators of alfalfa silage

Taylan AKSU[1,*], Shamil VALİDOV[2], Liliia KARİMOVA[3], Murat DEMİREL[4], Taner LEVENDOĞLU[5], Sibel ERDOĞAN[4], Mehtap GÜNEY[1]

[1]Van Yüzüncü Yıl University, Faculty of Veterinary Medicine, Van, Turkey

[2]Tatar Research Institute of Agricultural Chemistry and Soil Science, Federal Research Center «Kazan Scientific Center of Russian Academy of Sciences», Kazan, Tatarstan, Russian Federation

[3]Kazan State Agrarian University, Kazan, Tatarstan, Russian Federation

[4]Van Yüzüncü Yıl University, Faculty of Agriculture, Van, Turkey

[5]Van Yüzüncü Yıl University, Özalp Vocational School, Department of Medical Laboratory Technician, Van, Turkey

Received: 05.04.2021 • **Accepted/Published Online:** 22.07.2021 • **Final Version:** 25.08.2021

Abstract: To develop the approach for efficient alfalfa silage fermentation, *L. plantarum* strain RC1.4 (LP-RC1.4) was used as starter culture with or without sucrose. Trial groups consisted of ten repetitions for each treatment including (1) control, with added sterile water (10 mL); (2) LP, silages treated with LP-RC1.4 alone (10^6 cfu g^{-1} FM); (3) LP-S_1, silages treated with LP-RC1.4 (10^6 cfu g^{-1} FM) plus sucrose at 10 g/kg fresh matter; (4) LP-S_2, silages treated with LP-RC1.4 (10^6 cfu g^{-1} FM) plus sucrose at 20 g kg^{-1} fresh matter. Inoculation improved the quality of the silaging demonstrating a lower decrease of silage dry matter (DM) in comparison to the control silage. The CO_2 production was also lower in the inoculated silages. The addition of sucrose improved microbiological and biochemical parameters of the resulting alfalfa silage, simultaneously with the increased number of lactobacilli, raised the content of water-soluble carbohydrates, while the number of yeasts and molds decreased. It was concluded that LP-RC1.4 inoculation should be supported with sucrose to insure domination of LAB fermentation and enhance of the silage quality of alfalfa.

Key words: *L. plantarum*, sucrose, alfalfa silage, fermentation quality, aerobic stability

1. Introduction

Alfalfa (*Medicago sativa* L.) is an important roughage for ruminant animals. Alfalfa has favorable nutrient profile at the 1 / 10 flowering period; it is resistant to ensiling due to its insufficient dry matter (DM) and water-soluble carbohydrate (WSC) contents and high buffer capacity [1]. Another dominating factor for the silage fermentation process is the plant's epiphytic bacteria density and diversity. Epiphytic bacterial flora directly affects the course of fermentation process and fermentation end products [2,3]. Lactic acid bacteria (LAB) are an indisputable species in the variety of epiphytic bacteria in plants and are fully effective in the silage fermentation process. All bacteria in this group establish their dominance by producing lactic acid, the main / only product of silage fermentation. Although the number of lactobacilli in plants varies significantly, the clover plant has a severe vulnerability with 10 cfu g^{-1} (cfu: colony forming units) [4].

Proper LAB inoculation can be considered as a practical and effective way to achieve quality alfalfa silage fermentation with this approach [5]. Within the epiphytic bacterial flora, LAB have a lower potential for metabolic efficiency, so their growth is slower [6,7]. An important way to increase metabolic efficiency is to provide WSC support for LAB. Thus, it will be possible to dominate LAB fermentation in silage plants with low WSC content such as alfalfa [8,9,10]. *L. plantarum* is the most widely used species for silage starters as it promotes lactic acid fermentation, provides dynamic population growth, and provides antibacterial and antifungal activities as well as mycotoxin decontamination in silages [11,12,13].

The trial was performed to identify lactobacillus strains that could be used as a starter culture and evaluate its influence on quality of alfalfa silage fermentation with or without additional sucrose.

* Correspondence: aksuturkiye@gmail.com

2. Material and methods

2.1. Strains and growth conditions

All lactobacilli strains were cultivated in Rogosa broth (Difco, USA) at 37 °C without aeration. When needed, 20 g/L agar (Helicon Company, Moscow, Russia) was added to concentration. Yeast strains were maintained on YPD agar (Difco, USA).

2.2. Isolation of LAB

For isolation of LAB 100 mg of red clover leaf were placed in 10 mL of Rogosa broth amended with nystatin (Belmedpreparaty, Minsk, Belarussia) to concentration of 1000 per mL to prevent growth of concomitant yeasts. Samples were incubated at 37 °C for 48 h. The mixture of LAB was used for inoculation of juice of red clover leaves in the ratio of 1 : 100 (LAB suspension : juice). The juice was obtained by squeezing of freshly harvested leaves of red clover (*Trifolium sativum*) variety "Ranniy2" and sterilized using syringe filter with pore size 22 µm (Sartorius, Moscow, Russia). After 48 h of growth at 37 °C without aeration, bacterial suspensions were plated on Rogosa agar with nystatin added. Grown colonies were removed from plate with spatula and 2 mL of phosphate buffer saline (PBS) at pH = 8.0 (Amresco, OH, USA). The bacterial suspension in PBS was inoculated in the fresh juice in the ratio of 1:100. This procedure: inoculation in the clover juice and plating on Rogosa plate was repeated another two times. Finally, the third generation of the LAB grown in the clover juice was diluted in sterile PBS and plated on Rogosa agar to obtain single colonies. The colonies different in morphology were picked up for further characterization.

