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EFFECT OF PRE-FERMENTED JUICE, *LACTOBACILLUS PLANTARUM* AND *LACTOBACILLUS BUCHNERI* ON THE FERMENTATION CHARACTERISTICS AND AEROBIC STABILITY OF HIGH DRY MATTER ALFALFA BALE SILAGE

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ABSTRACT

The experiment was conducted to investigate the effects of pre-fermented juice (PFJ), *Lactobacillus plantarum* and *Enterococcus faecium* (LP), and *Lactobacillus buchneri* (LB) on the fermentation characteristics and aerobic stability of alfalfa bale silage. The herbage was wilted to 602.3 g/kg dry matter (DM). Treatments of alfalfa silage included (1) control; (2) PFJ: 2.6×10^5 colony-forming units (cfu/g); (3) LP: 1.0×10^6 cfu/g *Lactobacillus plantarum* and *Enterococcus faecium (*Pioneer 1188, USA) and (4) LB: 1.0×10^6 cfu/g *Lactobacillus buchneri* (Pioneer 11A44) and baled, 150 days. At the end of the ensiling period, three bales of each treatment group were opened, chemical and microbiological analyses were made. Consequently, lactic acid bacteria inoculants and PFJ increased the quality of alfalfa silages. In terms of aerobic stability, PFJ and LP used had a positive effect on CO_2 concentrations coliform bacteria and yeast. Also, LB inoculant decreased NDF content and increased *in vitro* organic matter digestibility of silages. A total number of 15 representatives of lactic acid bacterial strains were retained and among them 3 dominant genus were identified as *Lactobacillus plantarum (*46.66%), *Lactobacillus pentosaceus (*33.33%) and *Lactobacillus collinoides (*20%). It can be concluded that PFJ can be used as silage additive alfalfa bale silage in farm condition.

Keywords: Alfalfa bale silage; identification; *in vitro* organic matter digestibility; lactic acid bacterial inoculants; PFJ (pre-fermented juice).

INTRODUCTION

Ensiling has been known as a method to preserve the moist crops by controlling anaerobic fermentation (Bureenok *et al.* 2005). The success of the ensiling can be achieved when the number of lactic acid bacteria (LAB) is dominant in the fermentation, and the activity of clostridia is restricted. Therefore, an inoculation of LAB in the ensiling process has been recommended in order to make good quality silage.

The concept of using a microbial inoculant for silage involves adding fast-growing homofermentative LAB in order to dominate the fermentation, thereby producing higher quality silage. The most common homofermentative inoculant is *Lactobacillus plantarum.* It is generally considered that $1x10^6$ viable inoculant cells per gram is sufficient for bacterial additives to overwhelm the epiphytic LAB and predominant population in the silage (Gollop *et al*. 2005). In addition to *L. plantarum,* other *Lactobacillus* or *Pediococcus* species may be employed and *Enterococcus faecium* is also frequently used (*D*unière *et al*. 2013). Recently, in order to improve the aerobic stability and reduce fermentation losses of silage, heterofermentative LAB species, such as *Lactobacillus buchneri* was developed as silage additives (Keles and Demirci 2011, Arriola *et al*. 2011). Also, many researchers have reported that manipulating numbers of the epiphytic LAB can be improved by using fermented juice of epiphytic LAB as a silage additive obtained by macerating crop silage with water and anaerobic incubating for 2 days (Denek *et al*. 2011, Denek *et al.* 2012*,* Bureenok *et al*. 2012). It is natural, easy to prepare and have promising affects as a silage additive.

Commercial LAB inoculants contain one or more of these bacteria that have been selected for their ability to dominate the fermentation process (Widyastuti 2008). However, the properties of a bacterial strain vary even within the same species, and some strains are not effective in improving fermentative quality of silage as well (Woolford and Sawczyc 1984). However, inoculants sometimes do not improve silage quality because of limited substrates in the material crop, and because the inoculant strain does not grow well on the target crop.

It is well known that epiphytic LAB play a major role in natural silage fermentation, and their numbers become a significant factor in predicting the adequacy of silage fermentation and determining whether or not to apply bacterial inoculants to silage materials (Kozaki *et al.* 1992). However, from the silage fermentation and microbiological point of view, we know of no information available on the LAB composition of silage prepared, and the influence of epiphytic LAB from silage on fermentation quality. Therefore, the present

study set out to screen, isolate and identify the LAB colonizing silages prepared on farm conditions. In order to evaluate the relationships between natural populations of LAB and silage quality, the fermentation characteristics and chemical composition of silage samples were also studied.

