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LACTIC ACID BACTERIA AND ENZYME INOCULATION OF ITALIAN RYEGRASS-RED CLOVER SILAGE: ENHANCING MICROBIAL PROFILE, FERMENTATION QUALITY, AND DIGESTIBILITY

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Abstract: This study evaluated the effects of inoculating Italian ryegrass-red clover with a commercial additive containing lactic acid bacteria and enzymes (LABE) on the microbial profile, fermentation quality, nutritive value, and digestibility of silages. The experimental treatments were: (1) control (without LABE); (2) LABE1 (inoculated at 1.5×10^5 cfu/g of ensiled mass); and (3) LABE2 (inoculated at 3.0×10^5 cfu/g of ensiled mass). After a 60-day incubation period, nutrient composition, fermentation characteristics, microbiology, and digestibility were determined. The pH values of the first harvest Italian ryegrass-red clover silages were 4.59, 4.15, and 4.14 for the control, LABE 1, and LABE 2 groups, respectively ($P<0.05$), while the corresponding values for the second harvest silages were 4.90, 4.09, and 4.14 ($P<0.05$). In the control silages, the lactic acid contents were 7.60 and 3.68%, for the first and second harvests, respectively. Following LABE inoculation, these values increased to 8.02 and 10.01% for the first harvest LABE 1 and LABE 2 groups, and to 6.15 and 6.38% for the second harvest treatments, respectively ($P<0.05$). Ammonia nitrogen concentrations did not differ in the first harvest, however, in the second harvest, the LABE2 group had higher levels (101.54 g/L) compared to control (69.66 g/L) and LABE1 (81.25 g/L) groups ($P<0.05$). Microbiological analysis showed a decrease ($P<0.05$) in total aerobic mesophilic bacteria and enterococci populations in both harvests, while lactic acid bacteria counts were unaffected. Yeast-mold, and enterobacteria counts were below detection limits (<2.3 $\log_{10}$ cfu/g) in all groups. Furthermore, the *in vitro* neutral detergent fiber digestibility values of the first and second harvest control silages were 34.97 and 32.68%, respectively. LABE inoculation increased these values to 41.13 and 35.04% for the first harvest LABE 1 and LABE 2 groups, and to 42.45 and 39.26% for the second harvest treatments, respectively ($P<0.05$).

The population of lactic acid bacteria present in the natural microflora of fresh forage is often unpredictable. By overcoming this limitation, LABE improved the overall silage profile, as the enzymes accelerated fiber degradation, providing essential substrates for lactic acid bacteria. Ultimately, the LABE 2 treatment (inoculated at 3.0×10 cfu/g) produced the most optimal results. Consequently, these improvements in fiber digestibility are expected to positively affect overall animal performance, although further *in vivo* research is required to confirm these effects.

Key words: digestibility, fermentation, inoculant, Italian ryegrass-red clover silage, microbial profile

Introduction

The primary objective of silage production is to preserve green forage with high nutritional value while minimizing nutrient loss. In this process, in addition to the chemical composition of the ensiled mass, the microbial population and environmental conditions that stimulate or inhibit its growth significantly influence silage quality (Ávila and Carvalho, 2019). To ensure and enhance fermentation, the use of silage additives is generally recommended. Microbial inoculants and enzymes are increasingly used, as they are recognized as safe, natural products that do not pose environmental risks (Irawan et al., 2021; Liu et al., 2021). The addition of lactic acid bacteria (LAB) inoculants protect silage from spoilage by enhancing lactic acid (LA) fermentation, lowering pH, and thereby inhibiting the growth of undesirable microorganisms, ultimately improving its nutritional value (Wang et al., 2019). While inoculants primarily consist of LAB, whose main function is to rapidly reduce pH, decrease proteolysis, and increase the aerobic stability of the silage, enzymes are added to this mixture to degrade structural fibers. This enzyme addition increases carbohydrate availability, working synergistically with the bacterial combinations to enhance overall fermentation quality (Dong et al., 2025).

Italian ryegrass is a globally cultivated forage crop, widely grown in temperate, subtropical, and tropical plateau regions, and its silage is extensively used for feeding ruminants during the winter months (Yin et al., 2022). Green forage yields for the first, second, and third harvests are approximately 8.65, 8.48, and 9.21 t/ha, respectively, resulting in a total green herbage production of 26.34 t/ha (Şahin, 2019).

