Biotechnology in Animal Husbandry 37 (1), 65-73, 2021
Publisher: Institute for Animal Husbandry, Belgrade-Zemun
ISSN 1450-9156
UDC 631.563'633.32
https://doi.org/10.2298/BAH2101065D

# EFFECTS OF CUTTING STAGE AND BACTERIAL INOCULANT ON QUALITY OF THE RED CLOVER SILAGE

**Snežana Đorđević[1], Violeta Mandić[2], Nikola Đorđević[1]**

[1]Biounik d.o.o., Research and Development Center, Šimanovci, Republic of Serbia
[2]Institute for Animal Husbandry Belgrade-Zemun, Belgrade, Republic of Serbia
Corresponding author: Violeta Mandić, violeta_randjelovic@yahoo.com
Original scientific paper

**Abstract:** In this paper, the influence of cut at two maturity stages (the beginning of the flowering stage and mid bloom stage and bacterial inoculant "Silko za lucerku" (contains *Lactobacillus plantarum* and *Pediococcus* spp.) on the quality of red clover silage were presented. The commercial cultivar Nada selected at the Bc Institute in Zagreb was used for investigation. The silage was examined in mini-silos (glass jars of 1.5 l volume with plastic fermentation valve) in the laboratory. The chemical composition, energy and fermentation characteristics of silages were analyzed 90 days after ensiling. The values of dry matter, acid (ADF) and neutral detergent fibre (NDF), lactic acid and pH were significantly lower, while the crude protein content, total digestible nutrients (TDN), relative feed value (RFV), ammonia nitrogen in total nitrogen ($NH_3$-N/TN), acetic and butyric acids were significantly higher in the first cutting stage. The inoculation with inoculant "Silko za lucerku" improved the chemical, energy and fermentation parameters of silages. Inoculant-treated silage had lower contents of ADF, NDF, $NH_3$-N/TN, acetic and butyric acids and pH, and higher contents of dry matter, crude protein, TDN, RFV and lactic acid than control. Accordingly, timely cutting stage and application of microbial inoculant can contribute to a lesser loss of nutritional value of the forage and promote silage quality.

**Key words:** bacterial inoculant, maturity stage, red clover, silage, quality

## Introduction

The red clover silage is little used in ruminants' diets, although it can be a significant source of quality food like alfalfa. The alfalfa and red clover have similar crude protein, acid and neutral detergent fiber and mineral contents. However, the red clover contains polyphenol oxidases enzymes which inhibiting

plant proteases and proteolysis in the silo due to which red clover silage has more undegradable protein (25-35%) than alfalfa (15-25%) *(Hoffman and Broderick, 2001)*. It should be noted that the freshly forage of red clover contains phytooestrogen which negatively influences on development and function of reproductive organs, especially in sheep *(Hloucalová et al., 2016)*. This problem is solved by moving breeding animals from red clover crops. It has been determined that the red clover silage significantly increase performance and product quality of animal (higher milk yield, growth rate and higher amounts of isoflavonoids in milk) *(Steinshamn, 2008)*. However, the red clover as well as all forage legumes are difficult to ensile. They have high buffering capacity and low content of soluble carbohydrates *(Buxton and O'kiely, 2003)*. For that reason, it is necessary to apply chemical and bacterial additives for ensiling to ensure a stable fermentation. Essentially, bacterial inoculants improve the ensiling of legume by preventing the dry matter loss and ferment sugars to butyric acid *(Schmidt et al., 2009)* and the degradation of proteins to oligopeptides, free amino acids, ammonia and non-protein nitrogen *(Ohshima and Mcdonald, 1978)*. Lactic acid bacterial inoculant inhibits detrimental microbial activity, especially *Clostridium butyricum* in the silages *(Pys et al., 2002)*. The commercially available bacterial inoculants contain homofermentative and heterofermentative lactic acid bacteria. The homofermentative bacteria (*Lactobacillus plantarum, Pediococcus, Enterococcus and Lactococcus*) improve silage fermentation due to produced lactic acid and a faster drop in pH value *(Muck et al., 2018)*. Thus, they reduce dry matter losses and protein breakdown and the growth of undesirable microorganismsin silage. According to *Zielińska et al. (2015)*, it is recommended to use inoculants consisting of several bacterial strains because they increase content of lactic and volatile fatty acids, and aerobic stability. In general, the effect of bacterial inoculants depends on the plant species and the stage of maturity. *Mceniry et al. (2013)* found that the delaying the mowing time of red clover led to reduction of dry-matter digestibility, buffering capacity, crude protein and water-soluble carbohydrates and increasing concentrations of dry matter, neutral and acid detergent fibre. Also, *Kornfelt et al. (2013)* found the red clover silages harvested at late stage of growth has a higher dry matter, NDF, ADF and lactic acid and lower pH, acetic and butyric acids than red clover silages harvested at the early stage of growth.

