Short Communication

Increase in Rumen Fibrolytic Bacteria and the Improvement of Fiber Degradability of Ensiled Total Mixed Ration Assessed by *in vitro* Rumen Culture

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Abstract | Effect of ensiling total mixed ration (TMR) on the improvement of fiber degradability in response to change in rumen fibrolytic bacteria was evaluated by an *in vitro* rumen culture technique. The TMR for dairy cattle consisted of 45% of roughage and 55% for concentrates on a dry matter basis and it was ensiled for 40 days in summer. An *in vitro* ruminal incubation experiment was conducted using rumen fluid with pre-ensiled or the ensiled TMR samples in anaerobic fermentation bottles for 6 and 24 h. The methane production (p=0.02) and total short chain fatty acids production (p=0.01) in the endiled TMR culture were higher than that in the pre-ensiled TMR culture. Copy numbers of two of three major fibrolytic bacteria (*Ruminococcus albus* and *Ruminococcus flavefaciens*) in the culture were higher at 24 h culture of the ensiled TMR (p=0.007 for *R. albus*, p=0.002 for *R. flavefaciens*) than that of pre-ensiled TMR, whereas *Fibrobacter succinogenes* numbers seemed to increase earlier than did the other two species. Our results suggest that the ensiling process for TMR may affect activities three rumen fiber-degrading bacteria, followed by improved fiber degradation in the *in vitro* rumen culture.

Keywords | Ensiled TMR, Fibrolytic bacteria, *In vitro* rumen, Methanogen, Short chain fatty acids

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Ensiled total mixed ration (TMR), referred to as TMR silage, has attracted considerable research attention for practical applications owing to its advantages such as long-term preservation and favorable changes in animal palatability due to lactic acid fermentation (Xu et al., 2007; Wang and Nishino, 2008; Xu et al., 2010; Kondo et al., 2015). Changes in nutritional properties during ensiling have also been reported. For example, fiber digestibility has shown to be improved by ensiled TMR in an *in vivo* digestibility assessment (Cao et al., 2010), which possibly links to enhanced fiber degradation in the rumen. In depth clarification of this phenomenon should be useful for further understanding of the mechanism of rumen fiber digestion, which is balanced with rumen microbial kinetics (Zhou, 2015). Therefore, in this study, we applied real time PCR quantification technique of three representative fiber-degrading bacteria and methanogens to *in vitro* incubation tests, for monitoring the effects of ensiled TMR in view of the change in the degree of fiber degradability and in populations of these microbes.

The TMR composed of 38% (dry matter [DM] basis) roughage (23% Italian ryegrass silage, 8% sorghum silage, and 7% corn silage), 8% beet pulp, and 55% of commercial concentrates consisted of corn grain, soybean meal, barley grain, wheat bran, corn gluten feed, and additives, was prepared by a practical TMR blender. After mixing, subsamples were collected as a TMR before ensiling (referred to

as "pre-ensiled TMR") and stored at -30°C until use. For TMR ensiling, approximately 350 kg of TMR on a fresh matter basis TMR was wrapped by stretch film with a baling machine. The ensiled TMR was placed outside and fermented for approximately 40 days (ambient high temperatures of 30–35°C). Thereafter, ensiled TMR was unwrapped, mixed well manually and were collected (referred to as "ensiled TMR") and stored at -30°C until further use. Fermented products (lactic acid, short chain fatty acid [SCFAs]) and the pH of TMRs were examined from water extracts as previously described in (Kondo et al., 2015). The composition of major nutrients was analysed according to the official methods of AOAC (2002) and NRC (2001). An *in vitro* ruminal experiment was conducted according to the methods described by (Kondo et al., 2015). Pre-ensiled and ensiled TMRs were freeze-dried and ground to pass through a 1-mm screen. For each type of TMR, approximately 1 g of the sample was weighed into six 120-mL glass vials, of which three were used to determine DM and neutral detergent fiber (NDF) degradability, and the other three was used for analyses of microbial composition by real-time PCR, as described below. Rumen fluid collection, donor cattle, and post-sampling were followed with the paper. Fifty milliliters of this medium was dispensed into each vial and these vials were covered with rubber cap and an aluminum ring and then incubated in a water bath at 39°C. The *in vitro* ruminal study was replicated twice, and the mean values are presented. The volume of gas was measured at 6 and 24 h post incubation. At 24 h post incubation, contents of a vial were transferred into a centrifuge tube and centrifuged at 1,000× g for 10 min at 4°C. The residues from each tube were transferred into crucible and freeze-dried to determine DM degradability in the *in vitro* rumen culture. Then, dried residues were used for NDF determination to calculate NDF degradability.

