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FERMENTATION AND MICROBIAL DYNAMICS OF PERENNIAL GRASSES SILAGE PREPARED WITH BIOLOGICAL INOCULANT

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ABSTRACT

Ensilage provides an effective means of conserving green forage to supply as feed to ruminants. The fermentation process presented in the ensilage process depend on lactic acid bacteria (LAB). Silage quality is variable and the only way to effectively control the fermentation process, to improve the ensiling process and the quality of the resulting silage is to use an additive, mostly biological inoculants with LAB. The aim of this study was to evaluate the fermentation and microbial dynamics of perennial grasses silage with biological inoculant. Alfalfa and *Poaceae* mixed grasses were ensiled. Into grass silage was added biological additive, consisted of mixture of homofermentative and heterofermentative LAB and enzymes. Inoculant included strains *Lactobacillus plantarum, Pediococcus acidilactici, Pediococcus pentosaceus, Propionibacterium acidipropionici,* α-amylase, β-glucanase, cellulase and hemicellulase. The fermentative quality, chemical composition parameters and microbiological counts of silages at 7, 14, 21 and 60 days after ensilage were evaluated. Fermentation dynamics were examined using chemical analysis. The study showed higher values of dry matter, crude fat and NDF after supplementation of biological additive in all analyzed samples. Conversely, there was a reduction of the pH and water soluble carbohydrates concentration. Ensiling caused an increase of acetic acid concentrations as well ($p<0.001$). There were found significant differences ($p<0.05$) in contents of NEL between 21 and 60 days of ensiling after treatment and between the control group of fresh grass and 7 and 21 days after treatment as well. At the 7 day of fermentation process there was a significant increase in *Lactobacillus* spp. abundance ($p<0.001$) and on 60 day there was a decrease in *Clostridium* spp. abundance ($p<0.001$).

Key words: *Silage, Inoculant, Lactic acid bacteria, Enzyme, Fermentation.*

INTRODUCTION

Grass silage is an important ruminant feedstuff in dairy farms worlwide including Lithuania dairy farms. Different research studies concluded that individual elements of silage production - grass and other green plants vegetation phase, dry matter of fresh grass, used machinery, storage facilities, silage additives are the most important factors determining silage fermentation rates and impact on nutrient and energy levels and the hygienic properties of the feed (Santos et al., 2016). Silage making process can be explained very simply, it is actually very complex and dependant on many factors, such as the natural microbial population, harvesting conditions and the sugar content of the forage. Consequently, silage quality can be very variable and the only way to effectively control the fermentation process is to use an additive. Additives control or prevent certain types of fermentation, thus reducing losses and improving silage stability (Yitbarek et al., 2014). In most commercially available inoculants, homofermentative lactic acid bacteria (LAB) have been used because they are fast and efficient producers of lactic acid, improving natural silage fermentation (Weinberg et al., 1996). Heterofermentative LAB have attracted attention as an alternative additive to inhibit aerobic deterioration (Herkel et al., 2015). LAB as a biological silage additive provides stable feed value and secondary metabolic products during rapid anaerobic primary silage fermentation. They are able to ferment a large number of forage crops and also to reduce pH levels in fermented forages, which helps to suppress the growth of spoilage microorganisms. Furthermore, silage inoculants can enhance silage quality, nutritional recovery and shelf life of the inoculated product (Kim et al., 2021). The combination of different cultures of lactic acid bacterial species as a silage inoculant may be more beneficial than using a single species alone due to the differences in growth pattern and positive interaction among bacteria (Jatkauskas et al., 2013). Recently, inoculants containing homo- and hetero-fermentative LAB has become predominant additives, because the combination of both types of these LAB can reduce losses and increase the fermentation quality as well as aerobic stability of silage (Li et al., 2016). The possibilities of using enzymes help to improve nutrient digestion, utilization, and animal productivity and at the same time reduce animal fecal material and pollution. The enzyme amylase is useful for degrading starch into sugars. Cellulases or xylanases degrade cell walls into sugars. Sugars released by the enzymes increase growth of silage bacteria and, in some cases, fiber degrading enzymes also increase forage digestibility (Yitbarek et al., 2014). The aim of this study was to evaluate the fermentation and microbial dynamics of perennial grasses silage with biological inoculant containing LAB and enzymes.

