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Effects of different dietary concentrate to forage ratio and thiamine supplementation on the rumen fermentation and ruminal bacterial community in dairy cows

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Abstract. A subacute ruminal acidosis (SARA) model was induced gradually by increasing the proportion of dietary concentrate to evaluate the effect of thiamine supplementation on the structure of bacterial community in dairy cows. Three Holstein dairy cows with rumen cannula were randomly assigned to a replicated 3 × 3 Latin square design trial and received three diets during three successive 21-day periods in each square. The three dietary treatments were as follows: a low-concentrate diet (control), a high-concentrate SARA-induced diet (SARA) and a high-concentrate SARA-induced diet with 180 mg thiamine/kg DM (SARA+thiamine). Real-time–polymerase chain reaction assay was used to quantify the population variation of SARA-related ruminal bacteria in these cows. The results showed that SARA was induced gradually when cows were fed with the high-concentrate diets. The mean ruminal pH value was higher in the control cows than in those of SARA and SARA+thiamine groups, the mean was decreased in cows fed on SARA diet, and the depression was alleviated by supplemented thiamine and the difference was significant ($P < 0.05$) especially at 9-h and 12-h sample times (or 1 h and 4 h after the second feeding). The populations of *Streptococcus bovis* and genus *Lactobacillus* in cows from the SARA group were increased in log copies/μL by 3.62% and 4.65%, respectively, compared with the control group ($P < 0.05$). In contrast, in log copies/μL, populations of *Butyrivibrio fibrisovens* and *Megasphaera elsdenii* were decreased by 1.14% and 4.90%, respectively ($P < 0.05$). Thiamine supplementation led to an obvious reduction of *Strepococcus bovis* and *Lactobacillus* ($P < 0.05$), whereas the number of log copies/μL of *Megasphaera elsdenii* was dramatically increased ($P < 0.05$). There was no significant effect of thiamine supplementation on the number of log copies/μL of *Butyrivibrio fibrisovens* and *Selenomonas ruminantium* ($P > 0.05$). It was concluded that thiamine supplementation to high-concentrate diets at concentrations of 180 mg/kg DM could help alleviate SARA by increasing rumen pH and balancing the population of lactic acid-producing and -consuming bacteria.

Additional keywords: dietary NFC : NDF, SARA.

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Introduction

It is well established that thiamine is an essential cofactor required for carbohydrate metabolism, and the production of thiamine by the rumen microflora is normally adequate to supply ruminant's requirements (Breves *et al*. 1981; Miller *et al*. 1986). Subacute ruminal acidosis (SARA) is a common chronic digestive disorder in high-yielding dairy cows that receive highly digestible grain diets, and is defined as periods of moderately depressed ruminal pH, namely, lower than 5.8 (Penner *et al*. 2007). Current feeding practices in feedlot beef and high-producing dairy cattle use highly fermentable grain diets to increase growth rate and milk production, but because of microbial disturbances, they predispose cattle to digestive disorders such as SARA. It is estimated that 20% of early lactating dairy cows can be affected by SARA in intensive feeding systems, and this results in a decrease of feed efficiency and milk production, and SARA may even develop into chronic metabolic acidosis, causing hepatic abnormalities, diarrhoea and laminitis (Plaizier *et al*. 2008). Therefore, it is a major concern to prevent or alleviate the occurrence of SARA in dairy herds. In the past decade, several dietary strategies proposed for use in preventing SARA, such as sodium bicarbonate buffer and monensin iono-phones as well as probiotics, have been found to stabilise ruminal pH and improve animal production (Mutsvangwa *et al*. 2002; Paton *et al*. 2006; Chaucheyras-Durand *et al*. 2008; Desnoyers *et al*. 2009; Packer *et al*. 2011). However, none of these approaches has consistently maintained higher and stable ruminal pH. De Oliveira *et al*. (1997) and Tafaj *et al*. (2006) observed that the adequate status of thiamine in ruminants can be altered by

SARA conditions and this can lead to cerebrocortical necrosis, which is particularly associated with intensive feeding systems and high-concentrate diets in dairy cattle. Our previous research found some evidence that adding 180 mg thiamine/kg could alleviate SARA by increasing the rumen pH and decreasing the lactate concentration by regulating the structure of rumen microbial community *in vitro* (Pan *et al*. 2013). Moreover, effects of thiamine on the microbial community in the rumen of dairy cows during high-concentrate induced SARA are less understood, and there is very little information on use of thiamine for preventing SARA. Therefore, the objective of the present study was to determine the effects of different dietary concentrate levels and thiamine supplementation on the rumen fermentation and ruminal bacterial community in dairy cows.

