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In vitro assessment of the relationships between the digestion of different types of rice straw and bacterial community in the rumen

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Abstract: The aims of this study were to examine bacterial communities in relation to the rumen digestion of rice straw and to understand how concentrate supplements affect gut bacteria involving the digestion of a rice straw-based diet. The substrates were rice straw (RS) alone (experiment 1) and RS with 25% concentrates (barley and kidney beans) (experiment 2). The genomic DNA was collected to determine bacterial diversity by conducting denaturing gradient gel electrophoresis (DGGE). V6–V8 region group-specific (*Clostridium* and *Bacteroides*) primers were employed in the analyses. The DGGE band pattern was subjected to cluster analysis to demonstrate the similarity and difference between dietary treatments and solid-liquid fractions. Fibre digestibility, gas production, and volatile fatty acid (VFA) concentration were increased with incubation time. The differences between solid and liquid fractions were great in total bacteria, *Clostridium*, and *Bacteroides* communities. *Clostridium* and *Bacteroides* communities appeared unaffected by incubation time, whereas great differences existed between solid and liquid fractions throughout incubations (experiment 1). Barley and kidney bean supplements increased gas production and lowered rumen fluid pH, whereas changes in VFA concentration were significant only for kidney bean supplement. The *Clostridium*, and *Bacteroides* groups were affected by barley and kidney beans in the liquid fraction. However, the solid fraction was stable (experiment 2). These results indicate a rigid and stable community structure of *Clostridium* and *Bacteroides* groups involved in the digestion of rice straw-based diets in the rumen. Distinctive differences between solid and liquid fractions were described; hence, separate analyses of two fractions may greatly help understand the relationship between fermentation patterns and microbial communities in the rumen.

Keywords: *Clostridium*; *Bacteroides*; PCR; dgge; solid-liquid; concentrates

Ruminant animal production depends worldwide on forage as a main nutritional component (Wilkins and Jones 2000). In general, the forages are high in crude fibre and low in nitrogen and energy contents, which are poorly digested and have a low metabolism. On the other hand, the major forage digestion occurs in the rumen through microbial fermentation (Romney and Gill 2000). By continuously increasing the forage digestibility, we can limit digestible energy intake in ruminants because not

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even 50% of this fraction is readily digested and utilised (Hatfield et al. 1999). Rice straw (RS) is a by-product that consists of high fibre and low protein contents and has poor digestibility. Afghanistan is one of those countries where farmers can feed straw as the main diet to their cows during the dry season, and it is a big challenge for Afghan farmers. The exact numbers of livestock in Afghanistan are unknown, but the official estimates of about 3.7 million cattle and 18 million sheep and goats are considered present (Fitzherbert 2007). Most local farmers in Afghanistan feed their animals with low-quality feed that is high in fibre. To meet maintenance and production requirements of ruminants fed on low-quality roughage, we need to study the relationship between fibre digestion and the rumen bacterial community and investigate the changes in the bacterial community after high-fibre feeding.

To improve the meat and milk production of cattle, the nutritional density of diets should increase by feeding more concentrates and less forage (Plaizier et al. 2008). However, the effects of a highly concentrated diet would be demonstrated distinctively in changes in the gut bacterial community (Kleen et al. 2003). By increasing the availability of fermentable carbohydrates, microbial growth is stimulated. This increases the fermentation rate, providing the animal with increased energy for growth Nagaraja and Titgemeyer (2007). However, the proliferation of rumen cellulolytic organisms is directly correlated with the amount of fibre in the diet, and the replacement of fibre with more readily fermentable carbohydrates impacts these organisms and alters their dynamics. The relationship between fermentation patterns and microbial communities in the rumen must be studied to overcome the obstacles.

In this study, we collected a sample of rice straw from the provinces of Khost (altitude 1 100 m a.s.l.) and Nangarhar (altitude 590 m a.s.l.) in Afghanistan. We examined rumen bacterial communities in relation to the digestion of rice straw and the effect of concentrate supplements on gut bacteria involving the digestion of rice straw-based diet.

