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Effects of Ensiling Fermentation and Aerobic Deterioration on the Bacterial Community in Italian Ryegrass, Guinea Grass, and Whole-crop Maize Silages Stored at High Moisture Content

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ABSTRACT: The effects of storage period and aerobic deterioration on the bacterial community were examined in Italian ryegrass (IR), guinea grass (GG), and whole-crop maize (WM) silages. Direct-cut forages were stored in a laboratory silo for 3, 7, 14, 28, 56, and 120 d without any additives; live counts, content of fermentation products, and characteristics of the bacterial community were determined. 2,3-Butanediol, acetic acid, and lactic acid were the dominant fermentation products in the IR, GG, and WM silages, respectively. The acetic acid content increased as a result of prolonged ensiling, regardless of the type of silage crop, and the changes were distinctively visible from the beginning of GG ensiling. *Pantoea agglomerans*, *Rahnella aquatilis*, and *Enterobacter* sp. were the major bacteria in the IR silage, indicating that alcoholic fermentation may be due to the activity of enterobacteria. *Staphylococcus sciuri* and *Bacillus pumilus* were detected when IR silage was spoiled, whereas between aerobically stable and unstable silages, no differences were seen in the bacterial community at silo opening. *Lactococcus lactis* was a representative bacterium, although acetic acid was the major fermentation product in the GG silage. *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Morganella morganii* were suggested to be associated with the increase in acetic acid due to prolonged storage. *Enterobacter cloacae* appeared when the GG silage was spoiled. In the WM silage, no distinctive changes due to prolonged ensiling were seen in the bacterial community. Throughout the ensiling, *Weissella paramesenteroides*, *Weissella confusa*, and *Klebsiella pneumoniae* were present in addition to *L. plantarum*, *L. brevis*, and *L. lactis*. Upon deterioration, *Acetobacter pasteurianus*, *Klebsiella variicola*, *Enterobacter hormaechei*, and *Bacillus gibsonii* were detected. These results demonstrate the diverse bacterial community that evolves during ensiling and aerobic spoilage of IR, GG, and WM silages. (**Key Words:** Bacteria, Denaturing Gradient Gel Electrophoresis, Italian Ryegrass, Guinea Grass, Whole-crop Maize, Silage)

INTRODUCTION

Fermentation of silage depends on the competition between different groups of microorganisms. Desirable microorganisms are lactic acid bacteria (LAB), which usually dominate over the ensilage process, whereas a number of undesirable microorganisms could also grow and bring about anaerobic or aerobic spoilage. Enterococci, lactococci, and pediococci are generally thought to initiate silage fermentation, whereas in the late stages more acid-tolerant lactobacilli such as *Lactobacillus plantarum* and *Lactobacillus brevis* may replace them (McDonald et al., 1991; Lin et al., 1992).

The degrees of successful fermentation can be approximately evaluated by determining the composition of the fermentation products. If the silage has a high proportion of lactic acid, the proliferation of non-LAB bacteria may be effectively inhibited, reducing dry matter (DM) loss and protein degradation during storage (McDonald et al., 1991). Information on associated microorganisms can help understand how the ensilage process can be optimized for improving productivity because various microorganisms in addition to LAB can produce the major fermentation products (e.g. lactic acid, acetic acid, and ethanol).

In the analysis of the silage microbial community, culture-independent procedures are increasingly being used. Traditional culture-based assays may be biased by the

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growth characteristics of the microbes and can detect culturable cells only (Giraffa and Neviani, 2001). According to the estimates obtained from microbial ecology studies, only <10% of the microbial diversity present is culturable; hence, the use of selective media may lead to a non-representative assessment of the composition of the microbial community (Ampe et al., 1999). By using denaturing gradient gel electrophoresis (DGGE) analysis, we examined the microorganisms associated with fermentation and aerobic deterioration. The results of this analysis showed how conventional and non-conventional LAB species are involved in fermentation and aerobic stability (Li and Nishino, 2011a, 2011b) and how diverse bacteria are distributed in lateral and perpendicular directions within a bunker silo (Li and Nishino, 2011c).

Because high-moisture ensiling is best avoided in order to achieve acceptable fermentation, medium-moisture ensiling was examined in our previous studies (Li and Nishino, 2011a; 2011b). However, in large-scale ensiling, high-moisture crops can be used to perform time-saving operations. Furthermore, temperate grass, tropical grass, and whole-crop cereal silages should be analysed to understand how silage bacterial communities differ among different crop species, accounting for the differences in the interpretations of the fermentation patterns. Dairy production is expanding from temperate to tropical countries; hence, procedures for ensiling need to be optimized to account for the increase in animal production in hot environments (Nussio, 2005).

The objectives of this study were to identify the bacteria associated with anaerobic storage and aerobic spoilage of Italian ryegrass (IR), guinea grass (GG), and whole-crop maize (WM) silages. Changes in the fermentation and the bacterial community were examined from the early stages of ensiling in order to understand how microorganisms compete with one another in the process of establishing a stable community.

