Research Article

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Changes in nutrient composition and *in vitro* ruminal fermentation of total mixed ration silage stored at different temperatures and periods

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Abstract

BACKGROUND: Total mixed ration (TMR) is widely used for dairy cattle and needs to be prepared daily because it deteriorates rapidly. Ensiling TMR allows preservation and saves labour at the farm; however, silage fermentation may influence various nutritional components. The objectives of this study were to evaluate nutritional changes and *in vitro* rumen fermentation of TMR silage that was stored at different temperatures and durations on a laboratory scale in comparison with those of typical TMR before ensiling.

RESULTS: No distinct changes in crude protein (CP), neutral detergent fibre and non-fibrous carbohydrate contents were observed during silage fermentation. However, clear changes were observed in the soluble CP and soluble sugar fractions; solubilisation of the CP fraction in TMR silage was enhanced by prolonged storage and higher storage temperatures, and most soluble sugars were lost during ensiling. Short-chain fatty acid concentrations in the *in vitro* rumen from TMRs before and after ensiling were not significantly different; however, throughout incubation, NH_3-N concentrations from TMR silages were significantly higher than those from TMR before ensiling.

CONCLUSION: A higher ruminal NH_3-N concentration from TMR silage may be a result of a shortage of fermentable sugars and enhanced deamination of CP. Feeding TMR ensiled under a high temperature must be investigated to balance proteins and carbohydrates for rumen fermentation.

Keywords: total mixed ration; silage; soluble protein; *in vitro* ruminal fermentation

INTRODUCTION

Total mixed ration (TMR) has been widely used for dairy cattle because it contains roughage, concentrates, vitamins and minerals in ratios that are well balanced to meet the animal's requirements. However, TMR deteriorates easily because it has a high nutrient content and sufficient moisture content for the growth of bacteria, yeast and moulds.[1] Thus, most farmers must prepare TMR once or twice daily, and contractors who also do this must deliver TMR to farms almost every day. In contrast, ensiled TMR can be preserved; this saves labour for TMR preparation, and in some cases, it can be carried for long distances.[2] Large-scale preparation of TMR silage by a company or contractor leads to lower feed costs and the use of more high-moisture by-products, which deteriorate easily. Studies on TMR silage have increased in recent years, particularly in Asian counties.[3–10] Fermentation of TMR silage progresses with high amounts of organic acids [approximately 50 to >100 g kg^{-1} dry matter (DM)], despite the high DM content (approximately 400–600 g kg^{-1}).[2,3,8] Several studies have shown that TMR silage is stable after opening the silo, even under higher temperatures,[3,8,11] which leads to stable TMR feeding without being heated in hot climates.

Compared with crop silage, TMR silage typically has high contents of energy and proteins, thereby enabling it to meet the nutrient requirements of dairy cows. The nutritional value of TMR is based on a mixture of ingredients; therefore, some nutrients in TMR silage are expected to be lost during ensiling, and the balance differs between the time at which it is prepared and the time

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at which it is fed to animals. Respiration by plants and fermentation by microorganisms in crop silage produce smaller molecules from carbohydrates and proteins.[12–14] Because TMR silage can be prepared throughout the year, which is different from crop silage, the effect of temperature on the fermentation characteristics and nutrient changes in TMR silages should be understood. The ensiling period also affects these parameters. In particular, changes in carbohydrates and proteins during ensiling may influence the production of short-chain fatty acids (SCFAs) and concentrations of NH_3 in the rumen, which are highly related to energy and protein metabolism in ruminants.[15] Therefore, we investigated the nutritional changes and characteristics of rumen fermentation in TMR silages stored at different temperatures and for different durations in comparison with those in typical TMR before ensiling.

