International Journal of Agriculture and Biosciences 2024 xx(x): xx-xx.
https://doi.org/10.47278/journal.ijab/2024.105

RESEARCH ARTICLE
eISSN: 2306-3599; pISSN: 2305-6622

Effect of Addition of Fermented Green Juice, Bacterial Inoculant, Enzyme or Effective Microorganisms on Fermentation Quality of Timothy Silage

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ABSTRACT

A laboratory scale experiment was performed to investigate the efficiency of fermented green juice (FGJ) as a silage additive and compare it with other biological additives such as commercial lactic acid bacteria (LAB) inoculant, cellulolytic enzyme and three different kinds of effective microorganisms (EM). The organic acid composition and microbial flora of FGJ prepared from alfalfa and timothy were studied. After 2 days of incubation, FGJs recorded a low pH value, and the fermentation products included lactic acid and acetic acid; however, they did not include butyric or propionic acids. LAB count had significantly increased to a level of 10^8 cfu/g. Molds, yeasts, and enterobacterial counts were decreased to less than 3×10^3 cfu/g. Except for EM1, EM2, and EM3 treated silages, all silages, including controls, were well preserved with a slight difference in quality. Although the untreated silage was well preserved, the addition of timothy or alfalfa FGJ to timothy grass at the ensiling time resulted in higher lactic acid production, lower ($P<0.01$) pH value, and lower NH3-N % TN than the control silage. Compared to other additives, LAB inoculation had the highest efficacy in improving the fermentation quality of timothy silage, while enzyme addition had no effect, and all silages treated with EM were poor quality. This study showed that FGJ may effectively improve the fermentation quality of timothy silage as defined by a lower pH value and higher lactic acid production. No significant difference was found in the efficiency between timothy and alfalfa FGJs.

Keywords: Corn silage, Fermentation, Fermented green juice, Nutrition, Timothy silage

Article History
Article # 24-642
Received: 08-Jun-24
Revised: 23-Jun-24
Accepted: 25-Jun-24
Online First: 26-Jun-24

INTRODUCTION

Silage is the product formed when grass of high moisture (e.g., forage corn and forage legumes) is susceptible to spoilage by aerobic microorganisms and is anaerobically stored. The process, referred to as ensilage, occurs in a vessel called a silo. During this process, the grass undergoes acid fermentation in which bacteria produce acetic, butyric, and lactic acids from sugar present in the grass material, resulting in a lowering in pH, which prevents the development of spoilage microorganisms, almost all of which are intolerant of acid condition (Woolford, 1984; Li et al., 2017; Avila and Carvalho, 2020; Dong et al., 2022).

The first important aim of processing crops by natural fermentation is the establishment of anaerobic conditions. To achieve this goal, the most effective way is to store the crops in a hermetically sealed container, and under these circumstances, the oxygen present in the grass is rapidly eliminated by respiratory enzymes within the plant (McDonald et al., 1991a; Kung et al., 2018). The anaerobic environment stops the growth of yeast and molds, prevents plant respiration, and stimulates the growth of lactic acid bacteria (LAB) (Muck and Pitt, 1993; Abdelrahman et al., 2022).

The second principal aim is to discourage the activities of unwanted microorganisms, such as enterobacteria and clostridia. The latter microorganism is usually found on

Cite this Article as: Selim L, Ataku K and Tharwat M, 2024. Effect of addition of fermented green juice, bacterial inoculant, enzyme or effective microorganisms on fermentation quality of timothy silage. International Journal of Agriculture and Biosciences xx(x): xx-xx. https://doi.org/10.47278/journal.ijab/2024.105

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harvested grass in the form of spores but begins to multiply soon after the conditions in the silo change to anaerobic (McDonald and Whittenbury, 1973; Abd Samat et al., 2020). A clostridial spoilage is manifested by greater of butyric than lactic acid levels, ammonia nitrogen (NH3-N) levels greater than 10% of total nitrogen (TN), pH above 5.0, and an odor characteristic of butyric acid (rancid butter smell) or ammonia (Muck and Pitt, 1993; Stirling et al., 2022). The development of such microorganisms is unfavorable, as they form butyric acid and breakdown amino acids to a variety of outputs, which are of less nutritional impact.

