Pakistan J. Zool., vol. 49(6), pp 2271-2277, 2017. DOI: http://dx.doi.org/10.17582/journal.pjz/2017.49.6.2271.2277

Effect of Glucose and Lactic Acid Bacteria on the Fermentation Quality, Chemical Compositions and *in vitro* Digestibility of Mulberry *(Morus Alba)* Leaf Silage

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Article Information
Received 01 March 2017
Revised 15 April 2017
Accepted 03 May 2017
Available online 06 December 2017

Authors' Contribution
EBT wrote the manuscritp, JL analysed the data and YX participated in the design of the study. Zd and AAS reviewed the literature and TS revised the manuscript.

Key words
Mulberry silage, Glucose, Animal Feeding, Fermentation, Lactic acid bacteria.

ABSTRACT

To improve the fermentation quality and chemical composition of mulberry leaf (*Morus Alba*) silage was treated with glucose and lactic acid bacteria (LAB) additives. The groups were as follow: silage without additive as a control (c); glucose 1% fresh matter basis (G1); glucose 2% (G2); glucose 3% (G3); *Lactobacillus Plantarum* (LAB1); *Pediococuss acidilactici* (LAB2); *Lactobacillus rhamnosus* (LAB3). All the silos were incubated at 25°C. Then, 90 days later, the fermentation quality of tested silages was analyzed. No significant difference was found between the control and treated groups in term of dry matter (DM), neutral determgent fibre (NDF), acid detergent fibre (ADF) and water soluble carbohydrate (WSC). Acetic acid was significantly ($P<0.05$) low in G2 and G3, while propionic acid and yeast was significantly ($P<0.05$) low in LAB2. Gas production (GP 72) was significantly ($P<0.05$) high in LAB3. From the results of the present study, we concluded that addition of glucose and probiotic bacteria improved the chemical composition and ensiling characteristics of mulberry leaf.

INTRODUCTION

Conventionally, mulberry leaves are considered for sericulture in numerous places in the world. Its taxonomy is related to *Urticales* order, and *Moraceae* family, a genus of *Morus* (Miller *et al*., 2005). It is worth mention that species of *Morus* genus estimated 68 only in Asia (Datta, 2002) while in China, more than 1000 varieties were reported (Sánchez, 2002). The most known and nutritional used species are *M. nigra*, *M. indica*, *M. alba*, and *M. rubra*. It contains a high crude protein (15-25%) and satisfied digestibility percentage (75-85%). So, the biomass yield could reach 16-18 tons a year (Benavides *et al*., 1994). Mulberry is widely spreading in the farm animals to become a good source for feeding and supplementing ruminant animals. It has been reported that mulberry could be a rich protein source for herbivores (Vu *et al*., 2011). Also, the digestibility of mulberry produced the same energy values as alfalfa hay (Doran *et al*., 2007). Indeed, the nutrition advantages of mulberry leaf silage were evaluated better than other dry processing feeding pathways (Zhou *et al*., 2014). Usually, to produce hay, the fresh and green leaves are often collected during the rainy season; then used to be dried, which is slow and hard (Meeske *et al*., 1999). But the addition of glucose during the fermentation processes improves the forage quality and increases the supply of offered materials that help in bacterial growth (Shao *et al*., 2004). Also, some researchers reported that inoculation of more bacterial fermenters that are commercially available could enhance adequate fermentation outcomes (Weinberg *et al*., 1999). Therefore, this work was designed to investigate the effect of three different concentrations of glucose, with three varieties of lactic acid bacteria (LAB) on the fermentation quality of mulberry leaves silage and the chemical outcomes and its relation with *in vitro* digestibility of the mulberry leaf silage.