Materials and methods

Animals and sample collection

Three Holstein cows (650 ± 20 kg) fitted with ruminal cannula were allotted to a replicated 3 × 3 Latin square design (n = 6). The animals were fed with the following three diets: a low-concentrate diet [control: roughage to concentrate (R : C) ratio of 60 : 40, non-fibre carbohydrate (NFC) : neutral detergent fibre (NDF) = 1.19], a high-concentrate SARA diet (SARA: R : C ratio of 30 : 70, NFC : NDF = 2.30), and a high-concentrate SARA diet with 180 mg/kg thiamine (SARA+thiamine), where SARA was induced gradually by a high-concentrate diet. The composition and nutrient concentrations of diets are shown in Table 1. Cows were housed in individual tie-stalls and provided feed *ad libitum* at 8-h intervals daily (0600 hours, 1400 hours and 2200 hours) and free access to drinking water. All animals were cared for and handled in accordance with the protocol approved by Animal Care and Ethical Committee of Yangzhou University (No. 201206118). The trial was replicated with three successive 21-day periods in each square. At the end of each period, rumen digesta samples were collected from the three fistula cows at 0-h, 3-h, 6-h sample times (0 h, 3 h and 6 h after the first feeding), and 9-h and 12-h sample times (1 h and 4 h after second feeding) for pH determination. At each sampling time, the pH was measured immediately after collection, with a handheld pH electrode (Model B-4, Shanghai Chemical, China). Other rumen samples collected immediately 1 h after the morning feeding were used for DNA extraction.

DNA extraction

Community DNA was extracted from 0.5 mL aliquots of rumen fluid and digesta by the method described by Yu and Morrison (2004).

Real-time–polymerase chain reaction (PCR) assays

As demonstrated in Table 2, a set of PCR primers were designed according to GenBank accession numbers and validated for specific detection and quantification of *Strepococcus. bovis*, *Butyrivibrio fibrisolvens*, *Lactobacillus*, *Selenomonas ruminantium* and *Megasphaera elsdenii.*

Table 1. Ingredients and chemical composition of diets fed to the control and high-concentrate (SARA) or SARA+thiamine cows (DM basis, %)

Ingredient	Composition of experimental diets	
	Control	SARA
Guinea grass hay	14.66	6.08
Alfalfa hay	14.66	6.08
Corn silage	30.34	17.96
Concentrate mix[A]	40.34	69.88
Chemical composition		
Net energy for lactation (NE_L; MJ/kg)[B]	6.83	7.28
Crude protein (%DM)	14.31	16.63
Non-fibre carbohydrates (NFC, %) DM	46.79	58.39
Neutral detergent fibre (NDF, %DM)	39.32	25.39
NFC : NDF[C]	1.19	2.30
Ca (%DM)	0.49	0.47
P (%DM)	0.29	0.31

[A]Concentrate mix: 29% corn grain, 28% corn compressor, 12% DDGS, 12% soybean meal, 12% cottonseed meal, 2% $Ca(HCO_3)_2$, 0.7% $CaCO_3$, 2% $NaHCO_3$, 1% NaCl, 0.5% MgO and 0.8% premix. One kilogram of premix contains the following: 3125 mg $CuSO_4{\cdot}5H_2O$, 9375 mg $FeSO_4{\cdot}H_2O$, 14 375 mg $MnSO_4{\cdot}H_2O$, 125 mg $ZnSO_4{\cdot}7\ H_2O$, 0.3 mg Co, 0.2 mg Se, 6.25 mg I (as potassium iodide), 1 500 000 IU VA, 1 250 000 IU VD_3, 125 mg VE 3, 4500 mg niacin, 125 000 mg choline.

