THE EFFECT OF THE BACTERIAL INOCULANT ON THE ENSILED LUCERNE FERMENTATION CHARACTERISTICS, MICROBIAL POPULATION AND THE AEROBIC STABILITY IN THE MINI-SILOS

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Abstract. The aim of this study was to determine the effect of the inoculants containing homo-fermentative and hetero-fermentative lactic acid bacteria *Lactococcus lactis* and *Lactobacillus buchneri* on the ensiled in laboratory silo lucerne fermentation characteristics, microbial population and the aerobic stability. Combination of ensiled material and the inoculant was replicated five times when determining the chemical composition and aerobic stability in the silage. Addition of the inoculant resulted in a higher dry matter (DM) content ($P<0.01$), higher crude protein content ($P<0.05$) and a higher ($P<0.01$) total fermentation acids concentration. Lactic and acetic acids concentration were significantly higher ($P<0.01$) in inoculated silage when compare with the control silage. The inoculant has decreased ($P<0.01$) the levels of undesirable fermentation products such as the butyric acid, ammonia-N and ethanol and significantly ($P<0.01$) decreased dry matter loss. The inoculation significantly lowered the yeasts ($P<0.05$) and moulds count ($P<0.01$) and significantly increased lactic acid bacteria (LAB) count ($P<0.01$) compared to the control silage 120 days after ensiling as well as after 30 days of the aerobic exposure. Inoculated silage was more ($P<0.05$) resistant to aerobic deterioration.

Keywords: aerobic stability, fermentation, inoculant, lucerne, silage.

Introduction. Nowadays the bacterial inoculants are introduced to improve the silage fermentation process and the aerobic stability, as well as, to increase the nutritional value and the digestibility of the silage (DeBoever et al., 2013). As we know, there are two types of lactic acid bacteria's (LAB), homo-fermentative and hetero-fermentative. The main fermentation product for the homo-fermentative LAB is lactic acid, which consume a little energy in the feed. Hetero-fermentative bacteria without lactic acid produce higher amount of acetic acid and consume more feed energy. Under the aerobic conditions, the acetic acid, due to its antifungal properties, increases the stability of silage (Danner et al., 2003; Jatkauskas et al., 2012). The usage of silage inoculants containing hetero-fermentative LAB, mainly *Lactobacillus buchneri*, has been relatively new, but according to the researchers, this bacteria has a great influence on the silage aerobic stability (Hu et al., 2009; Kristensen et al., 2010; Tabacco et al., 2011; Wambacq et al., 2013).

When silage is used for feeding and is in contact with air, fermentation acids and other substrates are affected by aerobic bacteria, yeasts and moulds (Wilkinson et al., 2013). In the contact with air, yeasts are considered to be a very important group of undesirable microorganisms that become active and begin to demolish organic acids which preserve the silage. In addition to this, the increase of the pH in the silage results its heating, as well as, less acid-resistant and silage-containing microorganisms also become active. Epiphytic yeast in the silage can convert water-soluble carbohydrates into CO_2 and alcohols. Therefore, yeast activity can be stopped by increasing lactic and acetic acid concentrations (Driehuis et al., 2000; Dunière et al., 2013).

Most studies investigating the effect of the inoculant containing homo-fermentative and hetero-fermentative LAB have reported various changes in the chemical composition and the aerobic stability in a variety of silages. However, there is still a lack of information about the combination of quite new homo-fermentative LAB strains, the *Lactococcus lactis* together with the hetero-fermentative LAB *Lactobacillus buchneri*.

The purpose of this study was to determine the effect of homo-fermentative and hetero-fermentative lactic acid bacteria mix containing *Lactococcus lactis* and *Lactobacillus buchneri* on the ensiled lucerne fermentation characteristics, microbial population and the aerobic stability in the laboratory mini-silos. Was hypothesized that the mix of homo-fermentative and hetero-fermentative LAB inoculant would change the fermentation and microbial profile of the silage and can improve the aerobic stability of lucerne ensiled in laboratory silo.

Materials and methods

Experimental design. The experiment was conducted in 2016 - 2017 at the Institute of Animal Science of Lithuanian University of Health Sciences according to the DLG (Deutsche Landwirtschafts-Gesellschaft e. V. /internationally acknowledged German Agricultural Society) guidelines for the testing silage additives.

The experiment was carried out by using the random sampling method, where each laboratory storage capacity

was an experimental unit. Each combination of ensiled material and the inoculant was replicated five times when determining the chemical composition and aerobic stability of the silage. For the evaluation of pH drop after 3 days of ensiling mini-silages were prepared in glass jars capacity of 0.5 litre (Table 1). Therefore, two silage groups were formed: CON - control group (without an additive) and BIN - test group (added lactic acid bacteria).

