

## Ex-vivo cow rumen fluid fermentation: changes in microbial populations and fermentation products with different forages

Haitian Fang<sup>a</sup>, Omar Al-Marashdeh<sup>b</sup>, Huitong Zhou<sup>b</sup>, Andriy Podolyan<sup>b</sup>, Jonathan G. Hickford<sup>b</sup>, Grant R. Edwards<sup>b</sup>, Keith C. Cameron<sup>b</sup> and Long Cheng<sup>c</sup>

<sup>a</sup>Ningxia Key Laboratory for Food Microbial-Applications Technology and Safety Control, School of Agriculture, Ningxia University, Yinchuan, People's Republic of China; <sup>b</sup>Faculty of Agriculture and Life Science, Lincoln University, Lincoln, New Zealand; <sup>c</sup>Faculty of Veterinary and Agricultural Sciences, The University of Melbourne Dookie Campus, Victoria, Australia

### ABSTRACT

This study investigated the effects of three forage treatments [herbal plantain (PL), ryegrass-white clover pasture (RW) and root crop fodder beet (FB)] on ex-vivo rumen fluid fermentations. Quantitative real-time Polymerase Chain Reaction (qPCR) and PCR-single-stranded conformation polymorphism (PCR-SSCP) analyses were undertaken for aliquots collected from the fermentations. The FB treatment had the highest volatile fatty acid (VFA) and lowest pH compared with other treatments. The RW culture that had a higher fibre content, had higher *Fibrobacter succinogenes* (*F. succinogenes*) and anaerobic fungi levels when compared with the FB and PL. Protozoans were however the most abundant microorganisms in the FB cultures, and they had the highest water-soluble carbohydrate (WSC) content. The abundance of *F. succinogenes* increased in the RW fermentation up to 12 h, and the abundance of anaerobic fungi increased in the RW fermentation up to 24 h. In all the treatments, no effect was found on the overall abundance of bacteria. The findings confirmed that changes in rumen microbial community and fermentation products are partly related to the WSC and fibre content of two novel forages (i.e. PL and FB) increasingly used in animal production.

### ARTICLE HISTORY

Received 17 February 2018  
Accepted 26 June 2018

### KEYWORDS

Fodder beet; plantain; pasture; microbial population; VFA; qPCR; SSCP

## Introduction

The rumen is a diverse microbial ecosystem comprising bacteria, archaea, protozoa, and fungi (Jami and Mizrahi 2012; Newbold et al. 2015). The microorganisms play essential roles in digesting feeds and supplying nutrients and energy to host ruminant animals (Janssen and Kirs 2008; Belanche et al. 2012). It is accepted that feeding different forages to ruminant animals can induce changes in ruminal fermentation products and microbial populations (Burke et al. 2006; Busquet et al. 2006). However, despite numerous *in-vitro* studies describing ruminal fermentation profiles [e.g. volatile fatty acid (VFA) production] of commonly used forages in New Zealand (Roca et al. 2010), little research has been conducted to understand and compare how microbial populations and communities change during the fermentation of different forages.

Exploring changes in the structure, function and diversity of the rumen microbial populations in response to different feeding regimes, is an important part of assessing strategies to mitigate adverse effects and adapt to changes in the host's diet (Pitta et al. 2010; Stiverson et al. 2011). The use of traditional culture techniques to analyse the rumen microbiome and specific microbial populations are both time consuming and cumbersome. Tajima et al. (2001) made the first attempt to quantify ruminal microorganism populations using

a real-time Polymerase Chain Reaction (PCR) technique. They concluded the approach could offer a sensitive, efficient, simple and reliable means of quantifying populations. This PCR approach is now widely used (Fernando et al. 2010; Leng et al. 2011; Thoetkiattikul et al. 2013) and adapted for use in this study to quantify ruminal microorganism changes induced by three different feeds. PCR-single-stranded conformational polymorphism (PCR-SSCP) offers a simple, inexpensive and sensitive method for differentiating multiple DNA fragments containing different nucleotide sequences (Zhou et al. 2014). A PCR-SSCP approach allows investigation of the rumen microbial community and complements the microbial population quantification results derived from the use of real-time PCR. Together, PCR-SSCP and real-time PCR techniques would provide an improved understanding of how rumen microbial populations and communities change when incubating *ex-vivo* derived rumen fluid in a liquor with different forages. This study was therefore designed to compare diverse forages used in New Zealand animal production: commonly used perennial ryegrass/white clover pasture (RW; Cheng et al. 2016) and with two increasingly popular forages [herbal plantain (PL; Cheng et al. 2017) and root crop fodder beet (FB; Jenkinson et al. 2014)], for *in-vitro* fermentation and microbial dynamics.

**CONTACT** Haitian Fang  fanght@nxu.edu.cn  Ningxia Key Laboratory for Food Microbial-Applications Technology and Safety Control, School of Agriculture, Ningxia University, Yinchuan 750021, People's Republic of China

© 2018 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

## Materials and methods

### Forage harvesting

The FB (*Beta vulgaris* L. var. *iutea* Dc), PL (*Plantago lanceolata* L.) and RW (*Lolium perenne* L. /*Trifolium repens* L.) were harvested from the Lincoln University Research Dairy Farm (LURDF) Lincoln, New Zealand (43° 38'S, 172° 27'E). The PL and RW herbage were harvested to approximately 5 cm above the ground level, and the bulb of the fodder beet was chopped into small pieces. The forages were then freeze-dried and then ground through a 1 mm sieve (ZM200, Retsch).

### Rumen fluid collection, preparation and in-vitro fermentations

Rumen fluid (approximately 4000 mL) was collected from four fistulated Friesian × Jersey-cross dairy cows that were lactating and had been grazing PL at the LURDF. The fluid was collected into two pre-heated 2000 mL thermos bottles and rapidly transferred to the laboratory where it was blended and then filtered through four layers of cheesecloth into a pre-heated (39.5°C) flask. The flask was continuously purged with CO<sub>2</sub>.