Red clover is a versatile temperate forage crop cultivated extensively for hay, grazing, and silage production (McKenna et al., 2018). It is highly valued for its ease of establishment, rapid growth, high forage quality, and excellent soil improvement characteristics across a wide range of environmental conditions. Its maximum dry matter yield during the first harvest year in Europe can range from 9

to 18 t/ha without nitrogen fertilization, although these yields tend to decrease sharply after the second harvest year. A major nutritional advantage of red clover is that its digestibility declines more slowly with advancing maturity compared to grasses. Therefore, mixing red clover with grass species extends the optimal harvest window, yielding a highly digestible forage ideal for high-yielding dairy cows. Due to these characteristics, red clover is most commonly preserved as silage for the winter feeding of ruminants (Frankow-Lindberg, 2017).

Leguminous forages such as red clover are rich in protein but often lack sufficient water-soluble carbohydrates (WSC) for optimal LA production during ensiling. Furthermore, their high buffering capacity can delay pH decline, thereby increasing the risk of undesirable fermentation. Recent studies have confirmed that the fermentation dynamics and microbial community structure of red clover silage are highly sensitive to substrate composition and initial epiphytic microbiota (Dong et al., 2022). To overcome these limitations, mixing legumes with gramineous forages such as Italian ryegrass has been widely adopted as an effective strategy to improve fermentation quality and nutrient balance (Dündar and Mut, 2023). However, recent research on Italian ryegrass silage has shown that fermentation characteristics are strongly influenced by factors such as maturity stage and microbial composition, indicating that optimizing silage mixtures remains complex and system-dependent (Yin et al., 2023). In parallel, the use of silage additives has advanced considerably in recent years, with growing interest in combined microbial and enzymatic treatments. Recent studies have demonstrated that the synergistic use of LAB and fibrolytic enzymes can enhance fermentation quality, promote fiber degradation, and modify microbial communities more effectively than single additives (Liu et al., 2024; Fang et al., 2025). However, these studies have largely focused on low-quality crop residues or single-species silages, and their applicability to legume-grass mixtures remains unclear. More importantly, there is still limited information regarding the use of combined LAB and enzyme (E) inoculants (LABE) in Italian ryegrass-red clover silages, particularly concerning their integrated effects on fermentation dynamics, fiber degradation, and digestibility. Therefore, a clear research gap exists regarding the effectiveness of LABE inoculants in optimizing both fermentation quality and nutrient utilization in legume- gramineous silage systems. This study aims to address this gap by evaluating the effects of a LABE inoculant on the fermentation characteristics, fiber degradation, and digestibility of Italian ryegrass-red clover silage. We hypothesized that LABE application would enhance fermentation efficiency through accelerated acidification and improve digestibility by promoting plant cell wall degradation.

Materials and Methods

Silage material and experimental design

The experiment was conducted in a field in Amasya Province in the Middle Black Sea Region of Turkey (41˚ 2’ 5.48″ E and 36˚ 14’ 41.97″ N). A mixture of Italian ryegrass (*Lolium multiflorum* cv. Jivet) and red clover (*Trifolium pratense* cv. Suez), consisting of 80% and 20% seeds, respectively, was cultivated and sown at a rate of 4 kg/ha. The forage was harvested at the milk stage of Italian ryegrass, which served as the reference maturity point for the mixture (Mut et al., 2020). After harvesting, the forage from two separate harvests was wilted and chopped into pieces approximately 1.5 cm long.

The experimental treatments and the detailed composition of the commercial inoculant (Sil All 4×4, Lallemand Animal Nutrition, Blagnac, France; Lot No: V3136; Prod: 02/02/2020) were presented in Table 1.

Table 1. Experimental treatments and inoculant characteristics

	Control	LABE1	LABE2
Inoculant rate (cfu/g)	None	1.5×10^5 cfu/g of ensiled mass	3.0×10^5 cfu/g of ensiled mass
Inoculant	LAB: *Lactobacillus plantarum* CNCM l-3235, *Pediococcus pentosaceus* NCIMB 12455, *Pediococcus acidilactici* CNCM l-3237, *Propionibacterium acidipropionici* CNCM MA26/4 E: alpha amylase from *Bacillus amyloliquefaciens*, cellulase from *Trichoderma reesei*, xylanase from *Trichoderma longibrachiatum*, beta-glucanase from *Aspergillus niger*		

To ensure uniform distribution, the inoculant was first mixed into a minimal volume of distilled water, then applied to the chopped forage using a fine spray technique followed by vigorous mixing. For each treatment, the chopped forage mixtures were packed into laboratory-scale glass jar silos with a capacity of 1 L. Proper compaction to achieve optimal anaerobic conditions is essential for successful fermentation and minimizing aerobic spoilage. Following DLG (2012) guidelines, a target packing density of 200-240 kg dry matter (DM)/m³ is recommended. In this study, a density of 240 ± 5 kg DM/m³ was achieved, ensuring high-quality preservation and a stable fermentation environment. To prevent oxygen ingress and maintain anaerobic conditions, the glass jar silos were tightly filled with chopped forage and sealed using screw caps combined with plastic tape. A total of 15 silos were used for each harvest period, providing five replicates per experimental group. The silos were then stored in a temperature-controlled environment at 21±2°C for a 60-day fermentation period.