MATERIAL AND METHODS

Sampling

The experimental design was randomised and factorial. A total of 12 samples of only four types of rice straw (RS-1: rice straw of Nangarhar-sorkhrood, RS-2: rice straw of Nangarhar-behsood, RS-3: rice straw of Khost mandozai and RS-4: rice straw of Khost-shamal) were collected from the two provinces (Nangarhar and Khost) of Afghanistan (experiment 1). In the second connective experiment (experiment 2), one type of rice straw was selected; barley (25%) was used as carbohydrates and kidney beans (25%) were used as protein-rich supplements separately. All samples were used in triplicates in the two connective experiments. The major ingredients and chemical composition of the feed material are shown in Table 1. DM content was determined by oven-drying at 60 °C for 48 h at the Laboratory of Animal Science, Kabul University, Afghanistan. All samples were ground to pass a 1 mm sieve screen for analysis. The samples were transferred to Japan with a valid quarantine certificate. Rumen fluid was collected from Holstein dairy cows at Okayama Prefecture Livestock Institute, Japan. The cows were housed in a free stall barn and fed total mixed ration (TMR) silage, which was formulated to have 500–600 g/kg of dry matter (DM), 160–180 g/kg DM of crude protein (CP) (N × 6.25), and 720–740 g/kg DM of total digestible nutrients. Samples were collected in the morning. Daily minimum and maximum temperatures on the day of sampling were 9 °C and 19 °C. Rumen fluid was obtained using a flexible stainless spring tube (Lumenar stomach evacuator outfit, Fujihira Industry Co. Ltd, Tokyo, Japan). The rumen fluid

Table 1. Ingredient and chemical composition of diet used for *in vitro* digestibility

Ingredient	Chemical composition			
	DM (%)	CP (% of DM)	NDF (% of DM)	ADF (% of DM)
RS-1	92.70	6.52	67.50	40.09
RS-2	92.90	6.11	62.90	44.63
RS-3	88.20	6.65	64.40	40.31
RS-4	92.60	7.60	67.00	44.19
Barley	89.18	13.22	19.78	7.68
Kidney bean	86.85	24.23	12.73	6.70

ADF = acid detergent fibre; CP = crude protein; DM = dry matter; NDF = neutral detergent fibre; RS-1 = rice straw of Ningarhar sorkhrood, Afghanistan; RS-2 = rice straw of Ningarhar behsood, Afghanistan; RS-3 = rice straw of Khost mandozai, Afghanistan; RS-4 = rice straw of Khost shamal, Afghanistan

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was immediately transferred to the Laboratory of Animal Nutrition at Okayama University and strained through four layers of gauze into a flask.

In vitro incubation

A total of 87 glass vials of 50 ml volume were used for *in vitro* gas production technique as described by Pell and Schofield (1993). In experiment 1; we used 75 glass vials for the four types of rice straw and a blank in triplicate for 24, 48, 72, 96, and 120 h of incubation. In experiment 2, we used 12 glass vials for the rice straw (control), two concentrate treatments (barley and kidney beans), and a blank for 48 h of incubation. Feed samples of 0.5 ± 0.01 g were weighed into bottles. Consequently, 30 ml of the solution, 1 : 3 (v/v) proportions of ruminal fluid and buffer was added to each bottle. The buffer solution was prepared according to the formula for "synthetic saliva". The inoculum buffer mixture was continuously infused with CO_2 and maintained in a water bath at 39 °C prior to being dispensed. Once all bottles were filled, they were immediately gassed with CO_2 and closed with a rubber stopper and aluminium cap. Glass vials were shaken gently at each reading and placed in an incubator at 39 °C. Rumen liquor and reduced buffer solution were also included in each test as blanks for the correction of gas produced. The volume of produced gas was read by a pressure glass syringe described by El-Shazly and Hungate (1965) and recorded at 24, 48, 72, 96, and 120 h of incubation. The fermentation was stopped after each incubation period. The content was filtered through a nylon filter (40 µm pore size) and washed with distilled water (DW) five times. About 14 ml of mixed filter medium was used to analyse pH, volatile fatty acid (VFA) concentration, and population of liquid-associated bacterial communities. The residues were used for neutral detergent fibre (NDF) determination, scanning electronic microscopy (SEM) analysis, and the population of solid-associated bacterial communities.