Four fermentation jars from a commercial anaerobic incubator (DAISY II-200/220, ANKOM Technology Co. Ltd. NY, USA) were prepared containing 1596 mL of buffer solution. The buffer was made by mixing two parts; part A and part B in a 5:1 ratio to obtain a final solution of pH 6.8 at 39.5°C. Buffer solution A contained KH<sub>2</sub>PO<sub>4</sub> (10 g/L), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.5 g/L), NaCl (0.5 g/L), CaCl<sub>2</sub>·2H<sub>2</sub>O (0.1 g/L), and urea (0.5 g/L); and buffer solution B contained Na<sub>2</sub>CO<sub>3</sub> (15.0 g/L) and Na<sub>2</sub>S·9H<sub>2</sub>O (1.0 g/L) according to Martinez et al. (2006).

To prepare the artificial rumen liquor (Buffer + Rumen fluid) for the fermentations, 400 mL of the blended and strained rumen fluid was added to 1596 mL of the buffer in each fermentation jar. Freeze-dried FB, PL and RW (25 g) was assigned to three of the fermentation jars, with the remaining jar containing only rumen fluid liquor (made up to the same volume) as a control. The fermentations were then carried out at 39.5°C in the DAISY II-200/220 using an approach described by Martinez et al. (2006) and Anassori et al. (2012). The fermentations were all duplicated to enable assessment of experimental error.

### Measurements and analysis

The three forage types were also sub-sampled (100 g) and analysed for chemical composition using wet chemistry. The fibre content of forage was determined using an enzymatic-gravimetric method (McCleary et al. 2012) and the degradability of acid detergent fibre (ADF) and neutral detergent fibre (NDF) of the substrate was analysed using the Van Soest detergent procedure (Van Soest et al. 1991; Hindrichsen et al. 2006). Total soluble carbohydrate content was determined using the Anthrone reaction (Jermyn 1956; Pollock and Jones 1979) and digestibility was assayed using an *in-vitro* pepsin cellulase method (Clark et al. 1982).

The pH of the fermentations was recorded for each forage using a benchtop pH meter (Orion 2-Star, Thermo Scientific,

Beverly, USA), and the fermentations were sampled after 0, 4, 8, 12, 24, and 48 h of incubation. Samples were collected at each incubation time, two 1.5 mL samples for VFA and ammonia analysis, and one 18 mL sample for microbial DNA extraction. The fermentation jars were flushed with CO<sub>2</sub> and re-sealed after each sampling.

The 1.5 mL sample for ammonia-N (NH<sub>3</sub>-N) determination was immediately acidified, and centrifuged at 3000 g for 20 min at 4°C. The supernatants were then transferred into new tubes and frozen at -20°C until analysis. NH<sub>3</sub>-N was determined by an enzymatic UV method using a Randox ammonia kit and the Randox Rx Daytona analyser (United Kingdom). The VFA concentrations (total, acetate, propionate and butyrate) were measured using a Gas Chromatograph (GC: Shimadzu GC-2010, Japan) fitted with an SGE BP21 30 m × 530 μm × 1.0 μm wide-bore capillary column.

### DNA extraction from rumen fluid samples

Microbial genomic DNA was extracted from the 18 mL samples using a Bioline Isolated Fecal DNA kit (Bioline Reagent Ltd., UK) and following the manufacturer's instructions.

### PCR-single-stranded conformational polymorphism analysis

The microbial community in each of the cultures was analysed using a PCR-SSCP approach. A fragment (approximately 194 bp) of prokaryote 16s rRNA gene was amplified using total bacteria primers (Carberry et al. 2012) while a fragment (approximately 357 bp) of the eukaryote 18S rRNA gene was amplified using universal primers (see Table 1). Amplifications were performed in a 20 μL reaction containing 1 μL genomic DNA, 0.25 mM of each primer, 150 mM of each dNTP (Eppendorf, Hamburg, Germany), 3.5 mM Mg<sup>2+</sup>, 0.5 U of *Taq* DNA polymerase (Qiagen, Hilden, Germany), and 1× reaction buffer supplied with the polymerase enzyme. Amplification was carried out in an iCycler (Bio-Rad, Hercules, CA), with the thermal profiles shown in Table 1. A negative control without template DNA was included in all amplifications.

After confirming amplification using electrophoresis in 1% agarose gels, the PCR products were analysed using an SSCP approach. A 1.5 μL aliquot of each amplicon was mixed with 10 μL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol), and after denaturation at 95°C for 5 min, samples were rapidly cooled on wet ice and then loaded on 16 cm × 20 cm, 14% polyacrylamide gels. Electrophoresis was performed in 0.5× TBE buffer for 18 h using Protean II xi cells, either at 280 V and 22°C for 16S rRNA amplicons, or at 250 V and 25°C for 18S rRNA amplicons. The gels were silver-stained by the method of Byun et al. (2009).

### DNA sequencing and sequence analysis

Representative single-stranded DNA bands were excised as gel slices from the polyacrylamide gel, macerated, and then used as templates for re-amplification with the original rRNA primers. These amplicons were then sequenced in both directions at

**Table 1.** PCR primers and thermal profiles used in this study for the generic and specific rumen microbes.

Target	SSU rRNA <sup>a</sup>	Primer sequence (5'–3')	Amplicon size (bp)	Thermal profile	Reference
Universal primer <sup>b</sup>	18S	TCCGCAGGTTACCTACGGA GAGGCAATAACAGGTCTGTGATGC	357 <sup>d</sup>	94°C for 4 min; 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 45 s; 72°C for 5 min	In this study
General anaerobic fungi <sup>c</sup>	18S	GAGGAAGTAAAAGTCGTAACAAGGTTTC CAAATTCACAAAGGGTAGGATGATT	120	94°C for 2 min; 40 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 15 s	Denman and McSweeney (2006)
Protozoa <sup>c</sup>	18S	GCTTTCGWTGGTAGTGTATT CTTGCCCTCYAATCGTWCT	223	94°C for 2 min; 40 cycles of 94°C for 20 s, 52°C for 20 s and 72°C for 20 s	Sylvester et al. (2009)
Total bacteria <sup>b, c</sup>	16S	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	194	94°C for 2 min; 40 cycles of 94°C for 20 s, 55°C for 20 s, 72°C for 20 s	Carberry et al. (2012)
<i>Fibrobacter succinogenes</i> <sup>c</sup>	16S	GGTATGGGATGAGCTTGC GCCTGCCCTGAACTAC	446	94°C for 2 min; 40 cycles of 94°C for 20 s, 55°C for 20 s, 72°C for 30 s	Koike and Kobayashi (2001)

<sup>a</sup>SSU rRNA, small subunit rRNA gene targeted.

