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The survival of silage bacteria in the intestine was evaluated by identifying lactic acid bacteria in the feces of dairy cows given whole crop maize silage.

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ABSTRACT

One representative silage sample and three separate feces samples from dairy cows on three dairy farms in Hua Bei, China and three dairy farms in Kyushu, Japan were collected to evaluate the survival of lactic acid bacteria (LAB) in whole crop maize silage in the guts of dairy cows. Quantitative polymerase chain reaction and denaturing gradient gel electrophoresis were used to analyze the bacterial community makeup. All bunker-made corn silage samples, independent of dairy farm or sampling location, contained Lactobacillus acetotolerans. There were eight different LAB species found in the maize silage samples, but only three of them seemed to survive digestion: L. acetotolerans, L. pontis, and L. casei. Silage and feces L. acetotolerans populations were 106-7 and 103-4 copies/g, respectively, suggesting that competition in this niche may be tough and the population may drastically decline throughout the digestion process, even for the LAB species demonstrating possible survival in the gut. Because even surviving species might experience a significant population drop during digestion, it may be challenging for silage LAB to survive in the stomach of silage-fed dairy cows.

Key words: dairy cows, denaturing gradient gel electrophoresis, lactic acid bacteria, quantitative polymerase chain reaction, silage.

INTRODUCTION

Ensiling is a forage preservation method wherein epiphytic lactic acid bacteria (LAB) anaerobically metabolize water-soluble carbohydrates to organic acids. To improve silage fermentation and suppress aerobic spoilage after silo opening, inoculants pre- pared from selected LAB strains are often used. Although improved fermentation does not always lead to better animal performance, LAB inoculation has been shown to occasionally improve animal per- formance without having any apparent advantageouseffect on fermentation (Weinberg & Muck 1996). Such positive results suggested that silage LAB pro- vide a probiotic effect. Therefore, studies aimed at clarifying and developing probiosis through LAB inoculation are increasing.

Even if LAB inoculant strains have the potential for probiosis, their ability to compete with other silage microorganisms may vary according to the strain, the crop and the ensiling conditions, such as extent of wilting and the degree of anaerobiosis. In addition, the LAB species detected in silage inocu- lants differ from those in the ruminant gastrointes- tinal tract. *Lactobacillus plantarum*, *L. acidophilus*, *L. casei*, *L. buchneri* and *Enterococcus faecium* are the species commonly used in silage inoculant, whereas

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Streptococcus bovis, *L. vitulinus*, *L. ruminis*, *L. johnsonii* and *L. murinus* are usually found in the cattle gut (Krause *et al.* 2003; Hernandez *et al.* 2008; Nader-Maias *et al.* 2008). In our previous study, we fed non-inoculated, wilted Italian ryegrass silage to goats with and without concentrates, and determined the survival of silage LAB in rumen fluid and fecesby denaturing gradient gel electrophoresis (DGGE) analysis (Han *et al.* 2012). Although *Enterococcus* sp., *L. plantarum*, *L. brevis*, *L. murinus* and *Weissella cibaria* were found in silage, only *L. murinus* was detected in rumen fluid and feces regardless of feeding concentrates. *L. plantarum* and *L. brevis*, which are often used as silage inoculants, disappeared in the goat gastrointestinal tract. Therefore, we concluded that it may be difficult for silage LAB to confer probiosis.

In the present follow-up to our previous mini-silo and small ruminant study, we performed a practical survey to monitor LAB communities inhabiting bunker-made whole-crop maize silage and feces of silage-fed dairy cows. In order to represent the bacterial communities found in diverse silages produced in practice, samples were collected from three dairy farms in Hua Bei, China and three dairy farms in Kyushu, Japan. In addition to qualitative DGGE, quantitative PCR (qPCR) was performed in order to assess the survival rate of silage LAB in the gut. No information on the chemical composition and bacterial community of the pre-ensiled crop was obtained; however, such a survey may provide further insight into the use of silage for propagation and delivery of probiotic LAB in ruminants.

MATERIALS AND METHODS

Sampling

Silage samples were collected from six dairy farms in Hua Bei, China in August 2012 (farms 1, 2 and 3) and Kyushu, Japan in December 2012 (farms 4, 5 and 6). Farms 1, 2 and 3 were located in Shan Xi province, He Bei province and Beijing city, respectively, whereas farms 4, 5 and 6 were all in Kumamoto province. All farms produced whole-crop maize silage using bunker silos without any inoculants, and the silages had been stored for approximately 10 months (Hua Bei) and 4 months (Kyushu) at the time of sampling. Using disposable plastic gloves, samples were collected by digging into the silo face to approximately a 0.2 m depth and manually removing approximately 500 g of silage. Five samples(two outer samples from the top layer, two outersamples from the bottom layer, and one sample fromthe central part) were obtained from each bunkersilo and were thoroughly mixed to prepare the rep- resentative sample. In addition, fecal samples were obtained from the rectum of three dairy cows fed with maize silage from each farm. The proportion of maize silage in the total diet varied from 0.20 to 0.40 on a dry mater (DM) basis. Approximately 500 g of silage and 1 g of feces were put in a plastic bag and an Eppendorf tube, respectively, and kept on ice dur- ing transport to the laboratory.