MATERIAL AND METHODS

Samples collection

The silage samples were collected from one conventional (intensive) cattle farm in Lithuania, located in the central part of Lithuania (coordinates: 55.45860857021875, 23.6184147274186) during the year 2020. The

experiment was realized in practical conditions. The second-cut perennial grasses (Alfalfa and *Poaceae* mixed grasses) was harvested at initial flowering stage at July, 2020, and after 24h wilting, the silage mass was chopped on about 60 mm chop length using chopper harvester. Into grass silage was added biological additive, consisted of mixture of homofermentative and heterofermentative lactic acid bacteria (LAB) and enzymes. Inoculant was sprayed using a plant sprayer over the course of filling the silos. The inoculant was applied at recommended rate of 2 g/t of fresh forage. Inoculant included strains *Lactobacillus plantarum* CNCM I-3235 (≥ 1.00 x 1011 CFU/g), *Pediococcus pentosaceus* NCIMB 12455 (≥ 4.00 x 1010 CFU/g), *Pediococcus acidilactici* CNCM I-3237 (≥ 4.00 x 1010 CFU/g), *Propionibacterium acidipropionici* CNCM MA 26/4U (≥ 2.00 x 1010 CFU/g), Alpha-amylase (EC 3.2.1.1) from *Bacillus amyloliquefaciens* (≥ 3600 BAU), Cellulase (EC 3.2.1.4) from *Trichoderma longibrachiatum* (≥ 60 CMCU), Beta-glucanase (EC 3.2.1.6) from *Aspergillus niger* (≥ 1000 IU), Xylanase (EC 3.2.1.8) from *Trichoderma longibrachiatum* (≥ 1500 IU), organic sucrose, colloidal silica up to 250 g. Inoculant contained $5x10^{11}$ CFU per 1 g. After treatment grass was ensiled in trench silo. Laboratory analysis of control group (fresh grass) was made before ensiling. Laboratory analysis of treated silage samples was carried out at 7, 14, 21 and 60 days after treatment. The number of samples was three from each sampling at different periods. The samples were packed into plastic bags to avoid exposure to air and delivered to the laboratory. Chemical and fermentation analysis was conducted at Chemical Research Laboratory of the Lithuanian Research Centre for Agriculture and Forestry, Microbial counts analysis was made at Microbiology and Virology Institute at Lithuanian University of Health Sciences.

Chemical and fermentation analysis

Chemical analysis of examined silage samples was determined according reference methods of forage analysis. Crude fat (CF) content was determined according to Soxhlet method, crude protein (CP) according to Kjeldahl (AOAC 1990; Đorđević et al., 2016), crude fiber (CFB) according to Weende method, total nitrogen was measured as Kjeldahl nitrogen (LST EN ISO 5983-1:2005), neutral detergent fibre (NDF) and acid detergent fibre (ADF) according to Van Soest method, crude ash (CA) determined gravimetrically after biomass dry combustion at 600^{o}C. Element contents in DM (dry matter) were analyzed using near-infrared reflectance (NIR) spectroscopy (NIRS-6500). Metabolic energy (ME, MJ/kg DM), netto energy of lactation (NEL, MJ/kg DM) was calculated by a formula Nauman and Bassler (1993), organic matter digestibility evaluated according to the Hohenheim feed test (Naumann and Bassler, 1993). Fatty acid content evaluated according to gas chromatography method (Naumann and Bassler, 1993).

Microbial count analysis

20 g of silage samples were placed into plastic bags containing 180 ml of sterile physiological solution and mixed for 3 minutes using BagMixer (Interscience,

France). Serial dilutions of suspension were prepared in tubes with 9 ml of physiological solution. The total bacterial count was quantified by Tryptone Soya Agar (CM0131R, Thermo Scientific, Oxoid, UK), molds and yeasts counts were cultured on Sabouraud Dextrose Agar (PO1166A Thermo Scientific, Oxoid, UK), *Enterobacteriaceae* were quantified using Violet Red Bile Glucose Agar (PO5043A Thermo Scientific, Oxoid), *Clostridium* spp.were quantified using *Clostridium perfringens* agar (610147 Liofilchem, Italy) and *Lactobacillus* spp. were quantified using MRS agar (4017292 Biolife Milan, Italy).
After collecting complete analyze results of experiment, it was assessed the impact to inoculant on process of fermentation and changing nutrients in silage.

Statistic analysis

Statistical analysis was performed using the IBM SPSS Statistics Version 26. Differences in the test properties of the compared groups are expressed as means and RMSE (root mean square errors). For fermentation, chemical and microbial analysis was performed 1-way ANOVA analysis. The differences between the investigated groups were evaluated using Fisher's LSD criterion (α=5%). The differences were considered to be statistically significant when $p< 0.05$.