The aim of this research was to examine the influence of the cutting stage and bacterial inoculant on the quality of red clover silage.

## Materials and Methods

The red clover crop, genotype Nada selected at the Bc Institute in Zagreb, was taken for study. The forage was harvested at two maturity stages, at the beginning of the flowering (early; the first cut) and mid-bloom stage (late; the

second cut), in the first cut in the second year of exploitation during the growing season of 2017. After 24 h of wilting, the biomass was cut with a forage harvester to a length of about 2 cm and taken to the laboratory for testing. Two treatments were formed: "Silko za lucerku" where the green mass was treated with bacterial inoculant at a dose of 5 ml $t^{-1}$ green mass and control where the mass was treated with distilled water 5 ml $t^{-1}$ green mass. "Silko za lucerku" is a bacterial inoculant that contains homofermentative lactic bacteria *Lactobacillus plantarum* and *Pediococcus* spp. ($1x10^{10}$ CFU $ml^{-1}$). The hand-held sprayer was used to spray the inoculant and distilled water on the chopped mass. The green mass was compacted in mini-silos (glass jars of 1.5 l volume with plastic fermentation valve) and left in a dark place at room temperature around 22 $^{o}$C. The silos were opened and analyzed after 90 days. Each treatment contained three replicates. The chemical composition of red clover forages at early and late growth stages are shown in the Table 1.

**Table 1. The chemical composition of red clover forage before ensiling**

| Item | I cut | II cut |
|---|---|---|
| Dry matter (DM) (g $kg^{-1}$) | 280.0 | 480.0 |
| Crude protein (g $kg^{-1}$ DM) | 192.7 | 180.7 |
| Acid detergent fibre (ADF) (g $kg^{-1}$ DM) | 302.3 | 404.3 |
| Neutral detergent fibre (NDF) (g $kg^{-1}$ DM) | 436.5 | 516.5 |

The following parameters were determined:
- dry matter content (difference in weight before and after drying at 105 ° C in a oven to a constant mass);
- crude protein content (Kjeldahl method according to *AOAC, 1990*);
- neutral - NDF and acid detergent fiber - ADF (Van Soest method according to *Van Soest et al., 1991*);
- pH value (from silage extract using a Hanna Instruments HI 83141 pH meter);
- $HH_3$-N/total nitrogen (distillation method using a Kjeltec 1026 analyzer);
- lactic, acetic and butyric acid (using Gas chromatograph - GC-2014, Shimadzu, Kyoto, Japan according to *Faithfull, 2002*).

Total digestible nutrients (TDN) and relative feed value (RFV) calculated according to *Horrocks and Vallentine (1999)*:

TDN (%) = (-1,291 × %ADF) + 101.35;

RFV (%) = Digestible Dry Matter (DDM) × Dry Matter Intake (DMI) × 0.775;

DDM (%) = 88.9 - (0.779 x % ADF);

DMI (%) = 120 / (% NDF).

The experiment involved two factors, each at two levels. The factorial experiment is arranged in a randomized complete block design in 3 replications. The ANOVA was used to analyze the obtained data and Tukey test for differences between mean values at the level of $p \leq 0.05$. Statistical analysis was performed with Statistical Software Package SPSS 18 (IBM Corporation).