Chemical analyses

The DM content of silage was determined after oven drying at 60°C for 48 h. The pH value and fermentation products in water extracts were determined. Lactic acid, acetic acid and ethanol contents were measured by ion-exclusion polymeric high-performance liquid chromatography with refractive index detection as previously described (Han *et al.* 2012).

GGE

Extraction and purification of the bacterial DNA in silage was performed using a commercial kit (DNeasy Tissue Kit; Qiagen, Germantown, MD, USA). Bacterial DNA was purified from the fecal samples using the mini DNeasy Stool Kit (Qiagen). DGGE was per- formed as previously described (Han *et al.* 2012). In brief, a nested-PCR approach was used to detect the *Lactobacillus*-specific DNA, which involved an ini- tial PCR with the *Lactobacillus*-specific primers LAB159f (5⁰-GGAAACAG(A/G)TGCTAATACCG-3⁰) and LAB677r (5⁰-GGAAACAG(A/G)TGCTAATACCG-3⁰), followed by a second PCR with the GC-containing universal primers GC357f (5⁰-CGCCCGCCGCGC GCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACG GGAGGCAGCAG-3⁰) and 517r (5⁰-ATTACCGCGG CTGCTGG-3⁰), which amplified the variable region (V3) of the16S ribosomal RNA (rRNA) gene.

The GC-clamp PCR products were separated based on their sequences with a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Tokyo, Japan). The samples were applied directly onto 80 g/L polyacrylamide gels prepared in a denaturing gradient from 25% to 50% using 7 mol/L urea and 400 mL/L formamide as 100% denaturants.

Selected bands were excised from the DGGE gels, and the DNA was amplified by PCR using the 357f (without the GC-clamp) and 517r primers. After purifying the reaction products using a commercial clean up kit (Gene Clean Kit; Qbiogene, Carlsbad, CA, USA), the PCR products were cloned into the pTAC-1 vector, and the resulting plasmids were transformed into *Escherichia coli* DH5a competent cells (Dyna Express 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, CA, USA), and DNA sequences were ana-lyzed using an ABI PRISM® 3130 sequencer (Applied Biosystems Inc.).

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Quantitative PCR (qPCR)

Quantitative PCR was carried out on a MiniOpticon™ System (Bio-Rad Laboratories, Inc.). For quantification of both total bacteria and *L. acetotolerans*, 2 1L of DNA solution was added to 23 1L of a PCR mixture containing 12.5 1L of KAPA SYBR FAST Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA) and 1.0 1L of each 8 1mol/L primer. Primers of Laceto70f (5⁰-GAGCCGAACCAATTGATTACC-3⁰) and Laceto249r (5⁰-TTTAGCGACAGCTTACGCCG-3⁰) were used for quantification of *L.* acetotolerans,[21] and those of 357f and 517r (described earlier) were used for quantification of total bacteria. Serial dilution ser-ies of plasmids carrying the nearly full-length 16S rDNA gene of *E. coli* (for total quantification) and *L. acetotolerans* were used as known concentrations of the standard plasmid. The cycle parameters for total bacteria assay were as follows: 30 s at 95°C and 35 cycles of 15 s at 95°C, 20 s at 60°C and 30 s at 72°C. Those for *L. acetotolerans* assay were as follows: 30 s at 95°C and 40 cycles of 30 s at 95°C, 10 s at 58°C and30 s at 72°C. Copy number of each standard plasmid was calculated using the molecular weight of nucleic acid and the length (base pair) of the cloned plasmid.

Data analyses

The bacterial species were identified by Basic Local Alignment Search Tool (BLAST) with partial 16SrRNA gene sequences using the GenBank database, and their closest relatives were determined. The rRNAgene sequences determined in this study have been deposited in the DNA Data Bank of Japan (accession numbers AB853260–AB853311 for bands 1–52 and AB872144–AB872145 for bands 53–54, respectively).