LAB are also found on the harvested grasses and, similar to the enterobacteria, are facultative anaerobes. These microorganisms ferment the naturally occurring sugars (mainly fructose and glucose) in the grass to a blend of acids, but mainly lactic acid. The produced lactic acid elevates the pH concentration to a grade at which the unwanted microorganisms are banned (Woolford, 1984; Fang et al., 2022; Wang et al., 2022). The level of lactic acid formation is an important agent in preventing the growth of unwanted bacteria and in lowering fermentation losses, and this relies on the starting lactic acid bacterial number present on the ensiled grass and on the substrate availability. In the presence of oxygen, LAB produces less lactic acid but more acetic acid. Moreover, if sugars are not present, they will convert lactic acid to acetic acid (Muck and Pitt, 1993; Abdelrahman et al., 2022). There is evidence that in most silages, LAB may grow to reach maximum within 2 to 4 days and show a steady decline thereafter. It is therefore important to have dominance in the early stages of fermentation because the competition with undesirable microorganisms may be highest during the whole ensiling period (McDonald et al., 1991a; Kung et al., 2018).

To improve the process of ensiling many biological additives and chemicals have been used. Nowadays biological additives are most preferred over chemicals, because they are non-toxic, easy to use, do not present environmental risks, regarded as natural products and are non-corrosive. The epiphytic LAB populations can range from non-detectable to several million cfu/g of fresh forage. It was reported that the counts for epiphytic LAB quoted in more recent years are often considerably higher than those previously commonly accepted (McDonald et al., 1991b; Muck et al., 2018).

Fermented green juice is a macerated grass material for silage incubated anaerobically for 2 days in order to culture microorganisms adherent to the grass, which is supposed to include a number of species of domestic LABs and be used as a silage additive. It was also found that silage with good fermentation quality can be prepared from direct-cut alfalfa by adding fermented green juice (FGJ) of epiphytic LAB, a novel additive (Ohshima et al., 1997a,b). The effect of the addition of FGJ was also observed in the legumes in addition to grasses. Good quality grass silage can be obtained by the addition of FGJ regardless of the dilution rate and the additive volume (Masuko et al., 2002). Only a few data are available regarding the composition of FGJ.

The objective of this study was to investigate the usefulness of FGJ prepared from timothy or alfalfa as a silage additive and compare it with other biological additives such as commercial LAB inoculant, cellulolytic enzyme, and three different kinds of effective microorganisms (EM).

MATERIALS & METHODS

Preparation of Fermented Green Juice

Alfalfa (*Medicago sativa* L.) and timothy (*Phleum pratense* L.) were harvested at first flowering and heading stages, respectively. FGJs were prepared from chopped alfalfa or timothy by the following method: approximately 100g of each fresh herbage was macerated with 300mL of water using a blender. The macerate was filtrated through double cheesecloth, and each filtrate was diluted with 500mL distilled water to which 10g of glucose was added, then fitted with gas trap and kept at 30°C for 2 days (Ohshima et al., 1997a,b).

Preparation of Silage

Timothy was harvested and chopped into 1-5cm pieces. Each LAB, cellulase, or EM was diluted with distilled water in a certain amount to achieve the required concentration before being added to the grass. The additives were sprayed at a level of 5mL/500g of grass using a hand-operated sprayer and then mixed thoroughly. The silage additives were used as following design:

1. No additive (control)
2. Timothy FGJ 0.1%
3. Alfalfa FGJ 0.1%
4. LAB 10^6 cfu/g (Lactobacillus rhamnosus, Snow Brand Seed Co.)
5. Enzyme 0.01 % (cellulase enzyme derived from Acremonium cellulolyticus, Meiji Seika Co.)
6. EM1 0.1% (EM Research Institute, Inc.)
7. EM2 0.1% (EM Technical Research Institute Inc.)
8. EM3 0.1% (Kennoh Seeder Co.Ltd.)