MATERIALS AND METHODS

Preparation of total mixed ration and ensiling

The ingredients of TMR used in the present study are presented in Table 1. Italian ryegrass silage, sorghum silage and corn silage, ensiled for approximately 4, 11 and 11 months, respectively, were used as roughage. The DM contents of these silages were 466, 257 and 231 g kg^{-1}, respectively. The other ingredients, including concentrates and minerals, were purchased from feed companies. TMR was designed for dairy cattle, and the total digestible nutrient content was 730 g kg^{-1} DM, which was calculated from the Standard Tables of Feed Composition in Japan.[16] These ingredients were mixed in a practical scale (total of 2800 kg), and approximately 100 kg of TMR was used for the present study. TMR was packed into 1000-mL high-density polyethylene bottles (1.4 mm thickness), fitted with a rubber cap and gas-release bulb; this was maintained at 15 °C or 30 °C to mimic annual average and summer climates, respectively. The DM density of these silages was adjusted to approximately 330 kg DM m^{-3}, which was close to that of practical TMR silage in Japan. The silos were opened at 2, 5, 10, 30 and 90 days after ensiling. The silos were prepared in five replicates for 30 and 90 days and in three replicates for 2, 5 and 10 days. All bottles were weighed before and after ensiling, and DM loss during fermentation was calculated. An approximately 1 kg TMR sample was randomly collected five times during preparation before ensiling.

In vitro ruminal fermentation study

An *in vitro* ruminal study was conducted according to the methods described by Uddin *et al.*[17] TMRs before and after ensiling ($n = 5$ replicates/treatment) were dried at 60 °C for 48 h and ground to pass through a 1 mm screen. Approximately 1 g of sample was weighed into a 100 mL glass vial. Rumen fluid was collected via a rumen cannula before the morning feeding from two Holstein cows that were fed TMR of the same nutrient value as that in the present study. The fluid samples were pooled and filtered through four layers of cheesecloth. Glass vials were filled with 50 mL of medium consisting of one part of rumen fluid and two parts of McDougall buffer, incubated in a 39 °C water bath and shaken at 160 rpm. After 4, 8 and 24 h of incubation, 0.5 mL of the liquid medium in the vials was withdrawn with a sterilised syringe for SCFA and NH_3-N analyses. The *in vitro* ruminal study was replicated twice, and the mean values are presented.

Chemical analyses

DM contents of TMRs before and after ensiling were determined by oven-drying, as described above, without correcting for loss of volatiles. Fermented products (lactic acid, SCFA and NH_3-N) and the pH of TMRs were determined from water extracts. TMRs (30 g) were macerated with 210 mL distilled water and filtered to obtain a water extract. The pH values of the extracts were measured using a pH meter. Lactic acid and SCFA concentrations were analysed with a high-performance liquid chromatography (HPLC) system equipped with an ion-exclusion column using a post-column pH-buffered electron conductivity detection method (Shimadzu, Kyoto, Japan). NH_3-N contents in the extracts were determined using the steam distillation method. The compositions of the following nutrients were analysed in dried and ground samples, as described above. Organic matter (OM), crude protein (CP, total nitrogen × 6.25) and ether extract (EE) contents were measured according to the official methods of the Association of Official Analytical Chemists.[18] OM was calculated by subtracting the ash content (method no. 942.05). CP was determined using the Kjeldahl method (method no. 984.13), and the EE content was determined by Soxhlet extraction (method no. 920.39). Neutral detergent fibre (NDF) was determined according to the methods of Van Soest *et al.*,[19] using both sodium sulfite and heat-stable α-amylase and expressed as the ash-free form. The amount of soluble CP extracted with neutral buffer was determined using the method of Licitra *et al.*[20] Non-fibrous carbohydrate (NFC) contents were calculated using the following equation: NFC = OM − CP − NDF − EE.[15] Soluble sugars extracted with aqueous ethanol (800 mL L^{-1}) were analysed using the phenol–sulfuric acid reaction.[21] The SCFA concentration in liquid medium of the *in vitro* rumen was analysed by HPLC, as described above. The NH_3-N concentration in the medium was determined using the indophenol reaction.[22]

Table 1. Ingredients of the total mixed ration used in this study

Ingredient	Concentration (g kg^{-1} DM)
Italian ryegrass silage	138
Sorghum silage	78
Maize silage	70
Beet pulp	68
Steam rolled maize	208
Steam rolled barley	45
Wheat barn	44
Roasted soybean	48
Soybean meal	123
Corn gluten feed	45
Mineral mixture	12
Calcium carbonate	8
Sodium chloride	4
Magnesium oxide	4
Others	2
Total	1000

DM, dry matter.

