Asian-Aust. J. Anim. Sci.
Vol. 20, No. 11 : 1699 - 1704
November 2007
www.ajas.info

Fermentation Quality of Italian Ryegrass (*Lolium multiflorum* Lam.) Silages Treated with Encapsulated-glucose, Glucose, Sorbic Acid and Pre-fermented Juices

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ABSTRACT : This experiment was carried out to evaluate the effects of adding encapsulated-glucose, glucose, sorbic acid or pre-fermented juice of epiphytic lactic acid bacteria (FJLB) on the fermentation quality and residual mono- and disaccharide composition of Italian ryegrass (*Lolium multiflorum Lam*) silages. The additive treatments were as follows: (1) control (no addition), (2) encapsulated-glucose addition at 0.5% for glucose, (3) glucose addition at 1%, (4) sorbic acid addition at 0.1%, (5) FJLB addition at a theoretical application rate of 2.67×10^5 CFU (colony forming unit) g^{-1}, on a fresh weight basis of Italian ryegrass. Although control and encapsulated-glucose treatments had higher contents of butyric acid (33.45, 21.50 g kg^{-1} DM) and ammonia-N/Total nitrogen (114.91, 87.01 g kg^{-1}) as compared with the other treated silages, the fermentation in all silages was clearly dominated by lactic acid. This was well indicated by the low pH (4.38-3.59), and high lactic acid/acetic acid (4.39-22.97) and lactic acid content (46.85-121.76 g kg^{-1} DM). Encapsulated-0.5% glucose and glucose addition increased lactic acid/acetic acid, and significantly ($p<0.05$) decreased ammonia-N/total nitrogen, and the contents of butyric acid and total volatile fatty acids (VFAs) as compared with the control. However, there were higher butyric acid and lower residual mono-and di-saccharides on the two treatments as compared with sorbic acid and FJLB addition, and their utilization efficiency of water soluble carbohydrates (WSC) was lower than that of both sorbic acid and FJLB additions. Sorbic acid addition showed the lowest content of ethanol and ammonia-N/total nitrogen, and the highest content of residual fructose and total mono-and disaccharides as well as the higher lactic acid/acetic acid value. Sorbic acid addition decreased the loss of mono-and disaccharides, and inhibited the activity of clostridial and other undesirable bacteria, and greatly increased the utilization efficiency of fermentable substrates by epiphytic LAB. FJLB addition had the lowest pH value and the highest lactic acid content among all additive treatments, with the most intensive lactic acid fermentation occurring in FJLB treated silage. This resulted in the faster accumulation of lactic acid and faster pH reduction. Sorbic acid and FJLB addition depressed clostridia or other undesirable bacterial fermentation which decreased the WSC loss and saved the fermentable substrate for lactic acid fermentation. (**Key Words :** Italian Ryegrass, Additives, Fermentation Quality, Residual Mono-and Di-saccharides)

INTRODUCTION

The ensilage of forage crops is accompanied by a multitude of microbiological and biochemical changes. It depends on natural fermentation, in which the epiphytic lactic acid bacteria (LAB) convert water-soluble carbohydrate (WSC) into lactic acid (LA) under anaerobic conditions, and when pH decreases to a certain extent (approximately 4.2), the harmful microbiological activity is inhibited and the silage is well preserved for a long period (McDonald et al., 1991). Thus, the success of ensilage is principally dependent on the creation of anaerobic conditions in the silo and the presence at ensilage of both sufficient LAB and adequate WSC in the crop (Rooke, 1990). Although anaerobic conditions can be obtained by proper ensiling, such as chopping, compacting and sealing and so forth, there can still be considerable activity of aerobic microorganisms during the very early stages of ensiling and it is generally accepted that the activity of aerobic bacteria after ensiling is undesirable. These

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[a] Financial support for this research was provided by Grant-in-Aid of 2006BAD16B08-06 project in China.
Received July 5, 2006; Accepted January 12, 2007

organisms consume WSC and cause a shortage of fermentable substrate (Lacey, 1981; Alli et al., 1985).

