GRASSLAND SCIENCE

Japanese Society of Grassland Science

Japanese Society of Grassland Science ISSN1744-6961

ORIGINAL ARTICLE

A pilot examination of the fermentation products, aerobic stability and bacterial community of total mixed ration silage produced in Vietnam

Tu Tran Thi Minh[1,2], Van Nguyen Huu[3] and Naoki Nishino[1]

1 Graduate School of Environmental and Life Science, Okayama University, Okayama, Japan
2 Faculty of Agriculture, Engineering and Food Technology, Tien Giang University, My Tho, Vietnam
3 Faculty of Animal Science, Hue University of Agriculture and Forestry, Hue, Vietnam

Keywords
Aerobic stability; denaturing gradient gel electrophoresis; silage; total mixed ration; tropics.

Correspondence
Naoki Nishino, Graduate School of Environmental and Life Science, Okayama University, Kita-ku, Okayama 700-8530, Japan.
Email: j1oufeed@cc.okayama-u.ac.jp

Received 11 November 2013;
accepted 23 December 2013.

doi: 10.1111/grs.12041

Abstract

To evaluate the fermentation characteristics and aerobic stability of total mixed ration (TMR) silage produced in the tropics, rice straw (Rs) and corn stover (Cst) were mixed with molasses (M) and other feeds as TMRs, which were then preserved as silage in Hue, Vietnam. The silages were opened after 4 months, and the microbial counts, fermentation product levels and aerobic stability were determined. The bacterial community was assessed by denaturing gradient gel electrophoresis (DGGE). All the silages were well preserved, with lactic acid serving as the major preservative. However, the lactic acid content was low (5.99 g kg^{-1} dry matter [DM]) in the Rs-M silage, while the ethanol content was substantial (37.2 g kg^{-1}DM) in the Cst-M silage. Heating due to spoilage was observed at 115 and 81 h after silo opening in the Cst-M and Cst-TMR silages, respectively, whereas no heating was observed for 7 days after the opening of the Rs-M and Rs-TMR silages. Among the 14 bacterial strains identified in the silages by the DGGE analysis, only three were lactic acid bacteria. *Lactobacillus brevis* and *Weissella paramesenteroides* were detected in all silages, while *W. cibaria* was only detected in the Rs-M and Cst-M silages. In the aerobically unstable Cst-TMR silage, bands indicative of *Acetobacter pasteurianus*, *Staphylococcus* sp. and *Streptomyces* sp. were specifically observed. These results indicate that although desirable lactic acid fermentation can be expected in a TMR silage in a tropical environment, aerobic stability is lowered if Cst instead of Rs is used as the ingredient crop. The presence of *A. pasteurianus* in the air-tight laboratory silo was unusual, but it could account for the low aerobic stability of the Cst-TMR silage.

Introduction

To feed the current and increasing stock of ruminants in Vietnam, the utilization of forage crops and by-products needs to be optimized (Van 2012). Because biomass production is faster in the tropical regions than in the temperate regions, proper preservation is critical to enable stabilization of feed supply for both small- and large-holder farmers. Although ensiling is not commonly practiced in the tropics, attempts have been made to develop a suitable process for ensiling in this region (Nussio 2005). Fermentation of tropical silages may produce a large amount of acetic acid as the major preservative. Wilting and sugar addition have been shown to decrease the acetic acid fermentation (Nishino *et al.* 2012); however, prolonged ensiling may decrease the lactic acid level and increase the acetic acid level, even if lactic acid fermentation is observed when ensiling is initiated (Parvin and Nishino 2010). The difficulty in retaining lactic acid fermentation during prolonged storage of silage was also observed when by-products with high moisture and low sugar contents were ensiled alone (Schneider *et al.* 1995).

Furthermore, high ambient temperatures may suppress lactic acid production in the ensiling process and enhance aerobic deterioration after silo opening (Ashbell *et al.* 2002).

To obtain optimally fermented silage of acceptable aerobic stability, ensiling as a total mixed ration (TMR) is worth investigating. Spoilage does not occur in TMR silage for as long as 7 days, even in summer, although non-ensiled TMR easily deteriorates within 1–2 days (Nishino 2011). Resistance to aerobic deterioration was observed even when more than 10^6 colonfy-forming units (cfu) g^{-1} of yeast was estimated at silo opening, despite the fact that, typically, silages with over 10^5 cfu g^{-1} of yeast are prone to spoilage on exposure to air (McDonald *et al.* 1991). High aerobic stability was also noted in an Israeli study, in which large-scale TMR silage was prepared using cubic bales wrapped in stretch polyethylene film (Weinberg *et al.* 2011). Therefore, a good shelf life can be expected regardless of the ingredient composition and in both the tropical and temperate regions.