MATERIALS AND METHODS

Ensiling

First growth of IR and GG were harvested at the late heading stage on 19th May 2009 and 4th August 2009, respectively, and chopped using a forage cutter to approximate lengths of 25 mm. WM was harvested at the stage of half milk-line on 31st August 2009, and a precision-chop harvester was used to chop the crop to approximate lengths of 12 mm. The chopped crop (300 g) was packed in a laminated plastic pouch (Hiryu BN12, Asahi Kasei Pax, Tokyo, Japan) and then tightly packed using a vacuum sealer (SQ-303, Asahi Kasei Pax, Tokyo, Japan). The size, thickness, and oxygen permeability of the pouch were 270×400 mm, 0.075 mm, and 44 mL m^{-2} atm^{-1} per day, respectively. The silages were made in triplicate and stored at ambient temperatures for 3, 7, 14, 28, 56, and 120 d. No bacterial inoculant was made.

Aerobic spoilage test

After the silage was completely opened on d 14, 28, 56, and 120, half the content (150 g) was put into a polyethylene bottle (500 mL) without compaction. The top of the bottles was kept uncovered and exposed to air for 7 d in a room maintained at 25°C.

Chemical analyses and microbial enumeration

Silage samples were obtained at the time of opening the silo and after conducting the aerobic stability test. DM contents were determined by drying the material in an oven at 60°C for 48 h. The silage pH and lactic acid, short-chain fatty acid, and alcohol contents were determined from water extracts as described previously (Li and Nishino, 2011a). Water-soluble carbohydrates (WSC) were extracted with 800 g/L (v/v) ethanol, and the contents were determined by the phenol-sulphuric acid method (Parvin et al., 2010).

LAB counts were determined using de Man, Rogosa, and Sharpe agar and enterobacteria counts were obtained using violet red bile agar. Yeasts and moulds were enumerated on spread plates of yeast extract and malt extract agar (pH 3.5, obtained using sterilised lactic acid).

Denaturing gradient gel electrophoresis

Because preliminary experiments demonstrated that triplicate silages exhibited almost the same DGGE profiles, the DGGE was performed on 1 silage sample from 3 replications obtained at both the initial silo opening stage and after the aerobic stability test.

DGGE was performed as previously described (Li and Nishino, 2011a). In brief, the polymerase chain reaction (PCR) was used to amplify a variable (V3) region of the bacterial 16S rRNA gene by using the forward primer GC357f (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′) and the reverse primer 517r (5′-ATTACCGCGGCTGCTGG-3′). The GC-clamp PCR products were separated according to their sequences with a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Tokyo, Japan). The samples were applied directly onto 10% (w/v) polyacrylamide gels prepared in a 25 to 50% denaturing gradient using urea and formamide (7 mol/L urea and 40% (v/v) formamide as 100% denaturants).

Selected bands were excised from the DGGE gels, and the DNA was amplified by PCR by using the 357f (without GC-clamp) and 517r primers. After purifying the reaction products by using a commercial cleanup kit (GeneClean Kit, Qbiogene, Carlsbad, California, USA), the PCR products were cloned into the pTAC-1 vector, and the resulting

plasmids were transformed into *Escherichia coli* DH5α competent cells (DynaExpress TA Cloning Kit, BioDynamics Laboratory Inc., Tokyo, Japan). The sequencing reaction was performed using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems Inc., Foster City, California, USA), and the DNA sequences were analysed using an ABI PRISM 310 sequencer (Applied Biosystems Inc., Foster City, California, USA). BLAST searches of the GenBank database were performed to determine the closest relatives of partial 16S rRNA gene sequences.

Table 1. Characteristics of pre-ensiled crops

Item	Italian ryegrass	Guinea grass	Whole crop maize
Dry matter (g/kg)	212	170	253
pH	5.86	6.28	5.56
Water soluble carbohydrates (g/kg DM)	124	18.4	70.4
Lactic acid bacteria (log cfu/g)	<2.00	6.81	6.18
Yeasts (log cfu/g)	6.57	6.85	7.78
Enterobacteria (log cfu/g)	5.15	6.88	7.38

Data are means of duplicate analyses.

Data analysis

The data were subjected to analysis of variance, and differences due to storage period were determined using Tukey's multiple comparison. Differences between the microbial populations in the silage at silo opening and the populations in the silage after aerobic stability test were determined using Student's *t*-test. These analyses were carried out using the JMP software (ver. 7; SAS Institute, Tokyo, Japan).

RESULTS

The DM contents were 212, 170, and 253 g/kg and the WSC contents were 25, 19.3, and 70.4 g/kg DM for fresh IR, GG, and WM crops, respectively (Table 1). The epiphytic LAB count at the time of ensiling was more than 10^6 cfu/g in GG and WM, whereas the count was below the detectable level (<10^2 cfu/g) in IR.