Table 1. **Experimental design**

Groups	CON	BIN
Application of additive, cfu/g fresh forage	0	150 000 *Lactococcus lactis* and *Lactobacillus buchneri* (50:50)
Total mini-silos	15	15
Description	5 from each group for testing pH at day 3 (0.5-liter glass jars); 5 from each group for testing the chemical and microbiological composition after 120 days of storage (3-liter glass jars); 5 from each group for testing the aerobic stability for maximum 30 days or until all silages have crossed the threshold of $+3^0$C above the ambient temperature (3-liter glass jars).	

Mini-silos preparation. Lucerne (*Medicago sativa* L.) two years old second cut, at early bloom stage of maturity was cut by a mower conditioner and wilted up for 12 hours up to 320.6 g kg^{-1} dry matter (DM) content. Wilted crop was chopped by a forage harvester under farm conditions to 2-3 cm length, immediately delivered to the laboratory and ensiled.

The inoculant suspension was prepared by mixing 2.0 g of dry inoculant with 1 liter of drinking chlorine-free water, that 1 ml of the LAB in the suspension reached $1x10^8$ colony forming units (cfu) or $1x10^5$ cfu per 1 g of crop material. 1 ml of suspension was used per 1 kg of forage (added 3 ml chlorine-free water for uniform spraying). The same volume (4ml g^{-1} fresh forage) of chlorine-free water was used instead of the suspension in the control treatment. The samples of water and suspension were taken to determine the number of LAB according to the ISO 15214:1988 method. The suspension and water were sprayed into the fresh forage using a spray botlle and the forage was thoroughly mixed and putted into glass jars by periodical tamping. The density of the forage in the silo was in compliance with DLG recommendations, 0.2 kg DM per 1 litre volume. After the filling, the jars were immediately closed with tight caps and weighed. Filled mini-silos were stored at room temperature (≈20°C) for 120 days.

Sampling. Five samples of fresh forage (>500 g each) were taken prior to ensiling for the determination of the chemical composition and microbiological characteristics. After 3 days of fermentation, samples (>200 g each) of silage were taken (5 replication from each group) to evaluate the pH drop. After 120 days of fermentation, mini-silos were weighed for the determination of the DM loss and samples (>200 g each) were taken (n=5 for the each treatment) for the chemical and microbiological analyzes.

Aerobic stability test. The silage aerobic stability test (AST) was performed after 120 days after of the silages fermentation. 1 kg (1000 g) of the silage (5 replications from each treatment) was taken, and loosely was putted into polystyrene boxes. The boxes were covered with a lid with the diameter of 2 cm, so that the air could enter the silage mass freely. The temperature sensors were placed in the center of the silage mass and the temperature changes were recorded every six hours by the data loggers (MS4+, Comet system s.r.o.). Temperature sensor was putted into empty box to measure the ambient temperature. The boxes were kept in a constant room temperature (≈+20°C) for 30 days. The onset of the aerobic spoilage was marked from the point at which the temperature of the silo reached +3°C above the ambient temperature. At the end of the AST, each box was weighed (n=5) to determine DM loss and samples (>200 g each) were taken to evaluate the pH value and the population of yeasts, moulds and LAB.

Analytic methods. The content of DM of forage and silage were determined by oven drying at 60°C, equilibrated to room humidity overnight, milled through a 1 mm sieve and further dried at 105°C to constant weight. Silage DM content was corrected for volatile alcohols and fatty acids during the oven drying. Crude protein (CP) was analyzed by Kjeldahl-AOAC 984.13. Crude fat content was determined by extraction of Soxtec System while using the petrol ether 40-600C. Crude fat residue was determined gravimetrically after drying. Crude fiber content was determined with Fibercap (Foss Tecator) using sulphuric acid and Na hydroxide treatment. The Neutral detergent fiber (NDF) and Acid detergent fibre (ADF) concentrations were determined by using an Ankom200 fiber analyzer ("Ankom Technology", USA). Water-soluble carbohydrates (WSC) were determined by using the anthrone reaction assay from the herbage or silage extracts obtained from steeping fresh herbage or silage in water. Crude ash concentration was determined by ashing the samples in a furnace at 600°C. Buffering capacity of the forage was determined according to Playne and McDonald (1966), expressed as mEq of alkali required to change the pH from 4 to 6 per 1 kg of DM. Lactic acid, volatile fatty acids and alcohol concentrations were determined by gas-liquid chromatography. Gas-liquid chromatograph "GC-2010 Shimadzu" with wide-bore capillary column (Stabilwax®-DA 30 m, 0.53 mm, ID, 0.5 μm) was used according to Gas chromatography and Biochemistry analyzer official methods. Ammonia-N concentration was determined by direct distillation using the "Kjeltec Auto System 1030" (AOAC 941.04). The pH value was measured and recorded by using "Thermo Orion Posi-pHloSymp-Hony" electrode and "Thermo Orion 410"

meter. DM loss was estimated by measuring differences in mini-silos weights after ensiling (on day 0 after ensiling) and at the end of the ensiling period (on day 120 after the ensiling). All these chemical analytic methods and aerobic stability test were according Jatkauskas et al. (2013).