2.3. Elimination of siblings and heterofermentative lactobacilli strains among isolated LAB

To avoid isolation of the same strain in several copies, isolates originating from the same samples were compared using BOX-PCR and ERIC-PCR [14]. For these experiments, DNA was extracted from all isolates accordingly [15]. PCR mix comprised of 1 ′ buffer for Taq polymerase, 0.2 mM of each nucleotide triphosphate, 1 pM of each primer, 50 ng of template DNA and 5 units of Taq pol. Volume of PCR mixture was adjusted to 25 µL with deionized water. Amplification was carried out in the following temperature profile: 95°C for 2 min of initial denaturation were followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 45 °C for 20 s and elongation at 72 °C for 8 min. Amplified fragments were separated in 1% agarose in 0.5 ′ TBE and analyzed using QuantityOne software (Bio-Rad, USA). Profiles of amplified fragments were compared, and isolates from the same sample demonstrating identical fragment profiles were considered as siblings.

Selected strains were tested for CO_2 formation when grown on glucose. Strains, which do not produce were used for further work.

2.4. Identification of isolated LAB strains using 16S rRNA gene comparison

To identify isolated strains, fragment of 16S rRNA gene was amplified using primer pair 27fm (5'- AGA GTT TGA TCM TGG CTC AG -3') and R1522 (5'- AAG GAG GTG ATC CAG CCG CA -3') [16]. PCR mix comprised of 1buffer for Taq polymerase, 0.2 mM of each nucleotide triphosphate, 1 pM of each primer, 50 ng of template DNA and 5 units of Taq pol. Volume of PCR mixture was adjusted to 25 µL with deionized water. Amplification was carried out in the following temperature profile: 95 °C for 2 min of initial denaturation were followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 45 °C for 20 s and elongation at 72 °C for 1 min. Amplified fragments were separated in 1% agarose in 0.5 ′ TBE. The amplified fragments of the 16S rRNA gene were separated from concomitant amplification products using gel electrophoresis in 1 % agarose in 0.5 ′ TBE and were cut and purified from the gel using CleanUp Mini kit (Evrogen LLC, Moscow, Russia). The nucleotide sequences of the fragments of the 16S rRNA gene were determined in Evrogen LLC (Moscow, Russia). Similarity searches in GenBank were performed using BLASTN software against the sequences of 16S rRNA gene of bacteria available in GenBank [17]. The similarity of 99% was considered as a level acceptable for affiliation of the isolates to the species in question. Ten *Lactobacillus plantarum* strains (LP-A5.1, LP-A5.2, LP-12.2, LP-CW3, LP-CW5, LP-G1, LP-G2, LP-RC1.1, LP-RC1.4, and LP-3.1*)* were identified on the base of their 16SrRNA sequences. All isolation and identification tests were performed in Kazan State Agrarian University, Tatarstan, Russia. The *L. plantarum* strain RC1.4 (LP-RC1.4) was chosen as a starter culture due to its high acidification activity.

2.5. Plant and the silage groups

Alfalfa (*Medicago sativa* L.), second cut, at 1/10 bloom stage with 246.8 g dry matter, (DM) harvested from Van Yüzüncü Yıl University Agricultural Application and Research Center was tested as silage material. The amount of chopped alfalfa for each group was weighed out, sprayed (1) with 10 mL of sterilized water alone for the control, (2) with 10 mL of LP-RC1.4 alone for inoculanted alone (LP), (3) with 10 mL of LP-RC1.4 plus sucrose at 10 g/kg fresh matter (LP-S_1) and (4) with 10 mL of LP-RC1.4 plus sucrose at 20 g/kg fresh matter (LP-S_2), respectively. LP-RC1.4 was applied at a rate of 10^6 cfu/g to FM with 10 mL sterilized physiological saline. The amount of fresh mixed mass of alfalfa for each groups then filled into the 1 L of jars silo by hand. Each treatment group was designed from ten repetitions within itself. The jar-silos were incubated at room temperature for sixty days.

2.6. Chemical analyses

Silage samples were analyzed after 60 days of ensiling. After maceration of a total of 25 g of silage samples with 100 mL of sterilized water using an industrial blender,

the aqueous extract was divided in two portions for determination of pH and_fermentation products. pH values were immediatly measured using a potentiometer, and NH_3-N content was deterimined according to the study of Broderick and Kang [18]. For aerobic stability of silage, the silages were subjected to an aerobic stability test for 5 days in a system according to Ashbell et al. [19]. The aerobic deterioration indicators (pH, CO_2 production and microbial population) were tested. Acetic acid (AA), propionic acid (PA) and butyric acid (BA) were measured using GC (Hewlett Packard-USA. column: 60 – capillary column, detector: FID, elunt: 1 mL min^{-1} helium, temp: 250 °C). Lactic acid (LA) was determined using HPLC (column: CTO-20A, detector: DAD, 220nm, SPD-20A, eluent: 1 mL min^{-1}, 25 mM potassium phosphate, temp: 30 °C).

The WSC content was measured by spectrophotometric method based on the anthrone reagent reaction [20]. For chemical analyses, silage samples (20 g) were dried at 60 °C for 48 – 72 h. The contents of DM and crude protein (CP) were determined according to AOAC method [21]. Determination of the neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were performed by the method of Van Soest et al. [22].