To validate the *Lactobacillus*-specific PCR procedure and to describe the similarities and differences within and among the farm samples, the DGGE band profiles were analyzed using an image analysis system (Image J; National Institutes of Health, Bethesda, MD, USA). We used 26 bands (non-specific vs. *Lactobacillus*-specific) and 24 bands (within and among the farm samples) to compile a list of binary numbers and principal coordinate analysis (PCoA) was performed on the Bray-Curtis similarities matrix using the Primer version 7 with Permanova+ add-on software (Primer-E, Plymouth Marine Laboratory, Plymouth, UK).

Data for qPCR were subjected to a two-way analysis of variance with place of sampling and target of amplification as the factors. Tukey's multiple comparisons test was used to determine differences between silage and feces and between total bacteria and *L. acetotolerans* populations. All analyses were performed using JMP software (ver. 11; SAS Institute, Tokyo, Japan).

RESULTS

Silage fermentation characteristics

A remarkable difference in the DM content was observed between silage obtained from Hua Bei (221 g/kg) and silage obtained from Kyushu (299 g/kg) (Table 1). Although no statistical differences were seen, mean lactic acid, acetic acid and ethanol contents in Hua Bei silages were 60%, 12% and 188% greater, respectively, than the Kyushu silages. A small amount of butyric acid (<1 g/kg DM) was found in two high-moisture Hua Bei silages (silages 1 and 2), and 1,2-propanediol was detected in all silages exceptfor silage 6. Although the 1,2-propanediol content was lower than the ethanol content in Hua Bei silages,the opposite was seen in Kyushu silages.

Lactobacillus-specific and non-specific DGGE analysis

Bacterial communities detected using universal primers (non-specific) only and a combination of

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Table 1 Microbial counts and fermentation products of whole crop corn silage produced in bunker silos in Hua Bei, China and Kyushu, Japan

Sampling region	Hua Bei						Kyushu				*P*-value
Farm	No. 1	No. 2	No. 3	Mean	SD	No. 4	No. 5	No. 6	Mean	SD	
Dry matter (DM) (g/kg)	219	219	225	221	3.46	313	295	290	299	12.1	0.00
pH	3.92	3.51	3.52	3.65	0.24	3.98	3.94	3.88	3.93	0.05	0.11
Lactic acid bacteria (log cfu/g)	5.85	5.30	4.79	5.31	0.53	7.57	3.65	6.93	6.05	2.10	0.59
Yeasts (log cfu/g)	4.15	5.70	6.60	5.48	1.24	7.61	4.62	6.90	6.38	1.56	0.48
Lactic acid (g/kg DM)	59.5	129	97.3	95.3	34.8	47.1	57.2	73.7	59.4	13.4	0.17
Acetic acid (g/kg DM)	51.1	45.4	26.6	41.0	12.8	33.8	46.7	29.2	36.6	9.04	0.65
Propionic acid (g/kg DM)	14.7	0.00	0.00	4.90	8.49	0.00	0.00	11.7	3.91	6.77	0.88
Butyric acid (g/kg DM)	0.62	0.74	0.00	0.45	0.40	0.00	0.00	0.00	0.00	0.00	0.12
Ethanol (g/kg DM)	15.1	34.2	19.7	23.0	9.97	3.94	10.2	9.78	7.97	3.50	0.07
1,2-Propanediol (g/kg DM)	5.50	25.1	13.3	14.6	9.87	25.2	42.9	0.00	22.7	21.6	0.59
1-Propanol (g/kg DM)	16.2	10.5	3.60	10.1	6.31	2.39	0.78	2.49	1.89	0.96	0.09

Individual and summarized data are shown to describe the differences and similarities within and between the sampling regions.

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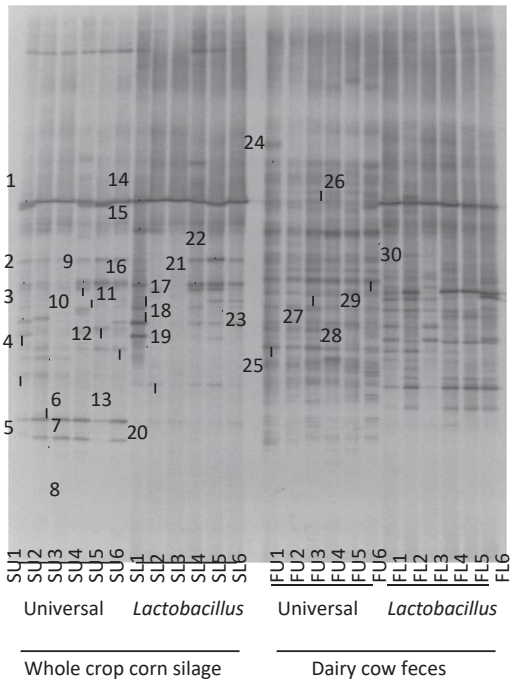