MATERIALS AND METHODS

Silage preparatioon: A second-cut alfalfa (*Medicago sativa L.)* was harvested at approximately 10% bloom mower conditioner on 10 June 2013. Cutting was carried out with a mower conditioner with flails (Kverneland Taarup 347; Kverneland, Nykoping, Sweden) and wilted for 24 h to a DM content of around 602.3 g/kg). There was no rainfall during harvesting and drying conditions were considered excellent. After wilting, fresh matter (FM) samples were taken from the field and the crop was baled with a high-density baler (Orkel GP 1260 Orkel AS, Fannrem, Norway; bale size 90x100 cm; cut length: 8-12 cm; bale colour: white).

Inoculants were supplied as freeze-dried powders by Pioneer (USA). The number of viable bacteria in the inoculants was determined by enumeration of diluted suspensions on DeMan±Rogosa±Sharpe (MRS) agar (Oxoid, Basingstoke, UK). The additive treatments were as followes: no additive (control treatment); PFJ: additional at a therotical application rate of 2.6×10^5 colony-forming units (cfu/g); LP: 1.0×10^6 cfu/g *Lactobacillus plantarum* and *Enterococcus faecium (*Pioneer 1188, USA) and LB: *Lactobacillus buchneri* (Pioneer 11A44). Additives were applied during baling with two nozzles attached to the baler pick up frame, pointing at the swath and spreading the additive on the crop as it was collected by the pick-up. All additives were applied at 2 L per ton FM. The bales were wrapped in 6 layers of 0.025 mm thick and 750 mm wide white plastic film (Trio Wrap, Trioplast AB, Sweden). Each bale was weighed (720-894 kg on fresh weight basis) and sampled to measure DM yield. The wrapped bales were weighed immediately and transferred to an open storage yard about 250m away with a clutch fork which picked the bales at both sides. Bales were prepared and stored outdoor at the feeding center. Ambient high temperatures were 25-28° C in spring and 33-36° C in summer. A total of 16 bales ensiled for 150 days.

Preperation of PFJ: PFJ was prepared according to the method described by Masuko *et al*. (2002). For this purpose, 200 g plant material was macerated with 1000 ml distilled water for 2 min in a high-speed blender. The macerate was filtered through two layers of cheesecloth, and aliquots of filtrate were collected in glass bottles to which molasses was added at 3 g per 100 mL(w/v) filtrate. These bottles were fitted with a gas trap and kept in a incubator for 48 h at 30° C.

Aerobic stability: All of the treatment silages at 150 days were subjected to an aerobic stability test in four 2L polyethylene terepthalate bottles for treatment at room temperature (25° C), which lasted for 5 days by the procedure of Ashbell *et al*. (1991).The system was constructed in two parts from recycled soft drink bottles (polyethylene terepthalate): the upper part (1 L) was filled with about 250 g (wet weight) of loosely packed silage, and the lower part with 100 ml of 20% KOH. Gas was exchanged through 1 cm holes in the upper part. Carbon dioxide produced during aerobic exposure was absorbed in the base and determined by titration with 1 N HCL. In addition, change in pH, yeast and mould counts served as indicators of aerobic spoilage. Chemical and microbiological analyses were carried out on the silage samples, initially and after 5 d exposure to air. Visual appraisal of the samples exposed to air was performed by a panel of 3 according to the extent of mould cover, texture and their odour. The panel evaluation was converted into a numeric scale from 1 to 5, with 1 being good quality silage with no apparent moulding and 5 being completely moulded samples (Filya and Sucu 2007). Visual appraisal is expressed using a scale of 1-5 where 1: good quality silage with no visible moulding, 2: a few small mould spots, 3: scattered mould spots, 4: silage with partially covered moulds, lumpy silage, 5: completely mould covered samples, unpleasant odour and silage particles sticking together.

Analytical analysis: Chemical analyses were performed on triplicate samples. Dry matter (DM) was determined by oven drying for 48 h. The pH in fresh material and silage samples was measured according to the British Standard method (Anonymous 1986). The ammonia nitrogen (NH_3-N) content of silages was determined, according to Anonymous (1986). The WSC content of silages was determined by spectrophotometer (Shimadzu UV-1201, Kyoto, Japan); after reaction with antron reagent (Thomas 1977). LA was determined by the spectrophotometric method (Koc and Coskuntuna 2003).