Chemical analysis of silages

Silage materials and also silage samples collected from the silos were dried at 60°C for 48 hours in a circulating air oven and ground to a 1 mm particle size. The DM, organic matter (OM), and crude protein (CP) contents were analysed using standard methods of AOAC (2006). All results for chemical composition and nutrient content were expressed on a DM basis. Fiber fractions, including ash-free acid detergent fiber (ADF_{OM}), ash-free neutral detergent fiber (NDF_{OM}), and ash-free acid detergent lignin (ADL_{OM}), were determined using an Ankom 200/220 Fiber Analyzer according to Van Soest et al. (1991). Total digestible nutrients (TDN), non-fiber carbohydrates (NFC), and total carbohydrates (TC) were calculated using the following equations:

TDN (%) = 105.2 – 0.68 × %NDF (Chandler, 1990).

NFC (%) = 100 – (%NDF + %CP + %Ether Extract (EE) + %Ash) (Weiss et al., 1992).

TC (%) = 100 – (%CP + %EE + %Ash) (Sniffen et al., 1992).

Determination of fermentation characteristics of silages

After the 60-day ensiling period, in order to determine the pH value of the experimental silages, a 25 g silage sample from each silo was homogenized in 100 mL of distilled water for 10 minutes (Polan et al., 1998). The pH of the silage extract was measured using a pH meter (Thermo Orion 710 A+, Thermo Electron Corporation). The LA concentrations in the silages were determined spectrophotometrically using the Barnett method (Barnett, 1951; Tekin and Kara, 2020). A standard lithium lactate curve (0.312–160 μg/ml; $R^2 = 0.95$) was used to calculate lactate equivalents. To express the results as a percentage of silage DM, the following equation was applied:

$$\text{LA, (\%) in DM} = \frac{\text{absorbance value} \times 10^{-2} \times (100 - \text{DM})}{\text{DM}}$$

For the analysis of volatile fatty acids, 1.5 mL of silage extract was acidified with 0.3 mL of 25% (w/v) meta-phosphoric acid. After centrifugation at 15,000 rpm for 15 minutes, the supernatant was transferred to vials for gas chromatography. Acetic (AA), butyric (BA), and propionic (PA) acid concentrations were measured using a Thermo Trace 1300 GC system coupled with an AI-1310 autosampler (Ersahince and Kara, 2017). Final values were quantified using Xcalibur software, with peaks identified by retention times and calculated as a percentage of the silage DM.

Determination of ammonia concentration in silages

Ammonia (NH_3-N) levels in the silage extract were quantified using a commercial enzymatic kit (K-AMIAR, Megazyme, Wicklow, Ireland). The analytical procedure involved mixing 0.10 mL of silage fluid with 2.0 mL of distilled water, then adding 0.3 mL of buffer (containing 0.02% w/v sodium azide and 2-oxoglutarate) and 0.2 mL of NADPH. Absorbance was measured at 340 nm using an UviLine 9100 spectrophotometer (Xylem Analytics Germany Sales GmbH & Co. KG, Mainz, Germany). After the initial measurement, a glutamate dehydrogenase suspension was added to the mixture, and the absorbance was measured again at 340 nm. The final ammonia concentration (g/L) was calculated according to the protocol provided by the manufacturers.

Microbiological analysis

Total aerobic mesophilic bacteria (TAMB), LAB, enterobacteria, enterococcus, yeasts and molds counts in the silages were determined using Plate Count Agar (PCA), de Man, Rogosa and Sharpe Agar (MRS), Violet Red Bile Glucose (VRBG) Agar, Slanetz Bartley Medium (SBM), and Rose Bengal Medium Agar (RO) (chloramphenicol selective supplement agar), respectively. Ten grams of silage were sampled and blended with 90 mL of sterilized distilled water containing peptone in sterile-filtered bags using a stomacher (Lab Blender 400, Sewart-England), then serially diluted in sterilized distilled water from 10^{-1} to 10^{-6}. To determine the number of TAMB, PCA was incubated at 37 ºC for 48 h. The number of LAB was determined by using MRS agar incubated at 30 ºC for 48 h in an anaerobic environment. Both VRBG for enterobacteria and SBM for enterococcus were incubated at 37 ºC for 48 h. RO agar for yeast and mold was incubated at 25 ºC for 5-7 days. Visible colonies were counted from plates at appropriate dilutions, and the number of colony forming units (cfu) was expressed per gram of fresh sample.

