EFFECT OF *L. PLANTARUM*, *PEDIOCOCCUS ACIDILACTICI*, *ENTEROCOCCUS FAECIUM* AND *L. LACTIS* MICROBIAL SUPPLEMENTATION OF GRASS SILAGE ON THE FERMENTATION CHARACTERISTICS IN RUMEN OF DAIRY COWS

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Summary. Trials were conducted at the Lithuanian Institute of Animal Science to determine metabolism changes in the rumen and blood profile of Lithuanian Black-and-White dairy cows fed different fermented silages. First cut 8 to 10 h wilted grass- legume sward (20% *Festuca pretense*, 30% *Trifolium pretense*, 50% *Lolium perenne*) with DM content – 348.3 g kg^{-1} was made in round bales either untreated (C) or treated with inoculant (a bacterial mixture of *Lactobacillus plantarum* Milab 393, *Pediococcus acidilactici* P6 and P11, *Eterococcus faecium* M74, and *Lactococcus lactis* SR3.54) at rate 5 × 10^5 colony forming units g^{-1} of fresh herbage (L). Treatment had no effect on chemical composition, digestible value of nutrients. Both ordinary made and inoculated silages were well fermented at opening with pH values 4.58 and 4.17, respectively. Addition of bacterial mixture resulted in a significant ($P<0.05$) increase in lactic acid concentration and markedly decreased the concentration of acetic acid in silage. Treatment had an effect on protein breakdown as measured by ammonia-N concentration, with values of 45.7 and 35.19 g kg^{-1} N for untreated and inoculated silages respectively.

The silages were offered *ad libitum* with a standard concentrate suplementation at a flat-rate (280 g for 1 kg milk) for ten Lithuanian Black-and-White dairy cows divided in two analogous groups for a period of 100 days. Inoculated silage led to higher infusoria count in rumen fluid during entire experiment compared to controls and at the end of the experiment, infusoria count in the rumen of the cows fed treated silage was by 13.9 % higher than in controls. Treated with the bacterial mixture silage had no effect on the rumen pH value and VFA concentration, however, at the end of the experiment the ratio of acetic acid to propionic acid was lower (on 1.19 %) in the rumen of cows offered treated silage compared to cows fed ordinary silage. Silage treated with microbial mixture was beneficial to rumen protein synthesis, whereas the content of protein nitrogen and that of total nitrogen were, respectively, by 5.17 mg l00 ml^{-1} ($P<0.01$) and by 3.37 mg l00 ml^{-1} ($P<0.01$) higher compared to controls. The content of ammonia-N was lower in the rumen fluid of cows offered inoculated silage during all experimental period. Blood metabolite content was unaffected by treatment and blood of animals of both groups corresponded to the physiological level.

Key words: silage, rumen contents, infuzoria count, volatile fatty acids, nitrogen, blood.

ŽOLIŲ SILOSO, PAGAMINTO SU PIENO RŪGŠTĮ PRODUKUOJANČIŲ BAKTERIJŲ *L. PLANTARUM*, *PEDIOCOCCUS ACIDILACTICI*, *ENTEROCOCCUS FAECIUM* IR *L. LACTIS* MIŠINIU, POVEIKIS MELŽIAMŲ KARVIŲ DIDŽIOJO PRIESKRANDŽIO FERMENTACIJOS RODIKLIAMS