Italian ryegrass silage fermentation characteristics and aerobic stability

2,3-Butanediol and ethanol were the predominant fermentation products in the IR silage (Table 2). Alcoholic fermentation was intensive in the initial stages of ensiling; the amounts of 2,3-butanediol and ethanol on d 3 were 58.2 and 27.7 g/kg DM, respectively, and after further increases by about 10 g/kg DM between d 3 and 7, the changes in the alcohol contents were small. The lactic acid content increased during the course of ensiling, from a low level on d 3 to a peak value on d 28. The acetic acid content was low in the IR silage. Butyric acid production was seen from the beginning of fermentation, and on d 120, the amount of butyric acid was more than that of lactic acid. The LAB numbers were counted at 10^7 cfu/g levels, regardless of the ensiling period. Although yeasts were enumerated at >10^7 cfu/g until d 28, the counts were below the detectable levels after d 56. Likewise, enterobacteria levels were >10^7 cfu/g up to d 14, after which they decreased to 10^5 cfu/g on d 28 and then became undetectable after d 56.

When 14-d and 28-d silages were exposed to air, the lactic acid content decreased and the pH value increased, indicating significant deterioration. The counts of LAB, yeasts, and enterobacteria had increased to 10^8 to 10^9 cfu/g

Table 2. Fermentation product contents and microbial counts of direct-cut Italian ryegrass silage determined at silo opening and after conducting a 7-d aerobic stability test

Item	At silo opening						SE	After 7-d aerobic stability test				SE	*t-test* for aerobic spoilage			
	3 d	7 d	14 d	28 d	56 d	120 d		14 d	28 d	56 d	120 d		14 d	28 d	56 d	120 d
DM (g/kg)	186^{a}	182^{abc}	182^{abc}	183^{ab}	173^{c}	176^{bc}	1.98	189	178	175	189	7.89	NS	NS	NS	NS
pH	5.54^{a}	5.47^{a}	5.04^{b}	4.97^{bc}	4.66^{d}	4.79^{cd}	0.05	6.72^{y}	7.05^{x}	4.73^{z}	4.81^{z}	0.17	**	**	NS	NS
LA (g/kg DM)	8.66^{c}	21.4^{bc}	30.0^{ab}	40.0^{a}	39.6^{ab}	25.1^{abc}	3.91	8.91^{y}	6.68^{y}	47.6^{x}	26.7^{xy}	4.48	**	**	NS	NS
C2 (g/kg DM)	6.16^{b}	5.19^{b}	4.44^{b}	6.11^{b}	6.91^{ab}	13.7^{a}	1.46	8.85^{xy}	6.22^{y}	11.7^{x}	13.8^{x}	3.57	NS	NS	*	NS
C3 (g/kg DM)	8.32^{a}	6.59^{ab}	4.91^{ab}	2.24^{b}	2.49^{b}	9.05^{a}	1.17	17.2^{x}	4.03^{y}	11.8^{xy}	6.05^{y}	4.45	**	NS	*	NS
2,3-BD(g/kg DM)	58.2^{b}	71.5^{ab}	67.0^{ab}	75.6^{ab}	64.3^{ab}	77.2^{a}	3.68	67.5^{xy}	12.8^{y}	80.7^{x}	68.3^{xy}	5.78	NS	**	NS	NS
C4(g/kg DM)	15.6^{b}	8.02^{b}	16.5^{b}	30.4^{a}	23.6^{ab}	36.7^{a}	1.21	12.0^{y}	5.69^{y}	41.2^{x}	35.5^{x}	5.28	NS	**	NS	NS
Ethanol (g/kg DM)	27.7^{b}	39.8^{a}	34.7^{ab}	39.3^{a}	33.7^{ab}	29.6^{b}	1.92	0.67^{y}	0.77^{y}	8.54^{x}	10.0^{x}	2.14	**	**	**	**
LAB (log cfu/g)	7.42	7.42	7.15	7.00	7.58	7.08	0.30	8.78^{x}	8.64^{xy}	8.58^{xy}	8.19^{y}	0.12	**	**	*	*
Yeasts (log cfu/g)	10.3	9.83	7.84	7.16	<2.00	<2.00	0.61	9.56^{x}	9.41^{x}	8.21^{xy}	7.68^{y}	0.37	**	**	-	**
ENB (log cfu/g)	8.74	8.87	7.10	4.90	<2.00	<2.00	0.17	9.30	9.00	<2.00	<2.00	0.12	**	**	-	-

Silos were opened after 3, 7, 14, 28, 56, and 120 d, and a 7-d aerobic stability test was conducted for the silages from the latter 4 silages.

Values with different letters ($^{a-b, x-y}$) within a column are significantly different from one another. NS, p≥0.05; * p<0.05; ** p<0.01.

DM = Dry matter; LA = Lactic acid; C2 = Acetic acid; C3 = Propionic acid; 2,3-BD = 2,3-butanediol; C4 = Butyric acid; LAB = Lactic acid bacteria; ENB = Enterobacteria.

