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DETERMINATION OF THE EFFECT OF A LACTIC ACID BACTERIA+ENZYME MIXTURE ON THE SILAGE QUALITY AND DIGESTIBILITY OF VETCH-OAT MIXTURE SILAGES

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Original scientific paper

Abstract: This study was carried out to determine the effects of the addition of lactic acid bacteria+enzyme (LBE) mixture on the fermentation characteristics and digestibility of Hungarian vetch-oat silages. An inoculant was used as additive which contains *Lactobacillus plantarum* CNCM l-3235, *Pediococcus pentosaceus* NCIMB 12455, *Pediococcus acidilactici* CNCM l-3237, *Propionibacterium acidipropionici* CNCM MA26/4U, alpha-amylase from *Bacillus amyloliquefaciens*, cellulase from *Trichoderma reesei*, xylanase from *Trichoderma longibrachiatum*, beta-glucanase from *Aspergillus niger* in its biological composition. While additive was not used for control, LBE1 and LBE2 groups were inoculated with LBE as 300 000 and 500 000 cfu/g of silage material, respectively. After 60-d of incubation, no significant difference was observed in pH values among silages, but the highest lactic acid value was detected in the LBE2 group silages ($P<0.01$). *In vitro* neutral detergent fiber digestibility (IVNDFD) values of the silages were 41.65, 44.14 and 47.38% for the control, LBE1 and LBE2 groups, respectively, and it was determined that there was a linear correlation ($r=0.945$) between the inoculant doses and the IVNDFD values. As a result, LBE improved fermentation characteristics and IVNDFD values of the Hungarian vetch-oat mixture.

Key words: digestibility, enzyme, lactic acid bacteria, vetch-oat silage

Introduction

Ensiling allows large amounts of forage to be conserved rapidly. The harvesting and storing process of ensiling is less dependent on the weather compared to haymaking. Corn, alfalfa, and other legumes, grasses, sorghum, and other alternative crops are commonly ensiled crops (Grant and Adesogan, 2018).

Lactic acid bacteria (LAB) play a crucial role in promoting a rapid and efficient fermentation process in silage. They convert plant sugars into lactic acid (LA) leading to a decrease in pH, which helps in preserving the forage and inhibiting the growth of undesirable microorganisms (Dunière et al., 2013). Futhermore, enzyme inoculants also play a crucial role in the breakdown of complex carbohydrates present in forage crops into simpler sugars. Both cellulolytic and other hydrolytic enzymes facilitate the degradation of plant cell walls, thereby increasing the availability of fermentable substrates for microbial fermentation (Irawan et al., 2021). This synergistic action not only improves the overall fermentation process and results in better silage quality (Dunière et al., 2013; Irawan et al., 2021), but also results in improved nutrient release and availability for animal digestion and utilization (McDonald et al., 1991; Ogunade et al., 2019). The application of enzymes at ensiling might have a beneficial effect on forages with low sugar contents (Nadeau et al., 2000) like as forage legumes which have higher crude protein but lower easily fermentable carbohydrate content than those of cereals. To our knowledge, the addition of lactic acid bacteria+enzyme (LBE) mixture for Hungarian vetch-oat silage has not been extensively explored. Therefore, the present study was conducted to assess the effect of lactic acid bacteria+enzyme mixture on the fermentation quality, nutrient composition, and *in vitro* digestibility values of Hungarian vetch-oat silage.

Material and Method

Silage Material and Experimental Design

The experiment was carried out at the field of Asarcık, Samsun, in the middle Black Sea (41.0346 N, 36.2451 E) on Hungarian vetch-oat mixture, grown for ensiling. The mixture with 75% Hungarian vetch seeds and 25% oat seeds was sown, and during sowing a composite fertilizer was applied. The Kansur variety of Hungarian vetch *(Vicia pannocia)* and a local variety of oats commonly grown in the region was used in the mixture. The Hungarian vetch-oat mixture was harvested at the early bloom stage of Hungarian vetch. Before ensiling, the silage material was wilted and chopped into approximately 1.5-2.0 cm size pieces. The commercial LBE mixture consisted of *Lactobacillus plantarum* CNCM l-3235, *Pediococcus pentosaceus* NCIMB 12455, *Pediococcus acidilactici* CNCM l-3237, *Propionibacterium acidipropionici* CNCM MA26/4U, and also it contained alpha-amylase from *Bacillus amyloliquefaciens*, cellulase from *Trichoderma reesei*, xylanase from *Trichoderma longibrachiatum*, beta-glucanase from *Aspergillus niger*. The inoculant was dissolved in a small amount of distilled water prior to ensiling, and it was sprayed evenly onto the silage material of LBE groups, and

thoroughly mixed for homogenization. Hungarian vetch-oat mixture was inoculated following as Control (without LBE), LBE1 (300 000 cfu/g of silage material), and LBE2 (500 000 cfu/g of silage material). The silage material was tightly packed into laboratory-scale silos with a volume of 1 L, ensuring no air pockets. A total of 15 silos, five silos for each group, were prepared and incubated at room temperature (21±2°C) for 60 days.