Control silage received distilled water at a similar rate without additive. Duplicate laboratory silos of 1000mL capacity containing 500g material were prepared for each treatment. Finally, sixteen silos were fitted with gas traps. Weights of the empty and full silos were recorded, and silos were then maintained at room temperature for 30 days.

Analytical Methods

The fermentation quality of FGJ was assessed by measuring the pH with a pH meter and determining the organic acid contents with liquid chromatography (GC 14-A, Shimadzu Co., Ltd.). The temperature of injector and detector was set at 210°C, and that of the column oven was programmed to increase from 120 to 190°C at a rate of 5°C min^{-1}. Microbiological analyses were enumerated in both herbage and FGJ.

When the silos were opened, each treatment was mixed thoroughly, and 100g sample was taken from each silo into a flask and filled up with distilled water until 500mL and stored in the refrigerator at 4°C for 24h. The material was then filtered using filter paper, and the filtrate was used for electrometric pH measurements. The filtrate was also analyzed for NH_3-N with steam distillation and for lactic acid and volatile fatty acid

determination with gas-liquid chromatography, as mentioned above. V-score was evaluated using the values of organic acids and NH_3-N (% TN).

The chemical composition of the grasses and silages was determined using ground samples oven-dried at 60°C for 24h. Dry matter contents were determined by oven-drying of the samples at 135°C for 2h. Crude protein (CP) was calculated by multiplying Kjeldal nitrogen by 6.25 (AOAC, 1990). Water soluble carbohydrate (WSC) content was estimated calorimetrically using anthrone. Data were subjected to analysis of variance and significance was declared at $P<0.05$ unless noted otherwise.

RESULTS

The pH values of both timothy and alfalfa were 5.93 and 6.05 respectively. LAB and aerobic bacterial count were nearly the same in both of them, 3.2×10^4 cfu/g and 4.8×10^6 cfu/g, respectively. Timothy was higher in mould and yeast count, while alfalfa was higher in enterobacterial count. The ideal level of dry matter (DM) content (28%) and sufficient substrate of WSC (11.4 % DM) were found in timothy grass, while alfalfa has low DM content (19%) and WSC (6.6% DM) (Table 1).

Table 1: The chemical composition, pH and viable counts of alfalfa and timothy

Parameters	Timothy	Alfalfa
Growth stage	heading	first flowering
Cutting time	June 30, 2002	June 30, 2002
Moisture (%)	72.0	81.0
CP (%DM)	6.31	14.5
WSC (%DM)	11.4	6.60
pH	5.93	6.05
LAB (cfu/g)	3.2×10^4	3.2×10^4
Aerobic bacteria (cfu/g)	4.8×10^6	4.9×10^6
Molds (cfu/g)	4.9×10^5	4.2×10^4
Yeasts (cfu/g)	$< 1.2\times10^5$	$< 1.2\times10^4$
Enterobacteria (cfu/g)	7.2×10^4	$> 1.2\times10^6$

CP, crude protein; WSC, water soluble carbohydrates; LAB, lactic acid bacteria

After 2 days of incubation, timothy FGJ recorded a lower pH value (3.55) than the pH of alfalfa FGJ (3.85). The LAB and aerobic bacterial count in both of the two FGJs had increased to the level of 10^8 cfu/g, but alfalfa FGJ had a higher number of both LAB and aerobic bacteria. The counts of molds, yeast, and enterobacteria had decreased after the incubation to a level of less than 10^3 cfu/g. Both Timothy and alfalfa FGJs organic acids included lactic acid and acetic acid and did not include propionic or butyric acid. Therefore, the total acid content was nearly the same. 2, 3 butanediol was detected in the two FGJs (Table 2).