[B]Calculated using NE_L values of feedstuffs from NRC (2001);

[C]Non-fibre carbohydrates to NDF ratio, where NFC = 100-(%CP + %NDF + %EE + %ash), other nutrients are measured values using method (AOAC 2005).

Table 2. Designed polymerase (PCR) primer sequences of the rumen bacteria

Rumen bacterium	Primer sequence	Amplicon size (bp)	GenBank Accession number
S. bovis	F:5′- CGATACATAGCCGACCTGAG-3′ R: 5′- TAGTTAGCCGTCCCTTTCTG-3′	235	*AF*135453.1
B. fibrisolvens	F:5′-GGAGCAAACAGGATTAGATACCC-3′ R:5′-TGACGACAACCATGCACCAC-3′	293	*EU*684229.1
Lactobacillus	F: 5′- AGCGAACAGGATTAGATACCC –3′ R: 5′-GATGGCACTAGATGTCAAGACC-3′	233	*AB*680529.1
S. ruminantium	F: 5′-GAGCGAACAGGATTAGATACCC-3′ R: 5′-TGCGTCGAATTAAACCACATAC-3′	194	*AB*198424.1
M. elsdenii	F: 5′-GACCGAAACTGCGATGCTAGA –3 R: 5′-CGCCTCAGCGTCAGTTGTC-3′	129	*JCM* 1772 [T]

Plasmids were constructed and verified by a real-time–PCR standard curve, using 10-fold serial dilutions. Real-time–PCR amplification and detection were performed using ABI 7500 system (Applied Biosystems, Foster City, CA, USA). The reaction was conducted in a final volume of 20 μL, containing the following: 10.4 μL SYBR® Premix ExTaq™ (TaKaRa, Dalian, China), 0.8 μL forward primer, 0.8 μL reverse primer, 6.0 μL distilled water, and 2.0 μL of DNA solution (50 ng/μL). PCR conditions for rumen bacteria above were as follows: DNA was initially denatured at 94°C for 5 min, followed by 30 cycles (denaturing at 94°C for 30s, annealing 56°C for 34s and extension at 72°C for 40 s). After the last cycle of amplification, an analysis of the product melting curve was performed to determine the specificity of amplification.

Statistical analyses

Statistical analysis was performed for data evaluation. Variables, least square means for rumen pH and bacterial population data were generated and tested at a significance level of $P = 0.05$. An analysis was performed by ANOVA using the mixed procedure of SAS version 9.2 (2002; SAS Institute Inc., Cary, NC, USA). The experimental data were analysed in a replicated 3 × 3 Latin square design, using following model:

$$Y_{ijkl} = \mu + T_i + S_j + C_{k(j)} + P_l + \varepsilon_{ijkl},$$

where Y_{ijkl} is the dependent variable, μ is the general mean, T_i is fixed effect of the treatments (i = 1, 2 and 3 for control, SARA, and SARA+thiamine, respectively), S_j is random effect of square (j = 1, 2 and 3), $C_{k(j)}$ is the effect of cow within square, P_l is period within square, and ε_{ijkl} is the random residual error. Means were separated using Duncan's multiple range tests.

Results

Induction of SARA model

The results in Table 3 show that ruminal pH in cows fed with a low-concentrate diet (control) was in the normal physiological range at 0- to ~12-h sample times after feeding. Lower pH values were observed in cows that received treatment SARA and SARA +thiamine than in the control. Ruminal pH fell below 5.8 in cows fed with SARA (high-concentrate) diets and this lasted more than 6 h (from 3-h to 12-h sampling times), indicating that SARA was induced successfully in cows receiving the SARA diet. In addition, ruminal pH in cows fed with SARA+thiamine diet was higher than that with SARA treatment and the differences were significant ($P < 0.05$) at 9-h and 12-h sample times (1 h and 4 h after second feeding).