Measurements

The chemical composition of the diet used *in vitro* incubation was analysed in duplicate for moisture (AOAC 1990), Kjeldahl nitrogen (AOAC 1990), from which CP was calculated as total N × 6.25. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined according to the procedure of Van Soest et al. (1991). The NDF procedure was modified to include incubation with a heat-stable alpha-amylase. The VFA content in the incubated mixture was quantitatively separated by a gas chromatograph (GC-14A; Shimadzu Co. Ltd, Kyoto, Japan) equipped with a capillary column coated with terephthalic acid modified polyethylene glycol (TC-FFAP; GL Sciences, Tokyo, Japan). Helium was used as a carrier gas at 0.5 kg × cm^{-2} pressure. The temperature of the injector and detector were set at 180 °C and 200 °C, respectively. Experimental feeds and their chemical compositions are shown in Table 1. For the rice straw morphology and ultrastructure, the control and incubated rice straw was dehydrated by a series of acetone solutions (30%, 50%, 70%, 80%, and 90%) and thrice in pure acetone for 15 min each. Samples were freeze-dried (Leica EM CPD300; Leica, Wetzlar, Germany), gold-coated (IB-3; Eiko, Hitachinaka, Japan), and observed under a scanning electron microscope (Model S-3400N; Hitachi, Tokyo, Japan).

DGGE-PCR

Microbial DNA was extracted using a repeat bead-beating plus column method of Yu and Morrison (2004). Briefly, solid and liquid samples (200 µl and 200 mg) were transferred into a fresh 2-ml screw cap tube. Cell lysis is achieved by bead beating in the presence of 4% (w/v) sodium dodecyl sulfate (SDS), 500 mM NaCl, and 50 mM EDTA. After bead beating, most of the impurities and the SDS were removed by precipitation with ammonium acetate, and then the nucleic acids were recovered by precipitation with iso-propanol. Genomic DNA was purified via sequential digestions with RNase and proteinase K, followed by the use of QIAamp columns. PCR was used to amplify V6–V8 region of the bacterial 16S rRNA gene, with the GC-clamp attached to the forward primer, U968-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-30) and L1401 (50-CGG TGT GTA CAA GAC CC-30). Amplification was performed in a standard reaction mixture containing 25 mM Tris (hydroxymethyl) methyl-

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amino-propane sulfonic acid (pH 9.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM dNTP, 2.5 IU Taq polymerase, 1.0 µM of each primer and the DNA template. The PCR was run with an initial denaturation at 95 °C for 10 min followed by 30 cycles of denaturation at 93 °C for 30 s; annealing at 65 °C (first 10 cycles), 60 °C (second 10 cycles), or 55 °C (last 10 cycles) for 30 s; and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min (Parvin and Nishino 2009). The reaction was conducted in a PCR thermal cycler (Dice; Takara Bio Inc., Shiga, Japan).

For the monitoring of the *Clostridium* and *Bacteroides-Provotella* specific community, we used nested PCR approach. This involved an initial PCR with Chis150f (5'-AA AGG RAG ATT AAT ACC GCA TAA-3'); ClostIr (5'-TT CTT CCT AAT CTC TAC GCA-3') (Hung et al. 2008), Bac303f (5'-GA AGG TCC CCC ACA TTG-3'); Bac708r (5'-CAA TCG GAG TTC TTC GTG-3') (Bartosch et al. 2004) primers that amplified the specific sequence. This was followed by a second PCR with GC-containing universal primers: GC357f (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCAC GGG GGG CCT ACG GGA GGC A GCAG-3') and 517r (5'-ATT ACC GCG GCT GCT GG-3'), which amplified the variable region (V3) of the 16S rRNA gene. Amplification was performed in a standard reaction mixture containing 25 mmol/l Tris (hydroxymethyl) methyl-amino-propane sulfonic acid (pH 9.3), 50 mmol/l KCl, 2.0 mmol/l $MgCl_2$, 0.2 mmol/l dNTP, 2.5 IU Taq polymerase, 1.0 µmol/l of each primer, and the DNA template. The first PCR run was performed with an initial denaturation at 94 °C for 2 min, followed by 35 amplification cycles (denaturation at 94 °C for 30 s; annealing at 61 °C for 1 min; and extension at 68 °C for 1 min), followed by a final extension at 68 °C for 7 minutes. The reaction was conducted in a PCR thermal cycler (Dice; Takara Bio Inc., Shiga, Japan). In the second round of PCR, an initial denaturation was performed at 95 °C for 10 min, followed by 30 amplification cycles (denaturation at 93 °C for 30 s; annealing at 65 °C (first 10 cycles), 60 °C (second 10 cycles) or 55 °C (last 10 cycles) for 30 s; and an extension step at 72 °C for 1 min), followed by a final extension step at 72 °C for 5 minutes.