1. *Lactobacillus acetotolerans*
2. *Lactobacillus pontis*
3. *Lactobacillus vini*
4. *Bacillus* sp.
5. *Lactobacillus casei*
6. *Curtobacterium flaccumfaciens*
7. *Acetobacter pasteurianus*
8. *Enterobacter* sp.
9. *Bacillus* sp.
10. *Bacillus* sp.
11. *Lactobacillus pontis*
12. *Lactobacillus helveticus*
13. Uncultured bacterium
14. *Lactobacillus acetotolerans*
15. *Lactobacillus pontis*
16. *Lactobacillus helveticus*
17. *Lactobacillus pontis*
18. *Lactobacillus vini*
19. *Lactobacillus reuteri*
20. *Lactobacillus casei*
21. *Lactobacillus pontis*
22. *Lactobacillus amylolyticus*
23. *Lactobacillus helveticus*
24. Uncultured bacterium
25. Uncultured bacterium
26. Uncultured bacterium
27. Uncultured bacterium
28. Uncultured bacterium
29. Uncultured bacterium
30. Uncultured bacterium

Figure 1 Bacterial communities in bunker-made whole crop maize silage and dairy cow feces determined using universal primers only (Universal) and a combination of *Lactobacillus*-specific and universal primers (*Lactobacillus*). SU and SL stand for silage bacterial community determined by universal and *Lactobacillus*-specific primers, and FU and FL stand for fecal bacterial
community determined by universal and *Lactobacillus*-specific primers, respectively.

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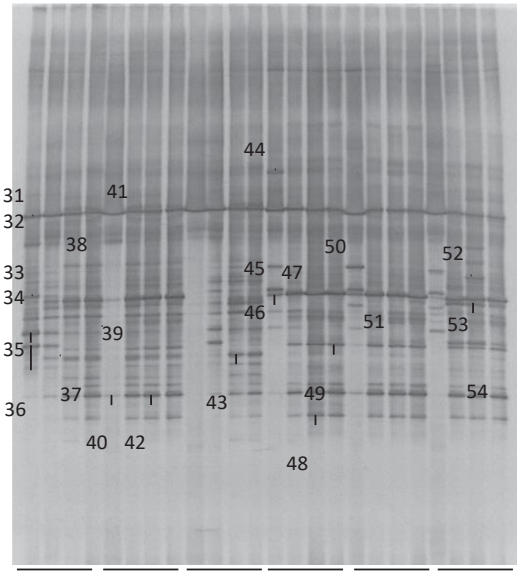
Lactobacillus-specific and universal primers are shown in Figure 1. The LAB species detected by the non- specific DGGE were *L. acetotolerans* (band 1), *L. pontis* (bands 2 and 11), *L. vini* (band 3) and *L. casei* (band5). In addition, several non-LAB species such as *Bacillus* sp. (bands 4, 9 and 10), *Curtobacterium fiaccumfaciens* (band 6), *Acetobacter pasteurianus* (band7) and *Enterobacter* sp. (band 8) were detectable bythe non-specific DGGE. If a *Lactobacillus*-specific DGGE was performed, these non-LAB species disap- peared or were replaced by LAB species at the same migration distance. Bands for *Curtobacterium fiaccum- faciens* (band 6), *A. pasteurianus* (band 7), *Enterobacter* sp. (band 8) and an uncultured bacterium (band 13) disappeared, whereas those for *Bacillus* sp. (bands 4and 9) were replaced by *L. reuteri* (band 19) and *L. pontis* (band 21), respectively. *L. acetotolerans* (band 1) and *L. vini* (band 3) were commonly detected by non-specific and *Lactobacillus*-specific DGGE at the same migration distance. Furthermore, *L. pontis* (band 15) and *L. helveticus* (band 23), which were not detected in the non-specific DGGE, were detected by *Lactobacillus*-specific DGGE. In contrast, band 16, which migrated to the same position as *L. pontis* band 2, was identified as *L. helveticus* by *Lactobacillus*-specific DGGE, and bands 5 and 20, which were both identified as *L. casei*, migrated close to but at a different position between the non-specific and *Lactobacillus*-specific DGGE.

Few bands in the dairy cow feces samples migratedto the same position in the non-specific and*actobacillus*-specific DGGE experiments. We examined seven distinctive bands present in the non-specific DGGE (bands 24–30), and all were identified as uncul- tured bacteria. Numerous changes in the band pat- terns were observed in the *Lactobacillus*-specific DGGE; the major bands were identified as LAB species.