It is well documented that addition of FJLB to silages is effective in improving the fermentation quality of silage, and often results in increased LA and reduction of ammonia-N (AN) even when the addition of commercial LAB was ineffective (Ohshima et al., 1997). In the present study, it was considered that FJLB would promote the onset of LAB fermentation, and increase the rate of LA production and pH reduction, and decrease the loss of WSC by restricting undesired bacterial activity. Woolford (1975) confirmed that sorbic acid had a strong inhibiting effect on the growth of yeast and moulds, and it is widely used as a preservative in the food industry and also as an ingredient of commercial silage additives to prevent aerobic deterioration after opening silos, (Hattori et al., 1996; Shao et al., 2004; 2005a). In this experiment sorbic acid was used as an additive to depress the loss of WSC by undesirable organisms (yeast, mould and aerobic bacteria) during the initial phase of ensiling, to save the WSC for lactic acid bacteria and improve the fermentation quality (Shao et al., 2003; Shao et al., 2004; 2005b). Glucose addition compensates the WSC loss caused by the initial plant respiration and undesirable bacteria activity (yeast, mould and aerobic bacteria) and ensures that sufficient WSC remains at the vigorous stage of LAB growth and produces lactic acid (LA). Several researchers have demonstrated the advantages of glucose as a silage additive to improve fermentation quality (Ohyama et al., 1975).

The largest decrease in WSC is due to consumption by plant respiration and undesired bacteria (yeast, mould or other aerobic bacteria), resulting in an insufficiency of fermentable substrate available for LAB in the early stage of ensiling. However, the largest growth of LAB also occurs during this period (Shao et al., 2002). Thus a medium (encapsulated-glucose) was made, which might be expected to give slower release rates of glucose into silage mass to coincide with early growth of LAB by providing additional substrate when needed.

The objectives of the present study were to evaluate the effects of addition of encapsulated-glucose, glucose, sorbic acid and FJLB on the fermentation quality and residual mono-and disaccharides of Italian ryegrass silages.

MATERIALS AND METHODS

Additive preparation

Pre-fermented juice epiphytic LAB (FJLB) : The FJLB was prepared from Italian ryegrass according to the following method; a 100-g sample of freshly cut Italian ryegrass was macerated with 300 ml of distilled water using a blender, the macerated sample was filtered through double layers of cheesecloth, and 200 ml of the filtrate was collected into a 500 ml-glass bottle containing 4 g of glucose. The glass bottle was fitted with a gas trap and maintained at 30°C for 3 days (Ohshima et al, 1997). After 3 days of anaerobic incubation, the pH value and the population of LAB of pre-fermented juice were determined just before silage treatment.

Encapsulated-glucose preparation : The encapsulated-glucose was prepared as follows: glucose powder was filled into a commercial capsule; each capsule contained 0.4 g glucose. A total of 8 pieces of encapsulated-glucose were mixed into the chopped Italian ryegrass just before ensiling into each silo, thus giving a theoretical application rate of 0.5% glucose on a FM basis.

Silage making

Italian ryegrass was cultivated in an experimental field at Nanjing Agriculture University in China. The first growth of Italian ryegrass was hand-harvested with a sickle at the vegetative stage on 16 April 2003. The harvested material was immediately chopped into about 1 cm lengths and then treated with additives. The silage treatments were as follows: (1) control (no addition), (2) encapsulated-glucose addition at 0.5% for glucose, (3) glucose addition at 1%, (4) sorbic acid addition at 0.1%, (5) FJLB addition at a theoretical application rate of 2.67×10^5 CFU g^{-1}, on a fresh weight basis of Italian ryegrass, respectively. After thorough mixing, 630 g of Italian ryegrass from each treatment was ensiled into a laboratory silo (1 liter capacity) in triplicate. Each silo was sealed with a screw top and kept at 25°C. All silos were opened after 30 days of storage.

