Asian-Aust. J. Anim. Sci.
Vol. 22, No. 4 : 528 - 533
April 2009

www.ajas.info

Silage Fermentative Quality and Characteristics of Anthocyanin Stability in Anthocyanin-rich Corn (*Zea mays* L.)

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ABSTRACT : The fermentative quality and quantitative change in anthocyanin of anthocyanin-rich corn (*Zea mays* L.) during storage and *in vitro* ruminal fermentation were studied. The anthocyanin-rich corn silages in bag silo, drum silo and round bale had good fermentative qualities, such as low pH (<pH 4), high lactic acid content (>5% DM) and butyric acid-free, and its quality was maintained for more than 370 d. The amount of anthocyanin in the anthocyanin-rich corn decreased after ensiling by about 45% (from 3.34 to 1.88 mg/g DM), but stayed constant after day 60. The *in vitro* incubation of the anthocyanin-rich corn with ruminal fluid revealed little degradation of anthocyanin. These results indicate that the anthocyanin had no negative effect on silage fermentation, and the anthocyanin-rich corn silage is utilizable for practical use as a feedstuff. Our results also demonstrate alteration of the anthocyanin content during storage, and show that anthocyanin-rich corn is a suitable antioxidant source for ruminants because of the high stability of the anthocyanin in ruminal fluid. (**Key Words :** Anthocyanin-rich Corn, Silage Fermentative Quality, Anthocyanin Stability, *In vitro* Ruminal Fermentation, Cattle)

INTRODUCTION

Although animals suffer oxidative stress, their natural antioxidant system combats free radicals (Miller et al., 1993). If this balance is disrupted, however, animals are considered to enter an oxidative stress status that may cause metabolic disorder (Miller et al., 1993; Davies, 2000). Castillo et al. (2006) observed that the plasma level of the total antioxidant status in lactating cows decreased during the lactation period. Thus, an antioxidant substance must be supplied to lactating cows.

Anthocyanin is a type of polyphenol that exhibits a potent antioxidant activity (Tsuda et al., 1994; Cevallos-Casals and Cisneros-Zevallos, 2003) and anthocyanin-rich corn (strain, Choko C922, *Zea mays* L.) for ruminant feed was recently developed in the Nagano Prefectural Chushin Agricultural Experiment Station, Japan. This corn is able to accumulate much more anthocyanin than commercial corns, and is thus considered an effective source of antioxidant for dairy cattle. However, there is little information on the characteristics of fermentation and the anthocyanin stability of anthocyanin-rich corn during ensilage and ruminal fermentation. In this report, we investigated the quality of silage fermentation and quantitative change in anthocyanin of anthocyanin-rich corn during both the preservation process and *in vitro* incubation with ruminal fluid.

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Received September 17, 2008; Accepted January 7, 2009

MATERIALS AND METHODS

Plant and animal management

Anthocyanin-rich (*Zea mays* L., Choko C922, 124-day relative maturity) and control (*Zea mays* L., 33N56; Pioneer Hi-Bred Japan, Tokyo, Japan; 112-day relative maturity) corns at the yellow ripe stage, grown in a field at the National Institute of Livestock and Grassland Science (Nasushiobara, Tochigi, Japan), were harvested (12-mm theoretical length of cut; MFH3200; Star Farm Machinery Mfg., Hokkaido, Japan), baled (MR-810; Takakita, Mie, Japan), and wrapped (SW1000; Takakita) with white plastic film as previously reported by Nishida et al. (2007). The bales were stored outdoors on the ground until being opened. Portions of forage materials were used to prepare the bag and drum silo silage.

Two Holstein steers (body weight, 490.5±4.9 kg (mean ±standard deviation)) were used for collection of rumen fluid. The animals received a diet that met the total digestible nutrients requirements for maintenance of the Japanese Feeding Standard for Beef Cattle (AFFRCS, 2000).