Statistical analyses

A completely randomised design was employed to allocate treatments into experimental units. Fermentation characteristics, nutrient composition and the *in vitro* ruminal fermentation parameters of TMRs before and after ensiling were analysed by one-way analysis of variance (ANOVA) and further tested using Tukey's test to compare treatment means. The TMR data before and after ensiling were compared using an orthogonal contrast test. The TMR data

Table 2. Fermentation characteristics of total mixed ration (TMR) before and after ensiling

Parameter	Before ensiling	After ensiling				SEM	Probability			
		30 days[1]		90 days[1]			Before versus after	Day[#]	Temp.[#]	Day × temp.[#]
		15 °C[2]	30 °C[2]	15 °C[2]	30 °C[2]					
Dry matter (g kg^{-1})	551	547	539	536	548	4.01	–	–	–	–
pH	5.79^{a}	4.44^{b}	4.28^{c}	4.26^{c}	4.20^{d}	0.006	***	***	***	***
Lactic acid (g kg^{-1} DM)	20.1^{c}	89.3^{b}	108.9^{a}	92.5^{b}	104.7^{a}	1.90	***	–	***	–
Acetic acid (g kg^{-1} DM)	12.6^{b}	32.8^{ab}	34.2^{ab}	39.3^{a}	31.0^{b}	1.73	***	–	–	*
Butyric acid (g kg^{-1} DM)	2.3^{a}	2.0^{a}	2.0^{a}	1.4^{b}	1.4^{b}	0.11	***	***	–	–
NH_3-N (g kg^{-1} total N)	21.0^{d}	32.6^{c}	46.0^{ab}	42.6^{b}	49.6^{a}	1.29	***	***	***	*
DM losses (%)		3.39	3.88	4.57	4.57	0.571	–	–	–	–

[1] Storage period of TMR silage.
[2] Storage temperature of TMR silage.
[a,b,c,d] Values with different letters in a row are significantly different ($P < 0.05$).
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. [#] Conducted only for samples after ensiling. DM, dry matter.

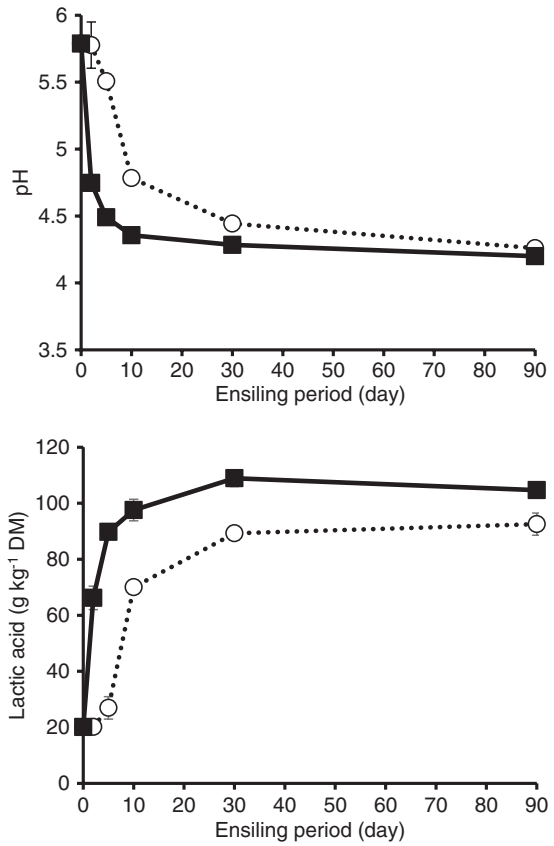

Figure 1. Changes in pH and lactic acid concentration of TMR silages stored at 15 °C (circles) and 30 °C (squares).

after ensiling were subsequently analysed by factorial ANOVA, in which the two fixed factors were the number of storage days and temperature. All statistical analyses were performed using SAS software ver. 9.3 (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

