Dose Response of Ruminal Microbial Flora and Metabolism of Sheep to Supplemental Polyacrylamide

Chen Mo[1], Luo Qiujiang[1*], Chen Yong[1], Liu Shimin[2], Zang Changjiang[1]

1. Laboratory of Animal Nutrition, College of Animal Science, Xinjiang Agricultural University, Urumqi 830052, China; 2. UWA Institute of Agriculture, The University of Western Australia, Crawley WA 6009, Australia

Abstract **[Objective]** The paper was to test the effects of polyacrylamide (PAM) on ruminal microbial flora, activities of fibre-degrading enzymes and voluntary feed intake (VFI) of sheep. **[Method]** Five of 2–3 years old male Small-tail Han sheep, with body weight of (51.5±4.1) kg and fitted with permanent rumen fistula were used. By a 5×5 Latin square design, sheep were fed a basal diet supplemented with PAM at 0 (control), 1.0, 2.0, 3.0 and 6.0 g/kg diet, respectively. **[Result]** PAM supplementation affected VFI and ruminal indexes, and the peak values of them were at 2.0 g/kg diet by 17.9% for VFI, 56.6% for total bacteria, 18.3% for endocellulase, 19.4% for exocellulase and 16.5% for cellubiase (all $P<0.05$), respectively, compared with control. The NH_3–N, protozoal counts, and the protease activity in rumen fluid were all linearly declined, but fungal counts increased linearly. No significant changes were found in pH, activities of xylanase and amylase of rumen fluid. The PCR copies of *Fibrobacter succinogenes*, *Ruminococcus albus*, *Butyrivibrio fibrisolven* and *Selenomonas ruminantium* increased to maximum of 21.4%, 22.7%, 22.6% and 20.6% (all above $P<0.05$), whereas these of *Prevotella bryantii*, *Clostridium aminophilum*, methanogens and microscopic counting of great coli decreased to maximum of 24.5%, 34.8%, 21.2% and 52.2% (all above $P<0.05$); but *Salmonella bovis* and *Prevotella ruminicola* were not affected by PAM. **[Conclusion]** PAM supplementation at 2.0 g/kg diet can reduce protozoa but increase bacteria, particularly those species for fibre digestion, and fungi in rumen. The effect of PAM on bacterial species was selective. These changes finally enhanced activities of enzymes for fibre digestion in rumen, and stimulated VFI of sheep, suggesting a potential application of PAM in ruminant production.

Keywords Polyacrylamide; Sheep; Microbial flora; Feed intake; Rumen

Partially decreasing the number of ruminal protozoa can result in an increase of voluntary feed intake (VFI), improvement of ruminal digestion in sheep[1–2], and promotion of productivity of animal[3–4]. Although supplementation of formalin or metronidazole have been found to be effective on reducing ruminal protozoal number and improving nutrition in sheep[2,5-6], formalin is toxic, and metronidazole is a forbidden feed additive, so they can not be used in farming practice. Alternatively, docusate (DOC) is an non-toxic anionic surfactant and can be used to manipulate ruminal microbe flora and metabolism in sheep[7] for increasing digestion and productive performances [8–10]. Therefore, DOC could be a potential feed additive for sheep production.

Here, we reported using of another reagent, polyacrylamide (PAM), also as an anionic surfactant, for manipulating protozoal number or microbial flora in rumen of sheep. PAM is usually used for water purification in factories, non-toxic, and does not de-polymerize significantly in heating conditions of cooking[11]. We hypothesized that PAM will have a similar effect to DOC does on ruminal protozoa and rumen metabolism in sheep, because of PAM also as an anionic surfactant. This hypothesis was tested in the present study with sheep supplemented with 4 levels of PAM in diet, and the ruminal microflora and activities of digestive enzymes in sheep, as well as VIF, were measured to assess the effect of PAM.

1 Materials and Methods

1.1 Animals and experimental design

The use of animals and the experimental protocol in this study was approved by the Animal Care Committee, Xinjiang Agricultural University (Urumqi, China), and the experimental procedures were in accordance with the University's guidelines for animal research.

Five Small-tail Han rams, 2–3 years old, with body weight (bw) of (51.5±4.1) kg, were installed with permanent rumen fistula for this experiment. Animals were kept in individual pens and were fed a diet containing concentrates and forages at a ratio of 4:6 *ad libitum*. Five treatments consisted of supplementation of PAM at doses of 0 (Control), 1.0, 2.0, 3.0, and 6.0 g per kg dry matter of diet, referring as treatments 1, 2, 3, 4, and 5, respectively. In the morning and afternoon feeding, PAM powder was weighed out according to the dosages, mixed into the concentrate and offered to animals, respectively. Five treatments were applied to those 5 animals according to a 5×5 Latin Square design. Each period lasted for 21 d, includ-

Received: 2022–05–08 Accepted: 2022–05–26
Supported by National Natural Science Foundation of China (31772625).
*Corresponding author. E-mail: qjl@xjau.edu.cn

ing 17 d for adaptation to the new dose of PAM, and following 4 d for sample collections of rumen fluid. At the end of each period, rumen fluid from another backup sheep fed the basal diet without any PAM supplementation was collected and dosed into the rumen of the experimental sheep, each 200 mL/d, for 3 d to recover the microbial flora in the rumen.

The powder of PAM was purchased from Jining Huakai Resin Co., Shandong, China. The product had a purity of ≥90%, a molecular weight of 3 000–22 000 KD and an ion density of 10%–50%.

1.2 The feeding and management of animals The ingredients and nutrient compositions of the basal diet for the animals are shown in Tab.1. The proportion of corn stalk (roughage) in the diet was about 60% on dry matter basis.

Sheep were kept in individual pens. The ration was divided to two equal portions, and fed at 09:00 and 19:00, respectively. The concentrate was fed first, followed with corn stalk. The amounts of feed offered and the residue were recorded daily for calculations of daily feed intake. The amount of feed offered was daily adjusted according to the feed consumption yesterday to allow for 2%–3% of diet residue for measuring VFI. The ratio of roughage to concentrates was maintained constant throughout the whole experimental period. The animals were free access to drinking water.