PCoA and similarity profile analysis

The efficacy of *Lactobacillus*-specific primers in detect- ing LAB communities in silage and feces was demon- strated using a PCoA and similarity profile analysis (Fig. 3a). Silage bacterial communities formed three distinct groups, and the band profiles for non-specific (SU1–6) and *Lactobacillus*-specific (SL1–6) amplifica- tion were regarded as different, except for that of silage 6. Likewise, bacterial communities in feces were evaluated as three different groups, with clear separation observed between non-specific (FU1–6) and *Lactobacillus*-specific (FL1–6) amplicons. Although the band profiles for non-specific amplicons were evaluated as one group, those for *Lactobacillus*- specific amplicons in the Hua Bei (FL1–3) and the Kyushu (FL4–6) samples were regarded as different.

The *Lactobacillus*-specific DGGE band patterns of dairy cow feces were similar within individual farms, whereas those for feces 1 of Hua Bei farm 1 (FH1) and feces 7 of Hua Bei farm 3 (FH7) were differ- ent from the other samples obtained in the same farms (Figs 2,3b). The LAB species detected at the same migration distance in silage and feces were



31. *Lactobacillus acetotolerans*
32. *Lactobacillus pontis*
33. *Lactobacillus helveticus*
34. *Lactobacillus pontis*
35. *Lactobacillus vini*
36. *Lactobacillus reuteri*
37. Uncultured bacterium
38. Uncultured bacterium
39. *Lactobacillus pontis*
40. *Lactobacillus casei*
41. *Lactobacillus acetotolerans*
42. *Lactobacillus casei*
43. *Weissella paramesenteroides*
44. *Lactobacillus fermentum*
45. *Lactobacillus pontis*
46. *Lactobacillus pontis*
47. *Lactobacillus pontis*
48. *Lactobacillus diolivorans*
49. *Lactobacillus casei*
50. *Lactobacillus amylolyticus*
51. *Lactobacillus helveticus*
52. Uncultured bacterium
53. *Lactobacillus casei*
54. *Lactobacillus casei*

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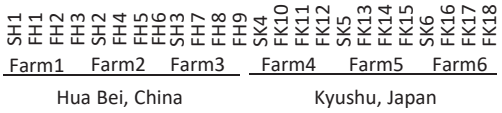
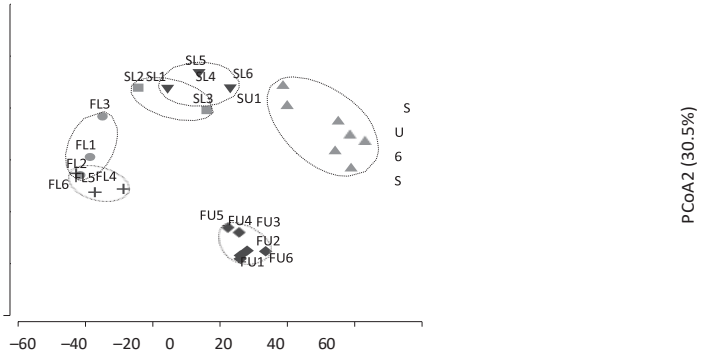


Figure 2 Lactic acid bacteria communities of bunker-made whole crop maize silage and dairy cow feces from six farms in Hua Bei, China and Kyushu, Japan. SH and SK indicate silage lactic acid bacteria community of Hua Bei and Kyushu farm samples, and FH and FK indicate fecal lactic acid bacteria community of Hua Bei and Kyushu farm samples, respectively.(a)Universal vs. *Lactobacillus*-specific