## Results and Discussion

The timing of silage cutting significantly affected the chemical, energy and fermentation parameters of red clover silages (Table 2). The dry matter content, ADF, NDF and pH significantly increased at the late cutting time for 45.1%, 22.5%, 0.5%, and 0.9% respectively, compared to early cutting time. Contrary, crude protein content, TDN, RFV, $NH_3$-N/TN, acetic and butyric acid significantly decreased at the late cutting time for 0.6%, 15.2%, 8.4%, 42.7%, 40.7% and 91.5%, respectively, compared to early cutting time. Similar results have also been found by *Kornfelt et al. (2013)* in red clover silage harvested at different stage of maturity. In general, the forage mass harvested at the early vegetative stage has a lower dry matter content *(King et al., 2012)* which can lead to loss of dry matter and nutrient due to the large effluent flow *(McGechan, 1990)* and increased clostridial activity in silage (*Wieringa, 1969)*. The higher protein content at the beginning of flowering result from the fact that young red clover plants have a higher share of leaves. During the vegetation period, under the influence of longer days and higher temperatures, morphological changes occur with the ageing of the plants: the leaves grow more slowly, the stem lengthens, the yield increases, and the quality decreases, especially the content of crude proteins, also, cell-wall content increases, and therefore NDF concentration *(Hatfield, 1993)*. Similar to our results, *Kuoppala et al. (2009)* found that the delaying red clover harvest resulted in reduced protein content and increased NDF content.

**Table 2. Effects of cutting stage on chemical, energy and fermentation parameters of red clover silages**

| Item | Cutting stage (A) | | F test |
|---|---|---|---|
| | I cut | II cut | |
| Chemical composition | | | |
| Dry matter (DM) (g $kg^{-1}$) | 238.7$^{b}$ | 434.7$^{a}$ | ** |
| Crude protein (g $kg^{-1}$ DM) | 175.9$^{a}$ | 174.8$^{b}$ | * |
| Acid detergent fibre (ADF) (g $kg^{-1}$ DM) | 269.2$^{b}$ | 347.5$^{a}$ | ** |
| Neutral detergent fibre (NDF) (g $kg^{-1}$ DM) | 417.8$^{b}$ | 420.0$^{a}$ | ** |
| Energy parameters | | | |
| Total digestible nutrients (TDN) (%) | 66.6$^{a}$ | 56.5$^{b}$ | ** |
| Relative feed value (RFV) (%) | 150.4$^{a}$ | 137.7$^{b}$ | ** |
| Fermentation parameters | | | |
| pH | 4.64$^{b}$ | 4.68$^{a}$ | ** |
| ammonia nitrogen/total nitrogen ($NH_3$-N/TN) (g $kg^{-1}$ TN) | 101.7$^{a}$ | 58.3$^{b}$ | ** |
| Lactic acid (g $kg^{-1}$ DM) | 67.9$^{b}$ | 81.8$^{a}$ | ** |
| Acetic acid (g $kg^{-1}$ DM) | 33.4$^{a}$ | 19.8$^{b}$ | ** |
| Butyric acid (g $kg^{-1}$ DM) | 4.7$^{a}$ | 0.4$^{b}$ | ** |

Means followed by the same letter within a row are not significantly different by Tukey's test at the 5% level; ** - significant at 1% level of probability and * - significant at 5% level of probability.

Inoculant significantly increased contents of dry matter, crude protein, TDN, RFV and lactic acid, and significantly decreased contents of ADF, NDF, $NH_3$-N/TN, acetic and butyric acid and pH value compared to control (Table 3).