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Santrauka. Norint nustatyti siloso, pagaminto su pieno rūgštį produkuojančių bakterijų mišiniu, įtaką didžiojo prieskrandžio metabolizmui ir sveikatingumui, LVA Gyvulininkystės institute atliktas bandymas su 10-čia Lietuvos juodmargių veislės melžiamų karvių. Ritininis silosas buvo pagamintas iš 8–10 val. pavytintos pirmos pjūties žolės (20 proc. *Festuca pretense*, 30 proc., *Trifolium pretense*, 50 proc. *Lolium perenne*), turinčios 348,3 g kg^{-1} SM. Pusė pašarui skirtų ritinių buvo pagaminta be jokių priedų (C), kita dalis – su bakterijų mišinio (inokulianto) (*Lactobacillus plantarum* Milab 393, *Pediococcus acidilactici* P6 ir P11, *Eterococcus faecium* M74, ir *Lactococcus lactis* SR3.54 bakterijų štamai) priedu – skiriant 5 × 10^5 ksv g^{-1} žalios masės (I). Siloso priedas neturėjo esminės įtakos siloso cheminei sudėčiai, maisto medžiagų virškinamumui. Skirtingai pagaminto siloso fermentacija buvo gera – pH svyravo tarp 4,58 (C) ir 4,17 (I). Inokulianto priedas patikimai ($p<0,05$) padidino silose pieno rūgšties kiekį ir žymiai sumažino acto rūgšties kiekį. Priedas sumažino baltymų skilimą silose, nes amoniakinio N kiekis I silose buvo 10,5 g kg^{-1} mažesnis nei C silose.

Dešimt melžiamų karvių, suskirstytų į dvi grupes pagal produktyvumą ir laktacijos mėnesį, 100 dienų buvo *ad libitum* šertos silosu, pagamintu su bakterijų mišinio priedu (grupė I) ir įprastai užraugtu silosu (grupė C). Karvėms buvo sušeriama po 280 g 1 kg pieno kombinuotųjų pašarų. Per visą bandymo laikotarpį infuzorijų buvo daugiau dižiajame prieskrandyje karvių, gavusių inokuliuotą silosą, nei karvių, šertų silosu be priedo. Bandymo pabaigoje I grupės karvių dižiajame prieskrandyje infuzorijų buvo 13,9 proc. daugiau negu kontrolinėje grupėje. Silosas su bakterijų priedu neturėjo esminės įtakos didžiojo prieskrandžio turinio pH ir LRR kiekiui. Bandymo pabaigoje acto ir propiono rūgščių santykis tiriamosios grupės karvių didžiajame prieskrandyje buvo 1,19 proc. žemesnis palyginti su kontroline grupe. Silosas su bakterijų priedu darė teigiamą įtaką baltymų sintezei, t. y. karvių, gavusių inokuliuotą silosą, didžiajame prieskrandyje baltyminio azoto ir bendro azoto buvo atitinkamai 5,17 ($p<0,01$) ir 3,37 ($p<0,01$) mg100 ml^{-1} daugiau, o amo-

niakinio azoto mažiau nei gavusių įprastai užraugtą silosą. Kraujo tyrimai parodė, kad abiejų grupių karvės buvo sveikos.

Raktažodžiai: silosas, didžiojo prieskrandžio turinys, infuzorijos, lakiosios riebalų rūgštys, azotas, kraujas.

Introduction. The cause of the improvement in animal performance following feeding with inoculated silage is unclear, but the results of feeding experiments suggest a possible probiotic effect of the LAB used in inoculants. One hypothesis is that certain LAB strains interact with rumen microorganisms to enhance rumen functionality and animal performance. Such a hypothesis is consistent with Fuller's (1989) definition of a probiotic: "Live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". To affect rumen microflora, LAB ingested by the animals along with the silage would have to survive under rumen conditions.

Inoculants for silage comprising mainly of lactic acid producing bacteria (LAB) are used to improve preservation efficiency. Silages treated with such inoculants may also have a positive probiotic effect on ruminant performance although the mechanism is as yet unclear. It is feasible that LAB interact with rumen microorganisms in such a way that their activity is enhanced and fiber degradability is improved. Another possibility is that LAB produce bacteriocins in the silage, which might inhibit detrimental microorganisms, both in the silage and in the rumen (Weinberg, 2004).