The GC-clamp PCR products were separated according to their sequences using a D-Code Universal Mutation Detection System (Bio-Rad Ltd, Tokyo, Japan). Briefly, the samples were applied directly into 8% (w/v) polyacrylamide gels in a running buffer containing 20 mmol/l Tris-acetate and 0.5 mmol/l EDTA-2Na (pH 8.5). The gels were then prepared with a denaturing gradient from 25–50% of urea and formamide [7 mol/l urea and 400 ml/l (v/v) formamide as the 100% denaturant]. Electrophoresis was then conducted at a constant voltage of 150 V for 8 h at 60 °C. After electrophoresis, the gels were stained using SYBR Green (Cambrex Bio Science Inc., Rockland, ME, USA) and photographed under UV illumination.

The DGGE profiles (gel images) were transferred to a personal computer, and the DNA bands were identified using the Gel-Pro Analyzer software (v6.0; Media Cybernetics, Silver Spring, MD, USA). The number 1 was assigned when a band was found at a certain position in a lane, and a 0 was assigned when no band was found at the same position in the other lanes. The data were then subjected to cluster analysis by Naoki and Yuji (2008).

Statistical analysis

The data were analysed using a completely randomised design with dietary treatments and repeated measurements for *in vitro* digestibility during each treatment. Statistical analysis was performed using the mixed procedure (v9.4; SAS Institute, Cary, NC, USA) and a model including diet, time, and diet × time effects. Differences between means owing to sampling times were determined using Tukey's multiple comparisons. All analyses were conducted using jmp software (v9.4; SAS Institute, Cary, NC, USA).

RESULTS

Fermentation parameters

Rice straw alone. The cumulative gas production was increased by the four types of straw within the incubation time. A significant difference existed between the types of rice straw at 72, 96, and 120 h of incubation. Higher gas was produced for RS-3, followed by RS-1, and the lowest was RS-2 (Figure 1). The NDF digestibility increased within the incubation time; however, there was no significant difference between each type of straw (Figure 2). The VFA concentration

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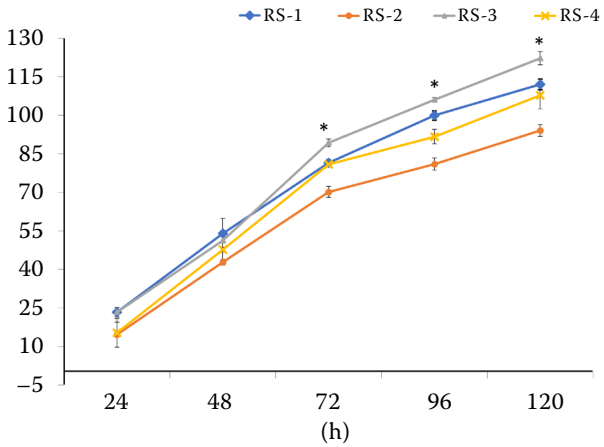

Figure 1. The cumulative gas production of rice straw (ml/g DM) in different incubation periods

RS-1 = rice straw of Ningarhar sorkhrood, Afghanistan; RS-2 = rice straw of Ningarhar behsood, Afghanistan; RS-3 = rice straw of Khost mandozai, Afghanistan; RS-4 = rice straw of Khost shamal, Afghanistan