**Table 3. Effects of inoculant on chemical, energy and fermentation parameters red clover silages**

| Item | Inoculant (B) | | F test | |
|---|---|---|---|---|
| | Control | "Silko za lucerku" | B | Interaction A × B |
| Chemical composition | | | | |
| Dry matter (DM) (g $kg^{-1}$) | 335.0[b] | 338.4[a] | ** | ** |
| Crude protein (g $kg^{-1}$ DM) | 170.8[b] | 179.9[a] | ** | nz |
| Acid detergent fibre (ADF) (g $kg^{-1}$ DM) | 365.7[a] | 251.1[b] | ** | ** |
| Neutral detergent fibre (NDF) (g $kg^{-1}$ DM) | 425.9[a] | 412.0[b] | ** | nz |
| Energy parameters | | | | |
| Total digestible nutrients (TDN) (%) | 60.6[b] | 62.5[a] | ** | ** |
| Relative feed value (RFV) (%) | 140.4[b] | 147.7[a] | ** | * |
| Fermentation parameters | | | | |
| pH | 5.02[a] | 4.30[b] | ** | ** |
| Ammonia nitrogen/total nitrogen ($NH_3$-N/TN) (g $kg^{-1}$ TN) | 110.2[a] | 49.7[b] | ** | ** |
| Lactic acid (g $kg^{-1}$ DM) | 59.2[b] | 90.5[a] | ** | ** |
| Acetic acid (g $kg^{-1}$ DM) | 28.3[a] | 25.0[b] | ** | ** |
| Butyric acid (g $kg^{-1}$ DM) | 4.9[a] | 0.2[b] | ** | ** |

Means followed by the same letter within a row are not significantly different by Tukey's test at the 5% level; ** - significant at 1% level of probability and * - significant at 5% level of probability.

A silage inoculant aims to supply a sufficient amount of selected strains to help optimum fermentation. The applied inoculant is a liquid inoculant contains *Lactobacillus plantarum* and *Pediococcus* spp. *Whiter et al. (1999)* pointed out that the microbial inoculants in a liquid form are more suitable for ensiling because the bacteria are added with their own moisture to help speed up fermentation. *Lactobacillus plantarum* belongs to the homofermentative lactic acid bacteria and convert sugars almost quantitatively to lactic acid. Also, *Pediococcus* spp. (belong to the family *Streptococcaceae*) ferment sugars (glucose, mannose and fructose) mainly in the lactic acid. Since these bacteria promote a rapid fermentation, the pH drops sharply in the inoculated silage and is prevented enterobacteria from breaking down the protein, as indicated by the lower ammonia content. *Kung and Muck (1997)* reported that in more than 60% of studies, the silages treated with homofermentative lactic acid bacteria had a lower pH, $NH_3$-N/TN, acetic and butyric acids and higher lactic acid content. Contrary, lactic acid fermentation in the control silage is slower because it is regulated only by epiphytic bacteria whose number in the forage before ensiling is small. According to *Schmidt et al. (2009)* and *Zhang et al. (2009)*, their number is less than 1% microbiome. The rate of 1 x $10^5$ CFU per gram of fresh forage will provides enough microorganisms for good

fermentation *(Cai et al., 1998)*. "Silko za lucerku" has a higher level than this, which means it has sufficient bacteria to positively influence silage fermentation. It is considered that if a silage inoculant has a lower level than this, or does not even specify a CFU count, then there may be insufficient bacteria to influence silage fermentation.

Only the crude protein content and NDF content were not significantly affected by the interaction of cutting time and application of inoculant. The lower values of ADF, NDF, pH, $NH_3$-N/TN, acetic acid and butyric acid and the higher values of dry matter, crude protein, TDN, RFV and acetic acid were recorded at both cutting in treatment "Silko za lucerku". These results indicated that the red clover forages independently of harvest time could be well fermented as good quality silage using lactic acid bacteria inoculant, such as "Silko za lucerku".

## Conclusions

The red clover silage harvested at the mid-bloom stage has a higher dry matter, ADF, NDF, lactic acid and pH and lower crude protein, TDN, RFV, $NH_3$-N/TN, acetic and butyric acids than red clover silage harvested at early flowering stage. The aplied inoculant containing homofermentative lactic acid bacteria positively affected the silage's chemical, energy and fermentacion parameters during the fermentation process. The inoculant enhanced the dry matter, crude protein, TDN, RFV and lactic acid content and decreased the NDF, ADF, $NH_3$-N/TN, acetic and butyric acids and pH, which resulted in a better silage quality.