At 6 and 24 h post-incubation, the culture fluid was separated into a fluid and particle fraction by filtration using a cell strainer (pore size of 100 µm; Falcon Yellow Nylon Mesh Cell Strainer, B & D Japan, Tokyo, Japan). Residues in the strainer and the filtrate were designated as particle and fluid fractions, respectively. An aliquot (0.2 g for the particle fraction and 200 µL for the fluid fraction) was used for microbial genomic DNA extraction, with the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Real-time PCR based quantitative analyses of three major fibrolytic bacteria (*Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens*) that associated with the particle fraction were performed as previously described (Lwin et al., 2012). The following primer pairs were used for the analyses: Fsf and Fsr for *F. succinogenes* (Tajima et al., 2001), 1281f and 1439r for *R. albus* (Koike and Kobayashi, 2001), Rfl (f) and Rfl (r) for *R. flavefaciens* (Koike and Kobayashi, 2001). The amount of the gene encoding the α-subunit of methyl co-enzyme M reductase in the fluid fraction was quantified according to a previous report (Denman et al., 2007). Specificity and upper- and lower- detection limit had been validated in previous reports. All data for *in vitro* rumen incubation were analyzed using unpaired student's t-tests with SAS software ver. 9.3 (SAS Institute, Cary, NC, USA). Significance was defined as $p < 0.05$.

Table 1: Average chemical composition of TMRs before and after ensiling used as substrates for *in vitro* ruminal fermentation

	Pre-ensiled TMR	Ensiled TMR
Dry matter (%)	54.4	53.0
Organic matter (%DM)	92.2	91.8
Crude protein (%DM)	15.4	15.9
NDF (%DM) [1)]	37.5	37.2
NFC (%DM) [1)]	37.7	36.2
Soluble sugars (%DM)	5.5	0.9
pH	5.6	4.3

[1)] **NDF**: Neutral detergent fiber; **NFC**: Non-fiber carbohydrates.

Chemical compositions and fermentation data of the tested TMRs (pre-ensiled and ensiled) were shown in Table 1. Nutritional values in both TMRs were similar except for soluble sugar and non-fiber extracts, which suggest that bacteria that contribute to silage fermentation (e.g., *Lactobacillus* species) utilized these readily available carbohydrates and converted these to lactate and acetate. Results of the *in vitro* cultivation experiment are shown in Table 2. Methane production and dry matter degradability in the ensiled TMR culture were higher than those in the pre-ensiled TMR culture. Total SCFA was also remarkably higher in the ensiled TMR culture than in the pre-ensiled one. These data clearly indicated that the ensiling process for TMR affects *in vitro* rumen cultivation characteristics by activating microbes that may be fiber-degrading bacteria, as NDF degradability was also higher in the ensiled TMR culture. Interestingly, all the three representative fiber-degrading bacteria studied herein showed trends of higher numbers in the ensiled TMR culture at different timings, i.e., at 6 h post incubation for *F. succinogenes*, and 24 h for *R. flavefaciens* and *R. albus* (Table 3). These bacteria are considered to have different ecological niches in fiber that have the specifically optimized conditions for their manifestation, for example, with respect to their ability of attachment and mode of action to fiber (Dehority, 2003; Koike and Kobayashi, 2001). Thus, ensiling TMR might induce changes in the fiber composition, which would in turn offer favourable conditions for the growth of these bacteria. For example *R. flavefaciens* showed greater changes along with incubation period and between the pre-ensiling and the ensiled TMRs in this study. The number of *R. flavefaciens* was undetectable level at 6h in both cultures, however, the numbers increased to 10^7 (the pre-ensiling

TMR) or 10^8 (the ensiled TMR). In addition, the number of *R. flavefaciens* in the ensiled TMR culture was 5 times higher than in the pre-ensiling TMR culture. Increased *R. flavefaciens* might enhance NDF degradability and production of total SCFA. Since *R. flavefaciens* produces hydrogen as fermentation products, higher number of *R. flavefasiens* by ensiled TMR would cause higher CH_4 production in the present study. As such, our results may in part contribute to the understanding of this mechanism. It is necessary to determine the physical and/or chemical changes that TMR fibers undergo during ensiling (Cao et al., 2011; Kondo et al., 2015).