1.3 Sample collection and pre-treatment During the 4 sampling days of each period, diet samples of the mixed concentrate, corn stalk and refusal for each sheep were collected every day, air-dried at room temperature, and pooled together at the end of each period. The samples were grounded through 1 mm sieve and used for analyses of DM and OM later. About 50 mL of rumen fluid was drawn via the rumen cannula from each sheep immediately before the morning feeding (0 h), and then respectively at 1.5, 3, 5, 7, and 10 h after feeding, so each animal had 4 replicate samples at each time point. After sampling at 0 h each day, 4.5 g PEG 6000, dissolved in about 100 mL water, for each sheep was injected into the rumen via ruminal cannula for determining the volume and turnover of rumen fluid.

The pH of rumen fluid was immediately measured after sampling. Then, the rumen fluid was filtered through double layer nylon bag of 100 mesh, and the nylon bag was gently squeezed. After mixing, three aliquots of 5 mL rumen fluid were taken, a drop of saturated mercuric chloride was added, and the samples were stored at −20 ℃ for the determination of NH_3–N, volatile fatty acids (VFA) and PEG. Three aliquots of 2.5 mL rumen fluid were mixed with an equal volume of 20% formalin and stored at 4 ℃ for classically microscopic counting of bacteria and protozoa. Another three aliquots of 1 mL rumen fluid were stored in liquid nitrogen for DNA extraction for measurement of the copy number of microbes. Additional three aliquots of 5 mL rumen fluid were mixed with an equal volume of phosphate buffer (50 mM, pH 6.0) to dilute rumen fluid and stored at −20 ℃ for determination of the activities of digestive enzymes. And 5 mL of rumen fluid, with a drop of saturated mercuric chloride, was frozen as a backup sample. Each sample from individual animal at given time was separately stored and determined.

Tab.1 The ingredients and nutrient compositions of the basal diet (DM basis)

Ingredients	Proportion//%	Nutrient composition②	Proportion//%
Cornstalk	59.50	Organic matter	91.35
Corn	24.72	Crude protein	12.01
Cottonseed meal	7.65	Cellulose	28.85
Rapeseed meal	2.91	Hemi–cellulose	21.37
Urea	0.72	Lignin	4.02
NaCl	0.40	Calcium	0.88
Limestone	2.10	Phosphorus	0.35
Mineral and vitamin premix①	2.00	Digestive energy//MJ/kg	11.24
Total	100		

Note: ① The mineral and vitamin premix provides nutrients per kg basal diet: vitamin A 1350 IU, vitamin D_3 270 IU, vitamin E 45 IU, iron 16 mg, manganese 10 mg, copper 8 mg, zinc 5 mg, iodine 8.5 mg, selenium 0.20 mg, cobalt 0.10 mg; ② The nutrient compositions were actually measured except for digestive energy.

1.4 Assays of samples

1.4.1 Microbial counting and metabolic index of rumen fluid. The pH of rumen fluid was determined with a pH-meter (model 510; Cyberscan). The concentration of NH_3–N was measured by the magnesium oxide distillation method from AOAC[12] and Mohsen *et al*.[13], and 3.0 mL of rumen fluid was used for each distillation. The concentration of VFA was determined by gas chromatography[14], and 0.1 mL of rumen fluid was used for each test. The method for PEG was referenced to Hyden[15] as described, and 1.0 mL of rumen fluid was used. The methods of microscopic counting and classification of rumen bacteria and protozoa were according to Chung *et al*.[16] and Dehority[17], but because of the difficulty of distinguishing between cocci and streptococcus, the counts of these two bacteria were pooled as one index.

Four rumen fluid samples with same sampling time were pooled, and 1 mL of sample was taken for DNA extraction by CTAB and physical crushing method [18]. Briefly, the samples were centrifuged at 4 ℃ and 12 000×g for 5 min, the supernatant was discarded, then 800 μL of sterilized PBS was added, mixed, and centrifuged again. Then the samples (sediments) were treated with 0.3 g of fine glass beads and 800 μL of CTAB, with ball milling instrument (concussion 2 min, interval 2 min,

for 3 times), and then RNA enzyme was added to incubate at 37 ℃ water bath to remove RNA for purification of DNA. Finally, the concentrations of DNA were determined by optical reading with a microplate reader and the OD_{280} of DNA was diluted with TE buffer (Tris·HCl 10.0 mM, EDTA 1.0 mM, pH 8.0) to $1.9 \leqslant 2.0$, with the OD_{260}/OD_{280} ratio of 1.6–1.8. The sample was then PCR-amplified following the primers of conserved or functional regions of bacteria, protozoa and fungi (Tab.2), provided by Shanghai Bioengineering Co., Ltd., China, and the copy numbers of rumen microbe were measured by real time fluorescent quantitative PCR determination using the corresponding recombinant plasmid confirmed by DNA sequencing as the standard[19]. Each sample was duplicate assayed, and the average value was used.

1.4.2 Digestive enzyme activities in rumen fluid. The activities of 7 digestive enzymes, namely endocellulase, exocellulase, cellobiose, xylanase, pectinase, amylase, and protease in rumen fluid were assayed.

Endocellulase activity was determined according to Agarwal *et al*.[28] and the substrate for endocellulase was 0.5% sodium carboxymethyl cellulose (CMC-Na) solution. Briefly, 0.5 g of CMC-Na was dissolved in 100 mL of 50 mM, pH 6.0 phosphate buffer. One mL of substrate solution was added to 10 mL graduated test tube and was put in 39 ℃ water bath for 30 min with oscillation. Then 1 mL of warmed diluted rumen fluid was added and incubated for exactly 3 min, and was taken out, and 2 mL of dinitrosalicylic acid (DNS) was immediately added to stop the reaction. Then the sample was put in boiling water bath for 10 min, followed with immediately cooling by running water. Sample was then topped up to exact 10 mL volume with distilled water, shaken and transferred to 10 mL centrifugal tubes, and centrifuged at 1 500×g for 10 min. The supernatant was used for the assay at OD_{550}[29].

The substrate for determination of the exocellulase activity was 0.5% microcrystalline cellulose suspension solution[28]: *i.e*., 0.5 g qualitative filter paper was cut into pieces and put into 200 mL beaker flask with 50 Mm, pH 6.0 phosphate buffer, topped to 100 mL volume. Glass beads were added and shaken on a horizontal shaker for 48 h. The determination procedure was the same as that for the endocellulase activity, but the amount of the substrate and the diluted rumen fluid were 1.0 and 0.6 mL, respectively.