2.7. Determination of in vitro dry matter (IVDM) and in vitro organic matter digestibility (IVOMD)

To determine the in vitro digestion of silage, an Ankom Daisy Incubator device fulfilling the role of an artificial rumen was used. Rumen fluid used for in vitro incubation was taken from a four-year-old cattle slaughtered in a slaughterhouse, consuming barley straw, cracked barley, and bran before morning feed. At this stage, a total of 40 bags were filled with 10 samples from each jar of silage and 1 blind sample for each silage group, with 0.5 g silage samples weighed and the bag sealed tightly. ANKOM method (ANKOM 2002 Technology Corp., Fairport, NY) was used with in vitro dry matter and organic matter digestibility levels were calculated using the following formula with the filter bag technique of Van Soest et al. [22].

The following equalities were used for in vitro true DM (IVTDMD) and OM digestibility (IVTOMD).

$$\% \text{ IVTDMD} = 100 - [\,(W_3 - (W_1 \times C_1)) \times 100\,] / (W_2 \times DM_{Feed})$$

$$\% \text{ IVTOMD}_{DM} = 100 - [(W_4) \times 100]/(W_2 \times \% DM_{Feed})$$

W_1 : tare of the bags

W_2 : weight of sample

W_3 : Nutrition amount in residue from NDF solution (fitler bag + sample)

W_4: organic material weight (calculated after inceration of fitler bags contained sample)

C_1 : Blind weight (empty bag weight after drying in the oven after removal from NDF device / original bag weight)

2.8. Microbiological analyses

The lactobacilli counts were determined by using MRS agar, and yeasts and molds were enumerated on spread plates of Yeast Extract Peptone Dextrose agar and Salt Czapek Dox agar, respectively. Incubation was applied 30 °C for 2 days for lactobacilli, and 28 °C for 3–5 days for yeast and mold, repectively. All counts for lactobacilli, yeast, and mold were converted to the logarithm of colony-forming units (log cfu g^{-1}) [23].

2.9. Statistical analysis

All data were performed by with one-way ANOVA using the GLM procedure of SAS [24], and duncan's multiple range test was used at 5 % significant level [25].

3. Results

3.1. Chemical and microbiological content of fresh alfalfa

The chemical composition, WSC content, and lactobacilli number of the fresh alfalfa are given in Table 1. The DM, OM, CP, NDF, and ADF values for fresh alfalfa were 246.86 g kg^{-1} DM, 899.75 g kg^{-1} DM, 148.10 g kg^{-1} DM, 318.65 g kg^{-1} DM, and 268.52 g kg^{-1} DM, respectively. The amount of WSC and lactobacilli count were also 25.36 g kg^{-1} FM and 3.31 cfu g^{-1} FM, respectively.

3.2. Nutritive value and in vitro digestibility of DM and OM of silages

Effects of LP-RC1.4 inoculation with or without sucrose on chemical composition, in vitro dry matter digestibility (IVDMD), and in vitro organic matter digestibility (IVOMD) values of silages samples are shown in Table 2. The greatest DM loses was observed in the control silage (5.5%), whilst the DM contents of the inoculated silages with or without sucrose were similar to that of fresh alfalfa. The bacterial inoculation with or without sucrose had no significant impact on the CP content of alfalfa silage. The inoculation of LP-RC1.4 starter culture reduced the

Table 1. The chemical composition, water-soluble carbohydrate content and Lactobacilli number of the fresh alfalfa (g kg^{-1}DM, unless otherwise stated).

DM	246.80
OM	899.75
CP	148.10
NDF	318.65
ADF	268.52
WSC, g kg^{-1} FM	25,36
Lactobacilli, cfu g^{-1} FM	3.31

DM: dry matter, OM: organic matter, CP: crude protein, NDF: neutral detergant fiber, ADF: acid detergant fiber, WSC: water-soluble carbohydrate, FM: fresh material.

Table 2. Chemical composition (g kg^{-1} DM, unless otherwise stated) and in vitro digestibility (%) of the alfalfa silages.

	Chemical composition					In vitro digestibility	
Treatments	DM[#]	OM	CP	NDF	ADF	IVTDMD	IVTOMD
Control	233.21^{b}	113.56^{c}	143.00	404.30^{a}	311.38^{a}	678.2	765.9^{c}
LP	237.20^{b}	116.51^{bc}	146.20	394.76^{a}	304.73^{b}	669.4	773.7^{ab}
LP-S_1	244.23^{a}	126.74^{ab}	150.45	361.88^{b}	269.12^{c}	681.8	776.0^{ab}
LP-S_2	247.81^{a}	128.83^{a}	151.43	330.24^{b}	247.76^{c}	689.7	788.0^{a}
SEM	1.32	2.06	1.65	5.60	5.26	10.58	9.30
P	.000	.013	.258	.000	.000	.315	.051

[#]: Fresh alfalfa silage;
LP, silages treated with LAB alone (LP-RC1.4, 10^6 CFU per g^{-1} FM); LP-S_1, silages treated with LAB (LP-RC1.4, 10^6 CFU per g^{-1} FM) and sucrose (10 g/kg FM); LP-S_2, silages treated with LAB (LP-RC1.4, 10^6 CFU per g^{-1} FM) and sucrose (20 g/kg FM). DM: dry matter, CP: crude protein, OM: organic matter, NDF: neutral detergent fiber, ADF: asit detergent fiber, IVTDMD: in vitro true dry matter digestibility, IVTOMD: in vitro true organic matter digestibility. The difference between values with different superscript letters $^{(a-c)}$ is statistically significant.