(b)Silage vs. Feces

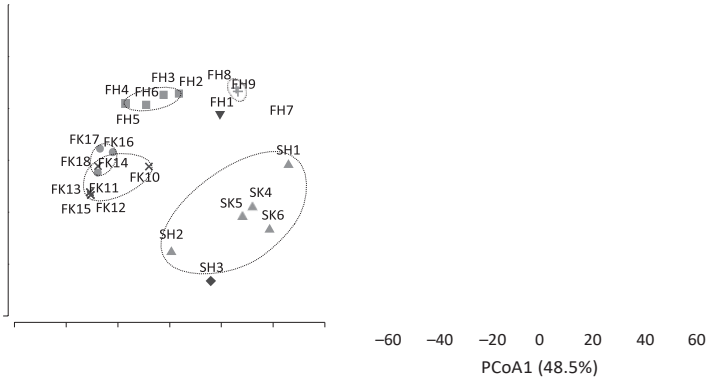


Figure 3 The principal coordinate analyses (PCoA) to explore the differences between non-specific and *Lactobacillus*-specific denaturing gradient gel electrophoresis (a) and to demonstrate that the lactic acid bacteria communities of silage and silage-fed dairy cow feces and of Hua Bei and Kyushu farm samples are related (b). SU and SL stand for silage bacterial community determined by universal and *Lactobacillus*-specific primers, and FU and FL stand for fecal bacterial community determined by universal and *Lactobacillus*-specific primers, respectively. SH and SK indicate silage lactic acid bacteria commu-nity of Hua Bei and Kyushu farm samples, and FH and FK indicate fecal lactic acid bacteria community of Hua Bei and Kyushu farm samples, respectively. The values in parenthesis are the percentages of the variation accounted for by the PCoA axes.*L. acetotolerans* (band 41), *L. pontis* (bands 39 and 47) and *L. casei* (band 42). No bands corresponding to *L. helveticus* (bands 33 and 51), *L. fermentum* (band 44) and *L. amylolyticus* (band 50) were observed in dairy cow feces. Several bands were detected exclu-sively in dairy cow feces; *Weissella paramesenteroides* (band 43) and *L. diolivorans* (band 48) were found in feces but not in silage. Although *L. pontis* (bands 39 and 47) and *L. casei* (band 42) were considered to be transferred from silage to feces, other bands for these AB species (bands 32 and 45 for *L. pontis* in silageand bands 49, 53 and 54 for *L. casei* in feces) werenot present in the counterpart samples.

The PCoA showed a clear difference in the LAB communities of the Hua Bei (SH1–3) and Kyushu (SK4–6) silage samples from farms, and the LAB communities of Hua Bei (FH1–9) and Kyushu (FK10–18) feces samples from dairy cows (Fig. 3b). Silage LAB formed one group irrespective of the source farm or the sampling region, except for silage from Hua Bei farm 3. Feces LAB formed four groups, whereas feces 1 of Hua Bei farm 1 (FH1) and feces 7 of Hua Bei farm 3 (FH7) were not grouped with any other samples. Fecal LAB from dairy cows of the Hua Bei and Kyushu farms were clearly distinct, and regional differences in the LAB communities appeared greater in feces than in silages.

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qPCR analysis

Total bacterial counts, as determined by qPCR, in maize silage and dairy cow feces were 10^8 10^9 and 10^9—10^{10} copies/g, respectively (Fig. 4). The population of *L. acetotolerans*, specifically, in silage was 10^6 ~~10^7~~ copies/g, whereas it was as low as 10^3 10^4 copies/g in feces. The proportions of *L. acetotolerans* relative to total bacteria in silage averaged 0.14 and and those in feces averaged 0.00017 and 0.00019 for Hua Bei and Kyushu samples, respectively.

DISCUSSION

In this study, we used the *Lactobacillus*-specific pri- mers and a nested-PCR procedure to analyze the

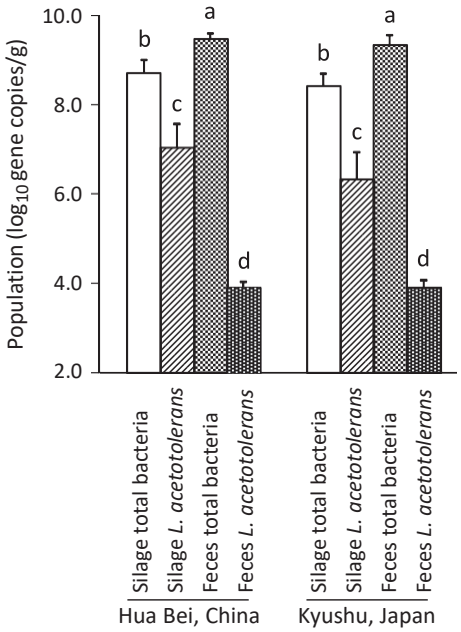

Figure 4 Quantitative PCR analysis to quantify populationsof total bacteria and *Lactobacillus acetotolerans* in whole crop maize silage and feces of silage-fed dairy cows.
Columns indicate mean values for silage (n = 3) and feces(n = 9) with standard deviations represented by vertical bars.
Tukey's multiple comparisons test was used to detect significant differences between total bacteria and *L. acetotolerans* populations in silage and feces (P < 0.05).Difference between populations from Hua Bei and Kyushu was not significant.

LAB community of silage and cow feces. Because the population of LAB among the total bacteria popula- tion in feces is small (Rudi *et al.* 2012), detecting LAB species may be difficult if diverse species are amplified by the universal primers. Indeed, in the non-specific DGGE of cow feces, many bands were identified as uncultured bacteria. In the silage LAB community analysis, we usually do not use *Lacto- bacillus*-specific primers because most bands can be attributed to known or cultivated species. Compar- ison of the two DGGE methods could show how *Lactobacillus*-specific amplification revises the infor- mation from the DGGE analysis, and how difficult it is to detect LAB species in cow feces without use ofthe genus-specific primers. This was supported by the finding that the bacterial count for *L. acetotolerans* was 10^{3-4} copies/g and its relative proportion to total bacteria in feces was about 0.0002, as determined by qPCR.