MATERIALS AND METHODS

Silage preparation

Mulberry leaves (*Morus Alba*) of 2-year old plants were collected from a field in Shitai County, Anhui province, China. Then an analysis of chemical contents

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0030-9923/2017/0006-2271 $ 9.00/0
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of fresh materials was implemented as shown in Table I. The leaves were chopped with lengths of 2 to 3 cm by hand forage chopper. The designed plan of experimental glucose treatments included: (C) control silage without additive; (G1) glucose 1% on fresh matter basis; (G2) glucose 2% on fresh matter basis; (G3) glucose 3% on fresh matter basis; LAB1 commercial lactic acid bacteria (L. *Plantarum, Ecosyl* MTD/1 CB*, Ecosyl* Products Inc. USA); LAB2 lactic acid bacteria (*Pediococuss acidilactici* GG13) GeneBank Accession Number: KF554251; (LAB3) lactic acid bacteria (*Lactobacillus rhamnosus* GG26) GeneBank Accession Number: KF554252 . The lactic acid bacteria were inoculated at 10^6 (CFU) g^{-1}. The preparation of silages was made according to a small-scale style of mix fermentation. After that, 80g of each Mulberry leaves batch was packaged into a laboratory hermitage which has 100 mL volume capacity that being bolted with a screw closer and then kept at room temperature for three months, each packaged were triplicated.

Table I.- The chemical compositions of Mulberry leaves before being ensiled.

Items	Value
pH	6.77
DM (g/kg FW)	424.92
WSC (g/kg DM)	80.31
CP (g/kg DM)	218.6
NDF (g/kg DM)	300.5
ADF (g/kg DM)	140.7
LAB (log cfu/g FM)	$4.1X10^5$
Yeast (log cfu/g FM)	$< 10^3$
BC (mEq/kg DM)	223.02

DM, dry matter; CP, crude protein; LAB, lactic acid bacteria; NDF, neutral detergent fiber; ADF, acid detergent fiber; WSC, water soluble carbohydrates; FM, fresh matter; BC, buffering capacity.

Investigation of chemical and microbiological contents

Mulberry raw leaves and those ensilaged were estimated for chemical outcomes and microbial mass. Also, the raw leaves dry mass composition and silage batch were assessed through drying collected samples for 48 h under 65°C until the weight become fixed, then the collected masses were pulverized and garbled through 2 mm sift to be ready for later examinations. The analysis of crude protein and Dry matter (DM) were carried out upon the guidelines of Official Analytical Chemists (AOAC, 1920). On the other hand, water soluble carbohydrates were tested by the colorimetric method reported by Arthur-Thomas (1977). Also, the determination of neutral detergent fiber (NDF) and acid detergent fiber (ADF) measures were made by the method of van Soest *et al*. (1991). Heat stable amylase was used in this analysis as well as sodium sulphite that helped an NDF and ADF finally to be expressed on a dry matter basis including residual ash. Besides that, to assess the fermentation indicators, kept batches were slowly opened and mixed thoroughly then 35g sub samples of each batch was diluted with 70 ml of distilled water and stored 24 h under 4 °C. The samples were filtered through filter paper and bilayer cheesecloth (Xinhua, Hangzhou, China), then the filtrate was used for the determination of pH, buffering capacity (BC), AN, LA, ethanol and volatile fatty acids (VFAs). To measure the pH of the silage, a glass electrode pH meter was used (HANNA pH 211; Hanna Instruments Italia Srl, Villafranca Padovana, Italy). Buffering capacity was determined by the hydrochloric acid–sodium hydroxide method of Playne and McDonald (1966), while the volatile fatty acids, ammonia-N, and lactic acid were determined as a method of Shao *et al*. (2005). V-score of NH3 –N/Total N and VFAs was used to estimate their concentrations by which the quality of fermentation was evaluated (Takahashi *et al*., 2005). Furthermore, the microbial mass was estimated by series dilution of 10 g of each sample with 90 ml sterile water. Then diluted samples were cultured on MRS agar medium, and Potato Dextrose Agar medium (Shanghai Bio-way Technology Co., Ltd.) and then the media were incubated under anaerobic condition 37°C for 72 h, and 30°C for 48–72 h, respectively. The lactic acid bacteria and yeasts were counted from the silages, and raw mulberry leaves samples (Guo *et al*., 2014). The all microbiological data were log10 transformed.