Chemical analyses

The chopped grass was immediately collected for the determination of contents of dry matter (DM), mono-and disaccharides compositions (fructose, glucose and sucrose), and the population of epiphytic LAB in the initial Italian ryegrass. After the silos were opened and the contents were mixed thoroughly, a 50 g sample was taken from each silo and 150 g of distilled water was added before being stored in the refrigerator at 4°C for 24 h. Then, the extracts were filtered through double layers of cheesecloth and a filter paper (Toyo No. 5A, Japan), and the filtrate was used for the determination of pH, ammonia-N (AN), LA, ethanol, and volatile fatty acids (VFAs). The pH of silage was measured using a glass electrode pH meter (Horiba Co, Japan). The LA content was determined using the method of Barker and Summerson (1941), and VFAs and ethanol contents were determined by gas chromatography (Shimadzu, GC-17A with 12 m capillary column, conditions: column temperature 100°C, injection temperature 250°C). Total nitrogen (TN) was determined by

Table 1. Characteristics of Italian ryegrass before being ensiled (g kg^{-1} DM)

Dry matter ($g\ kg^{-1}$)	Crude protein ($g\ kg^{-1}$ DM)	Glucose ($g\ kg^{-1}$ DM)	Fructose ($g\ kg^{-1}$ DM)	Sucrose ($g\ kg^{-1}$ DM)	Mono-and disaccharides ($g\ kg^{-1}$ DM)	LAB[1] CFU g^{-1} FM	LAB[2] CFU ml^{-1}	pH of FJLB
186.49	65.63	44.83	54.07	15.07	113.97	2.91×10^3	4.35×10^8	3.77

[1] The population of epiphytic lactic acid bacteria in initial fresh Italian ryegrass expressed as colony forming unit per g fresh matter (CFU g^{-1} FM).
[2] The population of pre-fermented juice of epiphytic lactic acid bacteria (FJLB) prior to being added to grass (CFU ml^{-1}).

Table 2. Chemical composition of Italian rygrass silages treated with some additives

Item	Treatments				
	Control	Encapsulated -0.5% glucose	Glucose (1%)	Sorbic acid (0.1%)	FJLB[3]
pH (SD)	4.38 (0.06)^{c,1}	4.17 (0.13)^{bc}	4.00 (0.20)^{b}	4.05 (0.15)^{b}	3.59 (0.01)^{a}
DM (SD) ($g\ kg^{-1}$)	154.90 (0.35)^{a}	164.44 (2.03)^{b}	177.46 (4.64)^{c}	183.15 (2.33)^{d}	182.06 (1.90)^{cd}
Lactic acid (SD) ($g\ kg^{-1}$ DM)	46.85 (10.80)^{a}	49.11 (5.77)^{a}	50.13 (12.24)^{a}	49.78 (8.03)^{a}	121.76 (3.67)^{b}
Acetic acid (SD) ($g\ kg^{-1}$ DM)	10.70 (6.16)^{b}	7.18 (4.36)^{a}	5.32 (0.75)^{a}	3.87 (0.15)^{a}	5.30 (2.07)^{a}
Propionic acid (SD) ($g\ kg^{-1}$ DM)	2.53 (1.26)^{b}	1.27 (0.68)^{ab}	0.59 (0.52)^{a}	0.45 (0.28)^{a}	0.12 (0.11)^{a}
Butyric acid (SD) ($g\ kg^{-1}$ DM)	33.45 (3.03)^{c}	21.50 (3.02)^{b}	8.50 (3.15)^{ab}	4.88 (2.61)^{a}	0.34 (0.22)^{a}
Ethanol (SD) ($g\ kg^{-1}$ DM)	2.42 (0.40)^{b}	2.09 (0.32)^{ab}	1.84 (0.10)^{ab}	1.26 (0.97)^{a}	1.67 (0.10)^{ab}
Total VFAs (SD) ($g\ kg^{-1}$ DM)	46.68 (8.66)^{c}	29.95 (10.26)^{b}	14.41 (6.16)^{ab}	9.20 (6.01)^{a}	5.76 (1.77)^{a}
AN/total N (SD) (g AN kg^{-1} TN)[2]	114.91(3.97)^{c}	87.01(8.01)^{b}	65.91 (12.99)^{a}	55.12 (16.37)^{a}	65.58 (4.64)^{a}
Lactic acid/acetic acid (SD)	4.39 (3.02)^{a}	6.84 (5.46)^{a}	9.42 (2.90)^{ab}	12.86 (0.83)^{b}	22.97 (6.52)^{c}

[1] Values followed by different letters in the same row show significantly differences at $p<0.05$.
[2] AN: ammonia-N, TN: total nitrogen.
[3] FJLB: pre-fermented juice of epiphytic lactic acid bacteria.