Table 2: *In vitro* fermentation results from TMR before and after ensiling

	Pre-ensiled TMR	Ensiled TMR	SEM	P value
Gas production (mL)	147	153	2	0.123
CH_4 production (mL)	14.5	16.8	0.5	0.023
Dry matter degradability (%)	39.0	41.2	0.5	0.004
NDF degradability (%) [1)]	40.2	49.1	1.9	<0.001
Total SCFAs (mM) [1)]	110.7	127.9	3.9	0.012
Acetate (mol% of total SCFAs)	55.1	57.4	0.6	0.051
Propionate (mol% of total SCFAs)	33.6	32.4	0.3	0.045
Butyrate (mol% of total SCFAs)	11.4	10.1	0.6	0.387

[1] **NDF**: Neutral detergent fiber; **SCFA**: Short chain fatty acid

Table 3: Microbial profile in *in vitro* rumen containing TMR before and after ensiling

	Pre-ensiled TMR	Ensiled TMR	SEM	P value
6 h incubation				
mcrA (× 10^4 copy/mL medium)	281	229	16	0.165
F. succinogenes (× 10^6 copy/g residue)	98	230	35	0.07
R. albus (× 10^4 copy/g residue)	30	36	3	0.47
R. flavefaciens (× 10^5 copy/g residue)	ND	ND		
24 h incubation				
mcrA (× 10^4 copy/mL medium)	907	1104	85	0.343
F. succinogenes (× 10^6 copy/g residue)	3416	4058	331	0.436
R. albus (× 10^4 copy/g residue)	32	78	10	0.007
R. flavefaciens (× 10^5 copy/g residue)	439	2191	373	0.002

ND: Not determined

Increased fiber degradation by fiber-degrading bacteria may occur, resulting in increased acetate production accompanied by hydrogen that was used for the reduction of CO_2 to generate methane. We also determined the relative numbers of methanogenic archaea in the respective cultures by quantifying the expression of *mcrA* gene involved in methane production. We observed a difference in the gene expression levels between the two groups but the difference was not significant, implying that relative activity of gene in respective cells of methanogenic archaea, but not absolute cell number, may be higher in the ensiled TMR culture than pre-ensiled TMR. Ensiled TMR using whole-crop rice and rice bran leads to low methane production *in vitro* and *in vivo*, compared to TMR which has not been ensiled (Cao et al., 2010, 2012), thereby resulting in reduced energy loss of the feed. However, we observed in this study that ensiled TMR resulted in more methane production compared to the pre-ensiled TMR (Table 2). As these results are conflicting with respect to increasing or decreasing methane production, in depth monitoring of the digestion kinetics of nutrients, as well as of microbial interactions within the ecosystem are required.

In this experiment, we succeeded to monitor populations of three fibrolytic bacteria in the *in vitro* rumen culture of ensiled TMR compared to pre-ensiled TMR. Increase in copy numbers of two *Ruminococcus* species may be involved in the increase in NDF degradability in the culture, and in the increase in methanogens by means of supplying hydrogen. However, relationship between fiber degradation activity in the rumen and rumen methane generation has yet been inconclusive, which is largely due to the complexity of the rumen microbial ecosystem (Flint, 1997; Russell and Rychlik, 2001). The contributions of other groups of microorganisms, including non-fibrolytic bacteria, protozoa, and fungi, should also be further evaluated. In addition, improvement in fiber digestibility does not necessarily lead to improvement of cattle productivity (Pinos-Rodríguez et al., 2008). Therefore animal feeding experiments are warranted to determine whether ensiling TMR increases feed efficiency due to the improvement of fiber degradability, or whether the effect may be offset by increasing methane emission.

AUTHOR'S CONTRIBUTION

All the authors contributed equally.

CONFLICT OF INTEREST

No competing financial or other interest exists.

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