Microbial composition was measured at National Food and Veterinary Risk Assessment Institute. The LAB, yeasts and moulds counts were determined by colony-count technique at 25°C using ISO 15214:1998 and ISO 21527-1:2008.

Statistical analysis. The statistical analysis of data was performed with the program package SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Results of descriptive statistics were expressed as the mean±standard deviation of the mean (mean±SD). Comparisons between the means were evaluated using Student's t-test. A probability of less than 0.05 was considered as a significant (P<0.05).

Results

Crop material analyzes. Mean DM content of wilted lucerne reached the targeted value of 32.06 % or 320.6 g kg^{-1} herbage. Lucerne had a crude protein concentration of 21.87 % of DM (218.7 g kg^{-1} DM) and WSC concentration of 5.10 % of DM (51.0 g kg^{-1} DM) or 1.64 % on a fresh matter basis (Table 2). Therefore, lucerne cultivar had low WSC content and high crude protein concentration. Buffer capacity of herbage was high (50.4 mEq kg^{-1} 100g DM^{-1}) and pH value reached 6.20 units. The calculated fermentability coefficient (FC) was medium (40.7). Consequently, lucerne was moderately difficult to ensile. Microbiological enumeration of herbage showed that the number of LAB, yeasts and moulds was typical for the epiphytic microflora characteristics for the lucerne forage in Lithuanian conditions. The number of epiphytic LAB was 4.96 log_{10} cfu g^{-1} of pre-ensiled fresh lucerne. Moulds and yeasts were detected at 4.72 log_{10} cfu g^{-1} and 4.60 log_{10} cfu g^{-1} respectively.

Table 2. **Chemical and microbiological composition of the fresh lucerne and silage after 120 days of storage**

Variable	n	Fresh forage	Silage	
		mean±SD	CON±SD	BIN±SD
Dry matter g kg^{-1}	5	320.6±7.28	292.46±6.31	302.0±1.43*
Dry matter, corrected for volatiles g kg^{-1}	5		302.8±6.44	314.9±2.0**
Crude protein g kg^{-1} DM	5	218.7±6.44	207.4±1.19	213.2±5.39*
Crude fat g kg^{-1} DM	5	17.5±1.93	23.1±1.76	22.9±1.34
Crude fibre g kg^{-1} DM	5	288.8±13.48	301.0±2.35	294.6±4.76*
Crude ash g kg^{-1} DM	5	81.4±5.81	86.8±3.11	85.6±4.22
WSC g kg^{-1}DM	5	51.0±1.87	5.4±0.55	5.6±1.14
NDF g kg^{-1} DM	5	442.2±13.59	446.2±12.15	444.6±14.06
ADF g kg^{-1} DM	5	339.4±14.05	367.4±11.06	361.8±8.35
Yeasts log_{10} cfu g^{-1}	5	4.72±0.13	2.47±0.32	1.1±0.13**
Moulds log_{10} cfu g^{-1}	5	4.6±0.23	1.97±0.07	1.3±0.21**
LAB log_{10} cfu g^{-1}	5	4.96±0.13	6.59±0.39	8.04±0.48**
Buffer capacity mEq 100 g DM^{-1}	5	50.4±5.81		
pH day 0	5	6.2±0.04		
pH day 3	5		5.69±0.05	4.9±0.06**
pH day 120	5		4.67±0.02	4.3 ±0.03**
Note. * - (P<0.05); ** - (P<0.01)				

Table 3. **Fermentation characteristics of ensiled lucerne**

Variable	n	CON±SD	BIN±SD
Total acids g kg^{-1} DM	5	64.1±4.64	100.4±4.92*
Lactic acid g kg^{-1} DM	5	40.5±4.89	66.2±3.95*
Acetic acid g kg^{-1} DM	5	19.0±1.95	32.4±2.42*
Butyric acid g kg^{-1} DM	5	3.51±0.57	0.44±0.11*
Propionic acid g kg^{-1} DM	5	1.0±0.30	1.31±0.20
Ethanol g kg^{-1} DM	5	9.76±0.66	3.93±0.63*
Ammonia-N g kg^{-1} total N	5	48.4±3.65	35.4±2.70*
DM losses g kg^{-1} DM	5	70.29±7.56	31.25±3.14*
Note. * - (P<0.01)			