The substrates for the activities of cellobiose[30], xylanase[28] and pectinase[31] were 0.5% salicin, 0.5% xylan and 0.5% pectin, respectively. The determination procedures were the same as described for the endocellulase activity assay, and the volumes of the substrate were 1.0 mL for all. But the diluted rumen fluid was 1.0 mL, 0.5 μL and 0.5 μL for cellobiose, xylanase, and pectinase, respectively[30].

The substrate for the amylase activity was 0.5 mL of 1% starch, with 60 μL rumen fluid. After incubation for 3 min at 39 ℃, the reaction was stopped by adding 1.0 mL of DNS and bathed in boiling water for another 10 min. After cooling by running water, the volume was topped exactly to 100 mL with distilled water, and leaving for 10 min. The value at OD_{540} was determined[32].

For determination of the protease activity, 1.0 mL of rumen fluid and 1.0 mL of 2% casein were preheated in 39 ℃ water bath for 2 min, respectively, and incubated together for another 3 min. After an addition of 2.0 mL of 0.4 M trichloroacetic acid and incubation in water bath at 39 ℃ for 20 min, the sample was centrifuged at 1 800×g for 10 min. One mL of the supernatant, 5.0 mL of 0.4 M sodium carbonate, 1.0 mL of diluted Folin reagent were added and mixed. The value at OD_{660} was determined after incubation at 39 ℃ for 20 min[33].

The procedures for the standard curves for various enzyme activities were the same as described for sample determination. The concentrations of glucose for the standard curves of the activities of en-

Tab.2 The primer sequences for rumen bacteria, fungi, and protozoa

Microorganisms	Forward primer (5'–3')	Reward primer (5'–3')	Amplicon//bp	References
Ruminococcus albus	CCCTAAAAGCAGTCTTAGTTCG	CCTCCTTGCGGTTAGAACA	175	Koike *et al*.[20]
Ruminococcus flavefaciens	CGAACGGAGATAATTTGAGTTTACTTAGG	CGGTCTCTGTATGTTATGAGGTATTACC	132	Denman *et al*.[21]
Salmonella bovis	TTCCTAGAGATAGGAAGTTTCTTCGG	ATGATGGCAACTAACAATAGGGGT	127	Stevenson *et al*.[22]
Ruminobacter amylophilus	CAA CCA GTC GCA TTC AGA	CAC TAC TCA TGG CAA CAT	642	Tajima *et al*.[23]
Fibrobacter succinogenes	GTTCGGAATTACTGGGCGTAAA	CGCCTGCCCCTGAACTATC	121	Denman *et al*.[21]
Butyrivibrio fibrisolvens	ACC GCA TAA GCG CAC GGA	CGG GTC CAT CTT GTA CCG ATA AAT	65	Stevenson *et al*.[22]
Selenomonas ruminantium	TGC TAA TAC CGA ATG TTG	TCC TGC ACT CAA GAA AGA	513	Bekele *et al*.[24]
Prevotella bryantii	ACT GCA GCG CGA ACT GTC AGA	ACC TTA CGG TGG CAG TGT CTC	540	Bekele *et al*.[24]
Clostridium aminophilum	ACG GAA ATT ACA GAA GGA AG	GTT TCC AAA GCA ATT CCA C	560	Patra *et al*.[25]
Methanogens	TTCGGTGGATCDCARAGRGC	GBARGTCGWAWCCGTAGAATCC	140	Denman *et al*.[21]
Total bacteria	GTGSTGCAYGGYTGTCGTCA	ACGTCRTCCMCACCTTCCTC	150	Maeda *et al*.[26]
Protozoa	GCTTTCGWTGGTAGTGTATT	CTTGCCCTCYAATCGTWCT	223	Karnati *et al*.[27]
Fungi	GAGGAAGTAAAAGTCGTAACAAGGTTTC	CAAATTCACAAAGGGTAGGATGATT	120	Denman *et al*.[21]

docellulase, exocellulase, and cellobiose ranged from 0 up to 0.9 mg/mL. The concentrations of xylose for the xylanase activity, galacturonic acid for the pectinase activity, maltose for the amylase activity, tyrosine for the protease activity, were 0–0.9, 0–0.9, 0–1.8, and 0–0.1 mg/mL, respectively.

1.5 Statistical analysis The data were subjected to the analysis of variance for a 5×5 Latin square design using the general linear models (GLM) of SPSS 18.0 statistical software (IBM, Armonk, NY). VFI analyzed with the model of $y_{ijk}=\mu+\alpha_i+\beta_j+\gamma_k+\varepsilon_{ijk}$ and the others with the model of $y_{ijkl}=\mu+\alpha_i+\beta_j+\gamma_k+\delta_l+\varepsilon_{ijkl}$.

Where, $y_{ijk\ (l)}$ is an observation; μ is the overall mean; α is the fixed effect of PAM supplementation (j=1–5); β is the random effect of animal (i=1–5); γ is the fixed effect of treatment periods (k=1–5); δ is the fixed effect of sampling times (l=1–6); $\varepsilon_{ijk(l)}$ is the residual error. The differences of those means are described, unless otherwise stated for the notable time-related patterns. Multiple comparisons among the treatment means were performed by Duncan's New Multiple Range Test. Statistical significance was declared at a level of $P\leqslant 0.05$.

2 Results and Analysis

2.1 VFI As shown in Tab.3, supplementation of PAM at 1.0, 2.0 and 3.0 g/kg diet increased the DM VFI by 8.8%, 17.9% and 8.2% (all above $P<0.05$), respectively, but reduced VFI by 5.5% at PAM dose 6.0 g/kg ($P<0.05$), showing a dose response of PAM on VFI of sheep, and a suitable dose of 2.0 g/kg diet for promoting VFI. The responsive patterns for the intakes of organic matter, the concentrate, and cornstalk to the PAM doses were as the same as those for the DM intake.

2.2 pH, NH3-N, and VFA in rumen fluid As shown in Tab. 4, PAM supplementation at 1.0, 2.0, 3.0 and 6.0 g/kg diet all had no significant influence on pH of rumen fluid at any time point; but the NH_3-N concentration linearly declined by 7.4%, 12.8%, 21.3% and 32.6% (all above $P<0.05$), respectively, with a linear regression of Y (NH_3-N, mg/100 mL rumen fluid) =−1.26 X (PAM, g/kg diet)+22.94, R^2= 0.785 ($P<0.001$).