Determination of *in vitro* digestibility values

Rumen inoculum was obtained from adult cattle at a local slaughterhouse in Samsun. Following collection, the fluid was filtered through four layers of cheesecloth and transferred into a CO_2-flushed thermos maintained at 39°C. The initial pH was measured at 6.25. For the digestibility analysis, approximately 0.5 ± 0.05 g of silage material and silage samples were weighed into Ankom F57 filter bags, which were subsequently completely closed using a heat sealer. In accordance with the Ankom Daisy incubator's operational protocol, a 48-hour incubation was performed to determine various *in vitro* metrics, specifically *in vitro* true digestibility (IVTD), *in vitro* true dry matter digestibility (IVTDMD), *in vitro* true organic matter digestibility (IVTOMD), and *in vitro* NDF digestibility (IVNDFD) % (Kılın and Selçuk, 2024).

Statistical analysis

Data were analyzed using one-way ANOVA considering treatment as the main effect. The normality of the data distribution was tested using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Levene's test was used to assess the homogeneity of variances. Mean values that were normally and homogeneously distributed were compared by one-way ANOVA followed by Tukey's test. Non-homogeneous mean values were compared using Tamhane's test. For non-normal distributions, the non-parametric Kruskal-Wallis test was used to compare means, and the Mann-Whitney test was subsequently applied to identify the sources of significant differences. Statistical analyses were performed using SPSS (Version 21.0, IBM Corp., Armonk, NY, USA). Differences among means were considered statistically significant at $P<0.05$.

Results and Discussion

In this study, the separate evaluation of each harvest provided a more accurate interpretation of inoculant effects, as variations in chemical composition between harvests are known to influence fermentation dynamics and treatment responses. Accordingly, the effects of LABE treatments were interpreted within each harvest to account for these compositional differences.

Nutrient composition and fermentation characteristics of silages

The nutrient and chemical compositions of the first and second harvest Italian ryegrass-red clover silages are presented in Table 2.

Clear differences between the chemical composition of the fresh forage and the resulting silages indicate that ensiling substantially modified nutrient fractions, largely depending on harvest. Although silage DM generally reflected that of the initial material, first-harvest silages exhibited slightly lower DM than the fresh forage, suggesting greater fermentation losses under high-moisture conditions. In contrast, second-harvest silages more closely preserved the initial DM content, likely due to reduced fermentation and effluent losses in drier forages during ensiling.

Table 2. Initial chemical composition and *in vitro* digestibility of first and second harvest Italian ryegrass-red clover green mass prior to ensiling, expressed as % of DM.

Chemical composition, %		
	The first harvest	The second harvest
Fresh DM	28.54	38.89
Ash	15.53	11.4
OM	78.34	80.66
EE	1.94	1.85
CP	16.91	17.63
ADF_{OM}	26.38	27.65
NDF_{OM}	45.79	49.51
ADL_{OM}	2.59	3.67
TDN	74.05	71.53
NFC	19.82	19.61
TC	65.62	69.12
In vitro digestibility, %		
IVTD	74.56	69.27
IVTDMD	72.90	66.62
IVTOMD	68.92	62.79
IVNDFD	40.84	32.47

DM: Dry matter, OM: Organic matter, EE: Ether extract, CP: Crude protein, ADF_{OM}: Acid detergent fiber-ash free, NDF_{OM}: Neutral detergent fiber-ash free, ADL_{OM}: Acid detergent lignin-ash free, TDN: Total digestible nutrients, NFC: Non-fiber carbohydrates, TC: Total carbohydrates, IVTD: *In vitro* true digestibility, IVTDMD: *In vitro* true dry matter digestibility, IVTOMD: *In vitro* organic matter digestibility, IVNDFD: *In vitro* neutral detergent fiber digestibility.