*$P < 0.05$

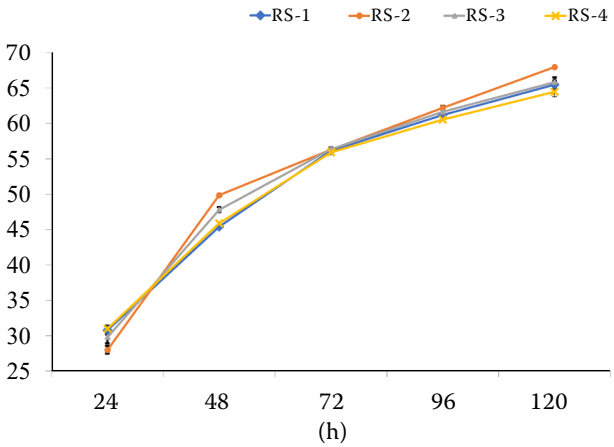

Figure 2. Rice straw NDF digestibility (mg/g DM) in different incubation periods

RS-1 = rice straw of Ningarhar sorkhrood, Afghanistan; RS-2 = rice straw of Ningarhar behsood, Afghanistan; RS-3 = rice straw of Khost mandozai, Afghanistan; RS-4 = rice straw of Khost shamal, Afghanistan

was increased within the incubation time for all types of rice straws. The total VFA (acetate, propionate and butyrate) concentration was affected by the types of rice straw, in which higher concentration was achieved by RS-1, followed by RS-3 during the incubation periods. The two types of straw (RS-1 and RS-3) had higher acetate concentrations during all incubation times, while propionic acid was only higher at 24 h of incubation (Table 2).

Rice straw and concentrates. The cumulative gas production was significantly increased by rice

Table 2. Volatile fatty acid (VFA) concentration (mmol/l) of four types of rice straw in different incubation periods

	Ingredient				*P*-value
	RS-1	RS-2	RS-3	RS-4	
24 h incubation period					
Acetic acid	31.38 ± 3.78	19.78 ± 1.58	21.59 ± 7.92	17.58 ± 1.04	*
Propionic acid	12.65 ± 1.75	8.02 ± 1.58	11.49 ± 4.94	6.30 ± 0.21	*
Butyric acid	3.81 ± 0.56	2.54 ± 1.03	2.05 ± 0.05	2.08 ± 0.62	NS
Total VFA	49.075	20.6	35.12	26.62	*
48 h incubation period					
Acetic acid	39.90 ± 0.59	30.15 ± 4.09	35.42 ± 7.94	28.34 ± 1.44	*
Propionic acid	17.64 ± 4.65	16.73 ± 1.42	18.55 ± 0.13	16.44 ± 1.65	NS
Butyric acid	3.41 ± 0.16	1.94 ± 0.26	3.48 ± 1.53	2.13 ± 0.19	*
Total VFA	60.95	48.81	57.44	48.9	*
120 h incubation period					
Acetic acid	73.24 ± 8.00	61.40 ± 13.29	63.44 ± 9.21	58.14 ± 6.56	*
Propionic acid	22.79 ± 7.53	22.20 ± 0.20	20.94 ± 0.04	21.24 ± 0.47	NS
Butyric acid	5.07 ± 0.41	4.09 ± 1.40	4.68 ± 2.38	4.96 ± 1.70	NS
Total VFA	105.34	87.92	89.17	84.49	*

NS = not significant; RS-1 = rice straw of Ningarhar sorkhrood, Afghanistan; RS-2 = rice straw of Ningarhar behsood, Afghanistan; RS-3 = rice straw of Khost mandozai, Afghanistan; RS-4 = rice straw of Khost shamal, Afghanistan

*$P < 0.05$

Data are presented as mean ± SD

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straw with barley (RS-BR) followed by rice straw with kidney beans (RS-KB) treatments at 48 h of incubation. The pH was decreased by supplements, but not significantly. The total VFA concentration was significantly enhanced by RS-KB and followed by RS-BR. Propionic and butyric acids were significantly increased by RS-KB and followed by RS-BR supplementation, whereas acetic acid was not affected by concentrate supplementation (Table 3).

Rice straw microbial degradation under SEM. The changes in rice straw morphology under exposure to different incubation periods were analysed under a SEM. In control (0-day incubation), the normal hyphae had a smooth surface (Figure 3). Meanwhile, treatment with different incubation periods resulted in degenerated and damaged shape abnormal hyphae and the damage size increased by 24, 48 and 120 h of the incubation periods, respectively.