Table 3. pH values, fermentation products (g kg^{-1} DM, unless otherwise stated), water-soluble carbohydrates contents (g kg^{-1} DM) and microbial counts (log $_{10}$ CFU per g FM) of alfalfa silage.

	Fermentation products							Microbial counts		
Treatments	pH value	LA	AA	PA	BA	NH_3-N (g kg^{-1} TN)	WSC	LAB	Yeasts	Molds
Control	5.63^{a}	24.56^{c}	21.43	3.63	2.25^{a}	78.32^{a}	9.86^{d}	5.51^{c}	3.45^{a}	2.03^{a}
LP	5.38^{b}	32.51^{b}	24.85	4.45	1.50^{b}	51.93^{b}	10.92^{c}	5.80^{b}	2.97^{c}	1.80^{c}
LP-S_1	5.23^{c}	40.92^{a}	23.78	4.22	1.21^{bc}	47.52^{bc}	13.21^{b}	6.08^{a}	3.11^{b}	1.91^{b}
LP-S_2	5.17^{c}	46.05^{a}	23.38	4.15	0.90^{c}	42.23^{c}	14.87^{a}	6.12^{a}	3.18^{b}	1.98^{b}
SEM	0.05	1.61	0.80	0.13	0.11	2.63	0.38	0.06	0.04	0.03
P	.000	.000	.329	.137	.000	.000	.000	.000	.000	.000

LP, silages treated with LAB alone (LP-RC1.4, 10^6 CFU per g^{-1} FM); LP-S_1, silages treated with LAB (LP-RC1.4, 10^6 CFU per g^{-1} FM) and sucrose (10 g/kg FM); LP-S_2, silages treated with LAB (LP-RC1.4, 10^6 CFU per g^{-1} FM) and sucrose (20 g/kg FM). LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid; NH3-N, ammonia nitrogen; TN, total nitrogen; WSC, water-soluble carbohydrates; LAB, lactic acid bacteria; SEM, standard error of the mean; The difference between values with different superscript letters $^{(a-c)}$ is statistically significant.

content of NDF in inoculated silages (from 404.30 g kg^{-1} DM in control alfalfa silages to 330.24 – 394.76 g kg^{-1} DM for the inoculated silages) and the content of ADF (from 311.38 g kg^{-1} DM in control alfalfa silages to 247.76–304.73 g kg^{-1} DM for the inoculated silages). Decreases in NDF and ADF content of alfalfa silages were more pronounced in silage with added sucrose ($p < 0.05$).

The IVDMD of silages ranged from 678.2 g kg^{-1} DM to 689.7669 g kg^{-1} DM, whilst the IVOMD were 773.7 g kg^{-1} OM and 788.0 g kg^{-1} OM, respectively. The bacterial inoculation with or without sucrose significantly increased the IVOMD of silages in comparison to the control ($p < 0.05$).

3.3. Fermentation quality and microbial count of silages

The effects of the LP-RC1.4 starter culture inoculation with or without sucrose on the pH value, fermentation products, water-soluble carbohydrate contents, and microbial counts of alfalfa silage are shown in Table 3.

The LP-RC1.4 inoculation with or without sucrose significantly decreased the silage's pH value ($p < 0.05$). Alfalfa ensiled without bacterial inoculant was characterized by high pH value (5.63), while the bacterial inoculation with or without sucrose had an important impact on pH value reduction to the range of 5.17– 5.38. The pH value reduction was more pronounced in silages due to increased sucrose addition ($p < 0.05$)

Table 4. Aerobic stability indicators (pH value, CO_2 production, (g kg^{-1} dry matter, unless otherwise stated) and microbial population) of the alfalfa silages at 5th days.

			Microbial Population, CFU, g^{-1}		
Treatments	pH value	CO_2	Lactobacilli	Yeasts	Molds
Control	6.08	2.87[a]	3.65[b]	4.27[a]	2.22[a]
LP	6.01	2.03[b]	4.54[a]	4.11[a]	1.59[b]
LP-S_1	5.97	1.92[b]	4.64[a]	2.52[b]	1.70[b]
LP-S_2	5.91	1.81[b]	4.72[a]	2.75[b]	1.68[b]
SEM	0.07	0.14	0.10	0.15	0.05
P	.857	.000	.000	.000	.000

LP, silages treated with LAB alone (LP-RC1.4, 10^6 CFU per g^{-1} FM); LP-S_1, silages treated with LAB (LP-RC1.4, 10^6 CFU per g^{-1} FM) and sucrose (10 g / kg FM); LP-S_2, silages treated with LAB (LP-RC1.4, 10^6 CFU per g^{-1} FM) and sucrose (20 g/kg FM). The difference between values with different superscript letters [(a-c)] is statistically significant.