macro-Kjeldahl procedures 7.033-7.037 (AOAC, 1984) and AN content with an ammonia electrode meter (Model IM-22P, Toa Electronics Ltd., Japan). The DM contents of the grass and silages were determined by drying in an oven at 60°C for at least 48 h (AOAC, 1984), whereas that of silages was determined by the removal of water using toluene distillation with ethanol correction (Dewar and McDonald, 1961). Mono-and disaccharide compositions of the grass and silages were determined by high performance liquid chromatography (HPLC) as previously reported (Shao et al., 2002). The population of LAB in the fresh grass and the FJLB was determined by the plate count method. Grass samples (10 g) were shaken well with 90 ml of sterilized distilled water, and 10^{-1}-10^{-8} serial dilutions were made in 0.85% sodium chloride sterilized solution. LAB were counted on an agar plate containing bromocresol purple and GYP-$CaCO_3$ agar after incubation in an anaerobic box (N_2:H_2:CO_2 = 80:10:10, TE-HER Hard Anaerobox, ANX-1, Hirosawa Ltd, Toky, Japan) at 30°C for 3 days. LABs were detected by yellowish colonies with a clear zone due to dissolving $CaCO_3$ (Masuko et al., 1992).

Statistical analyses

The experiment was a completely randomized design, with 4 treatment and 3 replicates. The statistical analysis included one-way analysis of variance with additive treatments as a factor and Fisher's least significant difference test; these were performed by ANOVA using the GLM procedure of the Statistical Analysis System (SAS, 1984). Significance was declared at $p<0.05$.

RESULTS

Table 1 shows the characteristics of the initial Italian ryegrass and FJLB. It contained 186.49 g kg^{-1} DM, 44.83 g kg^{-1} glucose, 54.07 g kg^{-1} fructose, 15.07 g kg^{-1} sucrose, 113.97 g kg^{-1} total mono-and disaccharides and 65.63 g kg^{-1} crude protein. The population of epiphytic LABs was 2.91×10^3 CFU g^{-1} FM for the original Italian grass and 4.35×10^8 CFU ml^{-1} for the FJLB, and the pH value of FJLB was 3.77.

The fermentation qualities with additive treatments are presented in Table 2. The addition of encapsulated-glucose caused only a slight decrease in pH and a small increase in LA content as compared with control silage; however, encapsulated-glucose addition significantly ($p<0.05$) decreased butyric acid, acetic acid and total VFA contents and AN/total N value. The glucose, sorbic acid and FJLB additions significantly ($p<0.05$) decreased pH and AN/TN and the contents of butyric acid, acetic acid and total VFAs as compared with the control. There were no significant ($p>0.05$)differences in acetic acid (AA) content between treated silages; however, there was a larger decrease for glucose, sorbic acid and FJLB additions than for encapsulated-glucose addition. Thus sorbic acid and FJLB additions significantly ($p<0.05$) increased the ratio of lactic to acetic acid (LA/AA), but glucose, encapsulated-glucose addition showed no significant increase ($p>0.05$). Propionic acid (PA) was slightly decreased in encapsulated silage, and the others were only detected. Sorbic acid addition significantly ($p<0.05$) decreased the ethanol content and the

Table 3. Residual mono-and disaccharides composition of Italian rygrass silage

Item	Treatments				
	Control	Encapsulated -0.5% glucose	1% Glucose	0.1% Sorbic acid	FJLB[2]
Fructose (SD) (g kg^{-1} DM)	7.44 (4.04)[a, 1]	13.91 (2.49)[a]	38.32 (13.14)[b]	53.48 (10.02)[b]	52.35 (9.72)[b]
Glucose (SD) (g kg^{-1} DM)	0.00 (0.00)[a]	0.00 (0.00)[a]	0.25 (0.43)[a]	0.00 (0.00)[a]	0.11 (0.20)[a]
Sucrose (SD) (g kg^{-1} DM)	3.10 (0.83)[a]	2.64 (0.45)[a]	4.29 (1.79)[a]	4.02 (0.54)[a]	3.01 (0.85)[a]
Mono-and disaccharides (SD) (g kg^{-1} DM)	10.54 (3.20)[a]	16.55 (2.04)[a]	42.86 (14.28)[b]	57.49 (10.56)[b]	55.47 (9.19)[b]

[1] Values followed by different letters in the same row show significantly differences at $p<0.05$.
[2] FJLB: pre-fermented juice of epiphytic lactic acid bacteria.

other additions showed only a slight decrease ($p>0.05$). All treated silages had significantly ($p<0.05$) increased DM content as compared with the control. FJLB significantly ($p<0.05$) increased LA content, whereas glucose, sorbic acid treatments showed only a slight increase.

