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# Additive Effects of Green Tea on Fermented Juice of Epiphytic Lactic Acid Bacteria (FJLB) and the Fermentative Quality of Rhodesgrass Silage

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**ABSTRACT :** Two experiments were carried out on a laboratory scale. The first involved a study of the effect of green tea on characteristics of fermented juice of epiphytic lactic acid bacteria (FJLB). FJLB was treated with 50 g/L of green tea products as follows: new shoot powder (FJLB+N), leaf powder (FJLB+L), commercial powder (FJLB+P), sterilized new shoot powder (FJLB+SN), sterilized leaf powder (FJLB+SL) or sterilized commercial powder (FJLB+SP). FJLB without any additive was also prepared (Untreated FJLB). After incubation, the number of microorganisms in FJLB were studied. Subsequently, these FJLB were applied at 10 ml/kg to chopped rhodesgrass to study their effects on fermentation. Compared with untreated FJLB, the addition of green tea increased ($p<0.05$) lactic acid bacteria (LAB) and also aerobic bacteria counts in FJLB. At 60 d of ensiling, all the FJLB treated silages were well preserved, pH and butyric acid content were lower ($p<0.001$) and lactic acid was higher ($p<0.001$) than that of the control. Lactic acid content was significantly higher ($p<0.001$) with treated FJLB than with untreated FJLB. FJLB treated with sterilized green tea decreased ($p<0.001$) the pH and the lactic acid content was higher ($p<0.001$) than that in the unsterilized green tea silages. (**Key Words :** Green Tea, Lactic Acid Bacteria, Rhodesgrass, Silage)

## INTRODUCTION

The microorganisms that grow naturally in forage crops are responsible for silage fermentation and influence silage quality. Silage made from grass grown in subtropical and tropical regions has been characterized as having a lack of lactic acid content and a high concentration of acetic acid, which suggests a deficiency of LAB (Ohmomo et al., 2002; Yahaya et al., 2004). Furthermore, some of these silages had high concentrations of lactic acid initially, but were unstable and regressed to low-quality silage during prolonged storage. In a previous study the fermented juice of epiphytic lactic acid bacteria (FJLB) was recommended as a silage additive for improving the fermentative quality of tropical grass silages (Bureenok et al., 2005 a, b). However, this is sometimes not effective because of the low LAB and low water-soluble carbohydrate (WSC) content of the grasses. Small numbers of aerobic bacteria, yeasts and moulds are also found in FJLB. These microorganisms may cause aerobic deterioration following exposure of the silage to air.

A method to improve the FJLB before use as an additive is required. It is well known that tea catechins are bactericidal and inhibit the growth of bacterial spores (Hara-Kudo et al., 2005), but they do not affect LAB (Hara, 1997). There are many reports on the antimicrobial properties of tannins and tea polyphenols (Ahn et al., 1991; Scalbert, 1991). This study was aimed to inhibit the growth of undesirable microorganisms in FJLB by the addition of green tea leaf powder during the incubation period. In the experiment, the application of FJLB treated with green tea as a silage additive and its effect on fermentative quality were examined. The experiments were conducted with rhodesgrass (*Chloris gayana*).

## MATERIALS AND METHODS

### Experiment 1

Japanese green tea was separated into new shoots and leaf parts, dried in an oven at 70°C for 24 h, and ground to pass a 1-mm screen. The new shoot consisted of the new tea shoot and the first two leaves. A dried commercial powder of green tea was supplied by a local factory in Okinawa prefecture, Japan. Sterilized green tea used in this study was

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**Table 1.** The lactic acid bacteria (LAB), aerobic bacteria and yeast number in the FJLB treated with any green tea in Experiment 1

| Treatments ($log_{10}$ cfu/ml) | LAB | Aerobic bacteria | Yeast |
|---|---|---|---|
| Untreated FJLB | 7.95$^{c}$ | 7.65$^{d}$ | 5.35 |
| FJLB+new shoot powder (FJLB+N) | 8.22$^{b}$ | 8.08$^{bc}$ | 5.41 |
| FJLB+leaf powder (FJLB+L) | 8.44$^{a}$ | 8.28$^{ab}$ | 5.48 |
| FJLB+commercial powder (FJLB+P) | 8.49$^{a}$ | 8.43$^{ab}$ | 5.38 |
| FJLB+sterilized new shoot powder (FJLB+SN) | 8.25$^{b}$ | 8.03$^{c}$ | 5.63 |
| FJLB+sterilized leaf powder (FJLB+SL) | 8.26$^{b}$ | 8.18$^{bc}$ | 5.64 |
| FJLB+sterilized commercial powder (FJLB+SP) | 8.42$^{a}$ | 8.28$^{ab}$ | 5.30 |
| SEM | 0.17 | 0.22 | 0.36 |