Crude protein (CP), ash and crude fiber (CF) were determined following the procedure of Association of Official Analytical Chemists (AOAC 1990). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) and acid detergent lignin (ADL) were analyzed according to the method of Van Soest (1982). Cellulose was calculated as the difference between ADF and ADL. *In vitro* organic matter (OM) digestibilities were estimated according to Aufrère and Michalet-Doreau (1988), with a three-stage technique: Pre-treatment with pepsin in hydrochloric acid (0.2% pepsin in 0.1 N HCl), starch hydrolysis, attack by cellulase (Onozuka R 10 from *Trichoderma viride*, Merck).

Microbiological analysis: For the quantitative microbial analysis, an aseptically weighed 10 g sample of g fresh matter or silage was suspended in 90 ml of physiological

saline containing 0.1% peptone and homogenized. The samples were analysed for counts of LAB (MRS agar, Oxoid; 30° C, 3 days, anaerobic incubation), total mesophilic bacteria (Plate Count Agar, Difco; 30° C, 3 days), clostridia (SFP Agar Base, Difco; 37˚C, 2 days, anaerobic incubation), as well as yeasts and moulds were determined by pour plating in malt extract agar (Oxoid CM59) that had been acidified, after autoclaving, by the addition of 85% lactic acid at a concentration of 0.5% vol/vol. Plates were incubated aerobically at 32° C for 48 to 72 h (Seale *et al.* 1990).

Morphological, physiological and biochemical tests: Morphological characteristics and Gram staining of LAB were examined after 24 h incubation on MRS agar. Catalase activity and gas production from glucose were determined by the methods of Temiz (2008). Carbonhydrate fermentation tests were carried out with the Analytical Profile Index (API 50 CH) strips (bioMerieux, Tokyo, Japan) of 49 different compounds and one control, according to the manufacturer's instructions, and reactions were determined after incubation at 30° C for 48 h (Lopez-Diaz *et al*. 2000).

Statistical analysis: Statistical analysis of the silage chemical analysis results included a one-way analysis of variance and Duncan's multiple range test performed with the Statistical Analysis System (2005) Software (SAS, Cary, NC).

RESULTS

The chemical and microbiological composition of the alfalfa forages prior to ensiling are summarized in Table 1. The fresh alfalfa contained 602.3, 181.9, and 42 g/ kg DM, CP, and WSC, respectively, and the pH was 5.75. The log numbers of colony forming unit (cfu)/ g FM of LAB and yeasts in the fresh material were 3.52 and 2.47, respectively. The pH and LAB counts of the PFJ were found 6.03 and 5.39 $\log_{10}$ cfu/g FM, respectively.

The chemical composition of the ensiled alfalfa silages are given in Table 2. The pH of all silages was lower than the fresh alfalfa. During fermentation, significant difference was observed between, the pH values of control and treatment silages ($P<0.05$). LP and LB treatment significiantly improved fermentation parameters in alfalfa silages with reduced pH ($P<0.05$) and an increased LA level ($P<0.01$) being notable. In the experiment, the WSCs in all silages decreased with the decrease in pH. The addition of PFJ had significantly lower WSCs compared with control and LAB treatments. No significant differences were observed between the control and additived silages with regard to DM and NH_3-N ($P>0.05$).

The microbiological composition of the alfalfa silage is given in Table 3. The addition of PFJ and LAB inoculant had no influence on LAB, total mesophilic bacteria (TMB), mold and yeast numbers of the silages. No differences were detected among treatments for microbiological composition.

Table 4 presents fiber composition, CP, CF and ash content of the ensiled alfalfa after 150 days. No differences were detected among treatments for ADF, ADL, and CF. Some differences were noted among treatments in ADF, ADL, and CF but were most likely a consequence of sampling variation. However, LP inoculant affected CP ($P<0.01$), and NDF contents ($P<0.05$).

Values for *in vitro* OM digestibility are given Table 5. The addition of LB at ensiling had significantly higher *in vitro* OM digestibility compared with the control, PFJ and LB silages ($P<0.05$).

Table 6 gives the results of the aerobic exposure test. pH change, CO_2, production and an increase mold and yeast numbers are indicators of silage deterioration. In present study, PFJ and LP treated silages decreased significantly CO_2 production in the alfalfa silages compared to control and LB silages. TMB and coliform bacteria counts were higher in the control and LB treated silages.