## Acknowledgements

The results of the research presented in this paper were financed by the Development Agency of Serbia (No 1-05-401-1068/2017) and Ministry of Education, Science and Technological Development of the Republic of Serbia, on the basis of the Agreement on the realization and financing of scientific research work of SRO in 2021 no. 451-03-9/2021-14/200022.

## Uticaj faze košenja i bakterijskog inokulanta na kvalitet silaže crvene deteline

*Snežana Đorđević, Violeta Mandić, Nikola Đorđević*

## Rezime

U radu je ispitivan uticaj dve faze košenja crvene deteline u proleće u drugoj godini eksploatacije (početak cvetanja (rana) i puno cvetanje (kasna)) i bakterijskog inokulanta "Silko za lucerku" (sadrži *Lactobacillus plantarum* i *Pediococcus* spp.) na kvalitet silaže. Za istraživanje je korišćena komercijalna sorta Nada selekcionisana u BC Institutu u Zagrebu. Silaža je analizirana u mini silosima (staklene tegle zapremine 1,5 l) u laboratoriji. Hemijski sastav, energetski i fermentacioni parametri silaže analizirani su 90 dana nakon siliranja. Vrednosti suve materije, kiselih (ADF) i neutralnih deterdžentskih vlakna (NDF), mlečne kiseline i pH bile su značajno niže, dok su sadržaj sirovih proteina, ukupna svarljiva hranljiva materija (USHV), relativna hranljiva vrednost (RHV), sadržaj amonijačnog azota u ukupnom azotu ($NH_3$-N/TN), sirćetne i buterne kiseline bili značajno veći u prvoj fazi košenja. Inokulacija sa inokulantom "Silko za lucerku" je poboljšala hemijske, energetske i fermentacione parametre silaža. Silaža tretirana inokulantom imala je niži sadržaj ADF, NDF, $NH_3$-N/TN, sirćetne i buterne kiseline i pH, i više suve materije, sirovih proteina, TDN, RFV i mlečne kiseline od kontrolne. Prema tome, pravovremena faza košenja useva i primena mikrobiološkog inokulanta može doprineti manjem gubitku hranljive vrednosti krme i promovisati kvalitet silaže.


**Ključne reči:** bakterijski inokulant, faza razvića, crvena detelina, silaža, kvalitet

## References


AOAC (1990): Association of Official Analytical Chemists (AOAC), 1990. Official methods of analysis,
Washington DC, USA, 1, 14, 684.

BUXTON D.R., O'KIELY P. (2003): Preharvest plant factors affecting ensiling. In: Buxton D.R., Muck R.E. and Harrison J.H. (eds) Silage science and technology, pp. 199–250. Madison, WI, USA: American Society of Agronomy.

CAI Y., BENNO Y., OGAWA M., OHMOMO S., KUMAI S., NAKASE T. (1998): Influence of *Lactobacillus* spp. from an inoculant and of *Weissella* and *Leuconostoc* spp. from forage crops on silage fermentation. Applied and Environmental Microbiology, 64, 8, 2982-2987.

FAITHFULL N. (2002): Methods in agricultural chemical analysis: A Practical Handbook, CABI Publishing, Wallingford.

HATFIELD R.D. (1993): Cell wall polysaccharide interactions and digestibility. In: Jung, H.G., Buxton, D.R., Hatfield, R.D., Ralph, J. (Eds.), Forage Cell Wall Structure and Digestibility. ASA, CSSA, and SSSA, Madison, WI, pp. 285-313.