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The fact that a number of the bands identified as non-LAB species in the non-specific DGGE were replaced by bands identified as LAB species in the *Lactobacillus*-specific DGGE indicated the presence of multiple DNAs in a non-specific DGGE band. Although DGGE analysis can provide a rapid and repeatable characterization of the bacterial commu- nity, the level of resolution is known to be low, and co-migration of DNA from multiple species may occur (Sekiguchi *et al.* 2001). Therefore, we usually clone re-amplified PCR products from DGGE bands before determining the DNA sequences. The fact that multiple bands detected at different migrating posi- tions belonged to the same bacterial species was alsoconsidered. Multiple heterogeneous 16S rRNA gene copies may exist in a bacterium and heteroduplexmolecules can form during the PCR amplification process (Muyzer & Smalla 1998), leading to band duplication in the DGGE analysis of the bacterial community.

high acetic acid environments (Entani *et al.* 1986; Matsushita *et al.* 1994); therefore, their growth

.*Lactobacillus pontis*, *L. helveticus*, *L. vini* and *L. reuteri* were only detected ῑn Hua Bei silage 1. *L. pontis* and *L. vini* have been previously isolated from fermented food/feed (Wiese *et al.* 1996; Rodas *et al.* 2006), but never from silage by plate-culture. *L. helveticus* is a common silage LAB, whereas *L. reuteri* is rarely detected in silage, even by culture-independent anal- ysis. *L. fermentum* and *L. amylolyticus* were exclusively detected in Kyushu silages. *L. fermentum* is a commonsilage LAB (Chen *et al.* 2012) and *L. amylolyticus* is known to metabolize starch into lactic acid (Bohak*et al.* 1998); therefore, detection of this species in maize silage is reasonable. Although the amounts of propionic acid, 1,2-propanediol, and 1-propanol found in all or some silages suggested the activities of *L. buchneri* and *L. diolivorans* (Oude Elferink *et al.* 2001; Krooneman *et al.* 2002), these two LAB species were not detected in our silage samples.

The LAB species detected in cow feces were *L. pontis*, *L. casei*, *L. acetotolerans*, *W. paramesenteroides* and *L. diolivorans*. *W. paramesenteroides* was detected in all cow feces from Hua Bei, but was not detectedin any maize silage. *W. paramesenteroides* is often seen in whole crop maize silage (Parvin *et al.* 2010), whereas it has not been reported in the gut. Like- wise, *L. diolivorans* is almost exclusively detected in fermented food/feed. Unlike *W. paramesenteroides* and

Three Hua Bei silages showed considerably low (approximately 200 g/kg) DM content. This may explain the higher concentrations of lactic acid, acetic acid and ethanol in the Hua Bei silages than those in the Kyushu silages, although the differences in the silage storage period (10 months for Hua Beivs. 4 months for Kyushu) should also be considered.

L. acetotolerans and *A. pasteurianus* have been found in bunker-made maize silages and both bacteria have often been detected in bunker-made maize silage by culture-independent analysis (Li & Nishino 2011; Wang *et al.* 2013). *A. pasteurianus* is thought to be one of the aerobic bacteria that initiate deterioration after silo opening (Spoelstra *et al.* 1988). However, the silages examined in this study were not obvi- ously spoiled and were considered acceptable based on the pH and fermentation product content.

L. acetotolerans and *A. pasteurianus* are resistant to

have been facilitated by the relatively high acetic acid content (26.6 51.1 g/kg DM)

L. diolivorans, *L. acetotolerans*, *L. pontis* and *L. casei* were considered to survive in the gut of dairy cows, because bands attributed to these species were detected at the same migration distance in both silage and cow feces. Our results were not straight-forward because there were other *L. pontis* (bands 32 and 45) and *L. casei* (bands 49, 53 and 54) bands that were not detected in silage or feces, and whether *L. acetotolerans*, *L. pontis* and *L. casei* were transferred from silage to feces or were indigenous inhabitants is not yet clear. In such a practical survey, it is often difficult to collect samples from the same herds that have never received silage.

Qualitative DGGE assessment demonstrated that about one-third (*L. acetotolerans*, *L. pontis* and *L. casei*) of the silage LAB were able to survive in the cowgut; therefore, silage may be regarded as an effectivevehicle for the propagation and delivery of probiotic LAB in ruminants. Although DNA-based assessment may overestimate the survival rate by detecting dead and injured cells, *L. acetotolerans* was found to show potential value as a probiotic based on this practical survey. Even so, the original *L. acetotolerans* count in silage (10^6 10^7 copies/g) was substantially decreased in feces (10^3 10^4 copies/g). Therefore, in addition to increasing the survival rate during the digestion rocess, the ability to sustain higher populations dur-ing long periods of ensiling may be required to con- fer probiosis in silage-fed cows.