Table 3. Fermentation product contents and microbial counts of direct-cut guinea grass silage determined at silo opening and after conducting a 7-d aerobic stability test

Item	At silo opening						SE	After 7-d aerobic stability test				SE	*t-test* for aerobic spoilage			
	3 d	7 d	14 d	28 d	56 d	120 d		14 d	28 d	56 d	120 d		14 d	28 d	56 d	120 d
DM (g/kg)	166^{a}	162^{ab}	167^{a}	152^{b}	153^{b}	151^{b}	2.40	166^{x}	162^{xy}	163^{x}	151^{y}	2.66	NS	*	*	NS
pH	5.96^{bc}	6.01^{bc}	5.58^{d}	6.67^{a}	6.19^{b}	5.80^{cd}	0.06	8.96^{x}	8.72^{x}	6.01^{y}	5.71^{z}	0.06	**	**	NS	NS
LA (g/kg DM)	14.0^{a}	7.67^{bc}	11.2^{ab}	2.96^{cd}	1.34^{d}	2.96^{cd}	1.15	0.00	0.42	0.32	1.53	0.49	**	**	NS	NS
C2 (g/kg DM)	26.0^{c}	39.6^{b}	42.1^{b}	44.9^{b}	56.8^{a}	57.8^{a}	2.15	0.00^{y}	2.13^{y}	50.0^{x}	54.5^{x}	5.77	**	**	NS	NS
C3 (g/kg DM)	0.60^{a}	0.00^{a}	1.00^{a}	2.26^{a}	3.25^{a}	2.32^{a}	0.90	0.00	0.00	0.86	1.63	0.60	NS	NS	NS	NS
2,3-BD (g/kg DM)	4.47	4.35	4.28	3.91	2.34	6.21	0.95	0.00^{y}	0.00^{y}	8.66^{x}	8.68^{x}	7.29	**	*	*	*
C4 (g/kg DM)	0.00^{b}	0.00^{b}	0.00^{b}	8.66^{a}	14.7^{a}	62.4^{c}	19.4	0.00^{y}	0.00^{y}	11.5^{x}	36.3^{x}	3.28	NS	NS	NS	**
Ethanol (g/kg DM)	3.20^{b}	7.09^{ab}	7.48^{ab}	10.3^{ab}	18.9^{a}	18.9^{a}	2.73	0.00^{y}	0.00^{y}	7.50^{x}	5.90^{x}	1.73	*	*	*	*
LAB (log cfu/g)	8.97	8.66	8.31	8.00	8.02	8.07	0.30	6.04^{y}	7.95^{x}	8.13^{x}	7.68^{x}	0.15	*	NS	NS	*
Yeasts (log cfu/g)	7.85^{ab}	7.32^{b}	7.14^{b}	8.22^{a}	8.12^{a}	8.15^{a}	0.26	7.21^{y}	8.86^{x}	8.83^{x}	7.66^{y}	0.33	NS	NS	*	*
ENB (log cfu/g)	7.01	6.84	6.76	7.52	7.10	6.83	0.21	7.06^{xy}	9.02^{x}	7.77^{xy}	5.38^{y}	0.60	NS	**	NS	*

Silos were opened after 3, 7, 14, 28, 56, and 120 d, and a 7-d aerobic stability test was conducted for the silages from the latter 4 silages.
Values with different letters ($^{a-b, x-y}$) within a column are significantly different from one another. NS, p≥0.05; * p<0.05; ** p<0.01.
DM = Dry matter; LA = Lactic acid; C2 = Acetic acid; C3 = Propionic acid; 2,3-BD = 2,3-butanediol; C4 = Butyric acid; LAB = Lactic acid bacteria; ENB = Enterobacteria.

from 10^7 cfu/g at silo opening. When 56-d and 120-d silages were subjected to aerobic stability tests, only a few changes were seen in the fermentation products, and thus, despite the increase in the yeast counts from undetectable levels at silo opening to 10^7 to 10^8 cfu/g levels, these silages were considered unspoiled. Small increases in the acetic acid and propionic acid contents were detected in the 56-d silage during the 7-d spoilage test.

Guinea grass silage fermentation characteristics and aerobic stability

Acetic acid was the predominant product in the GG silage (Table 3); the content was 26.0 g/kg DM on d 3 and then increased to 57.8 g/kg DM on d 120. Lactic acid, which was second to acetic acid in the initial stages of ensiling, decreased greatly after d 14, and only 3.0 g/kg DM was recorded on d 120. Distinctive changes were not observed in the 2,3-butanediol content over the course of ensiling. On the other hand, with prolonged ensiling, the ethanol content increased. With prolonged ensiling, butyric acid content also increased, and although it was not detected until d 14, the production was substantially enhanced from d 28, and finally, a level of 62.4 g/kg DM was observed on d 120. Large numbers of LAB, yeasts, and enterobacteria were detected at silo opening, regardless of the ensiling period.

After exposing the 14-d and 28-d GG silages to air, aerobic deterioration occurred, and the lactic acid, acetic acid, 2,3-butanediol, and ethanol contents decreased. Upon aerobic spoilage, the numbers of LAB and yeasts decreased, whereas that of enterobacteria increased. Aerobic deterioration did not take place in the 56- and 120-d silages. Although the counts of yeasts had increased in the 56-d silage after the 7-d spoilage test, those of LAB, yeasts, and enterobacteria decreased in the 120-d silage.