Chemical composition and fermentation parameters. The addition of the inoculant resulted in a higher by 3.8 % (P<0.01) DM (corrected for volatiles), higher by 2.7 % (P<0.05) crude protein content and less by 2.1 % (P<0.05) crude fiber content, compared to control silage. Inoculant treatment affected acidification level of silages. In inoculated silages pH value after 3 days and after 120 days of ensiling was by 0.79 and by 0.37 units,

respectively, lower (P<0.01) compared with the untreated control silage (Table 2). The inoculant treated silage had by 63.5 % higher (P<0.01) concentration of lactate and by 70.5 % higher (P<0.01) concentrations of acetate than did untreated silage. In the inoculant treated silage concentrations of butyrate, ammonia-N and alcohols were decreased by 8 time, 26.9 % and 2.5 time (P<0.01), respectively, compared to control silage (Table 3).

Microbial composition. Inoculant treatment increased lactobacilli and decreased yeast and mould numbers of lucerne silages compared with the control silage. As expected, higher count of lactobacilli were detected in the inoculated silage versus those without inoculant (8.04 log_{10} cfu g^{-1} *vs* 6.59 log_{10} cfu g^{-1} of fresh silage; P<0.01). The numbers of yeasts and moulds after 120 days of storage were lower in inoculated silages when compared with the control silage (1.10 *vs* 2.47 log_{10} cfu g^{-1} and 1.30 *vs* 1.97 log_{10} cfu g^{-1} fresh silage, respectively; P<0.01) (Table 2). The number of yeasts and moulds in inoculated silage after aerobic stability test (30 days air exposure) were by 27.9 % (P<0.05) and by 24.4 % (P<0.01), respectively, lower compared with control silage (Table 4). After 30 days air exposure bacilli count in inoculated silage was by 34.6 % higher (P<0.01) compare with control silage. Reduction in yeast and mould population during anaerobic phase of silage conservation and during aerobic exposure appears to be the main reason for the improvement aerobic stability of the inoculated silage compared to control silage. The inoculant treated silage deterioration was delayed 1.9 time (P<0.05) when compare with control silage.

Table 4. **Microbiological composition of the silage after AST**

Variable	n	CON±SD	BIN±SD
Yeasts after AST log_{10} cfu g^{-1}	5	3.76±0.70	2.71±0.37*
Moulds after AST log_{10} cfu g^{-1}	5	8.98±0.52	6.79±0.24**
LAB after AST log_{10} cfu g^{-1}	5	5.2±0.45	7.0±0.71**
DM losses after AST g kg^{-1} DM	5	91.2±3.19	57.0±3.39**
pH after AST (150 d)	5	8.73±0.13	6.54±0.17**
Note. * - (P<0.05); ** - (P<0.01)			

Aerobic stability. The results of the temperature changes during 30 days aerobic exposure are shown in Figure 1. Control silage had a temperature rise of more than 3^0C above the ambient temperature after 372 h air exposure, when inoculated silage had a temperature rise of more than 3^0C above the ambient temperature after 696 h air exposure. The aerobic stability of inoculated silages was improved by 324 h (13,5 days) when compare with control silage.