Table 1. Chemical composition of corn forage materials

Item	Type of corn	
	Control	Anthocyanin-rich
Dry matter (%)	28.7	27.1
Organic matter (% DM)	96.1	94.4
Crude protein (% DM)	7.4	8.3
Ether extract (% DM)	2.7	2.3
Acid detergent fiber (% DM)	22.9	20.6
Neutral detergent fiber (% DM)	40.6	38.9
Crude ash (% DM)	3.9	5.6
Anthocyanin (% DM)	0.04	0.34

and had free access to fresh water. Feed was provided in equal amounts twice daily at 09:00 and 17:00 h. The diet was made up of 50.0% timothy hay, 42.2% flaked maize grain, 8.0% soybean meal, 1.2% mineral mix, 0.3% vitamin mix, and 0.3% salt on a dry matter (DM) basis. Mineral mix was a commercial product (SuperMag100; Toyo Denka Kogyo, Kochi, Japan) containing 110 g/kg of P, 220 g/kg of Ca, and 100 g/kg of Mg. The vitamin mix (Vitaguard E20; Roche Vitamin Japan, Tokyo, Japan) contained 1,200 mg/kg of vitamin A, 10 mg/kg of vitamin D, and 20,000 mg/kg of DL-α-tocopherol acetate. The nutrient composition of the diet was 95.7% organic matter, 11.0% crude protein, 2.7% ether extract, 23.6% acid detergent fiber, and 39.9% neutral detergent fiber. The animal experiment in the present study was approved by the Animal Care Committee of the National Institute of Livestock and Grassland Science.

Experiment 1

This experiment was designed to compare the fermentative quality of the anthocyanin-rich corn in comparison to the control corn during ensilage. Samples (100 g fresh matter) of corn forage materials (see Table 1 for composition) were placed into plastic film bags (20 cm ×30 cm; Asahikasei Pax, Tokyo, Japan), and the bags were sealed using a vacuum sealer (SQ-202; Asahikasei Pax). The bags were kept in a dark place at ambient temperature (15-25°C) for 0, 2, 5, 7, 10, 15, 30 and 60 d. Corn silage was also prepared with a 50-L drum silo and stored for 60 d. The round bales of corn silage were stored for 80 and 370 d. After ensilage, a 10-g portion of silage was homogenized with 90 ml pure water for 1 min using a homogenizer (SH-IIM; ELMEX, Tokyo, Japan). The homogenate was passed through filter paper (No5A; Toyo Roshi, Tokyo, Japan) and the pH of the filtrate was measured. The filtrate was stored at -20°C until assayed for lactic acid, volatile fatty acids (VFA) and ammonia. DM was determined by drying at 75°C for 48 h. The experiment was performed in triplicate for the bag and drum silo, and in quadruplicate for the round bale for each storage time and corn type.

Experiment 2

The quantitative change of anthocyanin in the anthocyanin-rich corn during ensilage was investigated in this experiment. Silage bags of anthocyanin-rich corn were prepared as in Experiment 1. The bags were stored in a dark place at ambient temperature (15-25°C) for 0, 15, 30, 60, 120 and 180 d. The silage was freeze-dried and then ground with a 1-mm sieve for anthocyanin analysis.

Experiment 3

This experiment was undertaken to investigate if anthocyanin in corn is broken down during *in vitro* incubation with ruminal fluid. The 200-d stored silage (round bale) of anthocyanin-rich corn was air-dried at 60°C and milled through 1-mm mesh prior to use. Ruminal fluid (about 500 ml) was obtained from two steers before morning feeding via the mouth using a rumen catheter into an Erlenmeyer flask. The flasks were immediately sealed and transported to the laboratory at approximately 38°C. The fluids were passed through 4 layers of cheesecloth, and fluids from the two steers were mixed in equal volume. Culture fluid was prepared by mixing ruminal fluid and phosphate-bicarbonate buffer (McDougall, 1948; Abe, 1988), which was preliminarily purged with CO_2 gas (<10 ppm O_2), in a 1:4 ratio. *In vitro* incubation was performed in a glass tube containing 1 g of ground corn with 50 ml of culture fluid for 0, 12, and 24 h. The tube was kept at 38°C in a water bath and purged continuously with CO_2 gas. After incubation, the fermentation was stopped by sinking the tube into ice-cold water and the pH of the fluid was measured. The contents of the tube were lyophilized for the anthocyanin assay. The experiment was conducted in 4 replicates at each incubation time, and the incubations were repeated on 2 separate days.