CP concentrations remained largely stable between fresh material and silages, suggesting effective protein preservation during ensiling. In the present study, the most pronounced differences between fresh material and silages were observed in fiber fractions. In the first harvest, silage NDF and ADF values were substantially lower than those of the fresh forage, indicating partial degradation of structural carbohydrates during fermentation. In contrast, second-harvest silages showed fiber values much closer to the original material, which may be attributed to the constraining effect of increased lignification on cell wall degradation. The relative stability of ADL further supports that lignin is largely resistant to fermentation and limits the accessibility of digestible fiber. These compositional differences appear to be directly associated with fiber digestibility. The greater reduction in NDF and ADF in first-harvest silages corresponds with the higher IVNDFD observed in the fresh material (40.84% vs. 32.47%), suggesting that less lignified fiber is more susceptible to both ensiling-induced modification and ruminal degradation. Overall, the findings of the present study indicate that ensiling does not merely preserve the nutritional value of forage but selectively modifies fiber fractions, with the extent of these changes being strongly influenced by initial maturity. From a practical perspective, first-harvest forage appears to

offer greater potential for improving fiber utilization through ensiling, whereas second-harvest forage largely retains its inherent structural limitations.

Table 3. Chemical composition and fermentation characteristics of the first and second harvest Italian ryegrass-red clover silages, expressed as % of DM

	Control	LABE1	LABE2	SEM	P value
The first harvest					
Fresh DM, %	33.20^{a}	31.67^{b}	31.35^{b}	0.52	0.011
Ash, %	14.51	14.73	14.48	0.11	0.107
OM, %	76.02^{b}	76.62^{a}	76.83^{a}	0.08	0.001
EE, %	2.92	2.92	2.95	0.05	0.970
CP, %	17.24	17.80	17.25	0.20	0.086
ADF_{OM}, %	23.05	23.29	23.56	0.36	0.433
NDF_{OM}, %	34.76^{b}	35.85^{ab}	36.35^{a}	0.43	0.024
ADL_{OM}, %	1.81^{b}	2.08^{a}	2.00^{a}	0.06	0.025
TDN, %	81.56^{a}	80.81^{ab}	80.47^{b}	0.17	0.024
NFC, %	30.54^{a}	28.68^{b}	28.95^{b}	0.34	0.044
TC, %	65.31	64.54	65.31	0.16	0.073
pH	4.59^{a}	4.15^{b}	4.14^{b}	0.04	0.002
LA, %	7.60^{b}	8.02^{b}	10.01^{a}	0.45	0.004
AA, %	0.12	0.15	0.21	0.02	0.260
BA, %	0.010	0.009	0.009	0.00	0.214
PA, %	0.05^{b}	0.04^{a}	0.04^{a}	0.00	0.008
NH_3-N, g/L	75.53	78.17	76.77	0.67	0.301
The second harvest					
Fresh DM, %	35.69^{b}	36.42^{b}	36.83^{a}	0.17	0.014
Ash, %	11.61^{b}	11.94^{ab}	12.12^{a}	0.06	0.001
OM, %	81.81^{b}	82.56^{a}	82.64^{a}	0.12	0.011
EE, %	2.82	2.58	2.74	0.45	0.080
CP, %	18.39	18.64	18.20	0.11	0.316
ADF_{OM}, %	27.09	27.44	27.90	0.14	0.073
NDF_{OM}, %	43.89^{b}	46.24^{a}	46.63^{a}	0.38	0.001
ADL_{OM}, %	5.06	5.15	5.12	0.11	0.959
TDN, %	75.34^{a}	73.75^{b}	73.49^{b}	0.26	0.001
NFC, %	23.26^{a}	20.57^{b}	20.29^{b}	0.42	<0.001
TC, %	67.16	66.82	66.97	0.16	0.735
pH	4.90^{a}	4.09^{b}	4.14^{c}	0.10	0.002
LA, %	3.68^{b}	6.15^{a}	6.38^{a}	0.43	<0.001
AA, %	0.37	0.40	0.44	0.022	0.500
BA, %	0.010^{a}	0.009^{b}	0.009^{b}	0.00	0.007
PA, %	0.05^{a}	0.04^{b}	0.04^{b}	0.00	0.010
NH_3-N, g/L	69.66^{b}	81.25^{b}	101.54^{a}	4.89	0.009

DM: Dry matter, OM: Organic matter, EE: Ether extract, CP: Crude protein, ADF_{OM}: Acid detergent fiber-ash free, NDF_{OM}: Neutral detergent fiber-ash free, ADL_{OM}: Acid detergent lignin-ash free, TDN: Total digestible nutrients, NFC: Non-fiber carbohydrates, TC: Total carbohydrates, LA: Lactic acid, AA: Acetic acid, BA: Butyric acid, PA: Propionic acid, NH_3-N: Ammonia nitrogen