The fermentation characteristics and chemical composition of control and treated silages formed in this experiment are presented in Tables 3 and 4. With the exception of EM1, 2, and 3 treated silages, all silages, including the control silage, were well preserved with a slight difference in quality. The pH value of the control silage was 4.3. All treated silages except EM1 silage recorded lower pH than the control, but the lowest ($P<0.01$) value was obtained in silage treated with LAB followed by EM2, then Timothy and alfalfa FGJs.

The addition of FGJs, LAB, and EM2 enhanced the lactic acid production, but LAB-treated silage significantly had the highest lactic acid content ($P<0.01$). Acetic acid was detected in all silages, but the content of the control and LAB-treated silage was lower than FGJs and enzyme-treated silage ($P<0.05$). While EM1, 2 and 3 additions obviously increased the acetic acid content of the silages. Neither butyrate nor propionate was produced in any of the silages. 2, 3 butanediol was detected in the silages treated with EM1, 2, and 3, but 1, 2-propanediol was found only in the treated silage. As a result of these findings, Flieg's score of both control and LAB was the highest, followed by FGJs treated silages, while the addition of EM1, 2, and 3 resulted in a low Flieg's point. The proportion of NH_3-N was low in all the silages, but both FGJs and LAB-treated silages had the lowest value. Therefore, V-scores of all silages were high.

Table 2: The viable counts and fermentation characteristics of fermented green juice prepared from timothy and alfalfa

Parameters	Timothy FGJ	Alfalfa FGJ
LAB (cfu/g)	4.0×10^8	7.7×10^8
Aerobic bacteria (cfu/g)	3.9×10^8	5.4×10^8
Molds (cfu/g)	$< 3.0\times10^3$	$< 3.0\times10^3$
Yeasts (cfu/g)	$< 3.0\times10^3$	$< 3.0\times10^3$
Enterobacteria (cfu/g)	3.0×10^3	3.0×10^3
pH	3.55	3.85
Acetic acid (%)	0.03	0.04
Lactic acid (%)	0.21	0.18
Butyric acid (%)	0.00	0.00
Total acid (%)	0.21	0.23
Flieg's point	100	98.0
2,3-butanediol (%)	0.06	0.05

FGJ, fermented green juice; LAB, lactic acid bacteria.

DISCUSSION

By adding 5.0mL of timothy or alfalfa FGJ to 500g chopped timothy, it was calculated that 4×10^5 and 7.7×10^5 cfu/g were inoculated, respectively. This LAB level was less than the level added from the commercial LAB inoculant. Using FGJs prepared from timothy and alfalfa, the viable counts of the standing crops and FGJs were investigated. Both timothy and alfalfa had nearly the same level of epiphytic LAB 3.2×10^4 cfu/g, which drastically increased after 2 days of fermentation to a level of 4×108 and 7.7×10^8 cfu/g, respectively. These results well agree with the result of Ohshima et al. (1997a,b). The aerobic bacterial population also increased from 4.8×10^6 cfu/g in both to a level of 3.9×10^8 and 5.4×10^8 cfu/g in timothy and alfalfa FGJ, respectively. Molds, yeast, and enterobacterial counts decreased to a level less than 3×10^3 cfu/g, unlike the results of Masuko et al. (2002), where the yeast number increased in timothy FGJ. The pH value of Timothy 3.55 was lower than that of alfalfa FGJ 3.85, and both of them contained lactic acid, acetic acid, 2,3-butanediol, and no butyric acid.