The present study aimed to evaluate the fermentation characteristics and aerobic stability of TMR silage produced in Vietnam. Using local feed resources such as crop residues and the by-products from agriculture, food and beverage industries, two TMR silages and two simple molasses (M)-added silages were prepared. Bacteria associated with ensiling fermentation and aerobic spoilage were determined by culture-independent denaturing gradient gel electrophoresis (DGGE). This procedure has been shown to reveal the presence of diverse lactic acid bacteria (LAB) and non-LAB species in the silages (Muck 2012).

Materials and methods

Preparation of silage

Two types of laboratory-scale silages were prepared. The first type was a simple mixture of crop residue and M added at 50 g kg^{-1} dry matter (DM), and the second was a TMR mixture composed of crop residue, brewer's grains, rice bran, corn powder, peanut cake and M (Table 1). Rice straw (Rs) and corn stover (Cst) were used as the crop residue in both the silage types; hence, in total, four types of silages were prepared — Rs-TMR, Cst-TMR, Rs-M and Cst-M. Crop residues were manually chopped using knives into 10–20-mm cuts. Simple M-added and TMR mixture samples (300 g each) were packed in plastic pouches (Hiryu BN-12, Asahi Kasei Pax, Tokyo, Japan) and the air was removed by a vacuum sealer. The size, thickness and oxygen permeability of the pouches were 270 × 400 mm, 0.075 mm and 44 mL m^{-2} atm^{-1} per day, respectively. Silages were prepared in triplicate and stored at room temperature in Hue, Vietnam, for 4 months (from 26 May to 21 September 2012). Mean daily minimum and maximum temperatures during the period were 24 and 34°C, respectively. The silos were shipped by air to Okayama, Japan, opened, and then tested for the microbial counts and fermentation products. For the aerobic spoilage test, a 100-g sample of the silage was placed in a 500-mL plastic bottle and then exposed to air for 7 days in a 30°C incubator. The silage temperature was recorded every 10 min. The silage was considered to have deteriorated when the temperature reached 2°C above the ambient temperature (Wilkinson and Davies 2012).

Table 1 Composition of silages prepared with rice straw (Rs) or corn stover (Cst) and stored as a total mixed ration (TMR) or as a simple mixture with molasses (M)

	Rs-TMR	Cst-TMR	Rs-M	Cst-M
Ingredients (g kg^{-1})				
Rice straw	400	—	950	—
Corn stover	—	400	—	950
Brewers grains	150	150	—	—
Rice bran	150	150	—	—
Corn powder	150	150	—	—
Peanuts cake	80	80	—	—
Molasses	70	70	50	50
Composition				
Dry matter (g kg^{-1})	547	409	623	308
Lactic acid bacteria (log cfu g^{-1})	7.68	8.12	8.07	6.67
Yeasts (log cfu g^{-1})	7.68	6.62	8.45	7.90

Rs-TMR, silage prepared with Rs as a TMR; Cst-TMR, silage prepared with Cst as a TMR; Rs-M, silage prepared with Rs and M; Cst-M, silage prepared with Cst with M.

Chemical analysis and live counts of microorganisms

The DM contents of pre-ensiled mixed materials and silages were determined after drying the mass in an oven at 60°C for 48 h. The pH value and lactic acid, volatile fatty acid and alcohol content were determined in cold water extracts (Nishino *et al.* 2012). Lactic acid, acetic acid and ethanol contents were determined by an ion-exclusion polymeric high-performance liquid chromatography method with refractive index detection. A portion of the water extracts was passed through a 0.20-μm filter and 10 μL was injected into an ICSep COREGEL-87H column (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) containing a cation exchange polymer in the ionic hydrogen form. The mobile phase was 0.004 mol L^{-1} sulfuric acid

at a flow rate of 0.6 mL min^{-1} at 60°C. Enumeration of LAB was performed by the pour plate technique using de Man, Rogosa and Sharpe agar. Yeasts and molds were counted on spread plates of yeast extract and malt extract agar. All plates were incubated for 3 days at 30°C.