Also, other measured fermentation parameters of alfalfa silages such as the content of organic acids, amount of NH_3-N were the least preferred for silages untreated with the LP-RC1.4. The addition of starter culture with or without sucrose increased the content of LA (from 24.56 g kg^{-1} DM in control to 32.51–46.05 g kg^{-1} DM for inoculated silages). There was a numerical increase in the content of AA and PA for inoculated silages compared to those of the control. Also, the addition of starter culture with or without sucrose reduced the BA content (from 2.25 g kg^{-1} DM in control to 0.90–1.50 g kg^{-1} DM for inoculated silages). The addition of starter culture reduced the NH_3-N content (from 78.32 g kg^{-1} TN in control to 42.23–51.93 g kg^{-1} TN for the inoculated silages) and increased the content of WSC (from 9.86 g kg^{-1} in control to 10.92 g kg^{-1}–14.87 g kg^{-1} for the inoculated silages). The more pronounced WSC increase in sucrose silages was expected.

The lactobacilli population was significantly increased in the inoculated silages as 5.80, 6.08, and 6.12 cfu^{-1} g compared to the control (5.51 cfu^{-1} g), respectively ($p < 0.05$). Moreover, a more pronounced increase in lactobacilli counts was observed with the increase in sucrose addition level (from 5.80 cfu g^{-1} FM in the silages prepared with LP-RC1.4 alone to 6.08–6.12 cfu g^{-1} FM in the silage added with 1 or 2 % sucrose, respectively). Alfalfa silages prepared without any treatment were characterized by higher amounts of yeast and molds compared to those of the inoculated silages ($p < 0.05$). The addition of starter culture reduced the amounts of yeast (from 3.45 cfu g^{-1} FM in control to 2.97–3.18 cfu g^{-1} FM for the inoculated silages) and molds (from 2.03 cfu g^{-1} FM in control to 1.80–190 cfu g^{-1} FM for the inoculated silages). It was also determined that, in inoculated silages, increasing the sucrose level promoted lactobacilli count while it also significantly suppressed the number of yeast and mold ($p < 0.05$).

3.4. Aerobic stability indicators of silages

Aerobic stability indicators (pH, CO_2 production, and microbial population) of the alfalfa silages on the 5th days are shown in Table 4. pH value did not change, while the CO_2 production of alfalfa silages was decreased from 2.87 g kg^{-1} DM in control to 1.81 –2.03 g kg^{-1} DM in inoculated silages. While the excess lactobacillus count of inoculated silages (4.54–4.72 cfu g^{-1} FM) maintained in comparison to control silages (3.65 cfu g^{-1} FM), the yeast (from 4.27 cfu g^{-1} FM in control to 2.75–4.11cfu g^{-1} FM for inoculated silages) and molds' (from 2.22 cfu g^{-1} FM in control to 1.59–1.68 cfu g^{-1} FM for inoculated silages) numbers were lower in inoculated silages in comparison to those of control ($p < 0.05$), respectively.

4. Discussion

The formation of the desired silage fermentations depends on the amount and type of epiphytic microorganisms, as well as the DM content, buffering capacity and WSC of the silage feed [26]. The average WSC of raw alfalfa was lower than the 40–60 g/kg DM recommended by Mahanna [27] as adequate for the occurrence of good fermentation of silage. However, studies on alfalfa silage also reported WSC between 10 and 40 g/kg DM [28]. The chemical composition, WSC content, and the initial lactobacilli number in the fresh alfalfa (*Medicago sativa* L.) used the present trial were close in the values reported for alfalfa in some previous studies [1,9,10]. In addition, the LAB counts were lower than the minimum established by Muck [29] (5.0 log cfu/g fresh weight) as adequate for the occurrence of good fermentation of silage. In the present study, fresh

alfalfa (*Medicago sativa* L.) was, therefore, inoculated by LP-RC1.4 (10^6 cfu g^{-1} FM) with (1% or 2%) or without sucrose to provide a good-quality silage fermentation.

In the study, the LAB may have controlled the early active fermentation period, suppressing enterobacteria, clostridia, and other microorganisms and, thus, reduce proteolysis and fermentation DM losses [30]. The OM content of silages has also increased. These results, which were considered as a positive reflection of the high amount of WSC and intensive LAB fermentation on the nutritional properties of silage, were also important findings indicating that the silage quality has improved. The results were consistent with those of the previous studies where the alfalfa silage was inoculated by LAB species and supported by WSC [9, 31,32]. The reduction of CP content during the fermentation process was due to the plant and microbial proteolytic processes in the ensiled material, which change the nitrogenous compounds in silages [33], as not observed in our study. NDF is an indicator of the total amount of feed the animal can consume. As the amount of NDF increases, the amount of fiber increases the passage rate in the digestive tract of the feeds, and it takes longer to digest, so animals generally consume less feed [34]. ADF content of feeds is an indicator of the digestibility of roughages, and the digestion degree of feeds with low ADF content is higher [35]. In the present study, starter culture inoculation with or without sucrose decreased the content of NDF and ADF in silages compared to those of control. The fact that cell wall carbohydrate fractions that need their respective enzymes for digestion are treated with silage microbial enzymes is an important advantage of silage fermentation. The main reason for the decrease in the amount of NDF and ADF of inoculated silages is that this process was carried out by silage fermentation. Indeed, Aksu et al. [36] and Baytok et al. [37] indicated that decreases in the contents of NDF and ADF in inoculated silages were caused by increased cell wall digestion due to increased silage fermentation, which is supported by inoculant and/or WSC. These results are in compliance with those of previous studies where the alfalfa silage was inoculated with LAB species and supported with WSC [9, 31,32]. In contrast, Zhanget al. [8] reported that LAB and/or sucrose inoculation did not affect the content of NDF and ADF in alfalfa silages. The main reason for the changes in WSC content is that bacteria use these carbohydrates as substrates for growth. This mainly results in lactic acid synthesis [38]. As expected, the WSC concentrations of all silages were reduced during the fermentation due to the reduction of the inoculated silages content of ADF; the in vitro DM digestibility tended to increase as well as the in vitro OM digestibility increased remarkably in inoculated silages as reported previously [39].