PCR-DGGE

Rice straw alone. According to DGGE band profiles (Figures 4 and 5) and cluster analyses, the *Clostridium* and *Bacterioides-Prevotella* groups' bacterial community appeared unaffected by the incubation time of the solid and liquid-associated fractions. A remarkable difference existed between

Table 3. pH and volatile fatty acid (VFA) concentration (mmol/l) at 48 h of incubation of the rice straw and supplements treatment

VFA	Control (rice straw)	Supplements		*P*-value
		barley	kidney bean	
Acetic acid	35.94 ± 2.77	35.71 ± 2.02	35.89 ± 0.26	NS
Propionic acid	16.78 ± 2.22^{b}	18.07 ± 1.41^{ab}	22.05 ± 0.81^{a}	*
Butyric acid	3.43 ± 1.60	4.55 ± 0.09	5.63 ± 0.42	*
Total VFA	56.62 ± 6.69^{b}	58.34 ± 3.34^{ab}	63.58 ± 1.49^{a}	*
pH	6.71 ± 0.23	6.62 ± 0.13	6.62 ± 0.13	NS

NS = not significant

*P < 0.001; ^{a,b}different letters in the same row differ significantly

Data are presented as mean ± SD

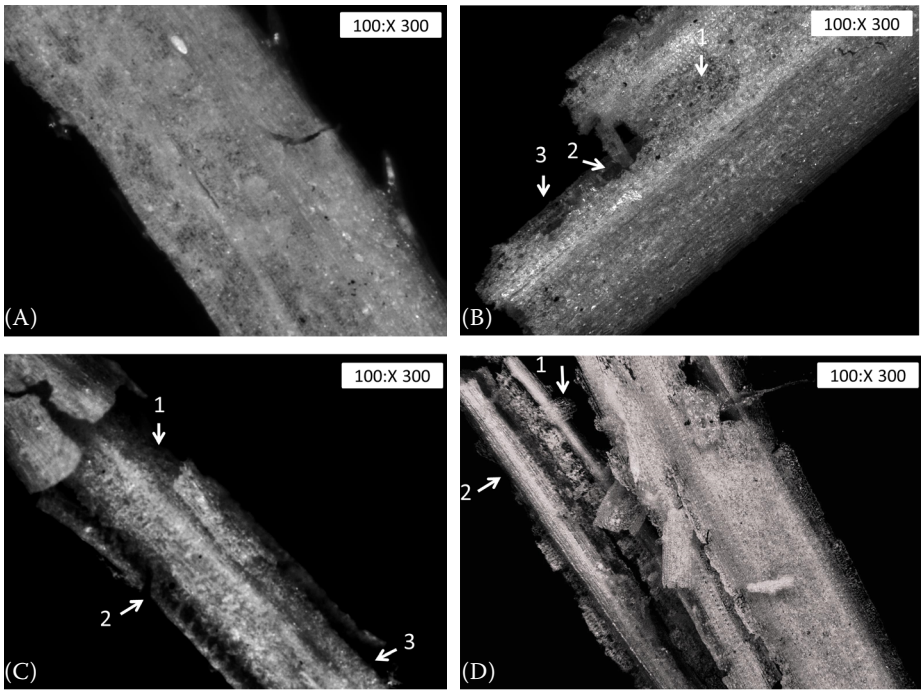

Figure 3. The microbial degradation structure of rice straw under scanning electron microscope after 0 h (A), 24 h (B), 48 h (C) and 120 h (D) of incubation