The residual mono-and disaccharide composition of silages is presented in Table 3. The addition of glucose, sorbic acid and FJLB showed significantly ($p<0.05$) higher contents of residual fructose and total mono-and disaccharides, and encapsulated glucose addition showed no significant increase ($p>0.05$) as compared with the control. The amounts of residual fructose and total mono-and disaccharides were in the following order: sorbic acid> FJLB>glucose>encapsulated-glucose>control. There were no significant differences in residual sucrose content which was present in only small amounts, and almost no residual glucose was detected in all silages.

DISCUSSION

Although control and encapsulated-glucose had higher contents of BA (33.45, 21.50 g kg^{-1} DM) and AN/Total N (114.91, 87.01) as compared with the other treated silages, all silages were clearly dominated by LAB as judged by their low pH values (3.59-4.38) and high LA/AA (4.39-22.97) and LA content (46.85-121.76 g kg^{-1} DM) (Catchpoole and Henzell, 1971). This was probably due to the sufficient fermentable substrates of mono-and disaccharides (113.97 g kg^{-1} DM) in Italian ryegrass used in this study which might lead to LA fermentation silages (Ridla and Uchida, 1998a, 1998b). The potential availability of sufficient fermentable substrates can be further explained as follows: although the mono-and disaccharides in the original Italian ryegrass were high (113.97g kg-1 DM), Italian ryegrass is one of the temperate grasses in which fructans are the most abundant source of WSC and can be hydrolyzed into glucose and fructose by plant enzymes during the very early stage of ensiling, which further increases the WSC for LAB fermentation.

Encapsulated-glucose addition

There were no marked differences in fermentation products and residual mono-and disaccharide compositions between the control and encapsulated-glucose treatments (Table 2). The absence of effect of encapsulated-glucose addition on the fermentation quality in this experiment was probably because the ratio of glucose addition (0.5% glucose on FM basis) was not enough to promote epiphytic LAB activity and cause further LA build-up and pH decrease. The activities of clostridial bacteria or other undesirable bacteria in encapsulated-glucose silages could still not be inhibited effectively. This was also reflected by high contents of BA, VFAs and AN/Total N in both control and encapsulated-glucose treatments. Low level of residual mono-and disaccharide content suggested that some mono-and disaccharides had been consumed by clostridia or other aerobic bacteria during ensiling, this being in agreement with the results of Tamada et al. (1996). In addition the number of epiphytic LAB in the original grass might have also affected the fermentation patterns of the silages. The insufficient number of viable epiphytic LAB ($<10^5$ CFU g^{-1} FM) could decrease the rate and extent of pH reduction and lactic acid production (Woolford, 1984; Hellings et al., 1985), resulting in a low utilization efficiency of WSC by LAB in the early stage of ensiling. Both control and encapsulated-glucose treatments showed significantly ($p<0.05$) higher BA and AN/Total N than other additive treatments. This was attributed to high moisture content, sufficient WSC and the low number of epiphytic LAB (3.46×10^3 CFU g^{-1} FM) in the original Italian ryegrass, which provided conditions which were adequate for development of clostridial bacteria and other undesirable bacteria during the period of ensiling (McDonald et al., 1991).