The extent of fermentation of WSC during ensilage into lactic acid and VFA can change the end-products of rumen fermentation. End-products of lactic acid fermentation in rumen may very depending on microbial population and rumen pH. The majority of published reports indicate that propionate is the main end-product of lactate fermentation with grass silage-based diets (Jaakkola et al., 1991). Diets based on restrictively fermented grass silages which are high in water WSC and low in lactate, favour a rumen fermentation pattern rich in butyrate or acetate and low in propionate. Silages low in WSC and high in lactic acid have increased the proportion of propionate in ruminal fluid (Cushnahan et al., 1995). Rumen fluid of animals feeding only silages were marked higher coefficients rumens transformations as well as higher number protozoa (Krzywiecki et al., 2003). In the majority of trials reported in the literature, the silages treated with inoculants appeared to be more digestible than the untreated silages (McDonald et al., 1991). It seems that the ingestion of silage fermentation end products may modify the rumen fermentation pattern and nutrient digestion, intake of forage and performance of the animals. The profile of VFA formed in the rumen also has environmental consequences because methane emissions by ruminants are involved in global climate change. There are uncertainties in the estimates, approximately 14 % of methane emissions may be caused by domestic animals, of which 97 % correspond to ruminants (Nordang, 1991).

The aim of this study was to assess how inoculated with *Lactobacillus plantarum*, *Pediococcus acidilactici*, *Eterococcus faecium* and *Lactococcus lactis* legume-grass silage with different level of fermentation end- products related with ruminal fermentation parameters.

Materials and methods. The second year's use, first cut grass-legume sward (20% *Festuca pretense*, 30% *Trifolium pretense*, 50% *Lolium perenne*) was harvested, prewilted 8-10 h, baled in round bales (1.2 m in diameter and 1.2 m high) and wrapped with 6 layers of stretch film. 45 round big bale silages were made without additive (C group) and 45 – treated with the bacterial inoculant (*Lactobacillus plantarum* Milab 393, *Pediococcus acidilactici* P6 and P11, *Eterococcus faecium* M74, and *Lactococcus lactis* SR3.54), (L group). The inoculant (dosage $5x10^5$ cfu/g fresh herbage) was applied using a commercial pump "HP-20". During the ensilage, samples of grass were collected to determine its chemical composition. Bales were opened after 82 days samples were taken and the fermentation quality (fermentation acids, ammonia N, pH), chemical composition (standard methods (AOAC, 1995) of the silages were measured.

Ten dairy cows of the Lithuanian Black-and-White breed were used in the experiment. A three-week pre-experimental period was used in which untreated silage was offered *ad libitum* together with the compound feed. Compound feed to cows was fed individually according to the milk yield (280 g for 1 kg milk). In experimental period (100 days) each group consisting of five cows was fed its respective silage *ad libitum* offered in two meals per day (Table 1). The weight of the offered silage was determined once weekly on two consecutive days and refusals were weighed back and subtracted when calculating daily intake. The amount of compound feed was recorded at each meal. Milking of cows was performed twice daily in the stable.

The rumen fluid was collected from three cows of each group using the pharynx probe with a steel tip in 2 hours after a.m. feeding once in the pre-experimental period and three times in the experimental period. The rumen contents was analyzed for infusoria count per 1 ml fluid in the Fux-Rozenthal chamber, total VFA by distillation with Markgham's apparatus and VFA ratio was determined with the gas chromatograph Chrom-5, pH-value was determined with the pH 526-meter, total nitrogen, protein nitrogen – according to the method of Kjelahl with apparatus Kjeltec System 1002, ammonia – by the method of Convey and Bright. Blood samples were taken from three cows from each group on the end of pre-experimental period and on the end of experimental period. Samples were taken through the indwelling catheters placed in the jungular vein starting 2 hours after morning feeding. Blood samples were analyzed for calcium, phosphorus, total protein, glucose in LVA Kaunas. The data were analysed by one-way ANOVA, and a mean comparison by Fisher'PLSD.