RESULTS AND DISCUSSION

After collecting complete analyze results of experiment, it was assessed the impact to inoculant on process of fermentation and changing nutrients in silage. Data in Table 1 shows that inoculant at different examination periods had highly significant impact on content of dry matter, crude fat and NDF after supplementation of biological additive in all analyzed samples. Conversely, there was a reduction of the pH and water soluble carbohydrates concentration. The data in Table 1 showed that starting pH of the fresh forage was 5.8 and the DM was 52.90%. The pH values at all experimental periods decreased and the final pH at the 60 days of ensiling after treatment with inoculant was 4.7. The result was statistically significant compared with the control group and to silage from each experimental periods ($p<0.001$). The final pH for the purpose of this assessment is controlled by 3 parameters. Primarily the DM controls the pH, but the acid that is formed utilises sugar as substrate, and the protein content of the forage defines the buffering capacity. The low pH shows the silage is stable and can not further develop undesirable microbes too in the in-silo (Kim et al., 2021). Kung et al. (2001) data suggests a pH of 4.7 at a DM of 35% on grass, or a pH of 5.0 on 55% legume silage which has a high buffering capacity. In our study a rapid drop in pH from 5.8 to 5.0 in the first week and then slow drop in pH to 4.7. In this study pH is perfectly reasonable and corresponds for the higher DM. There simply is not the free moisture available as the DM increases to produce the lactic acid to drop the pH. The final pH is higher as the DM increases (Driehuis et al., 2018). The lower pH in inoculated silage is important for conserving of nutrients and promoting homofermentative lactic acid bacteria. Generaly, the main effect of silage inoculant

was the increased production of lactic acid with significant reduction of pH (Đorđević et al., 2016, Stoškus et al., 2017).

Santos et al. (2016) concluded that low DM content in alfalfa silage is 35%, when high dry matter content is 45% respectively. In our study the dry matter content of silage was higher. The dry matter in fresh grass was 52.90% and at the ensiling process it increased to 57.80%. This result was statistically significant comparing to control group and to silage from each experimental periods ($p<0.001$). The DM of the forage increases the relative density of the forage decreases and the amount of air ensiled increases (Borreani et al., 2018). The fermentation occur once the ensiled air is converted to CO^2. In high DM silage the lagging in fermentation where the epiphytic bacteria are active and can be producing undesirable products is greater. Typically, for a 35% dry matter grass lactic acid and total volatile acids ratio is approximately 3:1 of (Kung et al., 2018). In our study DM content was higher than that ratio in at 50% in resulting a rapid anaerobiosis and then pH fall which stops loss of digestibility, metabolic energy and protein breakdown.

The NDF and ADF are important quality parameters of silage. High contents of NDF and ADF in silage adversely affect the quality and decreased digestibility (Đorđević et al., 2016). A decrease in NDF between fresh and ensiled samples has been reported by others (Ozduven et al., 2009; Đorđević et al., 2016) but in our study, NDF and ADF was higher at the period of 60 after treatment.

Table 1. Chemical composition of the silages at different periods of experiment

Variable	Control group	Days after treatment				SEM	p-value
		7	14	21	60		
DM, %	52.90^{a}	56.05^{b}	57.74^{c,d,e}	57.40^{d}	57.80^{e}	0.179	<0.001
pH	5.80^{a}	5.00^{b,c}	4.90^{c,d}	4.80^{d,e}	4.70^{e}	0.086	<0.001
CP, % DM	21.40^{a}	19.30^{b}	18.37^{c}	20.40^{d}	19.10^{b}	0.126	<0.001
CF, % DM	2.82^{a}	3.61^{b}	3.61^{b}	3.79^{c}	3.62^{b}	0.051	<0.001
CFB, % DM	21.20^{a}	23.57^{b}	23.09^{c}	22.80^{d}	24.10^{e}	0.085	<0.001
CA, % DM	11.00^{a}	10.87^{a}	10.42^{b}	10.60^{b}	10.90^{a}	0.090	<0.001
WSC, % DM	3.98^{a}	0.39^{b}	1.64^{c}	2.16^{d}	0.81^{e}	0.041	<0.001
NDF, % DM	38.20^{a}	40.75^{b}	42.57^{c}	41.80^{d}	44.70^{e}	0.091	<0.001
ADF, % DM	23.40^{a}	26.93^{b}	28.25^{c}	27.30^{d}	29.60^{e}	0.086	<0.001