The total VFA concentrations in rumen fluid of sheep for PAM supplementation at 1.0, 2.0, 3.0 and 6.0 g/kg diet increased by 4.5%, 7.3% ($P<0.05$) and 1.5%, and decreased by 4.0% (Tab.4), respectively, showing a significant increasing only at 2.0 g/kg diet of PAM and a declining at 6.0 g/kg diet, compared with control. This pattern of the total VFA was attributed to similarly dose-response patterns of acetic, propionic, and butyric acids, and the highest increases at PAM 2.0 g/kg diet were 6.8% (acetic), 8.8% (propionic), and 9.6% (butyric) (all above $P<0.05$), respectively.

2.3 Volume and turnover of rumen fluid The volume of rumen fluid of sheep ranged from 5.05 to 5.13 L (SEM 0.078) and the turnover rate was from 0.43 to 0.49 L/h (SEM 0.062). There was no significant difference in the volume and turnover rate for PAM supplementation.

2.4 Bacteria counts in rumen fluid

2.4.1 Classically microscopic count of bacteria. As shown in Tab.5, by PAM supplementation at 1.0, 2.0, 3.0 and 6.0 g/kg diet the counts of total bacteria in rumen fluid increased by 26.4%, 56.6%, 14.2% and −18.9% (all above $P<0.05$), respectively, showing a dose response to PAM supplementation with a highest increasing of count at 2.0 g/kg diet. The response pat-

Tab.3 The effect of polyacrylamide (PAM) supplementation on the voluntary feed intake of sheep (n=5)

VFI of sheep// g/(sheep·day)	PAM// g/kg diet					SEM	P-value
	0	1.0	2.0	3.0	6.0		
Dry matter	1 090.8^{c}	1 187.1^{b}	1 285.6^{a}	1 180.0^{b}	1 031.1^{d}	18.51	<0.001
Organic matter	996.4^{c}	1 096.3^{b}	1 164.7^{a}	1 083.4^{b}	941.8^{d}	16.94	<0.001
Mixed concentrates	434.6^{c}	470.6^{b}	515.8^{a}	469.9^{b}	406.3^{d}	7.36	<0.001
Cornstalk	656.2^{c}	716.5^{b}	769.8^{a}	710.0^{b}	624.9^{c}	11.16	<0.001

Note: Different lowercase letters in the same row represent extremely significant differences ($P<0.01$).

Tab.4 The effects of polyacrylamide (PAM) supplementation on pH, NH_3–N, and VFA in rumen fluid of sheep (n=5)

Item	PAM// g/kg diet					SEM	P-value
	0	1.0	2.0	3.0	6.0		
pH	6.55	6.53	6.60	6.55	6.49	0.028	0.086
NH_3–N// mg/100 mL	23.37^{a}	21.63^{b}	20.39^{c}	18.40^{d}	15.76^{e}	0.342	<0.001
Acetic acid// mmoL/L	65.71^{bc}	68.98^{ab}	70.19^{a}	67.11^{abc}	63.79^{a}	1.268	0.004
Propionic acid// mmoL/L	12.18^{b}	12.52^{b}	13.25^{a}	11.97^{bc}	11.58^{c}	0.197	<0.001
Butyric acid// mmoL/L	6.25^{b}	6.45^{b}	6.85^{a}	6.30^{b}	5.43^{c}	0.113	<0.001
Total VFA// mmoL/L	84.14^{bc}	87.96^{ab}	90.29^{a}	85.38^{b}	80.80^{c}	1.390	<0.001

Note: Each of the data is the average value of 5 sheep, 4 d each of sheep, and 6 time points per day; Different lowercase letters in the same row represent extremely significant differences ($P<0.01$).

terns of the counts of small bacillus, cocci +strepococcus, and new crescent bacteria in rumen fluid to PAM were similar to that of total bacteria, with highest increases of 87.9% (small bacillus), 53.5% (cocci +strepococcus) and 75.0% (new crescent bacteria) (all above $P<0.05$) respectively at 2.0 g/kg diet, compared with control. However, great coli count was linearly declined with PAM dose, with a linear regression of Y (×10⁹/mL rumen fluid) =−0.099 X (PAM, g/kg diet) + 0.815, R^2=0.875 ($P<0.001$), suggesting a selective effect of PAM supplementation on ruminal bacteria.

2.4.2 DNA copy of ruminal bacteria. As shown in Tab.6, by PAM supplementation at 4 doses, the DNA copies of total bacteria in rumen fluid increased significantly by 18.8%, 29.4%, 28.8%, and 12.2% (all above $P<0.05$), respectively, compared with control, showing a dose response and a highest value at 2.0 or 3.0 g/kg diet of PAM.

Among the bacterial species, compared with control, the response patterns of DNA copies of *R. albus*, *R. flavefaciens*, *F. succinogenes*, *B. fibrisolvens*, and *S. ruminantium* in rumen fluid to PAM were similar to that of total bacteria, with highest PCR values of 22.7% (*R. albus*), 22.5% (*R. flavefaciens*), 21.4% (*F. succinogenes*), 22.6% (*B. fibrisolvens*) and 20.6% (*S. ruminantium*) (all above $P<0.05$), respectively, at about 2.0 g/kg diet. And in fact there was no significant effect of PAM supplementation on the DNA copies of *P. bryantii*, *S. bovis*, *R. amylophilus* and *P. ruminicola*, except for some decrease at lower PAM supplementation. However, PAM supplementation decreased the DNA copies of *C. aminophilum* and methanogens to minimum by 34.8% and 21.2%, respectively. The above showed a different effect of PAM supplementation on various ruminal bacteria.