<sup>b</sup>Primers used for PCR -SSCP analysis.

<sup>c</sup>Primers used for quantitative real-time PCR measurement.

<sup>d</sup>Approximately 375 bp of the 18S rRNA gene (positions 4522 to 4897 in the *Saccharomyces cerevisiae* 18S rRNA gene, accession number JQ277730).

the DNA Sequencing Facility at Lincoln University, New Zealand. Sequence analysis was performed using DNAMAN (v. 6.0, Lynnon BioSoft, Vaudreuil, Canada). The BLAST algorithm was used to search the NCBI GenBank databases (<http://www.ncbi.nlm.nih.gov/>) for homologous sequences.

### Quantitative PCR

Primers and quantitative real-time PCR (qPCR) cycling conditions are given in Table 1. All primers were synthesized by Integrated DNA Technologies (IA, USA). Genomic DNA from the rumen fluid samples were diluted 1 in 10 with nuclease-free deionized water. Additionally, 5 µL aliquots from each genomic DNA sample were combined to create a pooled sample. This pooled DNA was used to generate standard curves by preparing 1 in 5 dilutions and including the dilution series with each qPCR run.

A typical 16 µL qPCR reaction contained 8.0 µL of SYBR<sup>®</sup>Pre-mix Ex Taq<sup>™</sup> (TaKaRa, Norrie Biotech, Auckland, New Zealand), 0.8 µL each 5 µM primer (250 nM final concentration), 1.5 µL of template DNA and nuclease-free deionized water to the final volume. No template control (NTC) samples were included with each run. All reactions were set up using the CAS-1200 Robotic liquid handling system (Corbett Life Science, BioStrategy, Auckland, New Zealand) and relative qPCR analysis was performed on a Rotor-Gene<sup>™</sup> 6000 real-time rotary analyser (Corbett Life Science). Fluorescence intensity was measured at the end of each extension step at 72°C and to confirm the specificity of the reaction, a melting curve analysis was

performed at the end of each run. Data analysis was carried out using Rotor-Gene<sup>™</sup> 6000 series software 1.7 (Corbett Life Science). The abundances for each gene were expressed as fold-changes relative to the control samples.

### Statistical analysis

All data were analysed using GenStat 16 (Lawes Agricultural Trust, Rothamsted, UK). Analysis of variance (ANOVA) was conducted to examine associations among rumen fermentation variables, and measured relative microbial abundance values, with the three dietary treatments included as fixed effects in the analysis. Significance differences between treatment means were identified using protected least significant differences (LSD) ( $P < .05$ ) following a significant ANOVA result. Mean separation was tested using the LSD method at the 5% level.

### Results

The composition of RW, PL and FB forages are presented in Table 2. RW had 208 g and 303 g NDF/kg DM more than PL and FB, respectively. The water-soluble carbohydrate (WSC) was 455 and 508 g/kg DM higher for FB than RW and PL, respectively.

The average pH, and concentrations of NH<sub>3</sub>-N and VFA produced across the period of incubation for the control, RW, PL and FB are presented in Table 3. The average pH ( $P = .024$ ) and the ratio of acetate to propionate ( $P = .008$ ) were highest, and concentration of total VFA ( $P = .015$ ), propionate ( $P = .009$ ) and acetate ( $P = .004$ ) were lowest, for the control compared with the other treatments. The average concentration of NH<sub>3</sub>-N was higher ( $P = .021$ ) for PL than other treatments, but similar in the control, RW and FB fermentations. The concentration of butyrate was lower ( $P = .004$ ) for the control compared to the other treatments, and higher ( $P = .004$ ) for FB, when compared to RW and PL. There was a significant treatment × time of incubation interaction (Table 3) for pH ( $P = .014$ ), concentration of ammonia-N ( $P = .05$ ), total VFA concentration ( $P < .001$ ), and propionate ( $P < .001$ ), acetate ( $P < .001$ ), and butyrate ( $P < .001$ ) concentration. The pH of all forages over 48 h incubation averaged at 6.37 (Figure 1(a)), while NH<sub>3</sub>-N and total VFA averaged at 15.6 mmol/l (Figure 1(b)) and 41.5 mmol/l (Figure 1(c)). The pH was higher and concentrations of total VFA, acetate and

**Table 2.** Chemical composition (g/kg DM) of perennial ryegrass/white clover (RW), plantain (PL) and fodder beet (FB).

	Perennial ryegrass/white clover pasture (RW)	Plantain (PL)	Fodder beet (FB)
Organic matter	905	885	918
Dry matter digestibility	802	825	947
Dry organic matter digestibility	771	773	945
Acid detergent fibre (ADF)	240	170	71
Neutral detergent fibre (NDF)	420	212	117
Crude protein (CP)	164	136	145
Water-soluble carbohydrate	153	100	608

**Table 3.** Ammonia (NH<sub>3</sub>-N) concentration, pH and volatile fatty acid (VFA) production of rumen liquid incubated *in vitro* without herbage or crop (control), or with perennial ryegrass/white clover (RW), plantain (PL) or fodder beet (FB) added.