Note. DM – dry matter, CP – crude protein, CF – crude fat, CFB – crude fiber, CA – crude ash, WSC – water-soluble carbohydrate, NDF – neutral detergent fibre, ADF – acid detergent fibre, SEM – standard error meaning.

a, b, c, d, e – means in row marked different letters differed statistically significant ($p<0.05$)

Results of energy characteristics are presented in Table 2. We found significant differences in content of ME and NEL as well. Higher contents of ME was found at all silage samples with inoculant at all experimental periods comparing to the control group. The highest levels of ME was found at 21 day after treatment and was statististically significant comparing to the silage sample at 60 day after treatment ($p<0.01$). There were observed significant differences ($p<0.05$) in contents of NEL after all days after treatment. The highest levels of NEL was in silage sample at 21 day after treatment. Herkel et al. (2015) reported similar results while Đorđević et al. (2016) reported that ME and NEL were not affected by inoculation treatment.

Table 2. Energy characteristics of the silages at different periods of experiment

Variable	Control group	Days after treatment				SEM	p-value
		7	14	21	60		
ME, MJ/kg DM	9.33[a]	9.69[b,c]	9.52	9.82[b]	9.46[a,c]	0.133	<0.001
NEL, MJ/kg DM	5.39[a]	5.64[b,c]	5.52[a,c]	5.72[b]	5.48[a]	0.106	<0.05

Note. ME – metabolic energy, NEL – neto energy for lactation, SEM – standard error meaning.
a, b, c – means in row marked with different letters differed statistically significant ($p<0.05$)

Data presented in Table 3 show that silage treated with inoculant had significant effect on all fermentation parameters resulting in excellent to very good fermentation value and silage stability (Figure 1). The lactic acid increased 14 days of ensiling after treatment with inoculant, but decreased at the 60 days after treatment statistically significant between these experimental periods ($p<0.001$), conversely decreased at 14 days of experiment, but increased at all other periods statistically significant ($p<0.001$). Butyric acid was generally not found in perenial grass silage samples. In this study according to DM content there were less moisture available to convert sugar to lactic acid, so as the DM increases end up producing a lower concentration of all fermentation products (Nielsen et al., 2007). The level of total acid was consistent at 44 – 48 g/kg DM which is actually high for the ensiled DM, with lactic acid accounting for approximatelly 70% of the total fermentation product which means it has been a driven homofermentative fermentation.

Table 3. Fermentation parameters of the silages at different periods of experiment

Variable	Days after treatment				SEM	p-value
	7	14	21	60		
Protein breakdown, %	62.0[a]	64.2[b]	64.9[c]	66.1[d]	1.473	<0.001
Digestibility of OM, %	71.0[a,c,d]	75.0[b]	73.0[b,c]	70.0[d]	1.132	<0.01
NH_3-N/TN,%	4.0[a]	4.0[a]	5.0[b]	6.0[c]	0.065	<0.001
TA, g/kg DM	44.0[a]	45.0[a]	46.0	48.0[b]	1.080	<0.05
LA, g/kg DM	70.7[a]	72.3[b]	69.8[c]	65.3[d]	0.141	<0.001
AA, g/kg DM	11.0[a]	10.0[b]	13.0[c]	14.0[d]	0.094	<0.001
BA, g/kg DM	< 1	< 1	< 1	< 1		

Note. TN – total nitrogen, TA – total acids, LA – lactic acid, AA – acetic acid, BA – butyric acid, DM – dry matter, SEM – standard error meaning.
a, b, c, d, – means in row marked with different letters differed statistically significant ($p<0.05$)