The DM content of the 80% Italian ryegrass (caramba)-20% Alexandria clover mixture silages was reported as 33.40% (Demiroğlu Topçu and Kahya, 2023). Oliveira et al. (2018) stated that the DM value of good quality silage was between 25-35%. The results for DM content in the current study are inline with previous studies. Regarding fiber fractions, LABE supplementation did not affect ADF content, and this result is consistent with previous studies (Coskuntuna and Gül, 2020; Marbun et al., 2020; Li et al., 2022; Şen et al., 2022; Kılın and Selçuk, 2024). In contrast, a significant increase in NDF content was observed alongside a decline in NFC values following LABE application. Silage inoculation with homofermentative LAB promotes LA fermentation and stimulates the degradation of non-structural carbohydrates (Zhao et al., 2019). This inverse relationship stems from active fermentation, where LAB preferentially utilize readily fermentable carbohydrates as substrates and could cause a decrease in easily fermentable carbohydrates (Oliveira et al., 2017; Cai et al., 2020). Consequently, the proportional increase in NDF likely reflects the depletion of soluble components. Rapid acidification is a critical factor for successful silage preservation, as it suppresses undesirable microbial activity and limits proteolysis (Pahlow et al., 2003). In this study, CP contents remained largely stable across treatments, which can be attributed to the presence of polyphenol oxidase (PPO) in red clover. It was reported that the presence of PPO enzyme can limit the natural proteolysis of forages mixed with red clover, thus reducing protein degradation during the ensiling process (Sullivan and Hatfield, 2006; Boller et al., 2010). Muck et al. (2018) stated that the use of additives containing PPO may limit protein losses caused by fermentation of forages. Unchanged in NH3-N concentration in the first harvest silages suggests that PPO can produce quinones that bind to proteins, which in turn reduces protein degradability during ensiling (Frankow-Lindberg, 2017). Thus PPO may have contributed to limiting proteolysis, thereby protecting true protein from excessive degradation. Nevertheless, NH_3-N concentrations were higher in the LABE2 treatment, particularly in second-harvest silages. Interestingly, while elevated NH_3-N levels can sometimes indicate clostridial activity (Doğan Daş et al., 2022), the higher concentrations observed in the LABE2 group must be interpreted differently. The absence of BA (<0.1%) and the negligible counts of Enterobacteriaceae (<2.30 $\log_{10}$ cfu/g) confirm that clostridial fermentation did not occur. Instead, the increased NH_3-N is more plausibly a result of enhanced enzymatic degradation of plant cell structures, which facilitated the release of intracellular nitrogenous compounds. The pH values and silage acid profiles further support this, as LAB are highly effective at producing LA, which has a greater acidifying capacity than other organic acids (Kung Jr et al., 2018;

Peng et al., 2021). Lactic acid concentration increased from 7.60 up to 10.01% of DM in the LABE-treated group of in the first harvest, and also 3.68 up to 6.38% of DM in the LABE-treated group of in the second harvest, indicating a more efficient fermentation compared to the control.The consistently low levels of undesirable fermentation products, such as PA and BA, across all treatments confirm that LABE application maintained fermentation stability, and this finding is consistent with the report of Okoye et al. (2023). Typically, the population of lactic acid bacteria present in the natural microflora of fresh forage is often unpredictable or numerically insufficient to rapidly dominate the fermentation process. Taken together, these findings suggest that overcoming this natural limitation through LABE supplementation, particularly at 3.0×10 cfu/g, improved silage quality primarily by enhancing fermentation efficiency and fiber digestibility rather than by altering overall nutrient concentrations. The observed increases in NDF and NH_3-N should therefore be interpreted not as indicators of reduced silage quality, but as consequences of active fermentation and enzymatic structural relaxation that ultimately enhanced nutrient availability for rumen microorganisms.

Microbiologic properties of silages

The microbiological characteristics of the first and second harvest Italian ryegrass-red clover silages are given in Table 4.

Table 4. Microbiology of Italian ryegrass-red clover silages (log_{10} cfu/g of fesh silage)

	Control	LABE1	LABE2	SEM	P value
The first harvest					
TAMB	9.29[a]	7.71[b]	7.61[b]	0.24	<0.001
Enterobacteria*	<2.30	<2.30	<2.30		
Enterococcus	7.34[a]	6.81[b]	6.65[b]	0.10	<0.001
LAB	7.14	7.47	7.66	0.24	0.150
Yeast-Mold*	<2.30	<2.30	<2.30		
The second harvest					
TAMB	8.41[a]	7.28[b]	6.73[b]	0.21	<0.001
Enterobacteria*	<2.30	<2.30	<2.30		
Enterococcus	7.07[a]	6.87[ab]	6.52[b]	0.09	0.030
LAB	7.42	8.02	8.06	0.15	0.180
Yeast-Mold*	<2.30	<2.30	<2.30		