Glucose addition

Glucose addition decreased significantly ($p<0.05$) the contents of BA and total VFAs and the AN/total N and pH values, whereas there were small increases in LA content and LA/AA which improved the Italian ryegrass fermentation quality. These results were similar to the findings of Ohyama et al. (1975) and may be due to supplying more fermentable substrate (1% glucose addition) compared with encapsulated-glucose addition, thereby stimulating the homofermentive LAB to produce more LA and decrease pH further. This suggests that,

although there was an inadequate population of epiphytic LAB in the initial Italian ryegrass, the larger amount of fermentable substrate addition (the first fermentation substrate glucose) may improve the fermentation quality of silage (Ohyama et al., 1975). However, there were lower ($p>0.05$) residual mono-and disaccharides and higher ($p>0.05$) BA content as compared with both sorbic acid and FJLB additions. This may be due to the inadequate population of epiphytic LAB in the initial Italian ryegrass (3.46×10^3 CFU g^{-1} FM), thus the rate of LA production and pH decrease were still not fast enough to inhibit the clostridial growth completely. These results indicated that glucose addition gave lower utilization efficiency of WSC by epiphytic LAB than the sorbic acid and FJLB additions. Adding fermentable substrate (glucose) to a mass of grass containing plant material with a low population of epiphytic LAB and a high moisture content was not the most efficient method of improving the fermentation quality.

Sorbic acid addition

Sorbic acid addition showed the lowest ethanol and AN/Total N, and the highest contents of DM and residual mono-and disaccharides, with small amounts of PA and BA. These results are in agreement with those of other workers (Alli et al., 1985; Weinberg et al., 1988, 1989). This indicated that sorbic acid addition was not only very effective in the inhibition of clostridia and other aerobic bacterial activity, but also stimulated homofermentative LAB activity to give efficient LA fermentation during the early stage of ensiling. There were significant ($p<0.05$) decreases in the loss of mono-and disaccharides and DM by the undesirable bacteria, resulting in the most efficient utilization of fermentable substrate by epiphytic LAB. Therefore, it was suggested that 0.1% sorbic acid addition is effective in improving silage quality; however, there was no significant ($p>0.05$) increase in LA content due to an insufficient epiphytic LAB population in the original Italian ryegrass.

FJLB addition

FJLB addition showed the lowest ($p<0.05$) pH value and the highest ($p<0.05$) LA content, indicating that FJLB addition had the most intensive LA fermentation of all the treated silages. This was also reflected by lower residual mono-and disaccharide content in FJLB addition than in sorbic acid addition, because more mono-and disaccharides might be utilized by LAB in the FJLB addition silage. Moreover, FJLB addition significantly ($p<0.05$) decreased AN/Total N and total VFAs with an absence of PA and BA content, and significantly ($p<0.05$) increased LA/AA, resulting in a large improvement in the fermentation quality of the silage. These observations indicated that FJLB addition to ensure rapid and vigorous LA fermentation resulted in faster accumulation of LA and reduction of pH values at earlier stages of ensiling, thus depressing proteolytic activity and avoiding the risk of clostridial or other undesirable bacterial fermentation of the silage. The high efficacy of FJLB addition also demonstrated the shortage of epiphytic LAB in the original grass (3.46×10^3 CFU g^{-1} FM). In addition, FJLB still showed significantly higher residual mono-and disaccharides than the other additive treatments except for sorbic acid addition, indicating that FJLB addition also increased the efficiency of utilization of mono-and disaccharides. These results are consistent with the reports of Ohshima et al. (1997) and can be explained as follows. Firstly, adding FJLB to silage supplied more abundant species and larger number of LAB, where some of these strains of LAB are more likely to adapt to the specific environment and enhance the LA production (Ohshima et al., 1997a,b,c). Secondly, the FJLB was prepared from Italian ryegrass, the same plant used for silage material, and might be more efficient in improving the quality of silage than that derived from other plant material, because the number and kinds of epiphytic LAB on different grasses were considered to be different Ohshima and co-workers (1997). Thirdly, some substances stimulating LAB proliferation in silages might be produced during the 3 days incubation used for FJLB preparation.

Based on the present study, the improvement in fermentation quality with additives was ranked in the following order: treatment with FJLB>sorbic acid>glucose> encapsulated-glucose>control. This suggested that adding a number of species of domestic LABs (FJLB) and an aerobic bacteria inhibiter (sorbic acid) to plant materials such as Italian ryegrass, which contained almost sufficient amounts of WSC but low DM content and a low population of epiphytic LAB in the present case, are more important and efficient than adding fermentable substrates (glucose and encapsulated-glucose) for improving the fermentation quality of the silage. In conclusion from the present study, sorbic acid and FJLB treatments are recommended for improving silage quality, and FJLB especially will be attractive for farmers, not only from its efficacy but also from an economical viewpoint.

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