In vitro *gas production*

Filter bags (ANKOM Technology, USA) were washed with acetone and dried for one day at 55°C. About 1 g ground sample was filtered. Bovine rumen fluid was collected, composited and passed through bilayer cheesecloth and then carried to the laboratory to be warmed at 39°C in a water bath and flushed with CO2 (Menke and Steingass, 1988). Rumen fluid was blended with buffer solution under the same warming condition and during progress ,CO_2 flushing for all tested samples gas production was measured from 0 to 72 h every 2 h. To evaluate DM digestibility, samples were rinsed gently with tap water then dried for 48 h at 65°C. The evaluation of *in vitro* neutral detergent fiber was investigated by filter bags of digested samples as described above. Finally, an appropriate gas production rate and extent of each feed were measured by the equation $V = b\ (1 - e^{-ct})$, reported by Ørskov and McDonald (1979). Symbols of this used equation are; (V), produced gas volume; (b), the potential gas production; (t), the consumed time; and (c), rate of produced gas, as well as, an iterative least square way was used to estimate (b) and (c) parameters through calculation of statistical regression.

Statistical analyses

General linear models Procedure (SAS Institute, Cary, NC, USA) was used to statistically analyze the experimental data. One-way Analysis of Variance (ANOVA) was used for analysis of fermentation quality and chemical composition data and significance results were evaluated when $P < 0.05$.

RESULTS

Table I shows the estimated chemical contents and an account of detected microbes isolated from raw mulberry leaves before ensiling. Also, a fresh material DM content was measured 424.92 g/kg fresh weight (FW) and water soluble carbohydrate (WSC) content was measured 80.31g/kg DM. The buffering capacity and CP concentration of fresh leaves were 223.02 mEq/kg DM and 21.86% DM, respectively. Epiphytic LAB on mulberry leaves was counted more than 1.0×10^5 CFU/g FM, while yeasts were counted less than 1.0×10^3 CFU/ g FM.

The chemical composition of mulberry leaves after ensiling is shown in Table II. All additive treatments were higher in water-soluble carbohydrate (WSC) as compared to control, while it was not significantly different (P>0.05).

As presented in Table III, the fermentation quality of mulberry leaves silage after three months of fermentation under room temperature was improved. After 90 days of ensiling, all additive treatments (except G1) decreased the pH value of mulberry leaves silage as compared with control, but the differences were significant only in G3 and LBA2 (P<0.05). The pH of G3 treatment was the lowest among all treatments after 90 days of ensiling, but the difference was not significant (P>0.05). On the other hand, all additive batches of mulberry leave silage (except LAB1) had increased lactic acid quantity, but the different was statistically non-significant. Also, acetic acid content in all additives treatments was expressed non-significantly different (P>0.05) in comparison with negative control. In glucose treatments (G1, G2, G3) after 90 day of ensiling, the amount of propionic acid was higher than lactic acid treatments (LAB1, LAB2, LAB3). The lowest amount of propionic acid was in LAB2, and the highest amount was in G3. Although Butyric acid amount among all treatments was noted lower than control, it was found statistically non-significant (P>0.05). The ratio of LA/AA after 90 days was significantly (P<0.05) high in G3. The LAB and yeast populations were not significantly different (P>0.05). Besides that the DM, NDF and ADF in all additive treatments were not significantly different (P>0.05) in comparison with negative control.

Table II.- Chemical compositions of mulberry leaves silage after 90 days of ensiling.