In this study, examined the LP-CR1.4 inoculant contributed to the intensification of lactic acid fermentation as it affected the decrease in the pH, the increase in lactic acid content and the reduction in the content of butyric acid in silage. as reported previously [9, 11]. Furthermore, sucrose addition improved the fermentation process of alfalfa silage, by indicating the better silage fermentation [8, 9]. The pH value is considered as a very important indicator for estimating fermentation profile and extent of fermentation quality of ensiled materials [31]. The pH values of inoculated silages were generally higher than that of control silages, indicating that inoculating can induce a better fermentation compared to uninoculated silages. It is generally desired that the pH value be around 3.8 to 4.2 for any quality silage, but this is usually not possible for silages from legumes. It is difficult to bring the pH value below 5 for legume silages, especially with a relatively low DM content [30]. However, in this study, while measured pH values ranged between 5.17 and 5.38 for treatment groups, it had 5.63 for control silage. Some researchers found that inoculated alfalfa, corn or sorghum silages had lower pH values than the control groups in their studies [40,41]. It can be speculated that a low pH value of inoculated alfalfa silage may be a result the efficiency of the strain of LAB used in the experiment as reported previously [1,8,32]. The LA production in quantity and LA rate in total acid produced in silo are important parameters for evaluating feed value of silage. In the present study, the increase in the content of lactic acid in the silage inoculated with LP-RC1.4 due to the increased amount of sucrose is associated with the support of sucrose to the initial amount of WSC of the silage material. Thereby, the activity of the LAB population was promoted. Sucrose also increased the amount of AA and PA. Numerous studies have reported that application of sucrose increased the total fermentation acid content of the silages [28,42,43]. In the trial, LP-RC1.4 inoculation with or without sucrose increased the lactic : acetic acid ratio of alfalfa silages. These findings were consistent with the findings that *L. plantarum* LP1 and LP2 [32] and *L. plantarum* L12FL5 [9] strains increased the lactic : acetic acid ratio of alfalfa silage. On the other hand, since the LABdominated the silage process, the lower content of BA and NH_3-N was formed in the inoculated silages. Rapidly pH decrease and increased acidification in inoculated silages may have reduced the content of BA and NH_3-N by preventing proteolytic activity. This result may be related to suppression of the growth of clostridia [32] due to high and rapid acidification in inoculated silages [9] . Also, Rongrong et al [9] also reported that the inoculation of *L. plantarum* with sucrose to alfalfa silages significantly reduced the relative abundance of Clostridia and Enterobacteria by promoting the LAB growth and increasing the organic acids content of silages.

In the trial, dominance in the number of LAB in inoculated silages was reflected as a noticeable suppression

of the number of yeast and molds. Most forages are habitats for yeasts and molds, but they are undesirable microorganisms for silage. As reported in previous studies [10,44], the addition of sucrose resulted in a relative decrease in the number of yeast and molds while increasing the number of lactobacillus. Lactic acid alone is not an effective antimycotic agent. Thus, a dominant homolactic acid fermentation may adversely affect aerobic stability [45,46]. Also, lactic acid can be used as a substrate for the growth of yeasts during aerobic exposure [46]. In the present study, the aerobic test (on the 5th day) of alfalfa silage showed that LP-RC1.4 inoculated silages had less CO_2 production. However, the inoculated silages were heavier populated with LAB and contained smaller numbers of yeasts and mold compared to those of control in 5th day after silages were opened. These findings were in agreement with the findings of the researchers who indicated that *L. plantarum* is an effective silage inoculum, especially with its dynamic population increase, antibacterial and antifungal activity, and decontamination of mycotoxin in silages [11,12,13,44]. On the other hand, acetic acid has a durable antifungal property, and its high concentration may have increased aerobic stability by depressing the growth of undesirable microorganisms such as yeast and mold (literature). Currently, in this study, the relatively higher acetic acid content in the inoculated silages supported this report.

Consequently, the results of the study indicated that inoculating of LP-RC1.4 alone (at 10^6 cfu g^{-1} FM) enhanced the silage quality parameters and aerobic stability of alfalfa silages. Furthermore, the supplementing inoculated silages with sucrose (at 1 % and 2 % levels) improved the silage quality parameters and aerobic stability more remarkably. It was, therefore, concluded that the LP-RC1.4 inoculation should be supported with at least 1 % sucrose in order to observe a more dominant LAB fermentation in alfalfa silages.

Acknowledgment

The authors thank the Rectorate of Van Yüzüncü Yıl University, Kazan State Agrarian University, and Tatar Research Institute of Agricultural Chemistry and Soil Science for all their support.