Sterilized by autoclaving at 120°C 15 min.
FJLB = fermented juice of epiphytic lactic acid bacteria.
Values in the same row followed by different letters are significantly different ($p<0.05$).

autoclaved at 120°C for 15 min before use as a FJLB treatment. The FJLB was made from rhodesgrass; 100 g of fresh crop at flowering stage was chopped and macerated with 300 ml of sterilized distilled water using a blender. The macerate was filtered through two layers of cheesecloth and 10 ml of the filtrate transferred to a test tube to which was added 30 g/L of glucose and 50 g/L of several types of green tea additives. Three replicates were prepared and incubated anaerobically at 30°C for 2 days. After incubation, the FJLB was filtered through a sterilized double layer of cheesecloth to remove the particulate green tea portion. The FJLB was kept for use as a silage additive. The FJLB treated with types of green tea additives were designed as follows:

i) Untreated FJLB
ii) FJLB+new shoot powder (FJLB+N)
iii) FJLB+leaf powder (FJLB+L)
iv) FJLB+commercial powder (FJLB+P)
v) FJLB+sterilized new shoot powder (FJLB+SN)
vi) FJLB+sterilized leaf powder (FJLB+SL)
vii) FJLB+sterilized commercial powder (FJLB+SP)

**Experiment 2**

The FJLB from experiment 1 were used as silage additives in this experiment. Rhodesgrass was harvested at the flowering stage and chopped into 1-2 cm lengths and 10 ml/kg fresh matter (FM) of each FJLB were packed into a plastic bag and sealed with a vacuum sealer. Three replicate bags were prepared for each treatment and kept at room temperature (22-27°C). The control silage was treated with an equivalent amount of sterilized distilled water. Silages were opened at 60 d after ensiling.

**Chemical analysis**

The dry matter (DM) content of the grass and silages was determined by oven drying at 70°C for 48 h. The dried sample was milled to pass through a 1.0 mm sieve. The samples were extracted with ethanol, and the concentration of water-soluble carbohydrates (WSC) was estimated by the Somogyi-Nelson method (Somogyi, 1952). The concentration of total nitrogen (N) was determined by the Kjeldahl procedure. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) concentrations were determined by methods described by Van Soest et al. (1991). NDF and ADF concentrations were calculated on an ash-free basis. Hemicellulose was calculated as the difference between NDF and ADF.

Fresh silages (20 g) were macerated with 70 ml of distilled water and stored in a refrigerator at 4°C for 12 h. The extract was filtered using No.5A filter paper (Toyo Roshi Co. Ltd., Tokyo, Japan). The pH value of the filtered extract was determined with a glass rod electrode pH meter. The filtrate was also used to determine ammonia-nitrogen ($NH_3$-N), lactic acid and volatile fatty acids (VFA). The $NH_3$-N content was determined using a steam distillation technique (Japan Grassland Farming Forage Seed Association, 1994). Lactic acid and VFA contents were determined using high performance liquid column chromatography (HPLC, Shim-pack SCR-102H, 300 mm× 8.0 mm i.d.; column temperature, 40°C; flow rate, 0.8 ml/min, Shimadzu Co. Ltd., Kyoto, Japan).

**Microbiological analyses of FJLB**

LAB were counted on bromocresol plate count agar (Eiken Co. Ltd., Tokyo, Japan) after incubating in an anaerobic pouch with Pack-Anaero (Mitsubishi Gas Chem Co. Ltd., Tokyo, Japan) at 35°C for 3-5 d. LAB were detected by a yellowish colony. Aerobic bacteria and yeast were counted on nutrient agar and potato dextrose agar (Nissui-Seiyaku Co. Ltd., Tokyo, Japan), respectively. The agar plates were incubated at 30°C for 5 d. Colonies were counted as viable numbers of microorganisms (colony-forming units (cfu) per millilitre).