Item	Treatment				SEM	p-Value		
	Control	RW	PL	FB		Treatment	Time <sup>d</sup>	Treatment × time
pH	6.6 <sup>a</sup>	6.3 <sup>b</sup>	6.3 <sup>b</sup>	6.2 <sup>b</sup>	0.06	.024	<.001	.014
NH <sub>3</sub> -N (mmol/l)	16.7 <sup>a</sup>	16.2 <sup>a</sup>	13.0 <sup>b</sup>	16.3 <sup>a</sup>	0.51	.021	<.001	.050
Total VFA (mmol/l)	21.1 <sup>b</sup>	44.1 <sup>a</sup>	47.0 <sup>a</sup>	54.0 <sup>a</sup>	3.91	.015	<.001	<.001
Propionate (mmol/l)	3.0 <sup>b</sup>	8.9 <sup>a</sup>	9.9 <sup>a</sup>	10.1 <sup>a</sup>	0.79	.009	<.001	<.001
Acetate (mmol/l)	15.9 <sup>b</sup>	30.1 <sup>a</sup>	31.7 <sup>a</sup>	35.9 <sup>a</sup>	2.83	.027	<.001	<.001
Butyrate (mmol/l)	2.3 <sup>c</sup>	5.1 <sup>b</sup>	5.4 <sup>b</sup>	7.9 <sup>a</sup>	0.45	.004	<.001	<.001
Acetate:propionate ratio	5.4 <sup>a</sup>	3.6 <sup>b</sup>	3.5 <sup>b</sup>	3.9 <sup>b</sup>	0.21	.008	.004	.121

<sup>a-c</sup>Means within a row with different superscripts differ (*p* < .05).

<sup>d</sup>Sampling points, during which rumen liquor was sampled (0, 4, 8, 12, 24, and 48 h of incubation).

propionate were lower for control than other treatments at 4, 8, 12, 24 and 48 h of incubation (Figures 1(a,c) and 2(a,b), respectively). The concentration of NH<sub>3</sub>-N was lower for PL than other treatments at incubation time of 4, 24 and 48 h, and NH<sub>3</sub>-N was also lower for PL than control and RW at an incubation time of 8 h (Figure 1(b)). The butyrate concentration was the highest for FB and the lowest for control at 4, 8, 12, 24 and 48 h of incubation (Figure 2(c)). The butyrate concentration increased from 1.9 at 0 h to 11.2 mmol/l at 48 h (Figure 2(c)).

The *F. succinogenes* population in the RW fermentations increased at least 9-fold between 0 and 8 h, while it tended

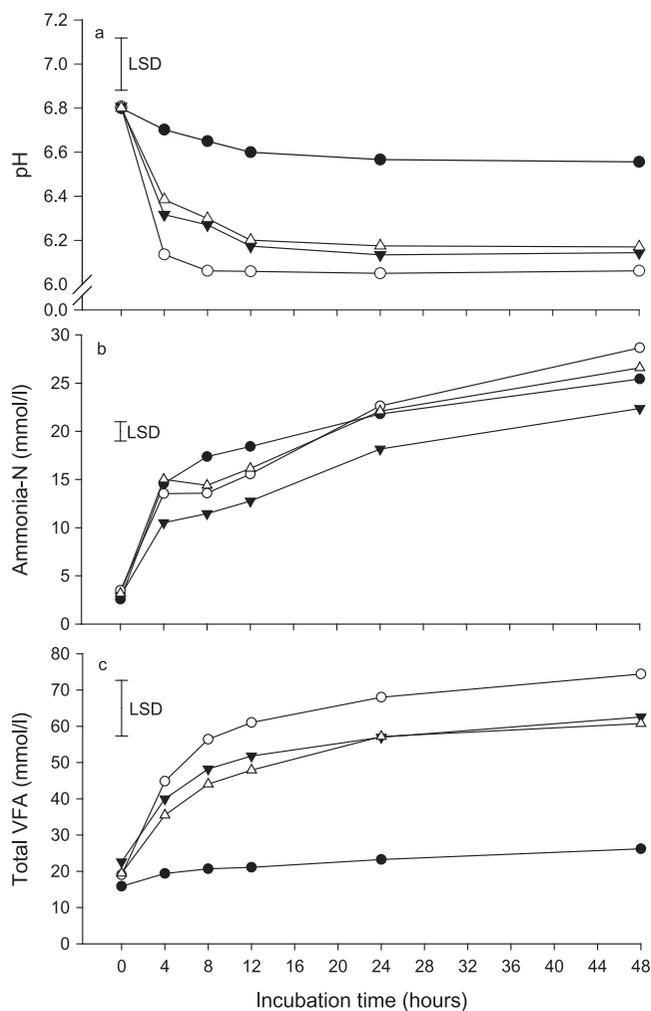
to be lower in PL than in the other two treatment groups (Figure 3(a)). Total bacterial counts increased for all three treatments between 0 and 8 h (Figure 3(b)), but declined after that. Anaerobic fungi levels in the RW fermentations increased at least 28-fold from 0 to 24 h, but then declined. In the other feed incubations, there was little difference between anaerobic fungi levels at different incubation times. Protozoa levels increased up to 8 h of incubation for the three feed supplemented fermentations, but then all declined as did the control levels of protozoa. The results suggest protozoans were the dominant type of microorganism in the FB cultures, but that anaerobic fungi were the dominant microorganisms in the RW and PL cultures.

The SSCP banding pattern for 16s rRNA was very complex and thus no attempt was made to sequence any of the bands (results not shown). Analysis then focussed on the SSCP banding pattern for 18s rRNA, with the PCR-SSCP profiles indicating that FB in the cultures resulted in different microbial communities growing (Figure 4). The microbial community appeared to have a large number of dominant groups, mainly protists and anaerobic fungal communities. The sequences obtained from sequencing SSCP bands were 100% similar to known sequences in GenBank and the species identified were distinct. They included: *Eremoplastron dilobum*, *Entodinium caudatum*, *Dasytricha ruminantium* and other uncultured protists, ciliates and fungi, including a *Piromyces* species.