Whole-crop maize silage fermentation characteristics and aerobic stability

Lactic acid production was intensive from the beginning of fermentation in WM silage (Table 4). The content on d 3 was 50.4 g/kg DM, and the peak value was shown on d 28. Although the acetic acid content was kept below one-third of the lactic acid content, it increased greatly after d 56 and reached the maximum on d 120. The ethanol content was stable from the beginning to the end of fermentation. The LAB count was the highest on d 3 and then decreased gradually to 10^6 cfu/g levels on d 120. Similarly, yeast numbers decreased during prolonged ensiling. Although enterobacteria were counted at 10^7 cfu/g levels in pre-ensiled crops, the counts were below the detectable levels throughout the ensiling period.

When the 14-, 28-, and 56-d WM silages were exposed to air, the lactic acid, acetic acid, and ethanol contents showed a decrease, whereas the pH values showed increases. Upon aerobic spoilage, the LAB and yeasts counts increased from 10^7 to 10^8 cfu/g to 10^9 cfu/g levels, and that of enterobacteria was determined to 10^4 cfu/g, although the levels at silo opening were $<10^2$ cfu/g.

Italian ryegrass silage bacterial community

The DGGE patterns were different between the time of ensiling and d 3 in the IR silage (Figure 1). Distinctive bands of *Pantoea agglomerans* (band 5), *Enterobacter* sp. (bands 6 and 9), *Enterococcus faecium* (band 7), and *Rahnella aquatilis* (band 8) were detected over the course of ensiling. A band indicative of *Clostridium butyricum*

Table 4. Fermentation product contents and microbial counts of whole-crop maize silage determined at silo opening and after conducting a 7-d aerobic stability test

Item	At silo opening						SE	After 7-d aerobic stability test				SE	*t-test* for aerobic spoilage			
	3d	7 d	14 d	28 d	56 d	120 d		14 d	28 d	56 d	120 d		14 d	28 d	56 d	120 d
DM (g/kg)	234^{b}	248^{ab}	266^{a}	239^{b}	231^{b}	237^{b}	5.68	255^{x}	241^{x}	252^{x}	256^{y}	9.90	NS	NS	NS	NS
pH	3.85^{b}	3.79^{bc}	3.73^{cd}	4.00^{a}	3.73^{cd}	3.65^{d}	0.02	6.46^{x}	6.24^{x}	6.21^{x}	3.59^{y}	0.07	**	**	**	NS
LA (g/kg DM)	50.4^{b}	64.2^{ab}	58.6^{ab}	72.5^{a}	65.9^{a}	69.9^{a}	3.26	7.53^{y}	3.38^{y}	6.72^{y}	74.6^{x}	2.23	**	*	**	NS
C2 (g/kg DM)	15.0^{b}	16.9^{ab}	14.0^{b}	18.3^{ab}	19.0^{ab}	29.9^{a}	1.06	5.28^{y}	5.90^{y}	5.89^{y}	22.7^{x}	0.73	**	*	**	NS
C3 (g/kg DM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2,3-BD (g/kg DM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C4 (g/kg DM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol (g/kgDM)	16.7	19.7	15.2	16.8	20.6	17.9	2.05	0.00^{y}	0.00^{y}	0.00^{y}	15.48^{x}	0.27	*	*	**	NS
LAB (log cfu/g)	8.92^{a}	8.76^{a}	8.28^{b}	8.13^{b}	7.87^{b}	6.23^{c}	0.10	9.19^{x}	8.98^{x}	9.64^{x}	6.88^{y}	0.15	*	*	**	NS
Yeasts (log cfu/g)	6.62^{c}	5.82^{d}	8.25^{a}	8.24^{a}	7.50^{b}	6.23^{cd}	0.15	9.63^{x}	9.77^{x}	9.82^{x}	4.64^{y}	0.15	*	*	**	*
ENB (log cfu/g)	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	-	4.94	4.41	4.61	<2.00	-	-	-	-	-

Silos were opened after 3, 7, 14, 28, 56, and 120 d, and a 7-day aerobic stability test was conducted for the silages from the latter 4 silages.
Values with different letters ($^{a-b, x-y}$) within a column are significantly different from one another. NS, p≥0.05; * p<0.05; ** p<0.01.
DM = Dry matter; LA = Lactic acid; C2 = Acetic acid; C3 = Propionic acid; 2,3-BD = 2,3-butanediol; C4 = Butyric acid; LAB = Lactic acid bacteria; ENB = Enterobacteria.

(band 10) was detected on d 7 and was detected up to the end of ensiling. When 14- and 28-d silages were examined after the 7-d aerobic stability test, several non-LAB species such as *Staphylococcus sciuri* (bands 12 and 13), *Bacillus subtilis* (band 15), and *Acinetobacter* sp. (band 17) were newly detected. Although distinctive spoilage was not seen, the DGGE patterns of 56- and 120-d silages appeared to be similar to those of aerobically spoiled 14- and 28-d silages, respectively.