The pH value of TMR before ensiling was 5.8, while the pH values of the silages after ensiling ranged from 4.2 to 4.4 (Table 2) ($P < 0.05$). The pH rapidly dropped during the ensiling period until 10 days after ensiling in TMR stored at 30 °C, and these values were maintained for 90 days (Fig. 1). The pH of TMR silage stored at 15 °C gradually changed and decreased from 30 to 90 days. Lactic acid was the dominant fermented product in TMR silage and was significantly higher in silages stored at 30 °C than in those stored at 15 °C ($P < 0.001$) (Table 2). When the ensiling period was prolonged from 30 to 90 days, the changes in the lactic acid content were small at both storage temperatures. The acetic acid content significantly increased with ensiling ($P < 0.05$), and a significant interaction effect was detected between the ensiling period and temperature ($P < 0.05$). A high organic acid content was a characteristic of TMR silage, although it also contains high DM.[2,9] According to previous studies using practical scales, lactic acid in TMR silage can be as high as 30–100 g kg^{-1} DM and it varies depending on the feed ingredients, season and ensiling method.[2,6,9,23] A high DM content suppresses fermentation in silage[13] because bacterial growth and metabolism are lower in a low-moisture environment. Specific bacteria are reportedly found in TMR silage but have not been isolated from crop silage.[23] Interactions among these specific bacteria and highly fermentable substrates in TMR could enhance organic acid production in TMR silages. Butyric acid, an indicator of undesirable fermentation, was found in low amounts. Although some differences were observed in the butyric acid content before and after ensiling, the changes were small. Fermentation by butyric acid bacteria such as *Clostridium* does not actively occur in high DM TMR silages because their water requirement is high.[12] The butyric acid in TMR silages was from crop silages, which are used in TMR as ingredients, and was not produced during the fermentation of TMR silages, as shown by Cao *et al.*[24] The storage temperature and duration affected the NH_3-N: total N (TN) ratio in TMR silages ($P < 0.05$); however, the ratio was 32.5–49.6 g kg^{-1} TN, which was lower than that of crop silage (100–200 g kg^{-1} TN).[12] These data indicate that compared with crop silage, deamination may be strongly suppressed in TMR silage. DM losses during ensiling were <5% in all TMR silages, and no effect of the storage temperature or duration was observed. DM losses while ensiling crop silage occur as a result of microbial fermentation, effluent and plant respiration, and losses have been estimated to be 7% to >40%.[12] Relatively lower loss of DM from TMR silage than those from crop silage can be explained by several factors. No effluent was observed from any of the TMR silages, which may have been

Table 3. Nutrient composition of total mixed ration (TMR) before and after ensiling

Parameter	Before ensiling	After ensiling				SEM	Probability			
		30 days[1]		90 days[1]			Before versus after	Day[#]	Temp.[#]	Day × temp.[#]
		15 °C[2]	30 °C[2]	15 °C[2]	30 °C[2]					
Organic matter (g kg^{-1} DM)	916^{ab}	918^{a}	915^{bc}	913^{c}	916^{b}	0.5	–	***	–	***
CP (g kg^{-1} DM)	155^{c}	163^{ab}	166^{a}	153^{c}	158^{bc}	1.4	**	***	*	–
Soluble CP (g kg^{-1} CP)	302^{d}	338^{cd}	354^{c}	401^{b}	455^{a}	8.8	***	***	***	*
NFC (g kg^{-1} DM)	364^{a}	349^{ab}	335^{b}	321^{b}	345^{ab}	6.5	**	–	–	–
Soluble sugars (g kg^{-1} DM)	52.6^{a}	7.1^{b}	7.2^{b}	7.0^{b}	7.2^{b}	0.72	***	–	–	–
Ether extract (g kg^{-1} DM)	30.4^{b}	30.1^{b}	32.9^{ab}	34.3^{a}	34.4^{a}	0.91	*	**	–	–
NDF (g kg^{-1} DM)	381	376	380	404	378	7.7	–	–	–	–

[1] Storage period of TMR silage.
[2] Storage temperature of TMR silage.
[a,b,c,d] Values with different letters in a row are significantly different ($P < 0.05$).
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. [#] Conducted only for samples after ensiling. CP, crude protein; DM, dry matter; NFC, non-fibrous carbohydrate.