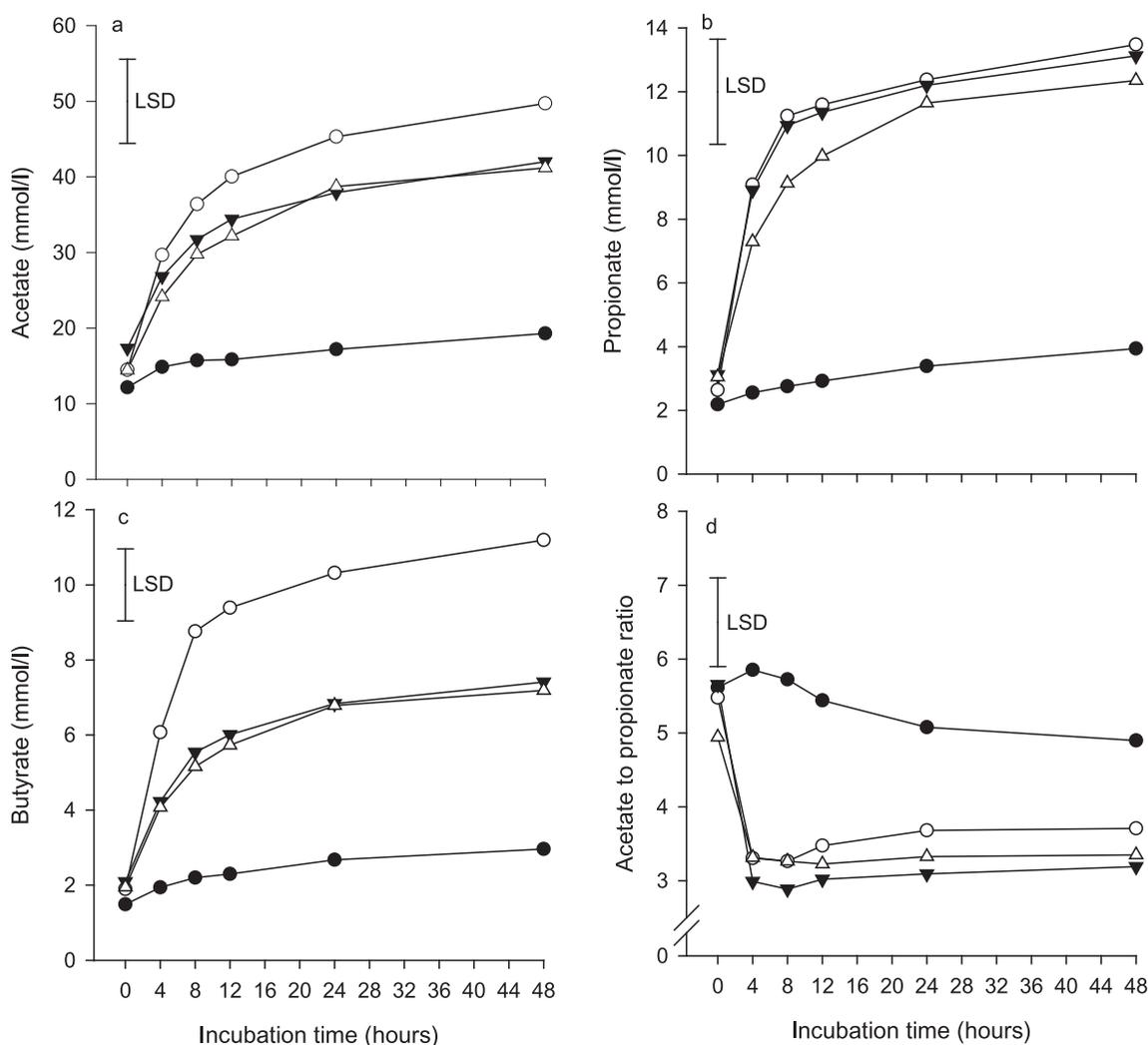
### Discussion

The chemical composition of three feed types used in this study were in line with previous publications (Cheng et al. 2016, 2017). The relative abundance of a microbial group in a given culture generally reflects its ability to adapt to the conditions and compete for the nutrients available (Belanche et al. 2012). In this study, microbial community dynamics in response to forage fibre or fermentable carbohydrates changes were assessed by quantitative real-time PCR. The abundance of measured microorganisms from all treatment groups in this study reached a peak prior to 24 h of incubation, indicating an active fermentation process took place. The difference in relative abundance and diversity of microbial species likely reflects the difference in the fibre degradation and carbohydrate fermentation for the three forages.

Anaerobic fungi and anaerobic bacteria, including *F. succinogenes*, are commonly found in the rumen (Krause et al. 2003) and they can release cellulolytic enzymes to break



**Figure 1.** (a) pH, (b) Ammonia-N (NH<sub>3</sub>-N) concentration, (c) and total VFA concentration, for control (●), fodder beet (○), plantain (▼) and perennial ryegrass/white clover pasture (Δ) evaluated *in vitro*.



**Figure 2.** (a) Acetate, (b) propionate, (c) butyrate concentrations, and (d) acetate to propionate ratio for control (●), fodder beet (○), plantain (▼) and perennial ryegrass/white clover pasture (Δ) evaluated *in vitro*.