Chemical analysis

The DM of fresh feed was examined by drying the sample at 100°C for 18 h. The feed samples and forage materials for chemical analysis were air-dried and ground to pass a 1-mm screen. The air-dried sample was dried at 135°C for 2 h to determine the DM. The crude protein, ether extract, crude ash, neutral detergent fiber and acid detergent fiber were measured by the methods of AOAC (2000) and Van Soest et al. (1991). Organic matter was calculated as the weight loss on ashing.

The amount of anthocyanin in the sample was measured by the method of Cacace and Mazza (2002) with a slight modification. Briefly, the sample was shaken with 1% HCl in methanol at 50°C for 24 h. The extract was centrifuged at 10,000×g for 10 min at 4°C, and the supernatant was passed through a 0.45 μm filter. The anthocyanin content of filtrate was quantified by measuring its optical density at 525 nm, with cyanidin-3-glucoside (Extrasynthese, Genay, France) as a standard, using a spectrophotometer (Ultrospec 3100;

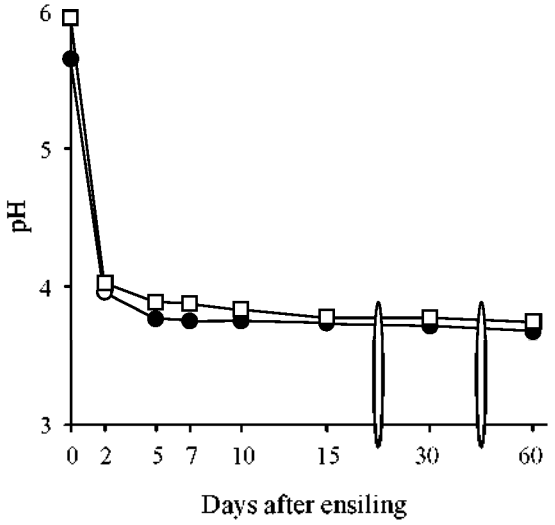

Figure 1. Changes in pH value during fermentation of silage. □, control corn; ○, anthocyanin-rich corn. Data are displayed as means with standard deviations. Filled circle indicates significant difference from the control ($p<0.05$).

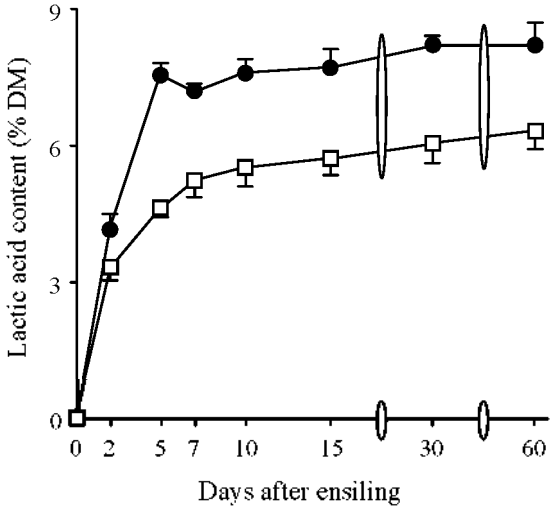

Figure 2. Changes in lactic acid content during fermentation of silage. □, control corn; ○, anthocyanin-rich corn. Data are displayed as means with standard deviations. Filled circle indicates significant difference from the control ($p<0.05$).

Amersham Pharmacia Biotech, Piscataway, NJ).

The lactic acid and VFA, and ammonia concentrations in the silage extract were determined by high-performance liquid chromatography (Hosoda et al., 2005) and by steam distillation using an automatic analyzer (Kjeltec Auto Sampler System 1035 Analyzer; Tecator AB, Hoganas, Sweden), respectively.

Statistical analysis

All statistical calculations were performed using the GLM procedure of SAS (SAS, 1988). Differences in the parameters each day were analyzed by ANOVA with the type of corn as a factor in the bag and drum silo silages of Experiment 1. In the round-bale silage of Experiment 1, significant differences between parameters were assessed by the Tukey's test following a significant main effect. In Experiments 2 and 3, the statistical significances of the anthocyanin amount between storage days or incubation times were detected using ANOVA, followed by Dunnett's multiple comparison test. A value of $p<0.05$ was considered to indicate statistical significance.