Table 4. Concentrations of short-chain fatty acids (SCFA) and ammonia nitrogen in *in vitro* rumen incubated with total mixed ration (TMR) before and after ensiling

Parameter	Before ensiling	After ensiling				SEM	Probability			
		30 days[1]		90 days[1]			Before versus after	Day[#]	Temp.[#]	Day × temp.[#]
		15 °C[2]	30 °C[2]	15 °C[2]	30 °C[2]					
4 h after incubation										
Total SCFA (mmol L^{-1})	39.8^{a}	37.7^{bc}	37.5^{c}	39.3^{ab}	38.5^{abc}	0.40	**	**	–	–
AP ratio	3.37^{a}	3.00^{bc}	2.67^{d}	3.09^{b}	2.88^{c}	0.048	***	**	***	–
NH_3-N (mg dL^{-1})	1.42^{c}	3.31^{b}	4.89^{a}	4.23^{a}	4.93^{a}	0.173	***	*	***	*
8 h after incubation										
Total SCFA (mmol L^{-1})	64.1	65.9	67.0	66.0	67.1	0.62	–	–	–	–
AP ratio	2.65^{a}	2.21^{b}	1.95^{c}	2.19^{b}	2.07^{bc}	0.033	***	–	***	–
NH_3-N (mg dL^{-1})	0.15^{d}	1.93^{c}	2.67^{b}	3.61^{a}	3.17^{ab}	0.141	***	***	–	**
24 h after incubation										
Total SCFA (mmol L^{-1})	102	98	103	102	103	1.5	–	–	–	–
AP ratio[5]	2.62^{a}	2.28^{b}	2.08^{c}	2.30^{b}	2.07^{c}	0.019	***	–	***	–
NH_3-N (mg dL^{-1})	4.65^{b}	6.67^{a}	7.75^{a}	7.41^{a}	7.09^{a}	0.466	***	–	–	–

[1] Storage period of TMR silage.
[2] Storage temperature of TMR silage.
[a,b,c,d] Values with different letters in a row are significantly different ($P < 0.05$).
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. [#] Conducted only on samples after ensiling. AP ratio, the ratio of acetic acid to propionic acid.

because of their high DM content. Losses due to plant respiration may also have been lower because the crops incorporated into TMRs had been ensiled; thus, most plant cells would not have been physiologically active.

Compared with fermentation products in TMR silages, it is more important to determine the nutrient composition because nutrients directly affect milk production by dairy cows. No distinct changes in OM, CP, EE or NDF contents were observed during silage fermentation (Table 3). There were no clear changes in the NFC content as a result of the ensiling period or temperature, whereas the soluble sugar content significantly decreased to <10 g kg^{-1} DM ($P < 0.05$). Starch, sugars, pectin and organic acids are included in the NFC fraction.[15] Soluble sugars are converted to lactic acid and acetic acid during silage fermentation; therefore, the NFC content did not change much; however, the components clearly changed. Bacteria in the rumen rapidly obtain energy as ATP from the breakdown of sugars but not from organic acids; thus, the energy supply from rapidly fermentable substrates may have been lower in TMR silages after ensiling than in those before ensiling. Hall *et al.*[25] reported on the neutral detergent soluble carbohydrate fraction in detail and showed that the components in the NFC fraction vary among feedstuffs. Detailed analyses of the NFC fractions in TMR silages are required to reveal rumen-fermentable carbohydrates.

Clear changes were observed in the soluble CP fraction, which increased from 302 g kg^{-1} before ensiling to 338–455 g kg^{-1} after ensiling on a CP basis. This result indicates that proteins in TMR were broken down to peptides, amino acids and NH_3 during ensiling. A high soluble CP intake by cattle causes excess NH_3-N in the rumen, leading to a higher urinary N loss.[15,26] The soluble CP fractions were significantly higher in TMR silage stored at 30 °C than in that stored at 15 °C after ensiling for 90 days ($P < 0.05$); however,