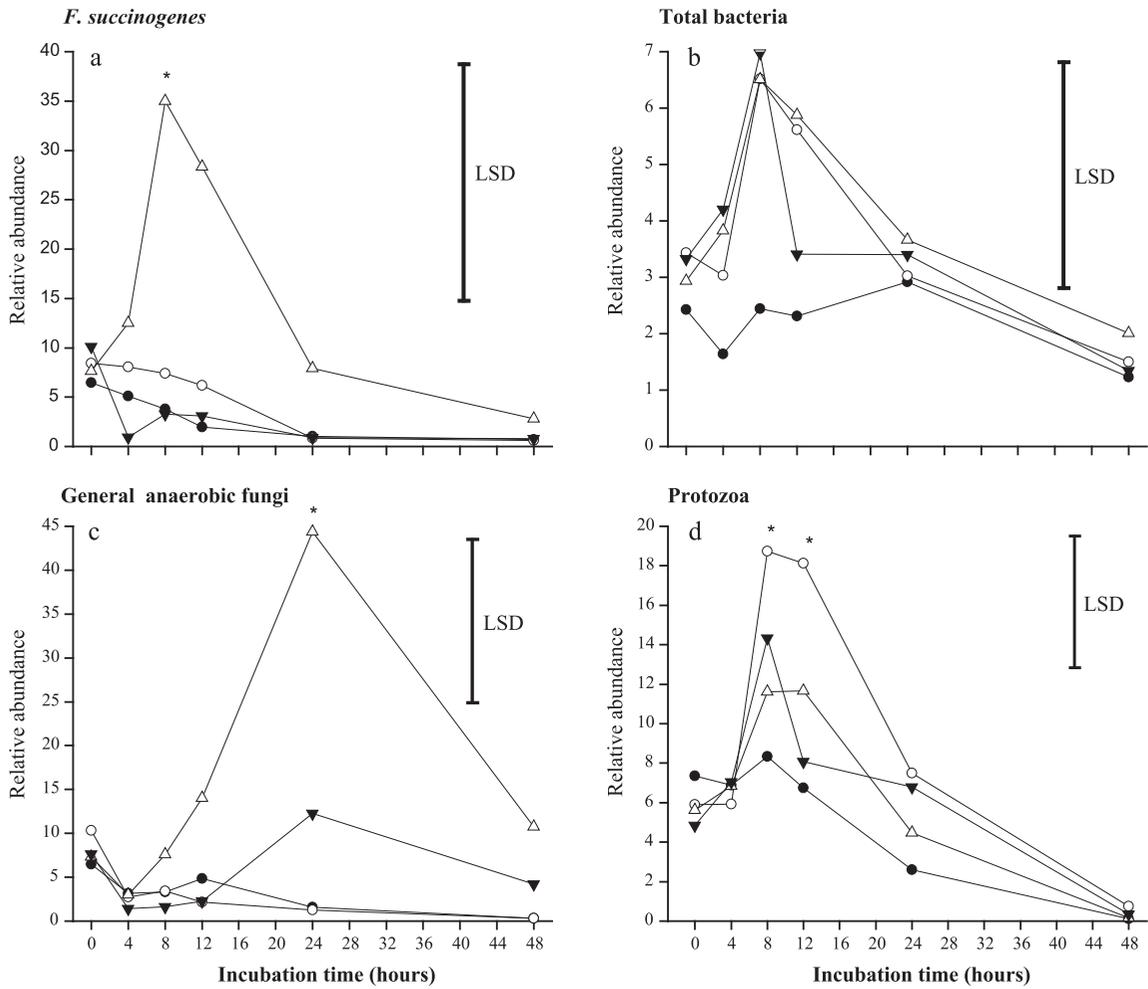
down fibre (Kamra 2005; Sun et al. 2008; Kebreab et al. 2009). In the current study, both anaerobic fungi and *F. succinogenes* were found to be the dominant microorganisms present in RW supplemented rumen liquor fermentations, and this finding is consistent with RW having the highest fibre (i.e. NDF) content compared with the other treatments. The fermentation product of *F. succinogenes* should mainly be acetate and propionate (Koike and Kobayashi 2001), and this likely explains the lack of a difference observed between acetate and propionate in PL and FB supplemented fermentations in this study.

The relative abundance of *F. succinogenes* and anaerobic fungi in RW peaked at 8 and 24 h respectively, and this is in agreement with Paul et al. (2010). It seems likely that the total bacteria population may have had a limited contribution to the overall difference in fermentation between the three treatment groups, as the total bacterial abundance was similar at all sampling times. Further, the three treatments produced a similar response, with their total bacterial population peaking at 8 h and then dropping.

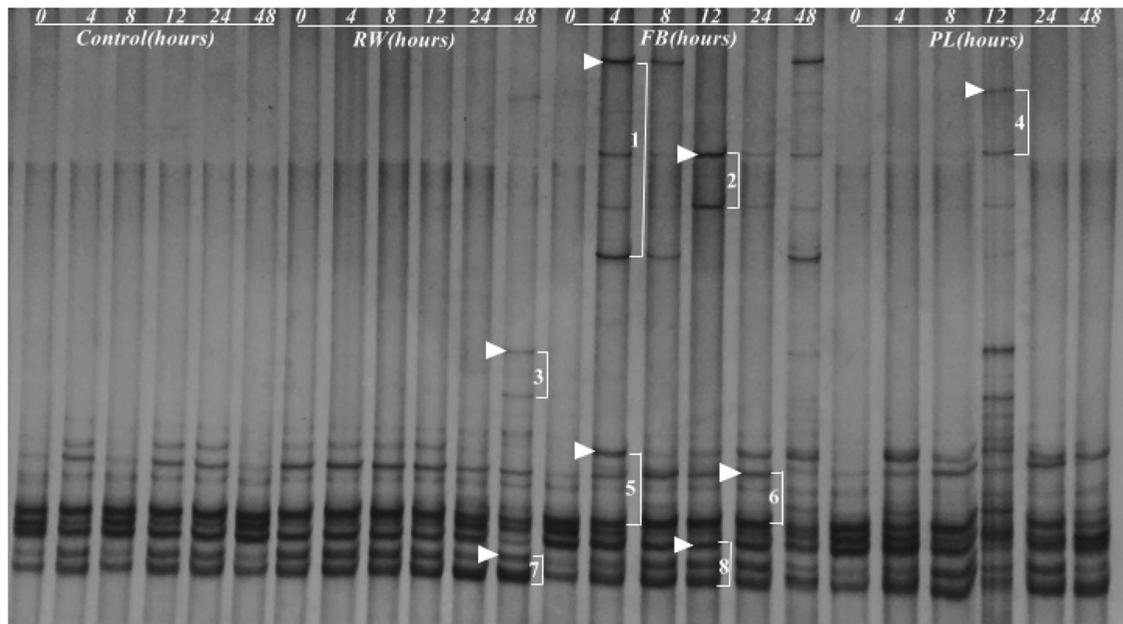
Protozoans live as commensals in the rumen, consuming bacteria and anaerobic fungi. They are mostly associated with

the presence of easily fermentable carbohydrates and proteins (Sylvester et al. 2009; Jami and Mizrahi 2012; Parmar et al. 2015). Protozoan populations have been observed to have positive relationships with butyrate and isobutyrate production in the rumen (Carberry et al. 2012; Newbold et al. 2015). It is clear that the greater abundance of protozoa and high butyrate levels may be due to the higher concentrations of readily fermentable carbohydrates present in FB (Newbold et al. 2015). An increased abundance of protozoa was found in FB supplemented fermentations, and these contained higher levels of WSC than the other forage treatments at both 8 and 12 h. The abundance of protozoa in RW was similar to those in PL at all sampling times and this is consistent with PL and RW having similar WSC levels. Butyrate is the main product of WSC fermentation in the rumen, and it was higher in FB, compared to both PL and RW between 8 to 48 h of incubation. As might be expected, WSC fermentation also resulted in the lowest pH and highest VFA concentration being detected in the FB samples.