The work was financially supported by the Research Fund of the Van Yüzüncü Yıl University (# TSG-2019-8016) and Kazan State Agrarian University Scientific Research Projects Coordinator (Cooperation agreement between Van Yüzüncü Yıl University (Turkey) and Kazan State Agrarian University, 01.06.2018), and Tatar Research Institute of Agricultural Chemistry and Soil Science (Russian Federation State target program #11021600147-1)

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Agarussi MCN, Rosinea OBP, de Paula RA, da Silva VP, Roseira JPS et al. Novel lactic acid bacteria strains as inoculants on alfalfa silage fermentation. Science Report 2019; 9: 8007. doi:10.1038/s41598-019-44520-9
2. Pahlow G, Muck RE, Driehuis F, Elferink SJWHO, Spoelstra SF. Microbiology of ensiling. In Buxton, DR, Muck, RE, Harrison JH (Editors) Silage Science and Technology. Madison, WI: Agron. Monogr. ASA, CSSA and SSSA; 2003. pp. 31–94.
3. Muck RE. Recent advances in silage microbiology. Agriculture and Food Science 2013; 22: 3-15.
4. Fabiszewska AU, Zielińska KJ, Wróbel B. Trends in designing microbial silage quality by biotechnological methods using lactic acid bacteria inoculants: a minireview World Journal of Microbiology and Biotechnology 2019; 35: 76. doi:10.1007/s11274-019-2649-2.
5. Oliveira AS, Weinberg ZG, Ogunade IM, Cervantes AAP, Arriola KG et al. Meta- analysis of effects of inoculation with homofermentative and facultative heterofermentative lactic acid bacteria on silage fermentation, aerobic stability, and the performance of dairy cows. Journal of Dairy Science 2017; 100 (6): 4587-4603. doi:10.3168/jds.2016-11815
6. Madigan MT, Martinko JM, Bender KS, Buckley DH, Stahl DA. Brock Biology of Microorganisms. 14th ed. San Francisco, USA: Benjamin-Cummings Publishing Company; 2015.
7. Avila CLS, Carvalho, BF. Silage fermentation—updates focusing on the performance of micro-organisms. Journal of Applied Microbiology 2019; 128: 966-984. doi:10.1111/jam.14450
8. Zhang Q, Yu Z, Wang X. Isolating and evaluating lactic acid bacteria strains with or without sucrose for effectiveness of silage fermentation. Grassland Science 2015; 61: 167-176. doi: 10.1111/grs.12097
9. Rongrong Li, Jiang D, Zheng M, Tian P, Zheng M et al. Microbial community dynamics during alfalfa silage with or without clostridial fermentation. Science Report 2020; 10: 17782. doi: 10.1038/s41598-020-74958
10. Ozduven ML, Cam AC. The effects of bacterial inoculants and/or enzymes on the fermentation characteristics and aerobic stability of alfalfa ensiled at different stages of maturity. International Journal of Current Research 2017; 9: 45983-45988.
11. Liu Q, Dong Z, Shao T. Effect of additives on fatty acid profile of high moisture alfalfa silage during ensiling and after exposure to air. Animal Feed Science and Technology 2018; 236: 29-38. doi:10.1016/j.anifeedsci.2017.11.022
12. Zielińska K, Fabiszewska A, Stefańska I. Different aspects of Lactobacillus inoculants on the improvement of quality and safety of alfalfa silage. Chilean Journal of Agricultural Research 2015; 75: 298-306. doi:10.4067/S0718 -58392 01500 04000 05

13. Guo XS, Ke WC, Ding WR, Ding LM, Xu DM et al. Profiling of metabolome and bacterial community dynamics in ensiled *Medicago sativa* inoculated without or with *Lactobacillus plantarum* or *Lactobacillus buchneri*. Science Reports 2018; 8: 357-366. doi:10.1038/s41598-017-18348-0

14. Louws FJ, Fulbright DW, Stephens CT, DeBruijn FJ. Specific genomic fingerprints of phytopathogenic Xanthomonas and Pseudomonas pathovars and strains generated with repetitive sequences and PCR. Applied Enviromental Microbiology 1994; 60: 2286-2295.

15. De S, Kaur G, Roy A, Dogra G, Kaushik R et al. A Simple method for the efficient isolation of genomic DNA from lactobacilli isolated from traditional Indian Fermented Milk (dahi). Indian Journal of Microbiology 2010; 50 (4): 412-418. doi: 10.1007/s12088-011-0079-4

16. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 1991; 173 (2): 697-703. doi: 10.1128/jb.173.2.697-703.1991

17. Altschul SF, Gish W, Miller W, Meyers EW, Lipman DJ. Basic local alignment search tool. Journal of Molecular Biology 1990; 215: 403-410.

18. Broderick GA, Kang JH. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. Journal of Dairy Science 1980; 63: 64-75. doi:10.3168/jds.S0022-0302(80)82888-8

19. Ashbell G, Weinberg ZG, Azrieli A, Hen Y, Horev B. A simple system to study the aerobic deterioration of silages. Canadian Agricultural Engineering 1991; 34: 171-175.

20. Ministry of Agriculture, Fisheries and Food. The Analysis of Agricultural Material. Reference Book, London, UK: H.M. Stationery Office,1986. pp. 427-428.

21. AOAC. Official Methods of Analysis, 15th ed., Association of Official Analytical Chemists, Arlington, VA, USA: The Association, 1990.