RESULTS

Experiment 1

Changes in the pH values of the bag silages after ensiling are presented in Figure 1. The pH values in both corn silages declined rapidly by day 2, and were kept under pH 4 from days 5 to 60. There were significant ($p<0.05$) differences in pH values between the control and anthocyanin-rich corn silages from days 5 to 60.

Figure 2 shows the changes in lactic acid concentrations of the bag silages after ensiling. The anthocyanin-rich corn silage exhibited a dramatic increase in lactic acid concentration which on all days after ensiling was significantly ($p<0.05$) higher in the anthocyanin-rich corn silage than in the control silage.

The silage quality in the drum silo stored for 60 d is shown in Table 2. Both silages were well preserved. In the silage of anthocyanin-rich corn, however, the pH value and the ammonia-N concentration were significantly ($p<0.01$) lower and the lactic acid concentration was significantly ($p<0.05$) higher than in the control silage. Butyric and

Table 2. Fermentation characteristics of corn silages stored for 60 d (50-L drum silo)

Item	Type of corn		
	Control	Anthocyanin-rich	SEM
Dry matter (%)	26.2	23.6*	0.7
pH	3.80	3.69**	0.01
Lactic acid (% DM)	6.89	8.50*	0.27
Acetic acid (% DM)	1.27	1.37	0.05
Butyric acid (% DM)	ND	ND	-
Propionic acid (% DM)	ND	ND	-
Ammonia-N (% DM)	0.10	0.08**	0.00

SEM: standard error of the mean, ND: not detected. * Indicate significant difference between the type of corn (* $p<0.05$, ** $p<0.01$).

Table 3. Fermentation characteristics of corn silages stored for 80 and 370 d (round bale)

Item	80 d		370 d	
	Control	Anthocyanin-rich	Control	Anthocyanin-rich
Dry matter (%)	27.0[a]*	23.9[b]	28.4[a]	27.8[a]
pH	3.73	3.70	3.63	3.69
Lactic acid (% DM)	6.3[ab]	7.7[a]	5.4[b]	5.7[ab]
Acetic acid (% DM)	1.2[b]	1.4[ab]	2.1[a]	1.4[ab]
Butyric acid (% DM)	ND	ND	ND	ND
Propionic acid (% DM)	0.0	0.0	0.2	0.1
Ammonia-N (% DM)	0.08[b]	0.07[b]	0.11[a]	0.10[a]

ND: not detected. * Values followed by different letters within a row are significantly different ($p<0.05$).

propionic acids were not detected in either of the silages.

The fermentation characteristics of silage in round bales stored for 80 and 370 d are presented in Table 3. The pH values in all silages were below 4. The values of lactate, acetate, propionate and ammonia-N for the anthocyanin-rich corn silage were similar to those of the control silage at each storage period. Butyric acid was not detected in any of the silages.

Experiment 2

The quantitative change in the anthocyanin content of the anthocyanin-rich corn during ensiling is shown in Figure 3 and at day 0 was 3.34 mg/g DM. The anthocyanin content of the corn silage started to decrease after ensiling, and at day 60, 120 and 180 was significantly ($p<0.05$) lower than at day 0. Thereafter, anthocyanin content in corn silage was maintained from days 60 to 180 at a steady level (1.88 mg/g DM) which was about 45% of that at day 0.

Experiment 3

Figure 4 illustrates the effect of *in vitro* incubation with ruminal fluid on the stability of anthocyanin in the corn. The pH of ruminal fluid at 12 and 24 h after onset of the incubation was significantly lower than that of unincubated ruminal fluid. There were no significant differences in the anthocyanin content of corn between incubation times (0, 12 and 24 h).