Protozoal and anaerobic fungal communities in the FB, RW and PL supplemented cultures were quite different to those in the control fermentation. Although the control group



**Figure 3.** Relative abundance of (a) *Fibrobacter succinogenes*, (b) total bacteria, (c) anaerobic fungi and (d) and protozoans under different treatment. Control (●), fodder beet (○), perennial ryegrass/white clover pasture (Δ), plantain (▼). \*Significant difference using Tukey HSD calculations ( $p < .05$ ).



**Figure 4.** PCR-SSCP profiles of the protozoal and anaerobic fungal communities from amplification of the 18S rRNA gene for four treatment groups at different sampling time point. Control, RW, FB, and PL stand for the control, perennial ryegrass/white clover pasture, fodder beet, and plantain, respectively. 1, *Eremoplastron dilobum* (Accession number: AM158472.1); 2, *Dasytricha ruminantium* (Accession number: AM158463.1); 3, Uncultured piromyces (Accession number: HQ000040.1); 4, *Entodinium caudatum* (Accession number: U57765.1); 5, Uncultured rumen protozoan (Accession number: KF893856.1); 6, Uncultured protist (Accession number: AB992253.1); 7, Uncultured ciliate (Accession number: AM158691.1); 8, Uncultured ciliate protozoan (Accession number: KF894051.1).

rumen fluid was harvested from cows that had ingested RW previously, the seven distinct strains found in the RW group suggest that the fermentation process may have enhanced the abundance of some protozoan species compared with the control. Interestingly, it was observed that only protozoan communities exhibited distinctive SSCP bands, and that there were more protozoan bands in FB and RW, than in PL and the control. This is likely to reflect a higher diversity of protozoan communities in the FB and RW supplemented cultures. Cost and time constraint precluded analysis of all the PCR-SSCP bands, and thus only distinct single bands and common patterns were selected for sequencing.

Interestingly, a *Piromyces* species was found. This organism can degrade cellulose in culture (Roger et al. 1992), and this supports the contention that RW and thus high NDF levels may have stimulated the growth of fibre degradation related microorganisms, such as the *Piromyces* spp., during the fermentation process. In comparison, the increased protozoa numbers and protozoan communities, and apparent dominance of *Entodinium* spp. are often found to be associated with an increased starch content in ruminant diets, and in general as the proportion of butyrate increases with increasing protozoa numbers in the rumen (Newbold et al. 2015). This agrees with the positive association between protozoa abundance and butyrate production in FB in this study. The high abundance of unclassified protozoa in the FB group could also reflect the highest content of soluble carbohydrate in this fermentation.

## Conclusions

The abundance of microbes in *in-vitro* cultures showed changes in all treatment groups. *F. succinogenes* and anaerobic fungi were found to be the dominant strains in the higher fibre RW treatment group compared to the FB treatment group. The populations of anaerobic fungi were higher in RW and PL treatment groups in the middle and late stage of culture, respectively. The FB treatment had the highest protozoa population, concentration of VFAs and NH<sub>3</sub>-N, and lower pH than RW and PL. The findings further confirm that changes in rumen microbial community and fermentation products are related to the WSC and fibre content of forages.

## Disclosure statement

No potential conflict of interest was reported by the authors.

## Funding

This work was financially supported by the Gene-Marker Laboratory at Lincoln University (New Zealand), Project of support local colleges and universities reform and development funds of China, National Natural Science Foundation of China (31301542) and China Scholarship Council (CSC No. 201408645035). We appreciate New Zealand AGMARDT Postdoctoral Fellowship to support Dr Long Cheng for this work.

## References

Anassori E, Dalir-Naghadeh B, Pirmohammadi R, Taghizadeh A, Asri-Rezaei S, Farahmand-Azar S, Besharati M, Tahmoozi M. 2012. *In vitro* assessment of