**Statistical analysis**

Experiment 1 data were subjected to analysis of variance with significant differences between means tested by Duncan's multiple range test. Experiment 2 data were subjected to analysis of variance with orthogonal contrasts being made between control vs. all additives, untreated FJLB vs. treated FJLB, and unsterilized vs. sterilized green tea groups. All experiments were analysed using the

**Table 2.** Chemical composition of rhodesgrass prior to ensiling for Experiment 2

| Item | |
|---|---|
| pH | 5.8 |
| Dry matter (g/kg) | 252.4 |
| Water-soluble carbohydrates (g/kg DM) | 55.6 |
| Neutral detergent fibre (g/kg DM) | 689.5 |
| Acid detergent fibre (g/kg DM) | 361.8 |
| Hemicellulose (g/kg DM) | 327.7 |
| Total nitrogen (g/kg DM) | 9.8 |
| Lactic acid bacteria ($log_{10}$ cfu/g) | 4.1 |

DM = Dry matter; Hemicellulose = NDF-ADF.

General Linear Model (GLM) of Statistical Analysis System (SAS, 1999).

## RESULTS

### Experiment 1

The LAB counts were $log_{10}$ 4.09 cfu/ml on FJLB and generally increased to about $log_{10}$ 7.96-8.49 cfu/ml within 2 days of anaerobic incubation (Table 1). The addition of any type of green tea increased LAB and aerobic bacteria counts in FJLB ($p<0.05$). However, the number of yeast was not different from untreated FJLB.

### Experiment 2

*Fermentative quality* : The chemical composition of the rhodesgrass before ensiling is shown in Table 2. The dry matter and WSC of the material were 252.40 g/kg and 55.60 g/kg of dry matter, respectively. The counts of LAB in the materials were approximately $log_{10}$ 4.09 cfu/ml at the time of ensiling. The chemical compositions of 60-day silages are shown in Table 3. All the FJLB-treated silages were well preserved, pH, acetic acid and butyric acid content were lower ($p<0.001$) and lactic acid was higher ($p<0.001$) than in the control silage. Although the total nitrogen concentration differed between the treated and control silages, the difference was small and there was no significant difference in DM concentration. The lactic acid content of untreated FJLB silage was lower ($p<0.001$) than that of all treated FJLB silages. Butyric acid was found in FJLB+N and FJLB+P silages, but the contents were not different from the control silages. The FJLB-sterilized green tea treated silage had the lowest pH as well as the lowest concentration of $NH_3$-N and the highest concentration of lactic acid. The control silage had a significantly ($p<0.001$) lower LA/AA as compared with the treated silages.

## DISCUSSION

### Effect of any green tea parts on microbial population

The addition of any type of green tea failed to inhibit the growth of aerobic bacteria and yeasts. On the contrary, the addition of green tea could stimulate the growth of microorganisms in FJLB, indicated by higher LAB and aerobic bacteria counts compared with the untreated FJLB. The number of LAB was similar between the treatments of green tea with or without sterilization. These results agree with the study of Kondo et al. (2004) who reported that green tea waste enhances the growth of lactic acid-producing bacteria and the lactic acid content of silage and suggested that green tea may contain nutrients for LAB which are stable after sterilization at high temperature. Tea is one of the richest sources of manganese (Mn) (Matsushita et al., 1993; Matsuura et al., 2001). *Lactobacillus plantarum* has a high Mn (II) requirement for growth (Archibald and Duong, 1984). Manganese has been recognized as a growth stimulator of LAB in fermented-sausage and American patents have been issued for using Mn salts in sausage and