2.5 Protozoan count in rumen fluid

As shown in Tab.7, PAM supplementation at doses of 1.0, 2.0, 3.0 and 6.0 g/kg diet linearly decreased the total protozoan count in rumen fluid in a dose-dependent manner; for the classically microscopic count, the declines were 24.3%, 47.6%, 69.1% and 85.5% (all above $P<0.01$), respectively, with a linear regression equation: Y (×10⁸ copies/mL)=−1.426 X+9.007, R^2=0.883 ($P<0.001$). The classically microscopic counts of *Holotrich*, *Entodinium* and *Diplodinium* showed similar response curves to PAM doses to the total protozoan count did. Using the DNA copy method, the protozoan copy declined by 23.5%, 31.2%,

Tab.5 The effects of polyacrylamide (PAM) supplementation on the classically microscopic count of bacteria in rumen fluid of sheep (n=5)

Item	PAM // g/kg diet					SEM	P-value
	0	1.0	2.0	3.0	6.0		
Great coli // ×10⁹/mL	0.90^{a}	0.72^{b}	0.54^{c}	0.43^{d}	0.29^{e}	0.022	<0.001
Small bacillus // ×10⁹/mL	11.30^{d}	15.8^{c}	21.23^{a}	16.68^{b}	9.39^{e}	0.198	<0.001
Cocci+streptococcus // ×10⁹/mL	98.59^{d}	123.18^{b}	151.31^{a}	109.65^{c}	80.6^{e}	1.367	<0.001
New crescent bacteria // ×10⁹/mL	1.92^{c}	2.80^{b}	3.36^{a}	1.91^{c}	1.18^{d}	0.076	<0.001
Total	112.71^{d}	142.49^{b}	176.45^{a}	128.67^{c}	91.45^{e}	1.414	<0.001

Note: Each of the data was the average value of 5 sheep, 4 d each of sheep, and 6 time points per day; Different lowercase letters in the same row represent extremely significant differences ($P<0.01$).

Tab.6 The effect of polyacrylamide (PAM) supplementation on the DNA copies of rumen bacteria of sheep (n=5)

Item	PAM // g/kg diet					SEM	P-value
	0	1.0	2.0	3.0	6.0		
Total bacteria // ×10¹⁰ copies/mL	4.69^{c}	5.57^{b}	6.07^{a}	6.04^{a}	5.26^{b}	0.138	<0.001
Ruminococcus albus // ×10⁸ copies/mL	2.77^{b}	3.40^{a}	3.32^{a}	2.82^{b}	2.70^{b}	0.089	<0.001
Ruminococcus flavefaciens // ×10⁷ copies/mL	3.95^{b}	4.56^{a}	4.74^{a}	4.84^{a}	4.70^{a}	0.114	<0.001
Fibrobacter succinogenes // ×10⁸ copies/mL	3.97^{b}	4.27^{b}	4.82^{a}	4.81^{a}	4.18^{b}	0.131	<0.001
Butyrivibrio fibrisolvens // ×10⁶ copies/mL	1.90^{bc}	1.85^{c}	2.33^{a}	2.13^{ab}	2.26^{a}	0.093	0.001
Selenomonas ruminantium // ×10⁶ copies/mL	3.93^{b}	4.74^{a}	4.65^{a}	3.98^{b}	3.36^{c}	0.121	<0.001
Prevotella bryantii // ×10⁷ copies/mL	1.55^{a}	1.17^{b}	1.49^{a}	1.70^{a}	1.53^{a}	0.070	<0.001
Clostridium aminophilum // ×10⁴ copies/mL	2.33^{a}	1.52^{b}	2.04^{a}	2.15^{a}	1.66^{b}	0.106	<0.001
Methanogens // ×10⁶ copies/mL	7.56^{a}	6.83^{b}	6.43^{bc}	6.59^{b}	5.96^{c}	0.206	<0.001
Salmonella bovis // ×10⁶ copies/mL	2.29	2.11	2.31	2.06	1.99	0.099	0.108
Ruminobacter amylophilus // ×10⁴ copies/mL	7.69^{a}	6.99^{b}	8.14^{a}	7.94^{a}	7.91^{a}	0.166	<0.001
Prevotella ruminicola // ×10⁶ copies/mL	2.17	2.13	2.21	2.22	1.95	0.080	0.123

Note: Each of the data is the average value of 5 sheep, 4 d each of sheep, and 6 time points per day; Different lowercase letters in the same row represent extremely significant differences ($P<0.01$).

33.7%, and 36.7% (all above $P<0.01$), respectively, with a linear regression equation of Y ($\times10^8$ copies/mL) =−0.386 X + 6.694, R^2=0.569 ($P<0.01$).

2.6 DNA copy of fungi in rumen fluid As shown in Tab.8, for PAM supplementation at 1.0, 2.0, 3.0, and 6.0 g/kg diet, the DNA copy of fungi in rumen fluid increased linearly by 14.4% ($P>0.05$), 22.9% ($P>0.05$), 35.1% ($P<0.05$), and 45.5% ($P<0.05$), respectively, with a linear regression equation of Y (10^7 copies/mL) = 0.381 X (PAM dose, g/kg) +5.460, R^2 = 0.495 ($P<0.001$), suggesting a linear increase of fungal amount of rumen by PAM supplementation.

2.7 Activities of digestive enzymes in rumen fluid When PAM at 1.0, 2.0, 3.0, and 6.0 g/kg diet was supplemented, the activities of digestive enzymes in rumen fluid are shown in Tab.9. Among them, the activities of endocellulase, exocellulase and cellubiase increased with PAM supplementation except at highest supplementation of 6.0 g/kg diet, and they showed a similar pattern of up-down to total bacteria number in response to PAM, with greatest increases of 18.3%, 19.4% and 16.5% (all above $P<0.05$) at 2.0 g/kg diet, respectively, compared with control. However, PAM supplementation did not significantly affect on the activities of xylanase and amylase. Furthermore, the activities of pectase and protease decreased with PAM supplementation, with highest decreases at 3.0 g/kg diet by 10.2% and 19.4% (both $P<0.05$), respectively, compared with control. The above showed a different effect of PAM supplementation and its dose on various digestive enzymes in rumen, with a promotion on activities of fibre degrading enzymes at suitable PAM dose.