Table 1. **Experimental design**

Group of cows	No of cows	Feeding pattern
Control (C)	5	Silage made without inoculant (DM content -336.8 g kg^{-1}, ME – 9.91 MJ kg^{-1} DM, crude protein – 137.9 g kg^{-1}DM; pH- 4.58; lactic acid – 27.59 g kg^{-1}DM, acetic acid- 12.26 g kg^{-1}DM, butyric acid – 4.71 g kg^{-1}DM; ammonia N – 45.57 g kg^{-1}N), compound feed (75% barley, 10% wheat, 15% soybean meal and vitamin-mineral concentrate *4923 Optima Dairy Extra)*
Inoculant (I)	5	Silage made with inoculant (DM content -328.4 g kg^{-1}, ME – 10.56 MJ kg^{-1} DM, crude protein – 139.5 g kg^{-1}DM; pH - 4.17; lactic acid – 46.09 g kg^{-1}DM, acetic acid – 13.66 g kg^{-1}DM, butyric acid- 2.22 g kg^{-1}DM; ammonia N – 35.19 g kg^{-1}N), compound feed (75% barley, 10% wheat, 15% soybean meal and vitamin-mineral concentrate *4923 Optima Dairy Extra)*

The feeding trial was performed in pursuance with the Lithuanian animal care, management and operation legislation (No 8-500, 6 November 1997).

Results and discussion. The inoculation of legume-grass sward made silage with lower by 15.4 g kg^{-1} ($P<0.05$) water soluble carbohydrates (WSC) content, higher by 18.3 g kg^{-1} ($P<0.05$) total fermentation acids and higher by 18.5 g kg^{-1} ($P<0.05$) lactic acid content, but lower by 2.49 g kg^{-1} butyric acid content compared with untreated silage. The ammonia N concentration was lower by 10.38 g kg^{-1} total N in the inoculated silage compared with untreated one. The use of biological additives in ensiling previlted material and improved fermentation quality were reported by many researchers (Muck and O'Kiely, 2002; Selmer-Olsen and Magne Mo, 1997; Weinberg, 2004; Olt et al., 2005).

The inoculation had a positive effect on the nutritional value of silages, however, the digestible energy of the inoculated silage was higher by 0.65 MJ g kg^{-1}DM ($P<0.01$) compared with untreated.

Inoculation gave the higher (by 2.37% cow^{-1} day^{-1}) dry matter intake than the untreated silage. Martinsson (1992) reported, that the inoculant-treated silage increased silage DM intake by 7% during weeks 4-12 of lactation. This large effect could be accounted for fully by the higher digestibility of the inoculant-treated silage.

Milk yield was affected due to the higher intake and the higher nutritive value of the inoculated silage. Average milk yield was higher by 6.47% for the inoculated silage diet compared with the untreated silage.

Table 2. **Microbiological and biochemical indicators**

Item	Group	At the end of pre-experimental period	Experimental period			Average in experim. period
			start	middle	end	
pH	C	6.65	6.53	6.50	6.49	6.51
	I	6.62	6.68	6.64	6.52	6.61
	$LSD_{0.05}$	0.072	0.244	0.376	0.500	0.113
	$S_{\bar{x}}$	0.178	0.606	0.941	1.264	0.530
Infusoria count. thous. ml^{-1}	C	429.6	402.3	508.3	457.8	456.2
	I	468.3	443.3	596.6	521.4[a*]	520.5*
	$LSD_{0.05}$	368.85	134.022	162.215	106.355	39.714
	$S_{\bar{x}}$	13.502	5.209	4.825	3.570	2.494
Total VFA. mmol $100ml^{-1}$	C	9.65	10.11	10.97	11.49	10.86
	I	9.46	9.61	10.71	11.19[a*]	10.50
	$LSD_{0.05}$	2.303	2.31	4.681	0.56	0.817
	$S_{\bar{x}}$	3.962	3.851	7.096	0.812	2.346

* - denotes significant at level 0.05

[a] – in comparison with pre-experimental period

During the experimental period infusoria count ranges from 429.6 thous. ml^{-1} to 521.4 thous. ml^{-1} in both groups, but average in the experimental period infusoria count in I group was by 13.98 % ($P<0.05$) higher than that in the C group. VFA concentration at end of experiment in the rumen of cows fed silage with *L. plantarum*, *Pediococcus acidilactici*, *Enterococcus faecium* and *L. lactis* was by 0.3 mmol $100ml^{-1}$ ($P<0.05$) lower compared with that of cows fed silage without inoculant. VFA concentration average in the experimental period in I group was by 0.36 mmol $100ml^{-1}$ lower compared to the C group. VFA concentration affected the pH values of the rumen content and in group I rumen pH value average in experimental period was by 0.10 unit higher compared with the C group. A studies (Weinberg et al., 2004) indicated that freeze-dried cultures of LAB used in silage inoculants

survived in rumen fluid; the pH of strained rumen fluid treated with LAB cultures was generally higher than that of uninoculated control rumen fluid throughout the 72-to 96-h incubation period.