Denaturing gradient gel electrophoresis

After opening the silo, the silage samples were stored at −30°C until DNA extraction. A 5-g sample was thawed and added to a 19 × volume (95 mL) of sterilized phosphate-buffered saline (pH 7.4), and the bacterial DNA was extracted and purified by using the DNeasy Tissue Kit (Qiagen, Germantown, MD, USA).

Polymerase chain reaction (PCR) was used to amplify a variable (V3) region of the bacterial 16S ribosomal RNA (rRNA) gene (Wang and Nishino 2013) using the forward primer GC357f (5′–CGCCCGCCGCGCGCGGCGGGCG GGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG –3′) and the reverse primer 517r (5′–ATTACCGC GGCTGCTGG–3′). The PCR analysis protocol consisted of an initial denaturation at 95°C for 10 min and 30 cycles of denaturation at 93°C for 30 s, followed by annealing at 65°C (first 10 cycles), 60°C (second 10 cycles) and 55°C (last 10 cycles) for 30 s, and finally by extensions at 72°C for 1 min and at 72°C for 5 min (Wang and Nishino 2013). These reactions were conducted in the TP-600 PCR thermal cycler (Takara Bio Inc., Otsu, Japan).

Amplicons were separated via DGGE by using the DCode Universal Mutation Detection System (Bio-Rad Ltd., Tokyo, Japan). The samples were applied directly onto 100 g L^{-1} polyacrylamide gels with 25–50% denaturing gradients, which were prepared using 7 mol L^{-1} urea and 400 mL L^{-1} formamide, respectively, as 100% denaturants. Electrophoresis was performed at a constant voltage of 150 V for 12 h at 60°C. After electrophoresis, the gels were stained with SYBR Green (Cambrex Bio Science Inc., Rockland, ME, USA) and photographed under UV illumination.

Cloning and sequencing of DGGE bands

Selected bands were excised from the DGGE gels and eluted overnight in 10 μL of sterilized water at 4°C. The extracted DNA was amplified using the 357f (without GC clamp) and 517r primers. The purified PCR products were cloned into a pTAC-1 vector, and the resulting plasmids were transformed into *Escherichia coli* strain DH5α-competent cells using the DynaExpress TA Cloning Kit (BioDynamics Laboratory Inc., Tokyo, Japan). The DNA sequences were analyzed using the ABI PRISM 310 sequencer (Applied Biosystems Inc., Foster City, CA, USA). The Basic Local Alignment Search Tool (BLAST) program and GenBank databases were used to determine the closest relatives of partial 16S rRNA gene sequences. Unknown sequences sharing more than 99% sequence identity with a sequence in the BLAST database were considered as identified.

Statistical analysis

Data for silages were subjected to two-way analysis of variance, with the composition of the mixture (TMR versus M addition) and crop type (Rs versus Cst) as the main factors. Differences were considered significant at $P < 0.05$. These analyses were performed using JMP software (ver. 7; SAS Institute, Tokyo, Japan).

Results

The DM contents of pre-ensiled mixture ranged from 308 to 623 g kg^{-1}, according to the composition, whereas LAB and yeasts were counted to be between 10^6 and 10^8 cfu g^{-1} regardless of the mixture composition (Table 1).

After ensiling, the pH values decreased to about 4.0 for all silages, except the Rs-M silage (Table 2). Compared with simple M addition, TMR preparation had a more pronounced effect on pH decline. However, there was not much difference in the pH between TMR preparation and simple M addition when Cst was used as the ingredient crop. In contrast, the lactic acid content was increased when Cst was used, and the difference in the lactic acid content between TMR preparation and simple M addition was greater when Rs was formulated. The lowest lactic acid level (5.99 g kg^{-1} DM) was found in the Rs-M silage, which had the highest DM (640 g kg^{-1}) and pH value (4.66) among the four silages. The acetic acid content was more in the TMR preparation when Cst was used. Substantial ethanol production (37.3 g kg^{-1} DM) was noted specifically in the Cst-M silage, whereas the levels were less than one-tenth (<3.51 g kg^{-1} DM) that in other silages. The LAB counts ranged between 10^3 and 10^5 cfu g^{-1} for the TMR preparation and simple M addition, respectively, and the use of Cst caused only a small increase in the LAB count when compared with the use of Rs. The yeast counts were approximately 10^5 cfu g^{-1} in all silages, whereas only a marginal difference was noted in the count between TMR preparation and simple M addition. After opening the silos, no spoilage was observed for more than 7 days in the Rs-containing silages. However, heating was observed after exposure to air at 115 and 81 h in the Cst-M and Cst-TMR silages, respectively.