- the digestibility of forage based sheep diet, supplemented with raw garlic, garlic oil and monensin. *Vet Res Forum*. 1:5–11.
- Belanche A, Doreau M, Edwards JE, Moorby JM, Pinloche E, Newbold CJ. 2012. Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. *J Nutr*. 142:1684–1692.
- Burke JL, Waghorn GC, Brookes IM, Chaves AV, Attwood GT. 2006. *In vitro* production of volatile fatty acids from forages. *Proc N Z Soc Anim Prod*. 66:50–54.
- Busquet M, Calsamiglia S, Ferret A, Kamel C. 2006. Plant extracts affect *in vitro* rumen microbial fermentation. *J Dairy Sci*. 89(2):761–771.
- Byun SO, Fang Q, Zhou HT, Hickford JG. 2009. An effective method for silver-staining DNA in large numbers of polyacrylamide gels. *Anal Biochem*. 385:174–175.
- Carberry CA, Kenny DA, Han S, McCabe MS, Waters SM. 2012. Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. *Appl Environ Microbiol*. 78(14):4949–4958.
- Cheng L, Judson HG, Bryant RH, Mowat H, Guinot L, Hague H, Taylor S, Edwards GR. 2017. Short communication: the effects of feeding cut plantain and perennial ryegrass-white clover pasture on dairy heifer feed and water intake, apparent nutrient digestibility and nitrogen excretion in urine. *Anim Feed Sci Technol*. DOI:10.1016/j.anifeedsci.2017.04.023.
- Cheng L, McCormick J, Logan C, Hague H, Edwards GR. 2016. Liveweight gain and urinary nitrogen excretion of dairy heifers grazing perennial ryegrass-white clover pasture, canola, and wheat. *Animal Prod Sci*. DOI:10.1071/AN15533.
- Clark T, Flinn PC, McGowan AA. 1982. Low-cost pepsin-cellulase assays for prediction of digestibility of herbage. *Grass Forage Science*. 37:147–150.
- Denman SE, McSweeney CS. 2006. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol Ecol*. 58:572–582.
- Fernando SC, Purvis HT II, Najjar FZ, Sukharnikov LO, Krehbiel CR, Nagaraja TG, Roe BA, DeSilva U. 2010. Rumen microbial population dynamics during adaptation to a high-grain diet. *Appl Environ Microbiol*. 76(22):7482–7490.
- Hindrichsen IK, Kreuzer M, Madsen J, Bach Knudsen KE. 2006. Fibre and lignin analysis in concentrate, forage, and feces: detergent versus enzymatic-chemical method. *J Dairy Sci*. 89(6):2168–2176.
- Jami E, Mizrahi I. 2012. Composition and similarity of bovine rumen microbiota across individual animals. *PLoS One*. 7(3):e33306.
- Janssen PH, Kirs M. 2008. Structure of the archaeal community of the rumen. *Appl Environ Microbiol*. 74(12):3619–3625.
- Jenkinson BAB, Edwards GR, Bryant RH. 2014. Rumen degradation characteristics of kale and fodder beet crops. *Proc 5th Australas Dairy Sci Symp*. 395–398.
- Jermyn MA. 1956. A new method for the determination of ketohexoses in the presence of aldohexoses. *Nature*. 177:38–40.
- Kamra DN. 2005. Rumen microbial ecosystem. *Curr Sci*. 89:124–135.
- Kebreab E, Dijkstra J, Bannink A, France J. 2009. Recent advances in modeling nutrient utilization in ruminants. *J Anim Sci*. 87:E111–E122.
- Koike S, Kobayashi Y. 2001. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus favefaciens*. *FEMS Microbiol Lett*. 204:361–366.
- Krause DO, Denman SE, Mackie RI, Morrison M, Rae AL, Attwood GT, McSweeney CS. 2003. Opportunities to improve fibre degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol Rev*. 27:663–693.
- Leng J, Zhong X, Zhu RJ, Yang SL, Gou X, Mao HM. 2011. Assessment of protozoa in Yunnan Yellow Cattle rumen based on the 18S rRNA sequences. *Mol Biol Rep*. 38:577–585.
- Martinez S, Madrid J, Hernandez F, Megias M, Sotomayor J, Jordan M. 2006. Effect of thyme essential oils (*Thymus hyemalis* and *Thymus zygis*) and monensin on *in vitro* ruminal degradation and volatile fatty acid production. *J Agric Food Chem*. 54(18):6598–6602.
- McCleary BV, DeVries JW, Rader JI, Cohen G, Prosky L, Mugford DC, Okuma K. 2012. Determination of insoluble, soluble, and total dietary fibre (CODEX definition) by enzymatic-gravimetric method and liquid chromatography: collaborative study. *J AOAC Int*. 95(3):824–844.

- Newbold CJ, de la Fuente G, Belanche A, Ramosmorales E, Mcewan NR. 2015. The role of ciliate protozoa in the rumen. *Front Microbiol.* 1313:1–14.
- Parmar NR, Kumar NJI, Joshi CG. 2015. Deep insights into carbohydrate metabolism in the rumen of Mehsani buffalo at different diet treatments. *Genom Data.* 6:59–62.
- Paul SS, Deb SM, Punia BS, Singh D, Kumar R. 2010. Fibrolytic potential of anaerobic fungi (*Piromyces* sp.) isolated from wild cattle and blue bulls in pure culture and effect of their addition on in vitro fermentation of wheat straw and methane emission by rumen fluid of buffaloes. *J Sci Food Agric.* 90(7):1218–1226.
- Pitta DW, Pinchak E, Dowd SE, Osterstock J, Gontcharova V, Youn E, Dorton K, Yoon I, Min BR, Fulford JD, et al. 2010. Rumen bacterial diversity dynamics associated with changing from Bermuda grass hay to grazed winter wheat diets. *Microbiol Ecol.* 59:511–522.
- Pollock CJ, Jones T. 1979. Seasonal patterns of fructan metabolism in forage grasses. *New Phytol.* 83:9–15.
- Roca FJ, Hutton PG, Pain SJ, Kenyon PR, Morris ST. 2010. Use of novel pasture species to reduce methane emissions from New Zealand's grazing ruminants. *Proc N Z Soc Anim Prod.* 70:77–79.
- Roger V, Grenet E, Jamot J, Bernalier A, Fonty G, Gouet P. 1992. Degradation of maize stem by two rumen fungal species, *Piromyces communis* and *Caecomyces communis*, in pure cultures or in association with cellulolytic bacteria. *Reprod Nutr Dev.* 32(4):321–329.
- Stiverson J, Morrison M, Yu ZT. 2011. Populations of select cultured and uncultured bacteria in the rumen of sheep and the effect of diets and ruminal fractions. *Int J Microbiol.* doi:10.1155/2011/750613.
- Sun XZ, Joblin KN, Andrew IG, Hoskin SO, Harris PJ. 2008. Degradation of forage chicory by ruminal fibrolytic bacteria. *J Appl Microbiol.* 105:1286–1297.
- Sylvester JT, Karnati SKR, Dehority BA, Morrison M, Smith GL, St-Pierre NR, Firkins JL. 2009. Rumen ciliated protozoa decrease generation time and adjust 18S ribosomal DNA copies to adapt to decreased transfer interval, starvation, and monensin. *J Dairy Sci.* 92:256–269.
- Tajima K, Aminov RI, Nagamine T, Matsui H, Nakamura M, Benno Y. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *Appl Environ Microbiol.* 67:2766–2774.
- Thoetkiattikul H, Mhuantong W, Laothanachareon T, Tangphatsornruang S, Pattarajinda V, Eurwilaichitr L, Champreda V. 2013. Comparative analysis of microbial profiles in cow rumen fed with different dietary fibre by tagged 16S rRNA gene pyrosequencing. *Curr Microbiol.* 67:130–137.
- Van Soest PJ, Robertson JB, Lewis BA. 1991. Methods for dietary fibre, neutral detergent fibre, and non-starch polysaccharides in relation to animal nutrition. *J Dairy Sci.* 74(10):3583–3597.
- Zhou H, Li S, Liu X, Wang J, Luo Y, Hickford JG. 2014. Haplotyping using a combination of PCR-single strand conformational polymorphism analysis and haplotype-specific PCR amplification. *Anal Biochemistry.* 466:59–64.