22. Van Soest PJ, Robertson JB, Lewis BA. Methods of dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. Journal of Dairy Science 1991; 74: 3583-3597. doi: 10.3168/jds.S0022-0302(91)78551-2

23. Seale D. Bacterial inoculants as silage additives. Journal of Applied Bacteriology 1986; 61: 9-26.

24. SAS, SAS User's Guide: Statistics, 5th ed. Cary, NC, USA: SAS Institute Inc., 1989.

25. Steel RGD, Torrie JH, Dickey DA. Principles and Procedures of Statistics: A Biometrical Approach. 3rd ed. New York, NY, USA: Mc Graw Hill Book Company, 1997.26. McDonald P, Henderson N, Heron S. The Biochemistry of Silage. 2nd ed. Marlow England: Chalcombe Publications; 1991.

27. Mahanna B. Proper management assures high-quality silages, grains. Feedstuffs 1994; 12-15.

28. Rodrigues PHM, De Almeida LFS, Lucci CS, Melotti L, De Lima, FR. Efeitos da adição de inoculantes microbianos sobre o perfil fermentativo da silagem de alfafa adicionada de polpa cítrica. Revista Brasileira de Zootecnia 2004; 33: 1646-1653.

29. Muck RE. Inoculant of silage and its effects on silage quality. In: Informational Conference with Dairy and Forage Industries. US Dairy Forage Research, Madison, WI; 1996. pp. 43–52.

30. Muck RE, Nadeau EMG, McAllister TA, Contreras-Govea FE, Santos MC, Kung Jr L. Silage review: Recent advances and future uses of silage additives. Journal of Dairy Science 2018; 101: 3980-4000. doi.org/10.3168/jds.2017-13839

31. Denek N, Can A, Avcı M, Aksu T, Durmaz H. The effect of molasses-based pre-fermented juice on the fermentation quality of first-cut lucerne silage. Grass and Forage Science 2011; 66: 243–250. doi: 10.1111/j.1365-2494.2011.00783.x

32. Zheng ML, Niu DZ, Jiang D, Zuo SS, Xu CC. Dynamics of microbial community during ensiling direct-cut alfalfa with and without LAB inoculant and sugar. Journal of Applied Microbiology 2017; 122: 1456-1470. doi:10.1111/jam.13456

33. Kung L, Shaver RD, Grant RJ, Schmidt RJ. Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. Journal of Dairy Science 2018; 101: 4020-4033. doi.org/10.3168/jds.2017-13909

34. Harper KJ, McNeill DM. The role iNDF in the regulation of feed intake and the importance of its assessment in subtropical ruminant systems (the role of iNDF in the regulation of forage intake). Agriculture 2015; 5: 778-790. doi:10.3390/agriculture5030778

35. Owens FN, Sapienza DA, Hassen A. Effect of nutrient composition of feeds on digestibility of organic matter by cattle: A review. Journal of Animal Science 2010; 88: 151-69. doi: 10.2527/jas.2009-2559

36. Aksu T, Baytok E, Bolat D. Effects of a bacterial silage inoculant on corn silage fermentation and nutrient digestibility. Small Ruminant Research 2004; 55: 249-252.

37. Baytok E, Aksu T, Karslı MA, Muruz H. The Effects of formic acid molasses, and inoculant as silage additives on corn silage composition and ruminal fermentation characteristics in sheep. Turkish Journal of Veterinary Animal Science 2005; 29: 469-479.

38. Muck RE. Silage microbiology and its control through additives. Revista Brasileira de Zootecnia 2010; 39: 183-191

39. Denek N, Can A, Avcı M, Aksu T. The Effect of fresh and frozen pre-fermented juice on the fermentation quality of alfalfa silage. Kafkas Universitesi Veteriner Fakultesi Dergisi 2012; 18 (5): 785-790, doi:10.9775/kvfd.2012.6396

40. Filya I, Muck RE, Contreas-govea FE. Inoculant effects on alfalfa silage: Fermentation products and nutritive value. Journal of Dairy Science 2007; 90:5108-5114. doi: 10.3168/jds.2006-877

41. Filya I. The effect of *Lactobacillus buchneri* and *Lactobacillus plantarum* on the fermentation, aerobic stability, and ruminal degradability of low dry matter corn and sorghum silages. Journal of Dairy Science 2003; 86: 3575-3581. doi: 10.3168/jds.S0022-0302(03)73963-0

42. Lima R, Lourenco M, Diaz RF, Castro A, Fievez V. Effect of combined ensiling of sorghum and soybean with or without molasses and lactobacilli on silage quality and in vitro rumen fermentation. Animal Feed Science and Technology 2010; 155: 122-131. doi: 10.1016/j.anifeedsci.2009.10.008

43. Heinritz SN, Martens SD, Avila P, Hoedtke S. The effect of inoculant and sucrose addition on the silage quality of tropical forage legumes with varying ensilability. Animal Feed Science and Technology 2012; 174: 201-210. doi:10.1016/j.anifeedsci.2012.03.017

44. Zhang T, Li L, Wang X, Zeng Z, Hu Y et al. Effects of *Lactobacillus buchneri* and *Lactobacillus plantarum* on fermentation, aerobic stability, bacteria diversity and ruminal degradability of alfalfa silage. World Journal of Microbiology and Biotechnology 2009; 25: 965-971. doi:10.1007/s11274-009-9973-x

45. Muck RE, Kung LJ. Effects of silage additives on ensiling. In: Proceeding from the Silage: Field to Feedbunk North American Conference; Hershey, USA; 1997. pp. 187-199.

46. Moon NJ. Inhibition of the growth of acid tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. Journal of Applied Bacteriology 1983; 55: 454-460. doi: 10.1111/j.1365-2672.1983.tb01685.x