TAMB: Total aerobic mesophilic bacteria, LAB: Lactic acid bacteria

Successful silage fermentation requires epiphytic LAB populations to reach at least 5 log cfu/g (Ennahar et al., 2003; Pang et al., 2012). This threshold was achieved in all experimental groups, indicating that the native microbiota and

ensiling conditions were sufficient to support effective LA fermentation. These findings are consistent with previous reports demonstrating adequate LAB development in well-managed silages (Ennahar et al., 2003; Pang et al., 2012; Bureenok et al., 2019). Moreover, the absence of a marked treatment effect on LAB counts aligns with the observations of Bureenok et al. (2019), who reported that LAB populations were not significantly influenced by enzyme and/or LAB inoculation. Indicators of silage hygienic quality further supported the effectiveness of the fermentation process. Yeast counts remained below the recommended upper limit of 6 log cfu/g (Kung et al., 2018), and mold counts stayed under the 4 log cfu/g threshold indicative of good production practices (Alonso et al., 2013). The yeast and mold counts in both LABE-treated and control silages are consistent with previous studies (Alonso et al., 2013; Ávila and Carvalho, 2019). These results suggest that rapid acidification during fermentation effectively suppressed the growth of undesirable microorganisms. Similar outcomes have been reported in a study showing that LAB and LAB+E inoculation reduce pH and increase LA concentration, thereby limiting yeast and mold proliferation (Uğurlu et al., 2022). Furthermore, lower yeast and mold counts are indicative of improved aerobic stability, as these microorganisms are primarily responsible for spoilage upon air exposure. Enterobacteria are generally present in poorly preserved silages, and some of those are capable of breaking down proteins, leading to the release of NH_3-N and biogenic amines (McDonald et al., 1991). Therefore, the presence of Enterobacteria in well-preserved silages is generally not expected because rapid LA production inhibits Enterobacteria growth. The negligible counts of Enterobacteriaceae in the current study is in agreement with previous study (Ávila and Carvalho, 2019).

Digestibility values of silages

There was an increase ($p<0.05$) in IVTD, IVTDMD, IVTOMD and IVNDFD in the LABE-supplemented silages (Table 5).

LABE supplementation significantly increased IVTD, IVTDMD, IVTOMD, and IVNDFD in both harvests, indicating a substantial improvement in nutritive quality. Roughage digestibility is a primary indicator of silage value, as it directly influences nutrient availability and animal performance (Şen et al., 2022; Liu et al., 2019). While several studies have reported no significant effects of LAB or enzyme additives on DM or organic matter digestibility (Li et al., 2022; Kılın and Selçuk, 2024; Bureenok et al., 2019; Hristov and McAllister, 2002; Muck et al., 2007; Özdüven et al., 2017) others have demonstrated positive responses, suggesting that the efficacy of such additives may depend on forage type, harvest stage, and additive composition (Weinberg et al., 1995; Ozduven et al., 2010).

Table 5. *In vitro* digestibilty values of the silages, expressed as %

	Control	LABE1	LABE2	SEM	P value
The first harvest					
IVTD	78.78^{b}	80.08^{a}	80.30^{a}	0.10	0.010
IVTDMD	76.57^{b}	78.20^{a}	78.43^{a}	0.08	<0.001
IVTOMD	73.28^{b}	75.04^{a}	75.26^{a}	0.08	<0.001
IVNDFD	34.97^{b}	41.13^{a}	42.45^{a}	0.60	<0.001
The second harvest					
IVTD	70.74^{b}	72.07^{a}	72.52^{a}	0.26	<0.001
IVTDMD	69.04^{b}	70.11^{ab}	71.01^{a}	0.27	<0.001
IVTOMD	65.38^{b}	66.71^{ab}	67.73^{a}	0.33	<0.001
IVNDFD	32.68^{c}	35.04^{b}	39.26^{a}	0.79	<0.001