DISCUSSION

It is well known that corn is one of the forages that show excellent fermentative quality during ensiling. The results from the present experiment using the bag and the drum silo silage revealed that the anthocyanin-rich corn, similar to the control corn, exhibited rapid and active silage fermentation such as fast accumulation of lactic acid and a lower pH value at earlier stages of ensiling, and good preservation at 60 d. To our knowledge, the fermentative quality of an anthocyanin-rich corn silage has never been reported. In local areas of Japan, however, red wine pomace from wineries, which is abundant in anthocyanin, is utilized as a silage for ruminants, and its silage has been reported to be well fermented (Nishimura et al., 1999; Yokoyama et al., 2006), which supports our present finding. Therefore, the

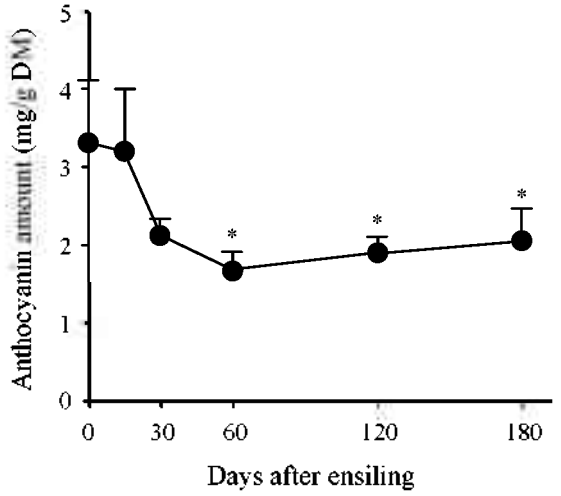

Figure 3. Change in anthocyanin content of anthocyanin-rich corn during ensilage. Data are displayed as means with standard deviations. Anthocyanin (mg)/corn sample (g DM). * Asterisks show statistical difference from the level at day 0 ($p<0.05$).

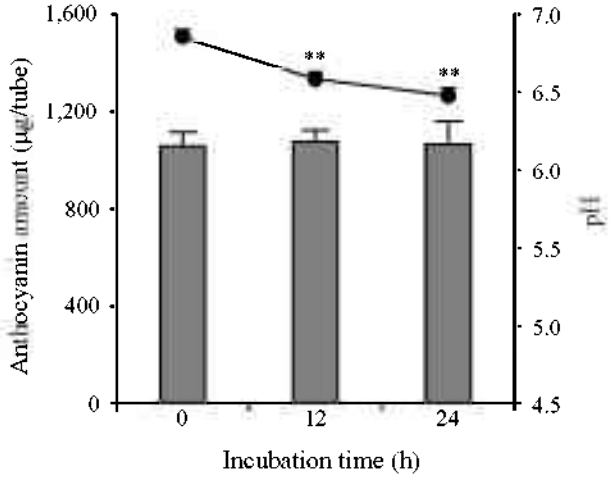

Figure 4. Changes in anthocyanin content (solid bar) and pH value (solid circle) in incubation tube during *in vitro* fermentation with ruminal fluid. Data are displayed as means with standard deviations. ** Asterisks show statistical difference from the level at 0 h ($p<0.01$).

anthocyanin content of the anthocyanin-rich corn would have no negative impact on the fermentative quality of corn silage.

In the present study, the anthocyanin-rich corn silage was prepared on a practical scale using a round-bale system (Shito and Yamana, 2002, 2004; Shito et al., 2005b) in addition to being prepared on a small scale. Shito et al. (2005a) reported that round-bale silages showed good fermentative quality as represented by a low pH value (4.0 or lower) and no detectable butyric acid, and their fermentative quality was maintained for at least 12 months. Consistent with their observations (Shito et al., 2005a), the round-bale silage of the control corn in the present study had excellent fermentative quality at days 80 and 370. Additionally, these fermentative qualities were not degraded by the abundance of anthocyanin. Thus, it is likely that anthocyanin-rich corn silage is an excellent feedstuff for practical use and has long-lasting stability of fermentative quality.