Table 3. Influence of dietary treatments on ruminal pH in cows
SARA, high-concentrate diet. a, b, c, Means within a column with different letters indicate a significant difference ($P < 0.05$), while the same letter indicates no significant difference ($P > 0.05$)

Dietary treatment	Sample time point				
	0 h	3 h	6 h	9 h	12 h
Control	6.52a	6.39a	6.13a	6.37a	6.56a
SARA	5.93b	5.67b	5.45b	5.63c	5.75c
SARA+thiamine	6.04b	5.82b	5.71b	5.78b	5.92b
s.e.m.	0.11	0.13	0.15	0.07	0.17
P-value	0.036	0.040	0.059	0.009	0.005

Verification of recombinant plasmid

The recombinant plasmids were verified by PCR amplification, with incubating medium as a template directly. As shown in Fig. 1, the gel electrophoresis results of PCR products were satisfactory with clear bands, correct placement and specific amplification, and were used for the construction of the calibration curve (data not shown).

Calibration curve and quantitative analysis of rumen bacteria

External standards for real-time–PCR were prepared from bacterial plasmids. For each standard, linear regressions derived from the threshold cycle [Ct] of each DNA dilution versus the log quality were calculated. Logarithms of the DNA concentration (copies/μL) were plotted against the calculated means, obtaining a straight line of equations $Y = -3.05X + 36.25$ (*S. bovis*, $R^2 = 0.996$); $Y = -3.35X + 36.77$ (*B. fibrisolvens*, $R^2 = 0.985$); $Y = -3.18X + 37.45$ (*Lactobacillus*, $R^2 = 0.987$); $Y = -2.93X + 36.25$ (*S. ruminantium*, $R^2 = 0.993$) and $Y = -2.99X + 42.51$ (*M. elsdenii*, $R^2 = 0.930$), where Y is the log of DNA concentration and X is the Ct, the equations above were used to quantify DNA from rumen fluid samples.

The profiles of related rumen bacterial population are illustrated in Fig. 2. The population of genus *Streptococcus bovis* and *Lactobacillus* of cows from the SARA treatment were increased ($P < 0.05$) in log copies/μL by 3.62% and 4.65%, respectively, compared with control. In contrast, log copies/μL of *B. fibrisolvens* and *M. elsdenii* were decreased by 1.14% and 4.90% ($P < 0.05$), respectively. Supplementation with thiamine led to a reduction of *S. bovis* profile ($P < 0.05$). However, the population of *M. elsdenii* was dramatically enhanced in the cows receiving the SARA+thiamine diet, compared with that of the cows receiving SARA without thiamine diet ($P < 0.05$). There was no significant ($P > 0.05$) effect of thiamine supplementation on the population of *B. fibrisolvens* and *S. ruminantium* in cows.

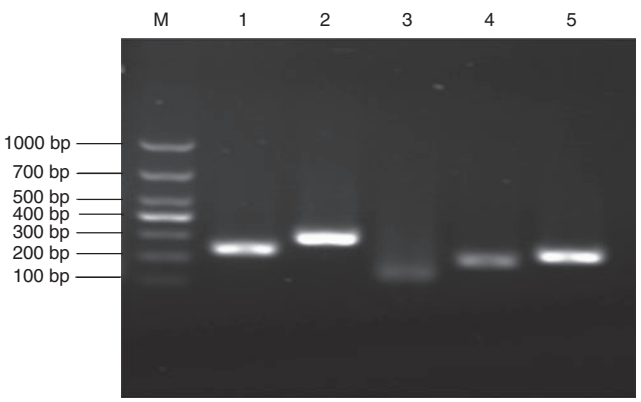

Fig. 1. Polymerase chain reaction (PCR) amplification production of positive cloning of rumen bacteria. M, DNA marker 1000; Lines 1~5, PCR products of positive cloning of *S. bovis*, *B. fibrisolvens*, *M. elsdenii*, *S. ruminantium*, *Lactobacillus*.