Compared to the silage with their material, the CP concentration of all silages was higher than that of Timothy. The treatment of grass with FGJs or LAB had no influence on nutrient recovery after ensilage compared to the control. Kennedy et al. (1989) observed no effect of inoculant on recovery of DM. Both untreated (control) and treated timothy silages resulted in good quality silages. A low pH, which did not exceed 4.3 or contained butyric acid, showed good fermentation in control silage. Moreover, the

Table 3: The chemical composition and losses of timothy silage treated with FGJ, LAB, enzyme or EM

Parameters	None	Timothy FGJ	Alfalfa FGJ	LAB	Enzyme	EM1	EM2	EM3	SE	P
DM (%)	27.5^{Aa}	27.4^{Aa}	27.4^{Aa}	27.2^{Aa}	26.6^{Bb}	25.8^{Cd}	26.3^{BCbc}	26.0^{BCcd}	0.13	0.00
DM recovery (%)	96.9^{A}	97.0^{A}	97.1^{A}	96.5^{A}	93.9^{B}	90.7^{C}	92.8^{BC}	91.5^{C}	0.46	0.00
Gass loss (%)	4.91^{B}	3.01^{D}	3.09^{D}	3.09^{D}	4.23^{C}	5.65^{A}	4.70^{B}	5.63^{A}	0.08	0.00
CP (%DM)	6.83^{b}	7.70^{a}	7.83^{a}	7.25^{ab}	7.76^{a}	7.36^{a}	7.58^{a}	7.44^{a}	0.23	0.17

DM, dry matter; CP, crude protein; FGJ, fermented green juice; LAB, lactic acid bacteria; EM, effective microorganisms. SE; standard error, A,B,C,D: P<0.01; a,b,c,d: P<0.05

Table 4: The fermentation characteristics of timothy silage treated with FGJ, LAB, enzyme or EM

Parameters	None	Timothy FGJ	Alfalfa FGJ	LAB	Enz.	EM1	EM2	EM3	SE	P
pH	4.31^{Aa}	3.91^{DCc}	3.95^{Cc}	3.55^{Ee}	4.09^{Bb}	4.24^{Aa}	3.82^{Dd}	4.08^{Bb}	0.03	0.00
NH_3-N (%TN)	5.88^{ABab}	3.56^{Dd}	4.78^{BCDbc}	4.22^{Dcd}	4.16^{Dcd}	6.14^{Aba}	6.22^{ABa}	6.95^{Aa}	0.37	0.00
Lactic acid (%)	0.85^{BCDc}	0.94^{BCbc}	1.04^{BCbc}	1.73^{Aa}	0.73^{CDd}	0.23^{Ee}	1.17^{Bb}	0.52^{DEd}	0.07	0.00
Acetic acid (%)	0.11^{Bc}	0.27^{Bb}	0.28^{Bb}	0.18^{Bc}	0.26^{Bb}	0.93^{Aa}	0.92^{Aa}	0.92^{Aa}	0.04	0.00
Butyric acid (%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-
Total acid (%)	0.95^{Ce}	1.21^{Ccde}	1.32^{Ccd}	1.91^{ABb}	0.99^{Cde}	1.16^{Cde}	2.09^{Aa}	1.44^{BCc}	0.10	0.00
2,3-butanediol (%)	0.00	0.00	0.00	0.00	0.00	0.14^{ab}	0.18^{a}	0.03^{b}	0.03	0.10
1,2- propanediol (%)	0.00	0.00	0.00	0.00	0.00	0.18	0.00	0.00	-	-
Flieg's point	100^{A}	96.5^{B}	97^{B}	100^{A}	90.5^{C}	50^{F}	67.5^{D}	55.5^{E}	0.35	0.00
V-Score	98.2^{A}	99.5^{A}	99.4^{A}	100^{A}	99.5^{A}	92.1^{B}	92^{B}	90.6^{B}	0.80	0.00

NH_3-N, ammonia nitrogen; FGJ, fermented green juice; LAB, lactic acid bacteria; EM, effective microorganisms. SE; standard error, A,B,C,D: P<0.01; a,b,c,d: P<0.05.