Determination of Nutrients and Fermentation Characteristics of Silages

The dry matter (DM) content of the silages was determined by drying the samples in a circulating air oven at 60°C for 48 hours. The dried samples were ground to achieve homogeneity for analysis. Obtained in this way was held in a circulating air oven at 105°C until a constant weight was reached. All results related to chemical composition and content of nutrients are expressed in absolute dry matter. The ash content was calculated after the organic matter portion of the samples was incinerated in a muffle furnace at 560°C for 4 hours. The crude protein (CP) content was determined using the Kjeldahl method. The values for acid detergent fiber (ADF), neutral detergent fiber (NDF), and acid detergent lignin (ADL) were analyzed using the Ankom 200/220 Fiber Analyzer following the method described by Van Soest et al. (1991). After 60-d of incubation, a total of 25 g sample of silage was taken from each silo and homogenized in approximately 100 ml of distilled water for about 10 minutes (Polan et al., 1998). The pH value of the silage liquid was measured using a pH meter. Silage samples were prepared following the method reported by Tjardes et al. (2000) and the detection of silage acids was performed using an HPLC following the procedure described by Canale et al. (1984).

Determination of Digestibility Values

The Ankom Daisy incubator was used to determine the *in vitro* digestibility values in the study. For the fermentation system, rumen contents were obtained from adult cattle slaughtered at a local abattoir in Samsun province. Immediately after slaughter, rumen contents were collected and filtered through four layers of cheesecloth into a thermos preheated to 39°C and filled with CO_2. The rumen fluid's pH was measured to be 6.25. The preparation of the buffers, 48-h of incubation procedure and the calculations of *in vitro* true digestibility (IVTD), *in vitro* true dry matter digestibility (IVTDMD), *in vitro* true organic matter digestibility (IVTOMD) and *in vitro* neutral detergent fiber digestibility (IVNDFD) values were done according to the operation manual of the Ankom Daisy incubator.

Statistical Analysis

The Shapiro-Wilk normality test was applied to all data in the study. The Levene's test for homogeneity was conducted for data that showed a normal distribution. One-way analysis of variance (ANOVA) was applied to data that exhibited homogenous distribution, and post-hoc multiple comparisons using the Tukey test were conducted to determine group differences. For data that did not show homogenous distribution, group differences were determined using the Tamhane test. The Kruskal-Wallis non-parametric test was applied to data that did not follow a normal distribution, and group differences were determined using the Mann-Whitney test. The SPSS software package was used for statistical analysis (SPSS 2012).

Results

The different amounts of LBE inoculant dramatically affected DM, CP, ash, LA and propionic acid (PA) values of 75% Hungarian vetch-25% oat mixture silages (Table 1). *In vitro* digestibilty values of the silages were presented in Table 2. There was an increase ($P<0.01$) in IVNDFD in the LBE-supplemented silages. There was a linear correlation ($r=0.945$) for IVNDFD value among the control, LBE1, and LBE2 groups were given in Figure 1.

Table 1. Effects of lactic acid bacteria+enzyme (LBE) additive on conservation characteristics (% on dry matter, unless otherwise stated) of 75% Hungarian vetch-25% oat mixture silages

Characteristics	Control x ± Sx	LBE 1 x ± Sx	LBE 2 x ± Sx	P value
Fresh DM %	23.17 ± 0.13^{a}	22.02 ± 0.61^{b}	22.33 ± 0.10^{b}	0.01
Ash %	9.77 ± 0.07^{b}	10.11 ± 0.12^{ab}	10.28 ± 0.11^{a}	0.01
CP %	14.43 ± 0.18^{b}	14.96 ± 0.09^{a}	13.82 ± 0.11^{c}	0.00
ADF_{OM} %	32.36 ± 0.53	31.33 ± 0.13	32.38 ± 0.20	0.07
NDF_{OM} %	44.09 ± 1.11	43.99 ± 0.76	45.31 ± 0.60	0.54
ADL_{OM} %	3.43 ± 0.18	3.09 ± 0.28	2.78 ± 0.07	0.10
pH	4.14 ± 0.03	4.19 ± 0.01	4.13 ± 0.00	0.16
LA %	17.31 ± 0.32^{b}	18.95 ± 0.29^{ab}	20.54 ± 0.85^{a}	0.00
AA %	1.60 ± 0.09	1.64 ± 0.05	1.59 ± 0.06	0.83
PA %	4.80 ± 0.07^{c}	6.40 ± 0.05^{b}	12.80 ± 0.01^{a}	0.01