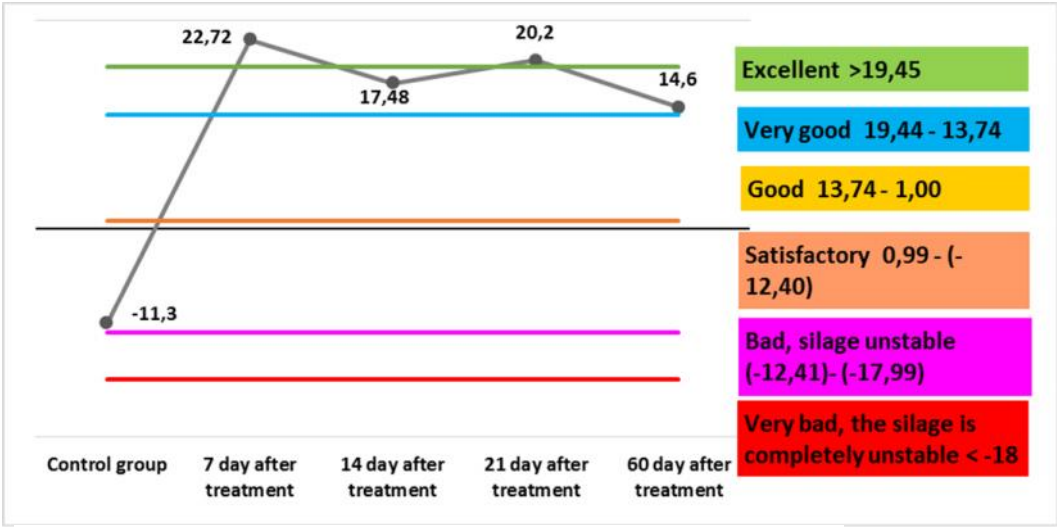

Figure 1. Relationship between fermentation value and silage stability at different periods of experiment

Microbiological composition of silage reveals counts of *Enterobacteriaceae*, *Lactobacillus* spp. *Clostridium* spp., yeast and molds. These indicators indicates to various silage failure processes. They are important because the results of the study can be used to determine whether the silage is safe to store and feed, whether it is overgrown or even spoiled, whether it contains a large number of dangerous microorganisms and is unsuitable for animal feed. Silage microflora can be categorized into two main groups, desirable and undesirable organisms. LAB are desirable microbes, while undesirable microorganisms (*Enterococcus*, yeast and molds) can cause anaerobic or aerobic spoilage during silage fermentation (Kim et

al., 2021). Clostridium species are gram-positive, obligate anaerobic spore-forming bacteria. Clostridia require relative high pH values (>4.5), high forage moisture concentration (>70%), and high water activity (from 0.952 to 0.971) for growth; hence, they are inhibited in silages if rapid acidification reduces the pH to 4 or below within 3 (Muck et al., 2003). The critical pH that inhibits clostridial growth varies with the plant moisture content (Driehuis et al., 2018). The microbiological composition of the corn silages is given in Table 4. Lactobacilli numbers of grass silages increased during the 14 day of fermentation. At the 7 day of fermentation process there was a significant increase in *Lactobacillus* spp. abundance ($p<0.001$) and on 60 day there was a decrease in *Clostridium* spp. abundance ($p<0.001$).

Table 4. Microbial composition of the silages at different periods of experiment

Variable	Days after treatment				SEM	p-value
	7	14	21	60		
TBC, log10 CFU/g	1.00^{a}	6.91^{b}	6.12^{b}	6.20^{b}	0.713	p<0.001
Lactobacillus spp. log10 CFU/g	7.43^{a}	7.64^{a}	6.19^{b}	4.39^{c}	0.162	p<0.001
Enterobacteriaceae log10 CFU/g	0.00	1.00	0.00	1.00	1.000	p=0.347
Clostridium spp. log10 CFU/g	4.86^{a}	7.17^{b}	5.78^{c}	0.00^{d}	0.117	p<0.001
Molds log10 CFU/g	0.00	0.00	0.00	0.00	0.00	p=1
Yeast log10 CFU/g	0.00^{a}	0.00^{a}	1.10	2.62^{b}	0.781	p<0.01

Note. TBC – total bacteria count, CFU – colony forming units, SEM – standard error meaning.
a, b, c, d – means in row marked with different letters differed statistically significant ($p<0.05$)

Our study results showed rapid decreasing of pH in resulting high levels of *Lactobacillus* spp. and the lowest levels of *Clostridium* spp. at the 60 days after treatment with inoculant. Before and during ensiling, management practices that favor rapid homolactic fermentations should be ensured because a rapid pH drop is critical to inhibiting *Clostridium* spp. and enterobacteria, which cause proteolysis and secondary butyric fermentation (Queiroz et al., 2018).

CONCLUSIONS

The study showed higher values of dry matter, crude fat and NDF after supplementation of biological additive in all analyzed samples. Conversely, there was a reduction of the pH and water soluble carbohydrates concentration. Biological inoculant with enzymes increased acetic acid concentration which had a significant impact on higher levels of *Lactobacillus* spp. abundance and decreased in *Clostridium* spp. abundance.

Our study showed that LAB and enzyme inoculation of perennial grass improved silage fermentation by increasing lactic acid and reducing pH value at the same time increasing ME and NEL.

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