NH_3-N level was lower than that suggested that well-preserved silage should have less than 8% of TN (Henderson, 1993; Campbell et al., 2020).

The fact that the untreated silages were well preserved may indicate that the WSC content in the original grass (11.4 % DM) was sufficient for LAB to produce lactic acid and reduce pH. Weinberg et al. (1995) summarized that the critical content of WSC needed to obtain satisfactory silage fermentation should be around 3-5 % DM. The good preservation of control silage may also be due to the fact that the DM content of the original grass was at an ideal level, since the DM content was 28%. McDonald et al. (1991a) recommended that the ideal crop for preservation as silage should contain an adequate level of fermentable substrate in the form of WSC and DM content above 20%. The number of epiphytic LAB in original grass might have also affected the fermentation quality of the control silage. Although the epiphytic LAB level was 3.2×10^4 cfu/g was not so close to the condition for the preparation of good silage 10^6 cfu/g (McDonald et al., 1991b: Ohmomo, 1996; Muck et al., 2018), it is assumed that the number of LAB was present at enough level and activity to sustain a satisfactory fermentation in the silo and to stop clostridial fermentation. Although the efficiency of the ensilage process is influenced by many factors, the size, diversity and activity of the epiphytic micro flora are of considerable significance (McDonald et al., 1991a,b; Kung et al., 2018).

The efficiency of biological additives varies with the microbiological and chemical composition of the fresh grasses, the ensiling technique, environmental conditions, and the properties of inoculants (Campbell et al., 2020). Compared to the control silage, both LAB and FGJ treatments of timothy silages resulted in further improved fermentation quality as defined by decreasing (P<0.01) pH value, increasing lactic acid concentration and further reducing the ammonia nitrogen concentration. Research conducted on inoculation of LAB at ensiling has shown variable effects on silage quality. Sometimes it was effective (Seale et al., 1986; Rook and Kafilzardeh, 1994; Kumai et al., 1990), and sometimes it was not (Ely et al., 1982). In this study, LAB inoculation produced higher (P<0.01) lactic acid, which resulted in a significantly lower pH value than FGJ-treated silage and also higher Flieg's point, while the V-scores were similar. Different from these results, Ohshima et al. **(**1997a,b), reported that FGJ had higher or similar efficacy to commercial LAB inoculant in improving alfalfa and napiergrass silages, respectively. On the other hand, the enzyme-treated silage produced lactic acid content even lower than the control silage (P<0.05) and, therefore, has a lower Flieg's point (P<0.01).

Fermented green juice succeeded in improving the quality of timothy silage, while enzyme addition had no effect, and this agrees with what was reported with high WSC grass, where there was no effect of the enzyme on the fermentation quality of the silage (Jaakkola et al., 1991; Wu et al., 2022). Although, the pH value of EM1, 2 and 3 treated silages did not exceed 4.2, and EM2 treated silage had lactic acid content as high as that of FGJ treated silage, they were badly preserved silages as indicated by their low Flieg's points. They produced acetic acid rather than lactic acid, and the probable reason for these results is the anaerobic conversion of lactic acid to acetic acid.

In conclusion, this study's results showed that FGJ may be effective in improving the fermentation quality of timothy silage, as defined by a higher lactic acid production and lower pH value. No significant difference was found in the efficiency between timothy and alfalfa FGJs.

Acknowledgment

The researchers would like to thank Dr. Hideki Terui, Department of Animal Nutrition, School of Dairy Science, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan for technical assistance. Genuine thanks to Dr. Nell Kennedy, Professor and Head of the Department of Biomedical English, School of Veterinary Medicine, Rakuno Gakuen University, for language revising, her continuous support and for her forceful discussions.

Conflicts of Interest Statement

The authors have no conflicts of interest to disclose.

Author Contributions

LS: concept, design, and writing the manuscript draft. LS and KA: practical work. MT: revised and edited the manuscript draft. All authors revised and approved the final manuscript for publication.

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