**Table 3.** Chemical composition of 60-d rhodesgrass silages in Experiment 2

| Item | Cont. | Unteated FJLB | Treated FJLB | | | | | | sem | Contrasts | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | FJLB+unsterilized | | | FJLB+sterilized | | | | Cont. vs. All additive | Untreated FJLB vs. All treated FJLB | Unsterilized tea vs. Sterilized tea |
| | | | N | L | P | SN | SL | SP | | | | |
| Dry matter (g/kg) | 262 | 264 | 257 | 260 | 258 | 262 | 262 | 259 | 2.5 | NS | NS | NS |
| Total N (g/kg DM) | 8.6 | 9.3 | 9.4 | 9.0 | 8.5 | 9.0 | 9.6 | 9.4 | 0.4 | * | NS | NS |
| pH | 4.89 | 4.69 | 4.15 | 4.52 | 4.24 | 3.99 | 3.85 | 4.27 | 0.23 | *** | *** | *** |
| Lactic acid (LA, g/kg DM) | 17.9 | 32.5 | 34.0 | 28.5 | 43.2 | 51.8 | 50.6 | 30.3 | 1.20 | *** | *** | *** |
| Acetic acid (AA, g/kg DM) | 12.9 | 11.5 | 11.6 | 11.4 | 12.7 | 10.1 | 9.7 | 11.9 | 0.47 | *** | NS | *** |
| Propionic acid (PA, g/kg DM) | 0.7 | 0.5 | 0.6 | 0.6 | 1.0 | 0.4 | 0.4 | 0.5 | 0.28 | NS | NS | * |
| Butyric acid (BA, g/kg DM) | 1.3 | 0.0 | 1.4 | 0.0 | 1.4 | 0.0 | 0.0 | 0.0 | 0.24 | *** | *** | *** |
| $NH_3$-N (g/kg TN) | 158.7 | 116.4 | 108.0 | 113.7 | 113.6 | 96.2 | 85.1 | 109.5 | 2.11 | *** | NS | * |
| LA/AA | 1.39 | 2.82 | 2.94 | 2.51 | 3.41 | 5.13 | 5.23 | 2.55 | 0.22 | *** | *** | *** |

NS: Not significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

DM = Dry matter; $NH_3$-N = Ammonia-nitrogen; TN = Total nitrogen. Treatment Abbreviations as in Table 1.

cucumber fermentation (Daeschel et al., 1987). The increase of LAB number by addition of green tea may be a result of a Mn effect.

**Effect of FJLB treated with any green tea on fermentative quality**

In this study, FJLB contained both LAB and numbers of aerobic bacteria and yeasts. However, all FJLB-treated silages had higher ($p<0.001$) lactic acid content, as well as a lower pH and butyric acid content than the control silage. The results suggest that the tea extracts increased the activity of lactic acid bacteria and consequently the amount of lactic acid produced. Among epiphytic LAB in fresh crops, the lactic acid-producing cocci (e.g., streptococci, leuconostocs, pediococci, lactococci, and enterococci) were present in higher numbers than lactobacilli (Cai et al., 1994). After ensiling, cocci groups will start lactic acid fermentation and create anaerobic environment suitable for the development of lactobacilli. The lactobacilli then grow and promote lactic acid fermentation (Cai et al., 1998). Therefore, after a few days of anaerobic incubation of plant juice, the lactic acid-producing cocci and lactobacilli were contained in FJLB. Thus, when such FJLB is applied as an additive, good silage will be obtained. As mentioned above, FJLB contained many kinds of microorganisms which could ferment sugar to not only lactic acid but also acetic acid, carbon dioxide and ethanol. Many studies have found that addition of FJLB could increase the acetic acid content of silage (Ohshima et al., 1997a,b; Shao et al., 2004). In contrast, this study found that the addition of untreated FJLB or FJLB treated with green tea lowered acetic acid as compared with untreated silages. This may possibly reflect that FJLB contains mainly homofermentative LAB, resulting solely in lactic acid production. The addition of FJLB treated with sterilized green tea greatly increased lactic acid and LA/AA and decreased pH as compared with the unsterilized treatment. The reason for this is unclear. This could probably be explained by the drying of unsterilized green tea at 70°C for 24 h, which may not be sufficient to kill some microorganisms which are resistant to this temperature and hence may survive the treatment process. Therefore, incubation of FJLB after addition of unsterilized green tea may also increase the number of these microorganisms, and eventually may compete or interrupt the fermentation. There were significantly ($p<0.001$) lower values of $NH_3$-N in the treated compared to untreated silages. This indicated that the growth of LAB to produce lactic acid was faster in these silages, resulting in lower pH. These conditions can inhibit the proteolytic activity of plant enzymes and other undesirable bacteria during the early stage of ensiling.

## CONCLUSION

The addition of any form of green tea could increase the number of LAB and aerobic bacteria in the FJLB solutions. These studies confirmed that addition of any green tea enhances the lactic acid content and improves the fermentative quality of silage. Further study is needed to clarify the green tea components that enhance lactic acid bacteria number and lactic acid production. It is also recommended that additional ensiling experiments be performed with different grass crops and different preparations of green tea supplement.

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