3 Discussion

Anionic surfactant, such as DOC[34], sodium lauryl diethoxy sulfate[35], had been long used as reagents for defaunation. But we found only partial depletion of ruminal protozoa by DOC was beneficial for improving VFI, digestion and nitrogen retention of sheep[7,10,36], and increasing absorbed amounts of nutrients such as DM, OM, total amino acids and lysine and ADG,

Tab.7 The effect of polyacrylamide (PAM) supplementation on the classical microscopic count and DNA copy of protozoa in rumen fluid of sheep (n=5)

Item	PAM// g/kg diet					SEM	P-value
	0	1.0	2.0	3.0	6.0		
By microscope//$\times10^5$/mL							
Holotrich	0.32^a	0.27^b	0.19^c	0.16^d	0.11^e	0.007	<0.001
Entodinium	9.43^a	7.11^b	4.91^c	2.78^d	1.22^e	0.048	<0.001
Diplodinium	0.46^a	0.34^b	0.25^c	0.20^d	0.15^e	0.008	<0.001
Total	10.21^a	7.73^b	5.35^c	3.15^d	1.48^e	0.052	<0.001
By PCR//$\times10^5$ copies/mL							
Total	7.69^a	5.88^b	5.29^c	5.10^c	4.87^c	0.145	<0.001

Note: Each of the data is the average value of 5 sheep, 4 d each of sheep, and 6 time points per day; Different lowercase letters in the same row represent extremely significant differences ($P<0.01$).

Tab.8 The effect of polyacrylamide (PAM) supplementation on DNA copy of fungi in rumen fluid of sheep (n=5)

Item	PAM// g/kg diet					SEM	P-value
	0	1.0	2.0	3.0	6.0		
Fungi//$\times10^7$ copies/mL	5.16^c	5.89^{cd}	6.34^{bc}	6.97^{ab}	7.51^a	0.300	<0.001

Note: Each of the data is the average value of 5 of sheep, 4 d each of sheep, and 6 time points per day; Different lowercase letters in the same row represent extremely significant differences ($P<0.01$).

Tab.9 The effects of polyacrylamide (PAM) supplementation on the activities of digestive enzymes in rumen fluid of sheep(n=5)

Activity of digestive enzymes mmoL/(mL·min)	PAM// g/kg diet					SEM	P-value
	0	1.0	2.0	3.0	6.0		
Endocellulase	1.26^c	1.35^b	1.49^a	1.45^a	1.23^c	0.018	<0.001
Exocellulase	1.44^d	1.53^c	1.72^a	1.64^b	1.47^d	0.020	<0.001
Cellubiase	1.82^c	1.96^b	2.12^a	1.92^b	1.75^d	0.018	<0.001
Xylanase	1.47	1.55	1.49	1.54	1.51	0.021	0.064
Pectase	1.67^a	1.62^{ab}	1.62^{ab}	1.50^c	1.58^b	0.021	<0.001
Amylase	1.13	1.20	1.21	1.16	1.19	0.022	0.076
Protease	0.36^a	0.35^a	0.31^b	0.29^b	0.31^b	0.011	0.007

Note: Different lowercase letters in the same row represent extremely significant differences ($P<0.01$).

and enhanced slaughter performance[8–9]. So we hypothesized that PAM, also as an anionic surfactant, at an appropriate dose, would also have similar effects on ruminal protozoa and rumen metabolism to DOC does in sheep. In the present experiment, we demonstrated that dietary PAM supplementation altered VFI, the rumen microorganism profile and fermentation pattern, and the digestive enzyme activities in sheep. The results support, in general, our previous hypothesis. There was no literature report of the effect of PAM supplementation on ruminant nutrition, but an anionic reagent, sodium dodecyl sulphate (SDS), was used for treatment of barley silage and NDF degradability was improved[37]. In another early report, supplementation of polymer gel PH20 increased body fat deposition and persistency of milk yield of dairy cows[38], possibly because PH20 has similar physical-chemical property to PAM. Therefore, we may propose that dietary supplementation of an appropriate amount of any selected anion surfactant can alter rumen microbial commune towards high enrichment of fibre degradable bacteria and low protozoal number, which stimulates digestion of fibrous components in the diet and VFI in sheep.

A significant finding in the present study is an up-down relationship between the PAM dose and the changes of ruminal microbial flora and metabolism and VFI in sheep. We used 4 doses of PAM, 1.0, 2.0, 3.0, and 6.0 g/kg diet, and noted that only supplementation of PAM at 2.0 g/kg diet was associated with the highest feed intake, VFA concentration, total bacterial counts, and the activity of enzymes involved in fibrous degradation in rumen fluid, although there were some responses of ammonia concentration, some minor species of bacteria, protozoa and fungi to PAM supplementation, the protease activity were dose related. Based on these results, we recommend that an appropriate dose of dietary supplementation of PAM in sheep should be 2.0 g/kg diet. This is compared with our previous research on DOC where the suitable dose for improving VFI and productive indexes in sheep was 0.8 g/kg diet[10]. Further research may focus on the relative effectiveness of PAM and DOC in sheep production.

The results in the present experiment showed that PAM supplementation altered the rumen microorganism components: total bacterial and fungal counts increased while protozoal number decreased. The linearly negative relationship between PAM dose and the protozoal number indicates that PAM may be poisonous to ruminal protozoa. We have also found DOC has a similarly detrimental effect on ruminal protozoa[7]. It appears that the anion surfactant is toxic to protozoa and may be used to manipulate the rumen microorganism components. It is well known that protozoa in the rumen engulf bacteria, so the reduction of protozoal population could be one of contributors to the increased bacterial count, as being observed in the present study. However, the quadratic relationship between PAM dose and the bacterial count in this experiment could imply that high doses of PAM are also detrimental to ruminal bacteria. Therefore, the appropriate dose of an anion surfactant needs to be defined when it is used to manipulate rumen microorganisms in sheep. The linearly positive relationship between PAM dose and the fungal count in the current study may be related with the decrease of protozoal population.

It was found in the present study that an appropriate dose of PAM enhanced the activity of those enzymes that are related to digestion of fibrous components in the diet, with similar pattern to total bacterial count. And the counts of some bacterial species could suggest that PAM supplementation at a proper dose differentiated bacterial species, and was likely in favour for those bacteria for fibre digestion. Indeed, we noted the significant increases of enrichment of *R. albus*, *R. flavefaciens*, *F. succinogenes* and *B. fibrisolvens*, predominant species (10^7–10^8/mL) of fibre-degrading bacteria in the rumen at PAM doses 1–3 g/kg. The increase of those bacteria and the enhanced activity of those enzymes associated with cellulose degradation resulted in more dietary fibre digested, inducing a higher VFA concentration in the rumen and VFI of sheep. No significant changes in the enrichments of *S. bovis* and *R. amylophilus*, starch-digestive bacteria, were consistent with no differences in the amylase activity between all treatments.