Guinea grass silage bacterial community

The DGGE patterns of the GG silage were also different from that of pre-ensiled crop from the beginning of fermentation (Figure 2). Many LAB species such as *L. plantarum* (band 22), *L. brevis* (band 24), *L. lactis* (bands 28 and 29), and *Lactococcus garvieae* (band 31) were detected in addition to non-LAB species such as *Klebsiella* sp. (band 21) and *Morganella morganii* (band 26). When the ensiling was prolonged, a number of new bands indicative of *L. plantarum* (band 25) and *Enterobacter cloacae* (band 32) were detectable. In spoiled 14- and 28-d silages, the number of DGGE bands increased during the 7-d spoilage test. In stable GG silages, however, the DGGE patterns appeared unchanged between before and after

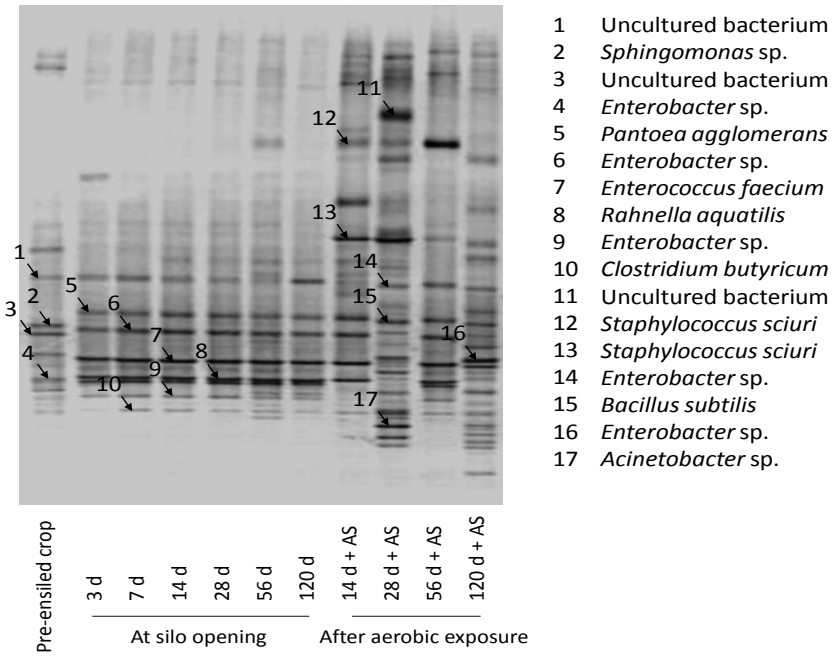

Figure 1. Bacterial communities in the ensiling process of direct-cut Italian ryegrass silage. Silos were opened after 3, 7, 14, 28, 56, and 120 d and a 7-d aerobic stability test was conducted for the silages from the latter 4 silages.

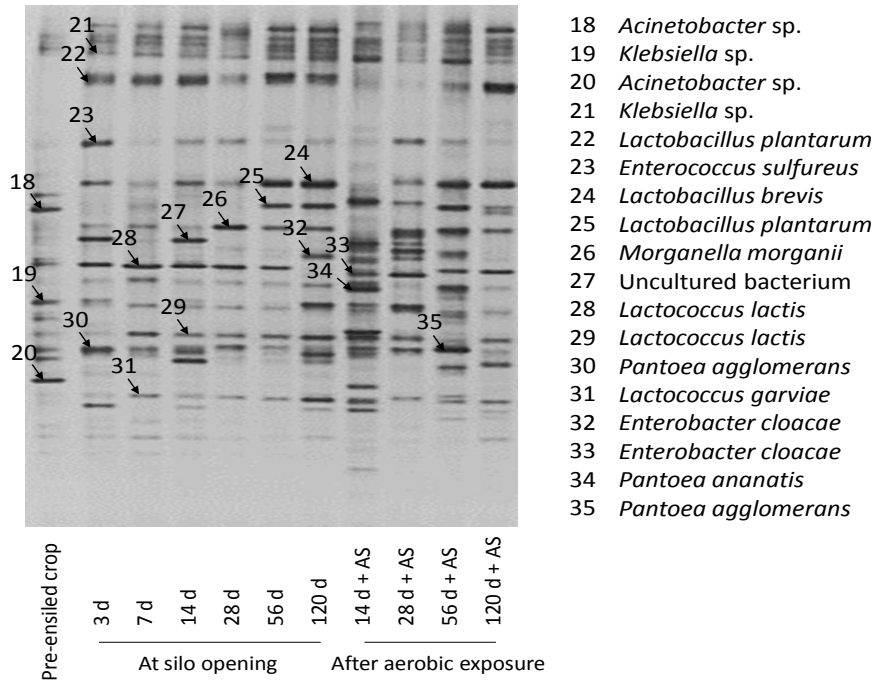

Figure 2. Bacterial communities in the ensiling process of direct-cut guinea grass silage. Silos were opened after 3, 7, 14, 28, 56, and 120 d and a 7-d aerobic stability test was conducted for the silages from the latter 4 silages.

exposure to air.