There were no significant effects of the type of silage on the proportions of acetic acid and butyric acid. But average in experimental period the proportions of propionic acid was by 1.14 (P<0.05) higher compared with C group (Table 3). Average in experimental period the ratio of acetic: butyric, acetic: propionic and (acetic + butyric): propionic in rumen of I group was, respectively, by 0.2; 0.17 (P<0.05) and 0.2 (P<0.05) lower compared with C group. Probably lactic acid of silage was transformed into propionate in rumen (Jaakkola et al., 1992; Saarisalo et al., 2004).

Table 3. **Molar proportions of VFA**

Item	Group	At the end of pre-experimental period	Experimental period			Average in experim. period
			start	middle	end	
Acetic acid	C	61.11	60.23	58.03	57.99	58.75
	I	61.28	57.25*	57.05	59.34	57.88
	$LSD_{0.05}$	2.04	2.067	2.673	12.123	2.422
	$S_{\bar{x}}$	0.548	0.578	0.763	3.396	1.274
Propionic acid	C	20.02	21.97	22.04	21.73	21.91
	I	20.86	23.06	23.18	22.92	23.05*
	$LSD_{0.05}$	3.181	3.587	2.507	3.267	0.846
	$S_{\bar{x}}$	2.558	2.618	1.822	2.405	1.153
Butyric acid	C	14.14	13.48	15.24	14.85	14.52
	I	13.56	14.60	15.21	15.13[a]	14.98
	$LSD_{0.05}$	1.075	3.705	3.642	2.372	0.968
	$S_{\bar{x}}$	1.275	4.337	3.931	2.600	2.012
Acetic: Butyric	C	4.32	4.49	3.82	3.93	4.08
	I	4.51	3.92	3.81	3.92[a]	3.88
	$LSD_{0.05}$	0.456	1.313	0.600	1.488	0.387
	$S_{\bar{x}}$	1.696	5.131	2.587	6.229	2.977
Acetic: Propionic	C	3.06	2.74	2.63	2.68	2.68
	I	2.95	2.48	2.46	2.59	2.51*
	$LSD_{0.05}$	0.375	0.328	0.396	0.627	0.137
	$S_{\bar{x}}$	2.054	2.064	2.557	3.905	1.619
(Acetic + Butyric) : Propionic	C	3.76	3.39	3.33	3.37	3.36
	I	3.60	3.12	3.12	3.25	3.16*
	$LSD_{0.05}$	0.522	0.531	0.554	0.584	0.157
	$S_{\bar{x}}$	2.33	2.684	2.825	2.897	1.476

* denotes significant at level 0.05.

No differences in ruminal pH, content and proportion of volatile fatty acids were observed in animals fed either restrictively or extensively fermented silages (Jaakkola et al., 1991).

The silage inoculated with *L. plantarum*, *Pediococcus acidilactici*, *Enterococcus faecium* and *L. lactis* increased content of total nitrogen and protein nitrogen at start, middle and end of experiment, respectively, by 1.69 *vs* 3.93 (P<0.05) mg 100 ml^{-1}, 6.30 (P<0.05) *vs* 8.72 (P<0.05) mg 100 ml^{-1} and 2.10 *vs* 2.85 mg 100 ml^{-1}compared to the untreated silage (Table 4). Feeding silage inoculated with biological additive was beneficial to rumen protein synthesis, i.e. the content of protein nitrogen and that of total nitrogen were, respectively, by 5.7 (P<0.01) and 3.37 (P<0.01) mg 100 ml^{-1} higher and the content of ammonia nitrogen was by 1.71 mg 100 ml^{-1} (P<0.01) lower compared to the untreated silage average in experimental period. Most of the studies comparing the effects of silage fermentation on rumen fermentation pattern sugests that the type of silage has a considerable influence on ruminal fermentation pattern of typical dairy cows (Krzywiecki et al., 2003; Miettinen, 1997; Keady and Steen, 1996). In addition, the differences in fermentation characteritics of silage can affect feed intake and consequently the total nutrient supply (Miettinen, 1997).