Table 2 Determination of fermentation product composition and microbial counts of the silages prepared with rice straw (Rs) or corn stover (Cst) and stored as a TMR or a simple mixture with molasses (M)

	Rs-TMR	Cst-TMR	Rs-M	Cst-M	SE	Recipe	Crop	Interaction
Dry matter (DM) (g kg^{-1})	537	402	640	291	2.38	NS	**	**
pH	4.12	3.86	4.66	3.95	0.01	**	**	**
Lactic acid (g kg^{-1}DM)	39.3	61.6	5.99	53.6	2.63	**	**	**
Acetic acid (g kg^{-1}DM)	10.3	15.2	3.59	10.0	0.67	**	**	NS
Ethanol (g kg^{-1}DM)	1.18	3.51	0.13	37.2	1.49	**	**	**
Lactic acid bacteria (log cfu g^{-1})	2.53	3.34	4.99	5.73	0.31	**	*	NS
Yeasts (log cfu g^{-1})	4.50	4.98	5.31	5.40	0.17	**	NS	NS
Heating after silo opening (h)	>168	81.0	>168	115	—	—	—	—

Mean values for triplicate silages are shown. Recipe, TMR preparation versus M addition; Crop, Rs versus Cst. SE, standard error; NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$.

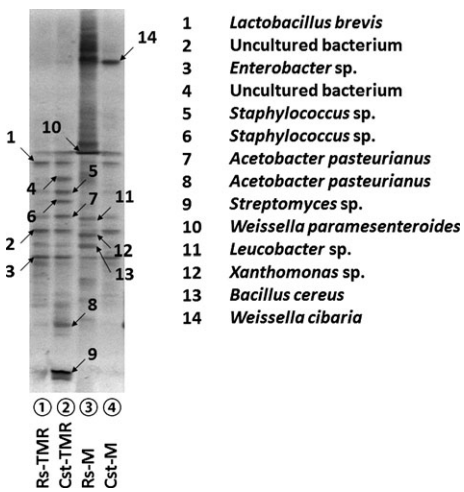

Figure 1 The bacterial community of silages prepared with rice straw (Rs) or corn stover (Cst) and stored as a total mixed ration (TMR) or a simple mixture with molasses (M). Denaturing gradient gel electrophoresis was performed at a constant voltage of 150 V for 12 h at 60°C, and a number of DNA bands were excised and sequenced. Rs-TMR; silage prepared with Rs as a TMR, Cst-TMR; silage prepared with Cst as a TMR, Rs-M; silage prepared with Rs and M, Cst-M; silage prepared with Cst and M.

The bands indicative of *Lactobacillus brevis* (band 1), *Enterobacter* sp. (band 3) and *Weissella paramesenteroides* (band 10) were detected for all four silages and that of *W. cibaria* (band 14) was detected only for the two simple M-added silages (Figure 1). The DGGE band patterns appeared different between TMR preparation and simple M addition, regardless of the type of crop added. Furthermore, a number of bacteria were observed in the Cst-TMR and Rs-M silages. Bands of an uncultured bacterium (band 4), *Staphylococcus* sp. (bands 5 and 6), *Acetobacter pasteurianus* (bands 7 and 8) and *Streptomyces* sp. (band 9) were detected only for the Cst-TMR silage, and those of *Leucobacter* sp. (band 11), *Xanthomonas* sp. (band 12), and *Bacillus cereus* (band 13) were detected only for the Rs-M silage. In the Rs-TMR and Cst-M silages, the DGGE band patterns were similar, except for presence of *W. cibaria* in the Cst-M silage sample.

Discussion

In all four silages, lactic acid was the major preservative. Typical acetic acid fermentation occurs when the tropical crops used have high moisture and low sugar contents (Nishino *et al.* 2012). Hence, pre-ensiled materials with acceptably high DM content (>300 g kg^{-1}) and sufficient sugar content (generated from the added M) may help avoid acetic acid fermentation. Although long storage can lower the lactic acid content and increase the acetic acid content in tropical ensiled materials (Parvin and Nishino 2010), these changes may occur over several months. Accordingly, the storage period in this study was sufficiently long.