In order to accurately provide anthocyanin to ruminant animals by feeding anthocyanin-rich corn silage, it is important to figure out the quantitative change in the anthocyanin content during storage. According to previous reviews (Francis, 1989; Mazza and Miniati, 1993), there are many factors affecting the stability of anthocyanin. Among those factors, high pH, oxygen, high temperature and light are associated with the stability of the anthocyanin in the corn silage during storage. In the present study, oxygen, temperature and light can be omitted as factors decreasing the anthocyanin content of silage during the storage period because the silage bags were evacuated, placed under low temperature conditions (15-20°C), and protected from light. With reference to pH, Cevallos-Casals and Cisneros-Zevallos (2004) studied the effect of pH on the stability of anthocyanin in an extract from anthocyanin-rich corn, and reported degradation of the anthocyanin above pH 3. Considering the results of Experiment 1, the silage was above pH 3 throughout the experiment, and therefore decreased amounts of anthocyanin observed during storage were perhaps attributable to the pH condition of the silage. However, the pH condition alone did not explain the reason for anthocyanin content in the silage not continuing to decline, and the reason for this observation is unclear. To understand anthocyanin stability during silage storage, the relationship between anthocyanin stability and lactic fermentation during ensiling should be determined, because anthocyanin is composed of anthocyanidin and sugar(s) and there is a possibility that sugar(s) of anthocyanin is used as substrate for lactic fermentation. In any case, there have been no reports investigating the quantitative alteration of anthocyanin in anthocyanin-rich materials during the ensilage period. Therefore, the finding from the present study would be a valuable indicator when anthocyanin-rich corn is used as a source of anthocyanin for ruminant animals as well as a nutrient.

The feeding of anthocyanin-rich feed to ruminant animals and the quantitative change of anthocyanin during ruminal fermentation have never been reported. In previous observations using monogastric animals, orally-administered anthocyanin was absorbed from the stomach and gut, and was then detected in the blood (Miyazawa et al., 1999; Passamonti et al., 2003; Talavéra et al., 2004). Additionally, Mazza et al. (2002) found that there was a positive relationship between serum anthocyanin concentration and the serum antioxidant capacity of human subjects who ingested anthocyanins. These previous observations lead us to presume that ingested anthocyanin is probably absorbed and exhibits a functional effect in ruminant animals if the anthocyanin is not broken down in the rumen. This is because the digestion and absorption functions in the abomasum and intestines of ruminants are analogous to those in the alimentary canal of monogastric animals (Dijkstra et al., 2005). Our present study revealed that the incubation of anthocyanin-rich corn with ruminal fluid did not cause degradation of the anthocyanin. Therefore, it appears that the anthocyanin in the anthocyanin-rich corn is protected from ruminal digestion and can therefore be absorbed by ruminant animals. Moreover, anthocyanin-rich corn seems to be suitable for providing an antioxidant substance to dairy cattle because of the stability of its anthocyanin in ruminal fluid. However, it is necessary to verify the prospective effect of anthocyanin-rich corn by an *in vivo* experiment using ruminants.

In summary, anthocyanin-rich corn in the bag and the drum silo exhibited good silage fermentation characteristics similar to the control corn. The anthocyanin-rich corn silage prepared practically also showed good fermentation and had a long-lasting stability in fermentative quality. These results demonstrated that the anthocyanin has no negative effect on silage fermentative quality and that the anthocyanin-rich corn is capable of practical use for feedstuff. Although the anthocyanin content of anthocyanin-rich corn decreased during ensilage, this observation is probably useful when anthocyanin-rich corn silage is fed to animals as a source of antioxidants. The present study also revealed that the anthocyanin in corn was not decomposed by ruminal fluid, which suggests that the anthocyanin-rich corn is appropriate to provide a functional substance for ruminants. Further study is needed to clarify if the feeding of anthocyanin-rich corn silage affects feed intake and productivity in dairy cattle.

ACKNOWLEDGMENT

The authors would like to thank Dr. K. Nonaka, Dr. T. Kida and Dr. M. Matsuo, Research Team for TMR Preparation and Feeding Technology, National Institute of

Livestock and Grassland Science (NILGS) for their valuable advice on the manuscript and for making the round bale silage. Thanks also go to Mr. K. Miki, Nagano Prefectural Chushin Agricultural Experiment Station, for providing the anthocyanin-rich corn seeds, the members of the Field and Animal Management Section, NILGS, for corn cultivation and animal management, and Ms. C. Nagasawa, Functional Feed Research Team, NILGS, for her skilled technical assistance in the lab experiment. This study was supported by a grant from the Animal Health Project of The Ministry of Agriculture, Forestry and Fisheries of Japan.

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