Items	Treatment								
	C	G1	G2	G3	LAB1	LAB2	LAB3	P value	SEM
DM (g/kg FW)	433.6^{a}	406.3^{a}	421.0^{a}	409.2^{a}	412.3^{a}	426.5^{a}	420.8^{a}	0.9688	0.7127
NDF (g/kg DM)	372.7^{a}	358.5^{a}	373.2^{a}	339.5^{a}	297.9^{a}	37.18^{a}	353.6^{a}	0.3944	0.9808
ADF (g/kg DM)	163.6^{a}	149.9^{a}	152.5^{a}	157.8^{a}	157.8^{a}	16.27^{a}	166.7^{a}	0.1175	0.1827
WSC (g/kg DM)	5.77^{a}	5.79^{a}	8.53^{a}	10.19^{a}	6.07^{a}	6.29^{a}	5.98^{a}	0.0971	0.5157

C, control; G1, Glucose 1%; G2, Glucose 2%; G3, Glucose 3%; LAB 1, commercial lactic acid bacteria; LAB 2, *Pediococuss acidilactici*; LAB 3, *Lactobacillus rhamnosus*; DM, dry matter; WSC, water soluble carbohydrates; NDF, neutral detergent fiber; ADF, acid detergent fiber. ^{a}Means, no significant differences among treatments.

Table III.- Fermentation quality of mulberry leaves silages after 90 days of ensiling.

Items	Treatment								
	C	G1	G2	G3	LAB1	LAB2	LAB3	P value	SEM
pH value	5.77^{ab}	5.87^{a}	5.54^{abc}	5.30^{c}	5.44^{bc}	5.37^{c}	5.44^{bc}	0.0010	0.0504
Lactic acid (g/kg DM)	5.21^{a}	5.58^{a}	6.37^{a}	9.21^{a}	5.13^{a}	6.67^{a}	7.73^{a}	0.3900	0.5375
Acetic acid (g/kg DM)	2.91^{ab}	1.96^{ab}	1.56^{b}	1.30^{b}	5.00^{a}	3.23^{ab}	4.15^{ab}	0.0095	0.3506
Propionic acid (g/kg DM)	1.78^{bcd}	3.69^{abc}	4.54^{ab}	5.09^{a}	1.43^{cd}	0.47^{d}	0.74^{cd}	0.0004	0.4358
Butyric acid (g/kg DM)	2.34^{a}	2.02^{a}	1.99^{a}	1.81^{a}	0.79^{a}	0.66^{a}	1.33^{a}	0.1696	0.2030
Lactic acid/ Acetic acid (g/kg DM)	1.95^{b}	2.91^{b}	4.13^{ab}	7.84^{a}	1.35^{b}	2.05^{b}	1.84^{b}	0.0012	0.5387
AN (g/kg TN)	12.23^{a}	14.13^{a}	11.4^{a}	12.68^{a}	14.55^{a}	14.29^{a}	12.35^{a}	0.2398	0.0093
LAB (log10 CFU/g FW)	7.14^{a}	7.05^{a}	7.11^{a}	7.11^{a}	6.95^{a}	7.03^{a}	6.96^{a}	0.9372	0.0482
Yeast (log10 CFU/g FW)	4.79^{b}	4.82^{ab}	4.84^{ab}	4.8^{ab}	4.83^{ab}	4.91^{a}	4.9^{ab}	0.0340	0.0136

AN, ammonia nitrogen; LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid. Values with different small letters show significant differences among treatments in the ensiling day (P<0.05). For other abbreviations and statistical details, see Table II.

Table IV.- Gas production kinetics, IVDMD and IVNDFD of mulberry leaves silages.

Item	Treatments								
	C	G1	G2	G3	LAB1	LAB2	LAB3	SEM	P
A (ml)	-7.90^{a}	-6.19^{a}	-5.43^{a}	-8.43^{a}	-9.96^{a}	-7.51^{a}	-9.28^{a}	1.22	0.3313
B (ml)	531.47^{ab}	790.70^{a}	604.27^{ab}	756.63^{ab}	496.33^{b}	509.7^{b}	684.67^{ab}	32.81	0.0091
C (ml)	0.0037^{a}	0.0019^{a}	0.0030^{a}	0.0036^{a}	0.0037^{a}	0.0039^{a}	0.0030^{a}	0.002	0.427
GP72 (ml)	88.5^{g}	94.9^{f}	101.2^{e}	111.5^{c}	108.9^{d}	117.8^{b}	125.2^{a}	0	<0.0001
IVDMD (g/kg)	437.2^{a}	389.1^{a}	397.5^{a}	428.7^{a}	421.4^{a}	399.2^{a}	413.5^{a}	0.28	0.5744
IVNDFD (g/kg)	512.4^{a}	417.6^{a}	392.1^{a}	356.6^{a}	246.4^{a}	286.1^{a}	407.2^{a}	2.06	0.3425