DM: Dry matter; CP: Crude protein; ADF_{OM}: Acid detergent fibre exclusive of residual ash; NDF_{OM}: Neutral detergent fibre exclusive of residual ash; ADL_{OM}: Acid detergent lignin exclusive of residual ash; LA: Lactic acid; AA: Acetic acid; PA: Propionic acid

Table 2. Effects of lactic acid bacteria+enzyme (LBE) additive on digestibility values of 75% Hungarian vetch-25% oat mixture silages

Digestibility%	Control x ± Sx	LBE 1 x ± Sx	LBE 2 x ± Sx	P value
IVTD	76.58 ± 0.67	77.35 ± 0.51	77.95 ± 0.25	0.21
IVTDMD	74.26 ± 0.69	75.41 ± 0.56	76.16 ± 0.28	0.08
IVTOMD	71.94 ±0.80	73.07 ± 0.66	73.77 ± 0.27	0.15
IVNDFD	41.65 ± 0.29^{c}	44.14 ± 0.66^{b}	47.38 ± 0.22^{a}	0.00

IVTD: *In vitro* true digestibility; IVTDMD: *In vitro* true dry matter digestibility; IVTOMD: *In vitro* true organic matter digestibility; IVNDFD: *In vitro* neutral detergent fiber digestibility

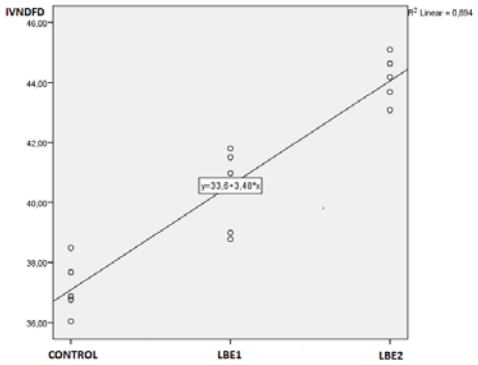

Figure 1. A linear correlation for IVNDFD value among groups
IVNDFD: *In vitro* neutral detergent digestibility; LBE: Lactic acid bacteria+enzyme

Discussion

To get good-quality silage, forages having high carbohydrate and low protein contents are generally preferred but this results in silage with high-energy but low-protein contents. However, a good feed material has to have a balanced energy and protein content. Therefore, energy-rich forages may be mixed with protein-rich forages or mixed planting (Şen et al., 2022). Kılıçalp et al. (2022) found that the LAB+Enzyme inoculant added to triticale and Hungarian vetch silages with different seed ratios resulted in lower crude protein contents in the groups with LAB+Enzyme compared to the control group. In the current study, the lower crude protein content of the LBE2 group compared to the control and LBE1 groups was similar to the findings of Kılıçalp et al. (2022). Some previous studies (Coskuntuna and Gül, 2020; Şen et al., 2022; Li et al., 2022; Marbun et al., 2020) reported that the addition of inoculant to silage material did not affect the ADF and

NDF contents of the silage. The results of the current study for ADF, NDF, and ADL were compatible with previous studies. Marković et al. (2018) stated that the pH values of silages made from 75:25 common vetch-oat mixture with or without inoculant were between 4.22-4.32. According to Weissbach (1996), to obtain well-fermented and stable silage with a dry matter content of 20%, pH values below 4.2 were necessary. The results of the current study were comply with this requirement.

In the silages, lactic acid produced by LAB is the primary acid and it contributes significantly to the decrease in pH during silage fermentation. Silages with low dry matter content (<30%) have higher LA concentrations (Kung Jr et al., 2018). In the current study, the lactic acidification in LBE2 silage was enhanced, which might be due to the dose of the inoculant that improved fermentation, resulting in higher LA and lower pH values compared to the other groups. The second silage acid is acetic acid (AA) and it can increase the stability of silage because of its negative effects on the growth of yeasts when silage is exposed to air. High concentrations of AA in silage (>4-6%) generally occur in undesirable silage fermentations dominated by bacteria such as enterobacteria and clostridia. The concentration of AA in grass silage is typically between 1% and 3% (on a DM basis) (McDonald et al., 1991). In the current study, the AA concentration in silages untreated or treated with LBE inoculant was similar and consistent with that of McDonald et al. (1991). In a previous study (Flores-Galarza et al., 1985) it was reported that propionic acid bacteria increased PA concentration and reduced the proliferation of yeasts and moulds of high-moisture maize silages. Kung Jr et al. (2018) mentioned that Propionibacteria that convert glucose and LA to PA and AA have been found in silages. In the current study, PA concentration was increased in the silages inoculated with the different doses of LBE, which might be because the LBE inoculant used in the study contained *P*ropionibacterium acidipropionici which produced PA and caused an increase in PA concentration. Butyric acid (BA) is an acid that should not be detected in well-fermented silage. The presence of BA in silage is associated with the metabolic activity of Clostridium species (Pahlow et al., 2003). It is stated that the amount of BA in high-quality silage should be less than 0.1% (Seglar, 2003). In this study, the presence of BA could not be determined, which is consistent with the findings of Pahlow et al. (2003) and Seglar (2003).