Instead of acetic acid fermentation, substantial ethanol fermentation was observed in the Cst-M silage. Ethanol fermentation has been reported in sugarcane silage prepared in tropical regions (Daniel and Nussio 2011); this can also occur in temperate regions if low moisture and high sugar grass is ensiled (Driehuis and Van Wikselaar 2000). Because M was added at 50 g kg^{-1}, the pre-ensiled Cst-M (DM 308 g kg^{-1}) had >70 g kg^{-1}DM of exogenous sugars, which presumably helped suppress acetic acid fermentation and facilitated ethanol production. Yeasts were probably responsible for the high ethanol content of the Cst-M silage. However, the count was not much different from other silage types either at ensiling or at silo opening. The bacterial community in the Cst-M silage, with the exception of *W. cibaria*, resembled that of Rs-TMR silage and *W. cibaria* was detected in the Rs-M silage. Because two Rs-containing silages had low ethanol content, the bacterial community cannot account for the enhanced ethanol production in the Cst-M silage. As

alcohol production is often accompanied by DM loss during storage and does not contribute to aerobic stability after silo opening (Huisden *et al.* 2009), ethanol fermentation should be avoided.

Although desirable lactic acid fermentation was seen in two TMR silages regardless of the crop type, aerobic spoilage occurred in the Cst-TMR silage. We tested aerobic stability for the TMR silage in multiple laboratory-scale experiments, but heating at as early time points such as 3.5 days after silo opening has not been noted. Because antifungal acetic acid level in Cst-TMR silage was the highest among four silages, it is difficult to explain why this silage was most unstable after exposure to air. However, the specific presence of *Staphylococcus* sp., *A. pasteurianus* and *Streptomyces* sp. in the Cst-TMR silage, was distinctive among the four silages. *A. pasteurianus* is an obligate aerobic bacterium known to play a role in initiating aerobic spoilage (Spoelstra *et al.* 1988). Crop specificity (for corn silage) is a distinctive feature of *A. pasteurianus* (Oude Elferink *et al.* 2001); therefore, the detection of *A. pasteurianus* in the Cst-TMR, but not in the Rs-TMR silage, was somewhat to be expected. However, although *A. pasteurianus* is often detected in corn silage produced in a bunker silo (Li and Nishino 2011; Wang *et al.* 2014), which is hard to protect from air ingress once opened due to the large surface area, this microorganism was never detected in an airtight pouch silo. This suggests that ensiling in tropical regions is more difficult than that in temperate regions.

Although lactic acid was the major preservative, the LAB species (*L. brevis*, *W. paramesenteroides* and *W. cibaria*) identified in the four silages were all hetero-fermentative and none of them were shown to inhibit aerobic spoilage at acceptable levels (Danner *et al.* 2003; Wang and Nishino 2009). In addition, although the presence of aerobic *Bacillus* spp. at silo opening was noted in the Rs-M silage, resistance to aerobic deterioration was observed. In this regard, the DGGE analysis failed to demonstrate the reason behind the desirable lactic acid fermentation as well as the high aerobic stability in the Rs-TMR and Rs-M silages.

In this study, we did not adjust the DM content of pre-ensiled mixture, although the DM content is one of the major factors that influence ensiling fermentation and aerobic stability. Silage of higher DM content is known to be more labile on exposure to air (Wilkinson and Davies 2012), whereas an opposite effect was noticed between Rs-containing and Cst-containing silages in the present study. Furthermore, the Rs-TMR and Rs-M silages (DM 537–640 g kg^{-1}) might have been subjected to further drying in a 30°C incubator during the aerobic spoilage test, which in turn could enhance the resistance to deterioration compared to the moist Cst-TMR and Cst-M silages (DM 291–402 g kg^{-1}). If the DM contents of the pre-ensiled materials were adjusted to the same level before ensiling, then the differences between Rs and Cst and between TMR preparation and simple M addition could be clearly evaluated. The effects of material composition and DM content on the aerobic stability need to be examined further to understand the factors involved in TMR-type mixed ensiling in the tropical environment.

References

Ashbell G, Weinberg ZG, Hen Y, Filya I (2002) The effects of temperature on the aerobic stability of wheat and corn silages. *J Ind Microbiol Biotechnol* 28: 261–263.