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

The digestibility results of the present study show a clear positive response to LABE supplementation. The observed enhancement in 48-h IVNDFD is particularly noteworthy and consistent with previous studies (Kılın and Selçuk, 2024; Guo et al., 2020). This improvement is likely due to the partial hydrolysis of plant cell wall components by exogenous enzymes during ensiling, which weakens the structural integrity of the fiber matrix (Getabalew et al., 2022). Similar improvements in cellulose fractions have been reported with LAB applications (Aksu et al., 2004; Addah et al., 2011). The positive response to LABE addition in the present study could be attributed to the breakdown of plant cell wall structures in the silage, which effectively liberates intracellular contents, thereby providing more substrates for rumen microorganisms. Oba and Allen (1999) stated that a 1-unit improvement in IVNDFD was positively associated with increases of 0.17 kg in dry matter intake (DMI) and 0.25 kg in 4% fat-corrected milk yield. In the present study, the increases observed in IVNDFD for for the first harvest silages, LABE1 and LABE2 treatments may increase DMI by approximately 1.05 and 1.27 kg/day and 4% FCM yield by 1.54 and 1.87 kg/day, respectively. For the second harvest silages, the corresponding increases are estimated at 0.40 and 1.12 kg/day for DMI and 0.59 and 1.65 kg/day for milk yield. Based on these relationships, the improvements in IVNDFD observed in LABE1 and LABE2 treatments could potentially translate into meaningful increases in feed intake and milk production compared to the control. In particular, the higher magnitude of IVNDFD improvement in LABE2 indicates a greater potential to enhance animal performance. These findings highlight the practical importance of improving fiber digestibility, as even moderate increases in IVNDFD can have significant impacts on animal productivity.

Conclusion

Inoculating Italian ryegrass-red clover silage with a combination of LAB and E at levels up to 3.0×10 cfu/g significantly improved fermentation characteristics and the microbial profile by significantly suppressing the growth of TAMB and Enterococcus while maintaining the lactic acid bacteria population, and enhanced *in vitro* digestibility. The observed improvements, especially in fiber degradation, show that LABE effectively optimizes the fermentation process and enhances the overall nutritive value of the silage. These findings indicate that LABE is a promising biological additive for grass-based silages, contributing to superior silage quality and greater fermentation stability. Future research should evaluate the effect of LABE-treated Italian ryegrass-red clover silages on animal performance parameters, such as feed intake, nutrient utilization, and milk or meat production. Additionally, investigating different enzyme formulations, inoculation rates, forage types and the interaction between LABE application and harvest maturity will provide deeper insights into optimizing its efficacy under practical farming conditions.

Inokulacija silaže italijanskog ljulja i crvene deteline mlečnokiselinskom bakterijom i enzimima: poboljšanje mikrobnog profila, kvaliteta fermentacije i svarljivosti

Emel Cam, Zehra Selcuk

Rezime

Ova studija procenjuje uticaj inokulacije silaže italijanskog ljulja i crvene deteline komercijalnim aditivom koji sadrži mlečnokiselinske bakterije i enzime (LABE) na mikrobiološki profil, kvalitet fermentacije, nutritivnu vrednost i svarljivost silaže. Eksperimentalni tretmani su se sastojali od: (1) kontrole (bez LABE); (2) LABE1 (inokuliran sa $1,5\times10^5$ cfu/g silirane mase); i (3) LABE2 (inokuliran sa $3,0\times10^5$ cfu/g silirane mase). Nakon perioda inkubacije od 60 dana, određeni su sastav hranljivih materija, karakteristike fermentacije, mikrobiologija i svarljivost. LABE inokulacija je povećala sadržaj mlečne kiseline ($P<0,05$) i u prvom i u drugom otkosu. Koncentracije amonijačnog azota se nisu razlikovale u prvom otkosu, ali LABE2 grupa je pokazala više ($P<0,05$) nivoe amonijačnog azota u drugom otkosu u poređenju sa ostalim grupama. Mikrobiološka analiza je otkrila smanjenje ($P<0,05$) ukupnih populacija aerobnih mezofilnih bakterija i enterokoka u obe žetve, dok je broj bakterija mlečne kiseline ostao nepromenjen. Broj kvasca, plesni i enterobakterija bio je ispod granica detekcije (<2,3 log10 cfu/g) u svim grupama.

Dodatno, LABE inokulacija je poboljšala vrednosti svarljivosti in vitro (P<0,05) u oba otkosa. Ovi rezultati ukazuju na to da LABE inokulacija u nivoima do $3,0\times10^5$ cfu/g silažne mase može poboljšati nutritivnu vrednost, mikrobiološki profil i svarljivost silaže od italijanskog ljulja i crvene deteline. Međutim, potrebna su dalja istraživanja kako bi se procenili njeni efekti na performanse životinja.

Ključne reči: svarljivost, fermentacija, inokulant, silaža od italijanskog ljulja i crvene deteline, mikrobni profil

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Conflict of interest
The authors declare no conflicts of interest.

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