It was interesting to note in the present study that PAM supplementation reduced NH_3-N concentration in the rumen in a dose-dependent manner, which is in consistency with the decline of the protease activity. These results imply that the degradation of dietary protein in the rumen was reduced by PAM supplementation, probably partial because of the detriment effect of PAM on protease[39]. Degraded protein to ammonia and amino acids provides a N source to the rumen microorganisms to support their growth; however, excessive degradation in the rumen could reduce the efficiency of dietary protein for the host. Based on the reduced ammonia concentration in the rumen, we propose that PAM supplementation may increase rumen-bypass protein in sheep. If so, this may be beneficial to the host. This proposal warrants further studies.

We used two methods, *i.e.*, classical microscopy and PCR amplification, to count the total numbers of protozoa and bacteria in rumen fluid in present study. Both approaches gave similar results, and the highly linear correlations were found between two methods, for the total bacterial count, R^2=0.387, P=0.001, n=25; and for the protozoal count, R^2=0.772, P<0.001, n=25. It suggests in future studies both methods could be available.

Among the bacterial commune, cocci +streptococcus was the dominant species, accounting for 85%–88% of the total count,

followed by small bacillus (10%–13%), and only small fractions of new crescent bacteria (1.3–2.0%) and great coli (0.3–0.8%). The PAM dose-response curves of the cocci +streptococcus, small bacillus, and new crescent bacteria counts were all quadratic, while the great coli count (Y, $\times 10^9$/mL) linearly declined with PAM dose (X, g/kg diet) (Y =–0.099 X +0.815, R^2= 0.875, P<0.001), showing the effect of PAM supplementation on bacteria was selective and it also suggested a direct effect of PAM on bacteria decrease, not only the engulfment of protozoa on bacteria.

It is known that PAM is safe, but the acrylamide (AA) as free monomer in PAM product is toxic. According to EU[40], the benchmark levels on AA in food are 50–850 μg/kg, among them, 50 μg/kg for wheat based bread, 350 μg/kg for biscuit and wafers, 500 μg/kg for French fries (ready-to-eat), 750 μg/kg for fried potato chips, and 800 μg/kg for ginger bread. However, the level of AA in sheep diet is below 500 μg/kg (the benchmark level of AA in food-grade PAM is below 250 μg/g[41], so 250 μg/g ×2 g/kg diet =500 μg/kg), which is similar to French fries (ready-to-eat). By calculation, the maximum amount of AA eaten by sheep (with bw of 50 kg, 1.5 kg diet per day) is 15 μg/kg bw/d.

4 Conclusions

The results revealed a dose response of ruminal microbial flora and metabolism of sheep to dietary PAM supplementation, and PAM at 2.0–3.0 g/kg diet DM, reduced protozoal number and increased bacterial number, particularly those species for fibre degrading, and fungal counts in the rumen of sheep. These changes resulted in enhanced activities of those enzymes for fibre digestion, an increase of VFA production and a decrease of NH_3-N in rumen fluid, and stimulated VFI of sheep. Although there is no report on any detrimental effect of PAM on ruminant animals, over-dosing, for example, 6.0 g/kg diet in sheep, should be avoided as a high dose of PAM will disturb rumen microbes and fermentation. PAM at 2.0 g/kg diet may be used as a novel reagent to manipulate rumen microbial flora for improving productive performances of ruminants.

Conflict of Interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

References

[1] LI A, LUO QJ, YIMANU M, *et al.* The effect of drinking formalin on the digestion and metabolism of rumen in sheep[J]. China Animal Husbandry and Veterinary Medicine, 2006, 33 (12): 3–7. (in Chinese)

[2] SHANGGUAN J, LUO QJ, CHEN Y, *et al.* Effects of oral administration of metronidazole on the ruminal microflora and metabolism of sheep [J]. Journal of Xinjiang Agricultural University, 2011, 34(4): 275–284. (in Chinese)

[3] ZHANG GQ, LUO QJ, YANG KL, *et al.*Effects of drinking formalin on intake, digestion and metabolism of lambs and ewes[J]. Journal of Xinjiang Agricultural University, 2008, 31(2): 1–5. (in Chinese)

[4] BAN WJ, LUO QJ, KONG FH, *et al*. The effect of oral administration of formalin on the digestion, metabolism and growth of lambs[J]. China Animal Husbandry and Veterinary Medicine, 2010, 37(12): 5–10. (in Chinese)

[5] KONG FH, LUO QJ, BAN WJ, *et al.* Effects of oral administration of formalin on the microbial population and metabolism of rumen in sheep[J]. Journal of Xinjiang Agricultural University, 2012, 35(5): 345–352. (in Chinese)

[6] CAI J, LUO QJ, WANG X, *et al.* Effect of the oral formalin and fauna-free on the microbiocoenosis and activities of digestive enzymes in the rumen of sheep[J].China Animal Husbandry & Veterinary Medicine, 2016, 43(10), 2578–2590. (in Chinese)

[7] LUO QJ, LI H, CHEN Y, *et al.* Effects of supplement of aerosol OT on the intake, rumen protozoon and bacteria of sheep [J]. Journal of Xinjiang Agricultural University, 2014, 37(2): 87–95. (in Chinese)

[8] LI H, LUO QJ, ZHOU FW, *et al.* The effect of supplement of aerosol OT on the growth, digestion, and metabolism of small-tail Han lambs[J]. China Herbivore Science, 2014, 34(4): 36–41. (in Chinese)

[9] LI H, LUO QJ, PAN R, *et al.* The effect of supplement of aerosol OT on the digestion and absorption in the former stomach and small intestine of sheep fed with concentrate-type diet[J]. China Animal Husbandry and Veterinary Medicine, 2015, 42(11): 2961–2968. (in Chinese)

[10] LI H, LUO QJ, ZANG CJ, *et al.* Docusate promotes digestion and absorption in sheep fed a roughage-based diet [J]. Appl. Anim. Sci, 2019, 35(3): 284–290.

[11] AHN JS, CASTLE. LTests for the depolymerization of polyacrylamides as a potential source of acrylamide in heated foods[J]. J. Agric. Food. Chem, 2003, 51(23): 6715–6718.

[12] Association of Official Analytical Chemists. Official Methods of Analysis, 16th Ed [M]. Washington, DC: Academy Press, 1995.