the result was not significant after ensiling for a shorter duration. Prolonging the ensiling period from 30 to 90 days also increased the soluble CP content ($P < 0.05$). Compared with the fermentation process shown in Fig. 1, changes in the protein fractions clearly occurred after acid was produced and the pH of TMR silage was stabilised. Protein degradation during ensiling of crop silage is thought to be caused by proteases from fresh plant material and microorganisms.[12] Although we did not determine protease activities in the ingredients, the contribution of these activities to protein degradation during ensiling would be low because these activities are much lower in silage than in fresh materials.[27] The proteolytic activity of grains during ensiling remains unknown. It has been reported that protease activities in grains gradually decline during maturation,[28] whereas they drastically increase after germination.[29] These findings suggest that protease activities in grains used as TMR ingredients are low because the grains are well matured and have not germinated. Heat processing grains by steaming and rolling partially deactivates enzymes; however, the effect of processing on these activities remains unknown. Protein breakdown caused by *Clostridium* and enterobacteria that produce proteases occurs in silage.[12] As described above, the possibility that *Clostridium* grew in TMR silages was low because of high DM. Moreover, butyric acid was not produced during ensiling in the present study. Cao *et al.*[9] reported that the number of enterobacteria are consistently low during TMR fermentation. Therefore, it seems that protein degradation by these bacteria is not a major factor during TMR ensiling. In contrast, lactic acid bacteria may have been predominant in TMR silages because of the high lactic acid content and low pH. Lactic acid bacteria are virtually non-proteolytic;[12] thus, their contribution to protein degradation in TMR silages would be extremely low. However, the soluble protein content increased from 30 to 90 days in the present study, suggesting that lactic acid bacteria may have had some effect on protein breakdown. Winters *et al.*[30] demonstrated that some lactic acid bacteria enhance protein degradation during ensiling. However, further studies on proteases of lactic acid bacteria are required to understand protein degradation in TMR silage.

In the *in vitro* rumen study, the SCFA concentration from TMR after a 4 h incubation before ensiling was slightly higher than that after ensiling; however, no significant difference was observed among the treatments after 8- and 24-h incubations (Table 4). These results indicate that the energy supply for ruminants from TMR silage after ensiling was not lower than that from TMR before ensiling. Lactic acid in the rumen is mainly metabolised to propionic acid as well as acetic and butyric acids.[31] The high lactic acid concentration in TMR after ensiling may have been metabolised to propionic acid in the *in vitro* rumen, as shown by the lower ratios of acetic and propionic acids from TMR silages. Throughout incubation, NH_3-N concentrations from TMR silages in the *in vitro* rumen were significantly higher than those from TMR silages before ensiling. These results are consistent with those of Cao *et al.*[24] who showed higher NH_3-N concentrations in the rumen of sheep fed a TMR silage than in that of sheep fed TMR before ensiling. No NH_3 absorption by the rumen wall or flow to the omasum occurred in the closed *in vitro* rumen system; thus, lowering the NH_3 content was highly dependent on bacterial uptake. NH_3 production in this system was mainly derived from the degradation of feed protein. The higher NH_3-N concentration from TMR silages in the *in vitro* rumen may have been a result of the rapid release of NH_3 from proteins and lower NH_3 uptake by bacteria. As described above, TMR after ensiling contained a higher soluble CP content than that before ensiling. Soluble proteins contain small N compounds such as peptides and amino acids, which rapidly release NH_3 into the rumen.[15] Similarly, fewer sugars remained in TMR silage, which led to a shortage of energy for rumen bacteria to take up NH_3. The NH_3-N concentration at the beginning of the incubation was 3.38 mg dL^{-1} (data not shown); thus, NH_3 may have been taken up by bacteria until 4 h of incubation when TMR before ensiling was used as the substrate. In contrast, the release of NH_3 from CP was higher than its uptake by rumen bacteria when TMR silages were used. These results suggest that N metabolism in the rumen of cows fed TMR silage after ensiling was altered in comparison with that in the rumen of cows fed TMR before ensiling.

CONCLUSIONS

TMR after ensiling had lower soluble sugar and higher soluble CP contents than that before ensiling. CP solubilisation was enhanced in TMR silage by both prolonged storage and higher storage temperatures. The presence of a higher ruminal NH_3 concentration from TMR silage during the *in vitro* ruminal incubation may have been caused by a shortage of fermentable sugars and enhanced deamination of soluble CP compounds. Further studies are required to investigate the nutritional changes in TMR silage on a practical scale and to identify N balance in ruminants fed TMR before and after ensiling.

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