HLOUCALOVÁ P., SKLÁDANKA J., HORKÝ P., KLEJDUS B., PELIKÁN J., KNOTOVÁ D. (2016): Determination of phytoestrogen content in fresh-cut legume forage. Animals (Basel), 6, 7, 43.
HOFFMAN P.C., BRODERICK G.A. (2001). Red clover forages for lactating dairy cows. Focus on Forage, 3, 11, 1-2.
HORROCKS R.D., VALLENTINE J.F. (1999): Harvested Forages. Academic Press, London, UK
KING C., MCENIRY J., RICHARDSON M., O'KIELY P. (2012): Yield and chemical composition of five common grassland species in response to nitrogen fertilizer application and phenological growth stage. Acta Agriculturae Scandinavica, Section B - Soil and Plant Science, 62, 644-658.
KORNFELT L., NØRGAARD P., WEISBJERG, M. (2013): Effect of harvest time of red and white clover silage on chewing activity and particle size distribution in boli, rumen content and faeces in cows. Animal, 7, 6, 909-919.
KUNG J.R.L., MUCK R.E. (1997): Effects of silage additives on ensiling. Proceedings from the Silage: Field to Feedbunk North American Conference, Hershey, 11-13 February 1997, NRAES-99, 187-199.
KUOPPALA K., AHVENJARVI S., RINNE M., VANHATALO A. (2009): Effects of feeding grass or red clover silage cut at two maturity stages in dairy cows. 2. Dry matter intake and cell wall digestion kinetics. Journal of Dairy Science 92, 5634-5644.
MCGECHAN M.B. (1990): A review of losses arising during conservation of grass forage: part 2. Storage losses. Journal of Agricultural Engineering Research, 45, 1-30.
MCENIRY J., KING C., O'KIELY P. (2013): Silage fermentation characteristics of three common grassland species in response to advancing stage of maturity and additive application. Grass and Forage Science, 69, 393-404.
MUCK R.E., NADEAU E.M.G., MCALLISTER T.A., CONTRERAS-GOVEA F.E., SANTOS M.C., KUNG L.JR. (2018): Silage review: Recent advances and future uses of silage additives. Journal of Dairy Science, 101, 5, 3980-4000.
OHSHIMA M., MCDONALD P. (1978): A review of the changes in nitrogenous compounds of herbage during ensilage. Journal of the Science of Food and Agriculture, 29, 497-505.
PYS J., MIGDAL W., PUCEK T., ZIVKOVIC B., FABJAN M., KOSOVAC O., RADOVIC C. (2002): Effect of lactic acid bacterial inoculant with enzyme and rolled barley additive on the chemicals composition and protein degradation of alfalfa silage. Biotechnology in Animal Husbandry 18, 3-4, 33-45.
SCHMIDT R., HU W., MILLS J., KUNG L. (2009): The development of lactic acid bacteria and *Lactobacillus buchnery* and their effects on the fermentation of alfalfa silage. Journal of Dairy Science, 92, 5005-5010.
STEINSHAMN H. (2008): The unique properties of red clover in the diet of ruminants. 1st Scientific Conference within the framework of the 8th European

Summer Academy on Organic Farming, September 3–5, 2008, Lednice na Moravě, Czech Republic.
VAN SOEST P. J., ROBERTSON J. B., LEWIS B. A. (1991): Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. Journal Dairy Science 74, 3583-3597.
WHITER A.G., KUNG JR. L., RANJIT N.K., TAVARES J.Y., ROBINSON, J.R. (1999): The interactions between silage dry matters and forms of bacterial inoculants. Journal of Dairy Science, 82, 125.
WIERINGA G.W. (1969): Influence of moisture and nutrient content of forage plants on fermentation processes. In: Conservation and Grassland Products, Proceedings of the 3rd General Meeting of the European Grassland Federation. Braunschweig, Germany, 133-137.
ZHANG T., Li L., WANG X., ZENG Z., HU Y., CUI Z. (2009): 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, 25, 965-971.
ZIELIŃSKA K., FABISZEWSKA A., STEFAŃSKA I. (2015): Different aspects of *Lactobacillus* inoculants on the improvement of quality and safety of alfalfa silage. Chilean Journal of Agricultural Research, 75, 3, 298-306.

Received 5 February 2021; accepted for publication 22 March 2021