[13] MOHSEN MK, EL-SANTIEL GS, GAAFAR HMA, *et al*. Nutritional evaluation of berseem 2-Effect of nitrogen fertilizer on berseem fed as silage to goats [J]. Researcher, 2011, 3(1), 25–30.

[14] HAMADA T, OMORI S, KAMEOKA K, *et al.* Direct determination of rumen volatile fatty acids by gas chromatography [J]. J Dairy Sci, 1968, 51(2): 228–229.

[15] HYDEN S. The use of reference substances and the measurement of flow in the alimentary tract[A]. in: Lewis D,Butterworths.Digestive physiology and nutrition of the ruminant [C]. London:Proceedings of the University of Nottingham, 1960: 35.

[16] CHUNG KT,HUNGATE RE. Effect of alfalfa fiber substrate on culture counts of rumen bacteria [J]. Appl. Environ. Microbiol, 1976, 32(4): 649–652.

[17] DEHORITY BA. Evaluation of subsampling and fixation rumen procedures used for counting rumen protozoa[J]. Appl. Environ. Microbiol, 1984, 48(1): 182–185.

[18] MINAS K, MCEWAN NR, NEWBOLD CJ, *et al.* Optimization of a high-throughput CTAB-based protocol for the extraction of qPCR-grade DNA from rumen fluid, plant and bacterial pure cultures[J]. FEMS Microbiol Lett, 2011, 325(2), 162–169.

[19] LWIN KO, HAYAKAWA M, BAN-TOKUDA T, *et al.* Real-time PCR assays for monitoring anaerobic fungal biomass and population size in the rumen[J]. Curr Microbiol, 2011, 62(4), 1147–1151.

[20] KOIKE S, KOBAYASHI Y. Development and

use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*[J]. FEMS Microbiol Lett, 2001, 204(2): 361–366.

[21] DENMAN SE, MCSWEENEY CS. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen [J]. FEMS Microbiol Ecol, 2006, 58(3): 572–582.

[22] STEVENSON DM, WEIMER PJ. Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR[J]. Appl. Microbiol. Biotechnol,2007, 75(1), 165–174.

[23] TAJIMA K, AMINOV RI, NAGAMINE T, *et al.* Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR[J]. Appl. Microbiol. Biotechnol, 2001, 67 (6): 2766–2774.

[24] BEKELE AZ, KOIKE S, KOBAYASHI Y.Genetic diversity and diet specificity of ruminal *Prevotella* revealed by 16S rRNA gene-based analysis[J]. FEMS Microbiol. Lett, 2010, 305 (1): 49–57.

[25] PATRA AK, YU Z.Effects of vanillin, quillaja saponin, and essential oils on in vitro fermentation and protein-degrading microorganisms of the rumen[J]. Appl. Microbiol. Biotechnol, 2014, 98(2): 897–905.

[26] MAEDA H, FUJIMOTO C, HARUKI Y, *et al.* Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, tetQ gene and total bacteria[J]. FEMS Immunol. Med. Microbiol, 2003, 39(1): 81–86.

[27] KARNATI SKR, SYLVESTER JT, NOFTSGER S, *et al.* Assessment of ruminal bacterial populations and protozoal generation time in cows fed different methionine sources[J]. J. Dairy Sci, 2007, 90(2): 798–809.

[28] AGARWAL N, KAMRA I, CHAUDHARY LC. Diurnal variations in the activities of hydrolytic enzymes in different fractions of rumen contents of Murrah buffalo[J]. J. Appl. Anim. Res, 2000, 18(1): 73–80.

[29] LEE SS, AHN BH, KIM HS, *et al.* Effects of non-ionic surfactants on enzyme distributions of rumen contents, anaerobic growth of rumen microbes, rumen fermentation characteristics and performances of lactating cows [J]. Asian Austral. J. Anim. Sci, 2003, 16(1): 104–115.

[30] PEIJI G. A simple method for estimating cellobiase activity by determination of reducing sugar[J]. Biotechnol. Bioeng, 1987, 29(7): 903–905.

[31] MILLER GL. Use of dinitrosalicylic acid as reagent for the determination of reducing sugars[J]. Anal. Biochem,1959, 31(3): 426–428.

[32] ENGVALL A.α-Amylase activity in rumen fluid of cows producing milk of low and normal fat content[J]. J. Dairy Sci, 1980, 63(12): 2012–2019.

[33] BROCK FM, FORSBERG CW, BUCHANAN-SMITH JG. Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors[J]. Appl. Environ. Microbiol, 1982, 44 (3): 561–569.

[34] ANKRAH PS, LOERCH C, KAMPMAN KA, *et al.* Effects of defaunation on in situ dry matter and nitrogen disappearance in steers and growth of lambs [J]. J. Anim. Sci, 1990 (68: 3330–3336.

[35] BURGGRAAF W, LENG RA. Antiprotozoal effects of surfactant detergents in the rumen of sheep[J]. New Zealand Journal of Agricultural Research, 1980, 23(3): 287–291.

[36] YU CC, LUO QJ, CHEN Y, *et al.* Impact of docusate and fauna-free on feed intake, ruminal flora and digestive-enzyme activities of sheep[J]. J. Anim. Physiol. Anim. Nutr, 2020 (104): 1043–1051.

[37] BAAH J, ADDAH W, OKINE EK, *et al*. Effects of homolactic bacterial inoculant alone or combined with an anionic surfactant on fermentation, aerobic stability and in situ ruminal degradability of barley silage[J].Asian-Aust. J. Anim. Sci, 2011, 24(3): 369–378.

[38] GARNSWORTHY PC, ALFORD RJ. Responses by grazing dairy cows given a polymer gel (PH20)[J]. Anim. Prod, 1988(46): 517.

[39] KRISTER HOLMBERG.Interactions between surfactants and hydrolytic enzymes [J]. Colloids and Surfaces B: Biointerfaces, 2017 (168):169–177.

[40] EU.European Commission Regulation Establishing Mitigation Measures and Benchmark Levels for the Reduction of the Presence of Acrylamide in Food (2017/2158) [EB/OL]. https://eur-lex.europa.eu/eli/reg/2017/2158/oj

[41] GB 3129-2014.National standard for food: Food additive polyacrylamide[S]. Beijing:2014.