Whole-crop maize silage bacterial community

Marked changes in the DGGE patterns were observed during the initial 3-d ensiling in the WM silage (Figure 3). Nearly all the bands detectable on d 3 were LAB; *Weissella paramesenteroides* (band 38), *L. plantarum* (bands 39 and 42), *L. lactis* (band 40), *L. brevis* (band 41), *Pediococcus pentosaceus* (band 47), *Pediococcus parvulus* (band 43), and *Weissella confusa* (band 45) were detected. Except the aerobically stable 120-d silage, the bands for *L. plantarum* and *P. parvulus* disappeared, whereas those for *B. subtilis*

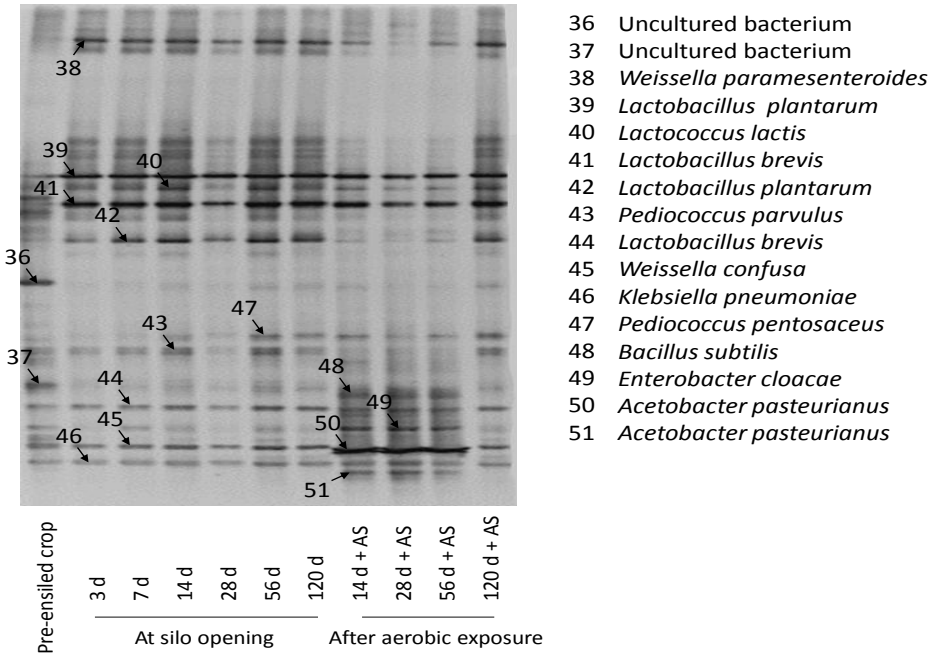

Figure 3. Bacterial communities in the ensiling process of whole-crop maize silage. Silos were opened after 3, 7, 14, 28, 56, and 120 d and a 7-d aerobic stability test was conducted for the silages from the latter 4 silages.

(band 48), *E. cloacae* (band 49), and *Acetobacter pasteurianus* (bands 50 and 51) newly appeared after the 7-d aerobic stability test.

DISCUSSION

Although sufficient levels of the WSC content were found in the pre-ensiled crops, 2,3-butanediol and ethanol production were enhanced in the IR silage, whereas intensive lactic acid production was seen in the WM silage. The bacterial community data were consistent with these differences; many types of enterobacteria were detected from the beginning of fermentation in the IR silage, whereas nearly all DGGE bands on d 3 were identified as LAB in the WM silage. Because epiphytic LAB numbers were $<10^2$ cfu/g in pre-ensiled IR, the enterobacteria detected on d 3 may have outcompeted the other bacteria and produced large amounts of 2,3-butanediol and ethanol. Notably, the enterobacteria found in the pre-ensiled IR were promptly replaced by enterobacteria that had adapted to the silage environment. This implies that, although increases in the enterobacterial count were small during the initial 3-d ensiling, the community structure was greatly altered. However, few differences were seen in the DGGE patterns of the IR silage after d 56, although the enterobacterial counts were below the detectable levels on d 56 and 120. Moreover, the changes observed in the DGGE patterns could not explain how the lactic acid content gradually increased until d 28 in the IR silage. DGGE analysis is qualitative or semi-quantitative, and thus limitations exist in understanding the changes in the metabolic activities of microorganisms. Nevertheless, the analysis was helpful in detecting the association of *C. butyricum* in the IR silage; the DNA band was visible from the beginning of fermentation, accounting for the production of butyric acid from the initial stages of ensiling.