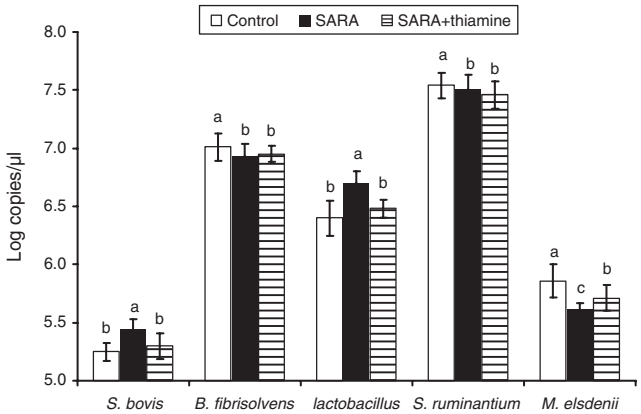

Fig. 2. Effects of a low-concentrate diet (control), a high-concentrate diet (SARA) and a high-concentrate diet with thiamine supplementation (SARA +thiamine) on rumen bacterial population. a, b, c, Different letters indicate a significant difference ($P < 0.05$), while the same letter indicates no significant difference ($P > 0.05$).

Discussion

SARA is the consequence of feeding grain diets to ruminant animals. The ruminal pH observed in the SARA diet corresponded to a higher rate of degradation and fermentation of the high-grain diet that produced more organic acids in the rumen, and a decline in ruminal pH occurred during SARA accordingly. Current definitions of SARA are based on the low rumen pH typically generated on high-grain diets (Oetzel 2003; Plaizier *et al*. 2008). However, there is no general agreement on the pH threshold that defines SARA, and moreover, rumen pH may not even be highly correlated with disease symptoms (Enemark *et al*. 2004; Khafipour *et al*. 2009). According to the definition of experimental SARA, a pH threshold value of 5.8 was used to define SARA as suggested by Kleen and Cannizzo (2012) and Penner *et al*. (2007). The pH values of cows on the SARA diet were below 5.8 for at least 9 h, and 6 h in cows on SARA+thiamine diet in the current experiment, this suggesting that SARA occurred in cows fed both the SARA and SARA+thiamine diets.

One of the most interesting findings of the present study was the bacterial population variation. The present real-time–PCR analysis shows distinct changes in the bacterial population profile in the rumen of cows fed different treatment diets. There was a more abundant population of *S. bovis* and *Lactobacillus* in cows fed the SARA diet than either the control or SARA+thiamine diets. Such changes in bacterial population profile and abundance may be due to the increased fermentable substrate present in the diet, favouring the growth of amylolytic and other starch-digesting bacterial species (Goad *et al*. 1998). The results suggest that supplementation with thiamine could alleviate SARA, and supports our previous findings *in vitro* (Pan *et al*. 2013). One possible reason for this is that thiamine promotes carbohydrate metabolism, where thiamine was in the form of thiamine pyrophosphatase (TPP) as the coenzyme of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase in the pathway of citric acid cycle, and, consequently, to reduce the accumulation of pyruvate and lactate. Also, SARA may be a result of decreasing profile of lactate-consuming bacteria (Russell and Hino 1985), where the growth of lactate-consuming bacteria such as *M. elsdenii* and *S. ruminantium* may be suppressed when SARA occurs, and the unbalanced rumen microflora may eventually result in SARA (Russell *et al*. 1981). Particularly, the real-time–PCR data also indicated that thiamine supplementation significantly reduced *S. bovis* and increased the *M. elsdenii* population profile compared with that of the SARA diet (Fig. 2). This implies that thiamine may have a function to stabilise ruminal pH value by improving the growth of lactate-consuming bacteria (such as *M. elsdenii*) and suppressing that of lactate-producing bacteria (such as *S. bovis*), which may help maintain the balance in the rumen bacteria and contribute to the remission of SARA. The pathway of thiamine regulating the bacterial growth and their metabolism is still unclear and further research is needed. Overall, this evidence may provide an alternative and method to manage SARA in the future.

Conclusions

The results of this study indicated that thiamine supplementation to high-concentrate diets at concentrations of 180 mg/kg DM could help alleviate SARA by increasing rumen pH and balancing the population of lactic acid-producing and -consuming bacteria.

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