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

Hongrong Wang\textsuperscript{A,D}, Xiaohua Pan\textsuperscript{A,B}, Chao Wang\textsuperscript{C}, Mengzhi Wang\textsuperscript{A} and Lihuai Yu\textsuperscript{A}

\textsuperscript{A}College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, China. \\
\textsuperscript{B}Faculté de Médecine vétérinaire, Université de Liège, Liège 4000, Belgique. \\
\textsuperscript{C}School of Clinical Medicine, Jiangsu University, Zhenjiang 212001, China. \\
\textsuperscript{D}Corresponding author. Email: hrwang@yzu.edu.cn

\textbf{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 \times 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 \textit{Streptococcus bovis} and genus \textit{Lactobacillus} in cows from the SARA group were increased in log copies/\textmu L by 3.62% and 4.65%, respectively, compared with the control group ($P < 0.05$). In contrast, in log copies/\textmu L, populations of \textit{Butyrivibrio fibrisoaves} and \textit{Megasphaera elsdenii} were decreased by 1.14% and 4.90%, respectively ($P < 0.05$). Thiamine supplementation led to an obvious reduction of \textit{Streptococcus bovis} and \textit{Lactobacillus} ($P < 0.05$), whereas the number of log copies/\textmu L of \textit{Megasphaera elsdenii} was dramatically increased ($P < 0.05$). There was no significant effect of thiamine supplementation on the number of log copies/\textmu L of \textit{Butyrivibrio fibrisoaves} and \textit{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.

\textbf{Additional keywords:} dietary NFC : NDF, SARA.

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\textbf{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 \textit{et al.} 1981; Miller \textit{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 \textit{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 \textit{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 \textit{et al.} 2002; Paton \textit{et al.} 2006; Chaucheyras-Durand \textit{et al.} 2008; Desnoyers \textit{et al.} 2009; Packet \textit{et al.} 2011). However, none of these approaches has consistently maintained higher and stable ruminal pH. De Oliveira \textit{et al.} (1997) and Tafaj \textit{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).

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, %)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Composition of experimental diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Guinea grass hay</td>
<td>14.66</td>
</tr>
<tr>
<td>Alfalfa hay</td>
<td>14.66</td>
</tr>
<tr>
<td>Corn silage</td>
<td>30.34</td>
</tr>
<tr>
<td>Concentrate mix^A</td>
<td>40.34</td>
</tr>
</tbody>
</table>

^AConcentrate mix: 29% corn grain, 28% corn coressor, 12% DDGS, 12% soybean meal, 12% cottonseed meal, 2% Ca(HCO3)2, 0.7% CaCO3, 2% NaHCO3, 1% NaCl, 0.5% MgO and 0.8% premix. One kilogram of premix contains the following: 3125 mg CuSO4 5H2O, 9375 mg FeSO4 H2O, 14375 mg MnSO4 H2O, 125 mg ZnSO4 7 H2O, 0.3 mg Co, 0.2 mg Se, 6.25 mg I (as potassium iodide), 1500 000 IU VA, 1250 000 IU VD3, 125 mg VE 3, 4500 mg niacin, 125 000 mg choline.

^BCalculated using NEc values of feedstuffs from NRC (2001);

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

<table>
<thead>
<tr>
<th>Rumen bacterium</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
<th>GenBank Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. bovis</td>
<td>F:5'- CGATACATAGCCGACCTGAG-3'</td>
<td>235</td>
<td>AF135453.1</td>
</tr>
<tr>
<td></td>
<td>R: 5'- TAGTATTAGCCGTTCCTTCTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. fibrisolvens</td>
<td>F:5'-GAGCAAACAGGATAGTACCC-3'</td>
<td>293</td>
<td>EU684229.1</td>
</tr>
<tr>
<td></td>
<td>R:5'-TGAGCAACATTAGCTACCC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>F: 5'-GATGGCATAGTACCC-3'</td>
<td>233</td>
<td>AB680529.1</td>
</tr>
<tr>
<td></td>
<td>R:5'-GATGACCATAGTACCC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. ruminantium</td>
<td>F:5'-GAGCGAACAGGATAGTACCC-3'</td>
<td>194</td>
<td>AB198424.1</td>
</tr>
<tr>
<td></td>
<td>R:5'-TGCGCTGAATTAAACCCACCATAC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. elsdenii</td>
<td>F:5'-GACCGAATCTGCGATGCTAGA-3'</td>
<td>129</td>
<td>JCM 1772 T</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CGCTCTACGCCGTAGTTGTC-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Plasmins 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 ExTag™ (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 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 x 3 Latin square design, using following model:

\[ Y_{ijkl} = \mu + T_i + S_j + C_{k(i)} + P_l + e_{ijkl} , \]

where \( Y_{ijkl} \) is the dependent variable, \( \mu \) 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), \( C_{k(i)} \) is the effect of cow within square, \( P_l \) is period within square, and \( e_{ijkl} \) is the residual random 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).

**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 \( Y = -2.99X + 42.51 \) (\( M. elsenii \), \( R^2 = 0.930 \)), 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 \( M. elsenii \) 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 \( M. elsenii \) 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.
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.

**Acknowledgements**

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