The analysis of blood samples indicated that the animals in both groups were healthy (Table 5). At the end of the experimental period the total protein content decreased by 0.26-0.44 g l^{-1} and that of phosphorus, calcium and glucose increased, respectively, by 0.12 (P<0.05)-

0.04, 0.01-0.06 and 0.77 (P<0.05) -0.74 (P<0.05) mmol l^{-1} in the animal blood in comparison with the end of the pre-experimental period, therefore there will no differences between groups of animals.

Table 4. **Nitrogen level in the rumen, mg 100 ml^{-1}**

Item	Group	At the end of pre-experimental period	Experimental period			Average in ex-perim. period
			start	middle	end	
Total nitrogen. mg 100 ml^{-1}	C	68.43	66.87	60.80	62.63	63.43
	I	67.92	68.56	67.10*	64.73	66.80**
	$LSD_{0.05}$	3.665	4.129	2.821	6.09	2.091
	$S_{\bar{x}}$	0.883	1.002	0.725	1.572	0.985
Protein nitrogen. mg 100 ml^{-1}	C	54.28	52.19	45.08	46.68	47.98
	I	53.97	56.12*	53.8*	49.53	53.15**
	$LSD_{0.05}$	1.117	2.762	5.692	9.667	2.744
	$S_{\bar{x}}$	0.339	0.838	1.892	3.303	1.664
Ammonia nitro-gen. mg 100 ml^{-1}	C	13.14	13.86	13.91	14.43	14.07
	I	13.06	11.42*	11.87*	13.79	12.36**
	$LSD_{0.05}$	5.145	1.683	1.832	2.155	0.808
	$S_{\bar{x}}$	6.454	2.188	2.336	2.51	1.876

* and ** denotes significant at level 0.05 and 0.01 respectively.

Table 5. **Blood profile**

Item	Group	End of pre-experimental period	End of experimental period
Total protein g l^{-1}	C	82.03	81.77
	I	81.97	81.53
	$LSD_{0.05}$	1.359	4.583
	$S_{\bar{x}}$	0.272	0.922
Phosphorus mmol l^{-1}	C	1.64	1.76*
	I	1.74	1.78
	$LSD_{0.05}$	0.186	0.029
	$S_{\bar{x}}$	1.813	0.267
Calcium mmol l^{-1}	C	2.58	2.59
	I	2.78	2.84
	$LSD_{0.05}$	0.534	0.385
	$S_{\bar{x}}$	3.271	2.337
Glucose mmol l^{-1}	C	2.13	2.90*
	I	2.11	2.85*
	$LSD_{0.05}$	0.108	0.386
	$S_{\bar{x}}$	0.838	2.204

* - denotes significant at level 0.05.

Conclusions

1. The use of biological additive in ensiling previlted material, rich in red clover, improved fermentation and silage quality, what resulted in rumen fermentation changes in dairy cows.

2. Infusoria count and VFA concentration in I group were, respectively, by 13.89 % (P<0.05) higher and 0.30 mmol 100ml^{-1} lower than those in C group. VFA concentration affected the pH values of the rumen content and in group I rumen pH-value was by 0.03 unit higher compared with the C group.

3. Feeding silage with *L. plantarum*, *Pediococcus acidilactici*, *Enterococcus faecium* and *L. lactis* was beneficial to rumen protein synthesis: the content of protein nitrogen and that of total nitrogen were, respectively, by 5.7 (P<0.01) and 3.37 (P<0.01) mg 100 ml^{-1} higher and

the content of ammonia nitrogen was by 1.71 mg 100 ml^{-1} ($P<0.01$) lower compared to the untreated silage.

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