A, GP from the immediately soluble fraction (ml); B, GP from the insoluble fraction (ml); C, GP rate constant; GP72, net gas production after 72 h incubation; IVDMD, *in vitro* dry matter digestibility; IVNDFD, *in vitro* neutral detergent fiber. Values with different small letters show significant differences among treatments in the ensiling day (P<0.05). For other abbreviations and statistical details, see Table III.

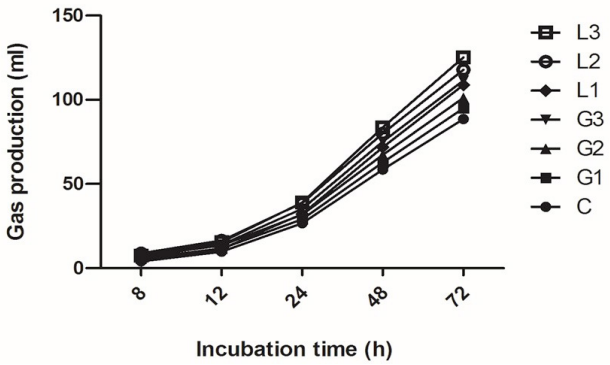

Fig. 1. Gas production profiles (mL gas 200 mg−1 DM) from in vitro fermentation of total Mulberry leaves silages for 72 h. For abbreviations, see Table II.

As presented in Figure 1, the differences of produced gas volume in vitro among all treated mulberry leave silages were statistically significant (P < 0.05) during the incubation period. Moreover, in vitro digestibility of prepared silage and other tested parameters were expressed with gas production kinetics in Table IV. There was no significant differences among a, b, and c (P > 0.05). Furthermore, GP was noted to be significantly influenced (P < 0.05) by additives during 72 h of incubation without any significantly detected effects on IVDMD and IVNDF (P> 0.05).

DISCUSSION

In this study, we found that silage quality and chemical composition of mulberry were affected. Many researchers concluded that a fresh material could be effectively foddered with a suitable dry matter composition, 30–50 g kg^{-1} DM of fermentable sugar, low BC, and lactic acid bacteria populations more than 1 ×10^{5} CFU g^{-1} FM (Weinberg, 2008). In the present work, the dry matters of mulberry leaves before ensiling was 424.92 g /kg^{-1} DM, while WSC was 80.31 g kg^{-1} DM, then lactic acid bacteria were counted more than 1 × 10^{5} CFU g^{-1} FM, suggesting that mulberry leaves produce good silage expect of DM content which considers high as compared to another kind of forages.

Mulberry leaves are not commonly used as a fresh material to make silage, so there is no much knowledge about the efficiency of presently available silage additives for the preservation of mulberry leaves as fermentation method. A lot of researchers considered that LAB inoculant and glucose additives are effective to ameliorate the quality of fermentation of silage. Specially pH value which is considered as a very important factor impacting and enhance the extent of fermentation quality of ensiled forage (Denek *et al*., 2011). The final low pH (4.19 or less) in both control and treated silages supposed to enhance good fermentation (Chen *et al*., 2015). However, in this research, the measured amount of pH ranged between 5.30 and 5.77 for G3 and control silage respectively which is higher than typical silage pH. So, the results of this research were noted in the same line of Ba and Giang which showed a high amount of mulberry leaves silage treated with molasses and rice bran (Ba and Giang, 2005). Here, we suggested that a high pH value of mulberry leaves silage may be because of the high dray matters content. Although many researchers suggested that the high dry matter content of ensiled forages decreases the number of LAB population because bacterial growth reduces under low water activity (Muck, 1990; Whiter and Kung, 2001; Rizk *et al*., 2005). Another study proved that the humidity during microbial growth of ensilages could be critically termed water activity (Albert *et al*., 1989). But, we have noted in our results that bacterial growth activity virtually depended on moisture degree of silages (Whiter and Kung, 2001). Moreover, Hristov and McAllister (2002) reported that grasses and forages composed of high dry matter content have slower fermentation rates than those containing lower dry matter contents (Hristov and McAllister, 2002). Besides that, the lowest pH in the present experiment was in G3 (5.3) which was significantly