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REFERENCES

Bohak I, Back W, Richter L, Ehrmann M, Ludwig W, Schleifer KH. 1998. *Lactobacillus amylolyticus* sp. nov., isolated from beer malt and beer wort. *System Applied Microbiology* 21, 360–364.

Chen MM, Liu QH, Xin GR, Zhang JG. 2012. Characteristics of lactic acid bacteria isolates and their inoculating effects on the silage fermentation at high temperature. *Letters in Applied Microbiology* 56, 71–78.

Entani E, Masai H, Suzuki KI. 1986. *Lactobacillus acetotolerans*, a new species from fermented vinegar broth. *International Journal of Systematic Bacteriology* 36, 544–549.

Han H, Takase S, Nishino N. 2012. Survival of silage lactic acid bacteria in the goat gastrointestinal tract as determined by denaturing gradient gel electrophoresis. *Letters in Applied Microbiology* 55, 384–389.

Hernandez JD, Scott PT, Shephard RW, Al Jassim RAM. 2008. The characterization of lactic acid producing bacteria from the rumen of dairy cattle grazing on improved pasture supplemented with wheat and barley grain. *Journal of Applied Microbiology* 104, 1754–1763.

Krause DO, Smith WJM, Conlan LL, Gough JM, Williamson MA, McSweeney CS. 2003. Diet influences the ecology of lactic acid bacteria and Escherichia coli along the digestive tract of cattle: neural networks and 16S rDNA. *Microbiology* 149, 57–65.

Krooneman J, Faber F, Alderkamp AC, Oude Elferink SJHW, Driehuis F, Cleenwerck I, *et al.* 2002. *Lactobacillus diolivorans* sp. nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable corn silage. *International Journal of Systematic and EvolutionaryMicrobiology* 52, 639–646.

Li Y, Nishino N. 2011. Monitoring the bacterial community of corn silage stored in a bunker silo inoculated with *Enterococcus feacium*, *Lactobacillus plantarum* and*Lactobacillus buchneri*. *Journal of Applied Microbiology* 110,1561–1570.

Matsushita K, Toyama H, Adachi O. 1994. Respiration chains and bioenergetics of acetic acid bacteria. *Advancesin Microbial Physiology* 36, 247–301.

Muyzer G, Smalla K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 73, 127–141.

Nader-Maias MEF, Otero MC, Espeche MC, Maldonado NC. 2008. Advances in the design of probiotic products for the prevention of major diseases in dairy cattle. *Journal of Industrial Microbiology & Biotechnology* 35, 1387– 1395.

Oude Elferink SJWH, Krooneman J, Gottschal JC, Spoelstra SF, Faber F, Driehuis F. 2001. Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. *Applied and Environmental Microbiology* 67, 125–132.

Parvin S, Wang C, Li Y, Nishino N. 2010. Effect of inoculation with lactic acid bacteria on the bacterial communities of Italian ryegrass, whole crop corn, guinea grass and Rhodes grass silages. *Animal Feed Science and Technology* 160, 160–166.

Rodas AM, Chenoll E, Macián MC, Ferrer S, Pardo I, Aznar R. 2006. *Lactobacillus vini* sp. nov., a wine lactic acid bacteriumhomofermentative for pentoses. *International Journal of Systematic and Evolutionary Microbiology* 56, 513–517.

Rudi K, Moen B, Sekelja M, Frisli T, Lee MRF. 2012. An eight-year investigation of bovine livestock fecal microbiota. *Veterinary Microbiology* 160, 369–377.

Sekiguchi H, Tomioka N, Nakahara T, Uchiyama H. 2001. A single band does not always represent single bacterial strains in denaturing gradient gel electrophoresis analysis. *Biotechnological Letters* 23, 1205–1208.

Weinberg ZG, Muck RE. 1996. New trends and opportunities in the development and use of inoculants for silage. *FEMS Microbiology Reviews* 19, 53–68.

Wiese BG, Trohmar W, Rainey FA, Diekmann H. 1996. *Lactobacillus panis* sp. nov., from sourdough with a long fermentation period. *International Journal of Systematic Bacteriology* 46, 449–453.

Spoelstra SF, Courtin MG, Van Beers JAC. 1988. Acetic acid bacteria can initiate aerobic deterioration of whole crop maize silage. *Journal of Agricultural Science* 111, 127– 132.

Wang C, Han H, Guo XY, Yu Z, Nishino N. 2013. A survey of fermentation products and bacterial communities in corn silage produced in a bunker silo in China. *Animal Science Journal* 85, 32–36.