Roughage digestibility is one of the most important parameters that directly reflects the quality of silage (Li et al., 2022; Liu et al., 2019). No significant difference was observed between the control and the silages inoculated with LBE in terms of the IVTD, IVTDMD, and IVTOMD values, which was consistent with Li et al. (2022) who reported the lack of effects of a commercial lactic acid bacteria-based inoculant as a silage additive on IVDMD. Another study conducted by Başkavak et al. (2008) indicated that LAB and enzyme inoculation did not affect the IVDMD and IVOMD values of wheat silage, but the addition of

inoculants tended to increase these values like the findings of the present study. However, in this study, there was an increase in IVNDFD value of the silages inoculated with LBE was consistent with the results of Guo et al. (2020), who reported that L. plantarum and E. faecalis increased IVNDFD. Similarly, in this study, it was found that LBE additions at different levels increased the IVNDFD at 48 h. The positive response to LBE addition in this study may be attributed to the disruption of plant cell wall structures. This disruption may effectively release intracellular contents, providing more substrate for binding and fermentation by rumen microorganisms. Oba and Allen (1999) stated that a 1-unit improvement in IVNDFD is positively associated with 0.17 kg of DM and 0.25 kg of corrected milk yield per 4% fat. In this study, increases of 5.97% and 13.75% were observed in NDFD values for silages in the LBE1 and LBE2 groups, respectively. According to Oba and Allen (1999), the increased IVNDFD values of the silages in the LBE1 and LBE2 groups compared to the silages in the control group potentially can resulted in an increase of 0.42 kg and 0.97 kg in DM intake and 0.62 kg and 1.43 kg in corrected milk yield per 4% fat, respectively.

Conclusion

Based on the obtained data, the mixture of Hungarian vetch-oat silage could be preserved without additive. However, the use of LBE inoculant up to 500 000 cfu/g had a positive response in the fermentation quality and IVNDF digestibility.

Određivanje uticaja mešavine mlečne kiseline i enzima na kvalitet silaže i svarljivost silaže mešavine grahorice i ovsa

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Rezime

Ovo istraživanje je sprovedeno da bi se utvrdili efekti dodavanja mešavine bakterija mlečne kiseline i enzima (LBE) na karakteristike fermentacije i svarljivost mađarske silaže od grahorice i ovsa. Kao aditiv je korišćen inokulant koji sadrži *Lactobacillus plantarum* CNCM l-3235, *Pediococcus pentosaceus* NCIMB 12455, *Pediococcus acidilacti* CNCM l-3237, *Propionibacterium acidipropionici* CNCM MA26/4U, alfa amilaze iz *Bacillus amyloliquefaciens*, celulaza iz *Trichoderma reesei*, ksilanaza iz *Trichoderma longibrachiatum*, beta-glukanaza iz *Aspergillus niger* u svom biološkom sastavu. Dok aditiv nije korišćen za kontrolu, LBE1 i LBE2 grupe su inokulisane sa LBE kao 300 000 i 500 000 cfu/g silažnog materijala, respektivno. Posle 60 dana inkubacije, nije primećena

značajna razlika u pH vrednostima među silažama, ali je najveća vrednost mlečne kiseline otkrivena u silažama grupe LBE2 (P<0,01). *In vitro* vrednosti svarljivosti vlakana neutralnog deterdženta (IVNDFD) silaža su bile 41,65, 44,14 i 47,38% za kontrolnu, LBE1 i LBE2 grupu, respektivno, i utvrđeno je da postoji linearna korelacija (r=0,945) između doza inokulanta i IVNDFD vrednosti. Kao rezultat toga, LBE je poboljšao karakteristike fermentacije i IVNDFD vrednosti mađarske mešavine grahorice i ovsa.

Ključne reči: svarljivost, enzim. bakterije mlečne kiseline, silaža grahorica-ovas

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Conflict of interest
The authors declare that they have no conflict of interest.

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