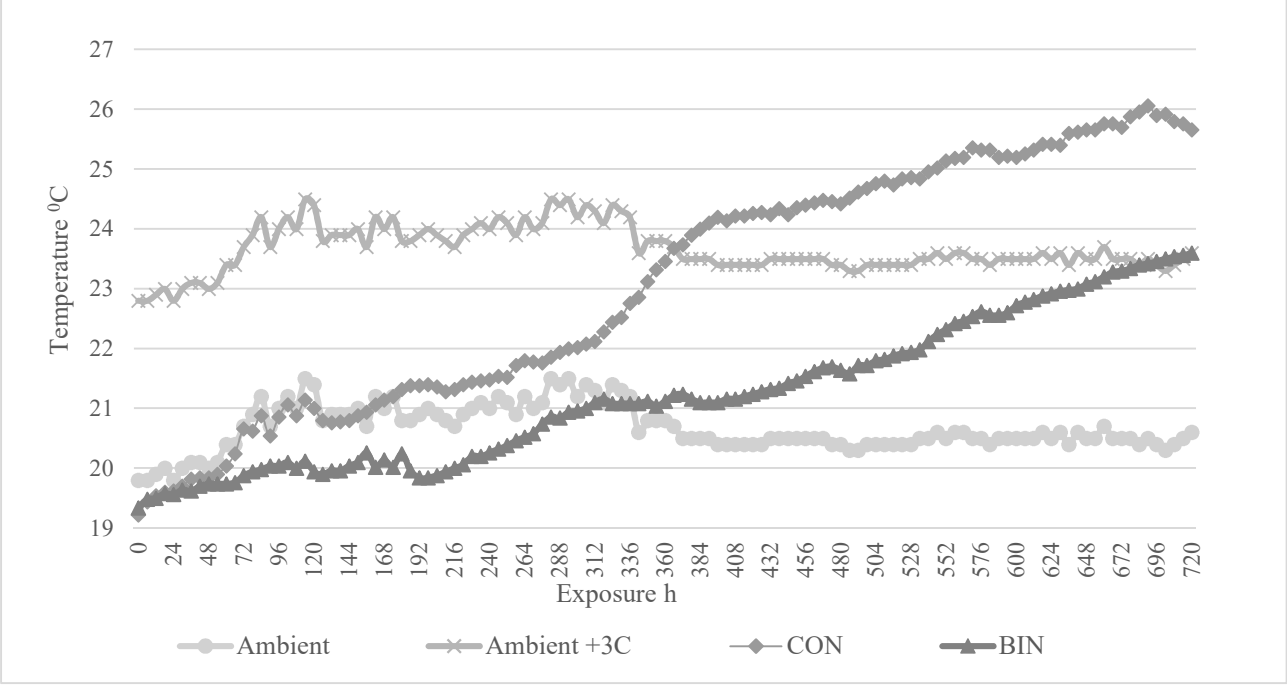

Figure 1. **Aerobic stability measurements**

Discussion. Our findings indicate that LAB inoculation of the lucerne increased the silage fermentation by reducing the pH value and accelerated the lactic acid and the acetic acid formation. Previous studies have reported that homo-fermentative LAB strains combination with *Lactobacillus buchneri* were efficient in improving

fermentation and resulted in a higher level of acetic acid (Jatkauskas et al., 2011; Witt et al., 2015).

The main indicator of protein breakdown are high level of ammonia-N. The lower ammonia-N concentration in the inoculated silage suggests that the inoculant reduced the proteolysis of plant proteins. These results confirm the recent research received earlier by Witt et al. (2015), but in contrast, some others studies reported that the addition of *L. buchneri* increased the ammonia-N concentration in lucerne and maize silages (Schmidt et al., 2009; Rabelo et al., 2017). Furthermore bacterial inoculant had a possitive affect on the chemical composition by preserved higher DM content and reduced DM loss.

The hygienic quality of silage is frequently connected with the occurrence of the micro-organisms. An epiphytic yeasts in a silage can convert WSC into CO_2 and alcohols (Dunière et al., 2013). In our study, the amount of ethanol was significantly lower in an inoculated silage than it was in the control group. Moreover yeasts and moulds counts tended to be higher in the control silage than in treated silage after 120 days of storage as well as after AST. The present study showing that inoculated silage had significantly better aerobic stability, compared with the control silage. These results are directly related to the inhibition of growth of yeasts and moulds and this can be achieved when *L. buchneri* is added in combination with homo-fermentative LAB (Kleinschmit et al., 2005). The current results agree with the others recent studies where *L. buchneri* combination with homo-fermentative LAB was superior in increasing aerobic stability (Reich and Kung, 2010; Jatkauskas et al., 2013; Li et al., 2016). In the other hand, more recent Jahnson (2017) reported that treating maize with a dual-purpose silage inoculant containing *Lactobacillus buchneri* and *Lactococcus lactis* applied at a rate of 2.5×10^5 cfu/g failed to improve the aerobic stability of corn silage stored for a short duration (<32 d).

However, our hypothesis that mix of homo-fermentative and hetero-fermentative inoculant would improve fermentation characteristics, microbial profile and the aerobic stability of ensiled lucerne in mini-silos was right.

Conclusions

1. Microbial inoculant generally had a positive effect on the lucerne silage characteristics in terms of a higher content of DM, a more efficient fermentation, resulting in lower pH, improved fermentation products profile and a lower DM loss compared with ordinary made silage.
2. In the inoculated silages ammonia-N levels were reduced, indicating that less protein was broken down resulting in higher nutritive value compared with negative control silages.
3. Lower concentrations of butyric acid, reduced number of yeasts and moulds and improved aerobic stability indicate better hygienic quality of inoculated silage compare to control silage.
4. Temperature changes during aerobic exposure indicated that inoculant treated silage deterioration was significantly delayed when compare with control silage.

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