different in comparison with negative control. The low pH mulberry leaves silage in G3 treatments was due to its high offered substrates for the lactic acid bacteria growth. Shao *et al*. (2004) showed that the pH of Guinea grass (*Panicum maximum Jac*) silage treated with glucose had significantly decreased in comparison with the control. On the other hand, mulberry leaves silage treated with lactic acid bacteria (*Pediococuss acidilactici* GG13) showed significantly lower pH than control, which may be due to the use of lactic acid bacteria that increases nutritive quantities and maintain silage quality through reduction of leaves respiration and enzymatic activity or by stopping of mischievous epiphytic microbial masses (Yuan *et al*., 2015). Hence, the inoculated lactic acid bacteria in this experiment could use wide types of substrates to produce large amounts of lactic acid in short time as reported by Yuan *et al*. (2015). The highest value of lactic acid measured was not statistically significant, but we noted that, in G3 treatment which was consisted of the lowest pH, and the highest value of LA/AA ratio, which might be assigned to high WSC quantity of fresh materials and high concentrate of glucose treatment (3%), thus allowed a high releasing of lactic acid by lactic acid bacteria, we noted this results on the line with Shao *et al*. (2005) which showed that glucose additives increased the lactic acid amount as compared to the control in the Guinea grass silage, but the difference was significant. While the glucose treatment was noted a lightly higher PA quantity rather than the other supplied silages, that pointed to possible clostridial bacteria activity or some heterofermentative LAB activity occurring from organic bacteria (Shao *et al*., 2005).

The in vitro produced gas was originally utilized to forecast degradability of rumen and metabolizable energy (ME) composition of feedstuffs of farm animals (Menke and Steingass, 1988). Several studies have assessed the digestibility feeds successfully through the feeds organic matter degradability and gas produced relationship (Hassanat *et al*., 2007; Negesse *et al*., 2009). Rumen's digestion rate is usually evaluated according to produced gas, and also the produced gas could affect the rate of DM intake and passage. Furthermore, the gas production (GP) after 72 h of incubation of silages supplemented with additives was increased in comparison with the negative control, which may be owing to the reduction of nutrients loss (Kozelov *et al*., 2008; Li *et al*., 2014). There are no significant differences in IVDMD and IVNDFD among all tested treatments which were as similar as de Jesus Ferreira *et al*. (2013) who described that DM digestibility was not impacted by microbial inoculation in silage of elephant grass. In contrast with Weinberg *et al*. (2007) who used 10 different origin of lactic acid bacteria (LAB) to compare IVDMD and IVNDFD of corn silages. The results showed that IVDMD were enhanced with some bacterial inoculants, whereas IVNDFD were not, so, it was summarized that this might be owing to some solubilization of hemicellulose through ensiling, which ameliorated IVDMD mass but the digestibility of the residual NDF did not change or even decreased.

CONCLUSION

There is a positive potential of producing well preserved and high nutritional silage from mulberry leaves. Upon the findings of this work, we can summarize that adding glucose at the rate of 3% of fresh matter basis and lactic acid bacteria (*Pediococuss acidilactici* GG13) had a positive effects on quality of fermentation and digestibility of mulberry leaves silage in vitro. In addition, the high pH indicated that mulberry leaves silage was not able to be stored for a long time.

ACKNOWLEDGEMENTS

This work was supported by Jiangsu Independent Innovation (CX (15)1003-3) and the National Key Basic Research Program of China (2016YFC0502005).

Statement of conflict of interest

Authors have declared no conflict of interest.

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