In tropical ensiling, acetic acid rather than lactic acid often becomes the predominant fermentation product (Catchpoole and Henzell, 1971; Parvin and Nishino, 2009). High-moisture preparation and prolonged ensiling may intensify this acetic acid fermentation (Nishino et al., 2012); hence, the use of direct-cut GG and assessments from the initial to the late stages of fermentation could help in understanding which types of bacteria are involved in the tropical grass silage. However, the bacteria associated with the enhanced acetic acid production from the beginning of fermentation were not clearly identified. Although the appearance of *L. plantarum* (band 25) and *M. morganii* (band 26) after d 28 could account for the increase in the acetic acid content in the late stages of ensiling, no distinctive changes were seen in the DGGE patterns between d 3 and 7, during which marked changes in the lactic and acetic acid contents occurred. We have speculated that *L. plantarum* is involved in the increase in acetic acid content as a result of prolonged ensiling (Parvin and Nishino, 2009; Nishino et al., 2012), because *L. plantarum* can metabolize lactic acid to acetic acid under sugar-deficient conditions (Lindgren et al., 1990). The same metabolic process may have occurred in this study because *L. plantarum* (band 22) was detected from the initial stage to the end of GG ensiling. Nevertheless, the increase in the acetic acid content was much greater than the decrease in the lactic acid content; hence, lactic acid metabolism could account for a part of the enhancement of acetic acid fermentation. In this regard, the amino acid-degrading activities of *M. morganii* and other bacteria might have contributed to the increase in the acetic acid content. However, although a distinctive increase in the butyric acid content was observed after d 56, no bands indicative of *Clostridium* spp. were seen in the bacterial community of the GG silage.

In the case of WM ensiling, the fermentation products data were consistent with the bacterial community data and accounted for the enhanced lactic acid production from the beginning of fermentation. This is because almost all DGGE bands were detected as LAB species and few changes were seen from the prolonged ensiling in both the amount of the fermentation products and the DGGE band patterns. Small increases in the lactic acid and acetic acid contents during long storage periods may indicate sustained activities of hetero-fermentative LAB species.

In this study, the results of DGGE analysis helped to account for the differences in the composition of the fermentation products among the IR, GG, and WM silages; however, these results could not explain how acetic acid fermentation is enhanced in the GG silage. The changes in the bacterial community that had taken place in the first 3 d of ensiling could be considered interesting findings. Regardless of the silage crops, the DGGE patterns on d 3 were different from that on the day of ensiling. Although $>10^6$ cfu/g levels of LAB were counted in the GG and the WM at the time of ensiling, the composition of LAB species was greatly altered during the first 3 d of ensiling. Given that DGGE analysis is not quantitative, small populations may be difficult to detect. Therefore, the prompt changes in the DGGE patterns found at the very beginning of fermentation suggest that, although a large number of LAB were enumerated in the pre-ensiled crop, many of these may be outcompeted by the LAB species that are adaptable to the silage environment.

Differences were also seen between the silage crops in the bacterial species that appeared upon aerobic spoilage. *Enterobacter* sp. was detected in common among all 3 spoiled crop silages, *Bacillus* sp. was present in the spoiled IR and WM silages, *S. sciuri* was observed in the spoiled IR silage, and *A. pasteurianus* was detected in the spoiled WM

silage. Because we examined the bacterial community before and after the 7-d aerobic stability test, we could not ascertain whether these bacteria were spoilage-initiating or follow-up species. Yeasts, moulds, and acetic acid bacteria are considered the main microorganisms associated with aerobic spoilage; however, the populations of *Bacillus* spp. and enterobacteria may also increase when the silage is spoiled (Woolford 1990; Driehuis and Oude Elferink, 2000). *A. pasteurianus* is known to show strong crop specificity with respect to the corn and cereal silages it inhabits (Oude Elferink et al., 2001), which was reasonably confirmed in this study.

Aerobic spoilage did not take place in the IR and GG silages unloaded on d 56 and 120, and in the WM silage unloaded on d 120. The main fermentation products that inhibit yeasts and moulds are acetic, propionic, and butyric acids in the silage; hence, the increased acetic and butyric acids in the IR and GG silages and the increased acetic acid in the WM silage could function as inhibitory substances. Because the GG silage showed high acetic acid content (>26.0 g/kg DM) from d 3, explaining why the 14- and 28-d silages were spoiled would be difficult. However, the GG silages had high pH values (>6.0); hence, the proportion of undissociated acetic acid was calculated to be approximately 0.10. In contrast, the 120-d WM silage showed a low pH of 3.65, and therefore, >0.90 of the acetic acid was present in the undissociated form. For the suppression of microorganisms, undissociated acid is of primary importance, and dissociated molecules have minor effects (Courtin and Spoelstra, 1990; Muck et al., 1991). Therefore, the results in this study reconfirm the importance of undissociated acids in spoilage inhibition.

CONCLUSIONS

Changes in the live counts, fermentation product contents, and composition of the bacterial communities were examined from d 3 to d 120 in the course of ensiling of the IR, GG, and WM silages. DGGE analysis reasonably accounted for the types of bacteria that dictated alcoholic fermentation in the IR silage and lactic acid fermentation in the WM silage. Although the analysis did not help in identifying the bacterial species responsible for acetic acid fermentation in the GG silage, metabolic changes rather than changes in the community structure were suggested to be involved in the fermentation. When marked changes were detected in the amount of the fermentation products, distinctive changes were also found in the bacterial community structures. However, we could not ascertain whether these were spoilage-initiating or follow-up bacteria. Although our results demonstrate the usefulness of DGGE analysis in elucidating the ensiling process, data from plate cultures are also helpful for adding quantitative information to the data from DGGE analysis.

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