Daniel JLP, Nussio LG (2011) Contribution of silage volatile compounds for the animal nutrition. In: *Proceedings of the 2nd International Symposium on Forage Quality and Conservation*, 16–19 Nov 2011, São Pedro, 279–306.

Danner H, Holzer M, Mayrhuber E, Braun R (2003) Acetic acid increases stability of silage under aerobic conditions. *Appl Environ Microbiol* 69: 562–567.

Driehuis F, Van Wikselaar PG (2000) The occurrence and prevention of ethanol fermentation in high-dry-matter grass silage. *J Sci Food Agric* 80: 711–718.

Huisden CM, Adesogan AT, Kim SC, Ososanya T (2009) Effect of applying molasses or inoculants containing homofermentative or heterofermentative bacteria at two rates on the fermentation and aerobic stability of corn silage. *J Dairy Sci* 92: 690–697.

Li Y, Nishino N (2011) Monitoring the bacterial community of maize silage stored in a bunker silo inoculated with *Enterococcus faecium, Lactobacillus plantarum* and *Lactobacillus buchneri*. *J Appl Microbiol* 110: 1561–1570.

McDonald P, Henderson AR, Heron SJE (1991) *The Biochemistry of Silage*. Chalcombe Publications, Welton, Lincoln, 1–340.

Muck RE (2012) Microbiology of ensiling. In: *Proceedings of the 16th International Silage Conference* (Eds Kuoppala K, Rinne M, Vanhatalo A), MTT Agrifood Research Finland, University of Helsinki, Hämeenlinna, 75–86.

Nishino N (2011) Aerobic stability and instability caused by bacteria. In: *Proceedings of the 2nd International Symposium on Forage Quality and Conservation*, 16–19 Nov 2011, São Pedro, 127–141.

Nishino N, Li Y, Wang C, Parvin S (2012) Effects of wilting and molasses addition on fermentation and bacterial community in guinea grass silage. *Lett Appl Microbiol* 54: 175–181.

Nussio LG (2005) Silage production from tropical forages. In: *Silage Production and Utilisation* (Eds Park RS, Mayne CS, Keady TWJ), Wageningen Academic Publishers, Wageningen, 97–107.

Oude Elferink SJWH, Driehuis F, Becker PM, Gottschal JC, Faber F, Spoelstra SF (2001) The presence of *Acetobacter* sp. in ensiled forage crops and ensiled industrial byproducts. *Med Fac Landbouw Univ Gent* 66: 427–430.

Parvin S, Nishino N (2010) Succession of lactic acid bacteria in wilted rhodesgrass silage assessed by plate culture and denaturing gradient gel electrophoresis. *Grassl Sci* 56: 51–55.

Schneider RM, Harrison JH, Loney KA (1995) The effects of bacterial inoculants, beet pulp, and propionic acid on ensiled wet brewers grains. *J Dairy Sci* 78: 1096–1105.

Spoelstra SF, Courtin MG, Van Beers JAC (1988) Acetic acid bacteria can initiate aerobic deterioration of whole crop maize silage. *J Agric Sci Camb* 111: 127–132.

Van NH (2012) Grassland system in Vietnam: history, present condition and prospects. In: *Proceedings of the 4th Japan-China-Korea Grassland Conference*, 30 Mar–1 Apr 2012, Aichi, 30–33.

Wang F, Nishino N (2009) Association of *Lactobacillus buchneri* with aerobic stability of total mixed ration containing wet brewers grains preserved as a silage. *Anim Feed Sci Technol* 149: 265–274.

Wang C, Nishino N (2013) Effects of storage temperature and ensiling period on fermentation products, aerobic stability and microbial communities of total mixed ration silage. *J Appl Microbiol* 114: 1687–1695.

Wang C, Han H, Gu X, Yu Z, Nishino N (2014) A survey of fermentation products and bacterial communities in corn silage produced in a bunker silo in China. *Anim Sci J* 85: 32–36.

Weinberg ZG, Chen Y, Miron D *et al.* (2011) Preservation of total mixed rations for dairy cows in bales wrapped with polyethylene stretch film – A commercial scale experiment. *Anim Feed Sci Technol* 164: 125–129.

Wilkinson JM, Davies DR (2012) The aerobic stability of silage: key findings and recent developments. *Grass Forage Sci* 68: 1–19.