The phenotypically characteristics of LAB strains are shown in Table 7. A total of 15 strains isolated from the alfalfa silages. All isolates were gram-positive, catalase-negative, rod shaped bacteria. The 7 strains isolates were identificated as *Lactobacillus plantarum,* 5 strains were alloted to *Lactobacillus pentosaceus,* 3 strains were identificated as *Lactobacillus collinoides.*

DISCUSSION

Silage fermentation is a complex process which depends on many factors. The forage characteristics that contribute to a good fermentation are: dry matter content, physiological properties of epiphytic bacteria and, most importantly, the quantity of soluble carbohydrates (Zanine *et al.* 2010). The decline in pH values inhibit the spoilage microorganism proliferation, which allows the silage nutritive values to be preserved. Thus, the best silage forages are the ones with high soluble carbohydrates contents, which should be sufficient to promote the fermentation and produce enough acid to preserve the silage. In the present experiment, content of WSC in all preensiled alfalfa forages (4.2% DM, Table 1) was lower than the 6 to 7% content recommended theoretical requirement to achieve well preserved fermentation (Wang *et al*. 2009). Thus the alfalfa without additives was adequate for producing good quality silages.

In the experiment, LAB inoculants improved some fermentation parameters of alfalfa silages. Generally the addition of LAB inoculants at ensiling is intended to ensure rapid and vigorous fermentation that

results in faster accumulation of lactic acid (LA), lower pH values at earlier stages of ensiling, and improved forage conservation. Well preserved alfalfa silage is characterized by lower pH, greater LA content, lower contents of NH_3-N (Muck and Kung 1997, Zhang *et al*. 2009). In this study, after 150 days of ensiling, alfalfa silages treated with LP and LB properly improved the silage fermentation quality with markedly lower contents of pH and NH_3-N and markedly higher LA content as compared with the control silage. Many studies have shown the advantage of such inoculants (Adesogan *et al*. 2003, Nkosi *et al.* 2009, Vakily *et al*. 2011).

In some studies, LAB inoculants decreased cell wall contents of silages (Nadeau *et al.* 2000, Filya 2002, Polat *et al.* 2005). In contrast to these researcher's findings, some reports show that inoculants did not decrease significantly cell wall contents of silages (Meeske *et al*. 1999, Zahiroddini *et al*. 2004). At the end of the ensiling period, treatment with LP significantly decreased NDF concentration alfalfa silages compared with the control, PFJ and LB silages in present study. All of the additives did not affect the ADF, ADL and CF content of alfalfa silage compared to control silage, which is in agreement with past findings (Ranjit and Kung 2000, Kleinschmit *et al*. 2005).

There are various reports indicating that LAB inoculant did not effect ruminal DM and OM degrabilities or digestibility of silages (Arriola *et al.* 2011, Mohammadzadeh *et al.* 2012, Postulka *et al.* 2012). However in some studies, LAB and PFJ treated silage improved, degradability or digestibility (Keles and Demirci 2011, Denek 2011, Bureenok *et al*. 2012, Haghparvar *et al.* 2012). In the present study, the *in vitro* OM digestibility were higher in LB silages treated with control, PFJ and LP silages.

The effect of LB on silage fermentation has been well known since 1996 when an increase in aerobic stability was first observed in LB inoculated silages (Mari *et al.* 2009, Kristensen *et al.* 2010, Tabacco *et al.* 2011). In our experiment control and LB silages had high contents of both residual WSC and mold and yeast count therefore, tended to spoil more upon aerobic exposure, as indicated by more intensive CO_2 production, but did not change in pH test. The results revealed that aerobic deterioration of the control and LB silages was more intensive than PFJ silages.

Many studies (Lin *et al.* 1992a, Tjandraatmadja *et al*. 1994, Santos *et al.* 2011) have reported that *lactobacilli* are the dominant microbial population on forage crops and contribute to silage fermentation. The *lactobacilli* play a more important role in fermentation processes and effectively promote LA fermentation for a longer time than do lactic acid-producing cocci (e.g., *enterococci*, *streptococci*, *leuconostocs*, *weissella*, and *pediococci*). Generally, silage can be well preserved when the *lactobacilli* reach at least 10^5 cfu/ g of FM (Hellings *et al*. 1985). In this study all silages were well preserved, as would be expected with 24 h wilted alfalfa material. *Lactobacillus plantarum* and *Lactobacillus pentosaceus* are usually found living in association with forage crops and silages (Muck 1989, Lin *et al*. 1992b). In this study on alfalfa silages, found as the predominant species *Lactobacillus plantarum* and *Lactobacillus pentosaceus.*

Conclusions: The results obviously confirmed that using of PFJ and LAB inoculant additive some how would be one of the ways to improve the fermentative quality of silage in the alfalfa. In terms of aerobic stability, PFJ and LP used had a positive effect on CO_2 concentrations coliform bacteria and yeast. Also, LB inoculant decreased NDF content and increased *in vitro* organic matter digestibility of silages. It can be concluded that PFJ can be directly used as silage additive alfalfa bale silage in farm condition.

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