1–3 = degradation degree of the tissues

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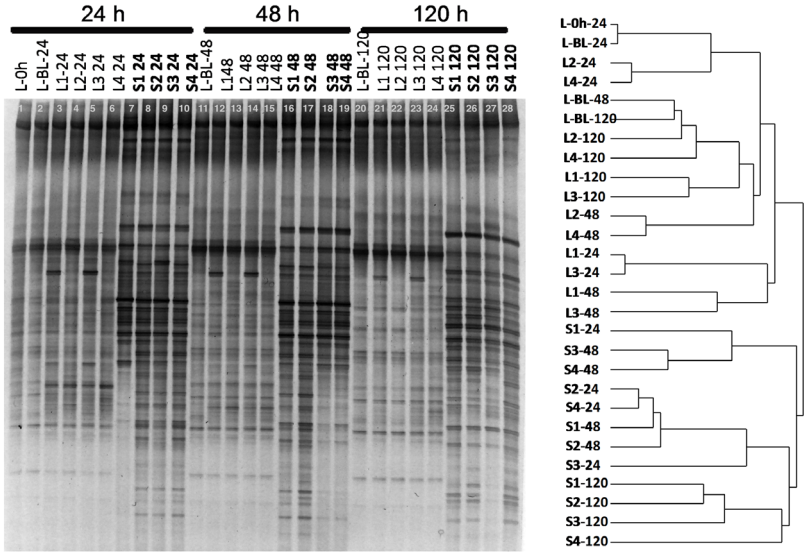

Figure 4. Liquid- and solid-associated bacterial communities determined by DGGE employing group-specific primers for *Bacteroides-Prevotella*
The first letter stands for liquid- (L) and solid- (S) associated bacteria, the numeral (1–4) on the second position represents the type of rice straw, and the following description in parenthesis means 24, 48 and 120 h of incubation

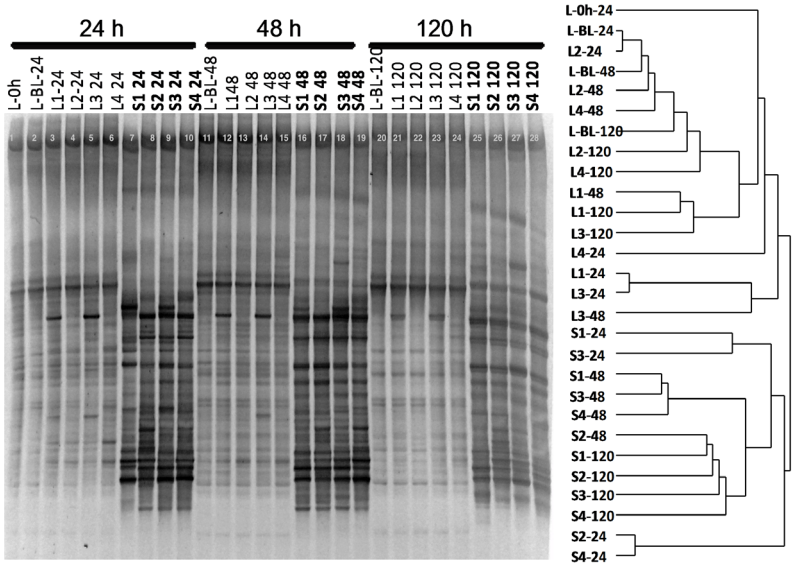

Figure 5. Liquid- and solid-associated bacterial communities determined by DGGE employing group-specific primers for *Clostridium*
The first letter stands for liquid- (L) and solid- (S) associated bacteria, the numeral (1–4) on the second position represents the type of rice straw, and the following description in parenthesis means 24, 48 and 120 h of incubation

the solid and liquid fractions throughout incubation; the density of the dominant bands was higher with the solid fraction. A small difference existed between the four types of rice straw. The liquid-associated bacterial community had a different band pattern in RS-1 and RS-3 compared to RS-2 and RS-4 overall incubation, while in the solid fractions, the difference was only by 24 h and 48 h of incubation.

Rice straw and concentrates. Looking at the V6–V8 region (Figure 6) band patterns, the bacterial community profile was grouped and separated by the solid and liquid fractions, where the effect of barley and kidney beans was unclear on the total bacterial community profile. In the group-specific bacterial community profile, a clear difference was observed between the solid and liquid fractions of the *Clostridium* where *Bacteroides-Prevotella* was not clear. The solid-associated community looked stable and was not affected by BR or KB, typical for the V6–V8 region, *Clostridium,* and *Bacteroides-Prevotella* group analyses. Liquid-associated communities determined by *Clostridium* and *Bacteroides-Prevotella* group were affected by supplements. R + BR and R + KB had different community structures from rice straw (RS) in the *Bacteroides-Prevotella* group; however, only R + KB differed from RS in the *Clostridium* group. Also, the difference in the bacterial community between barley and kidney beans was found only with the *Clostridium* group in the liquid fraction.

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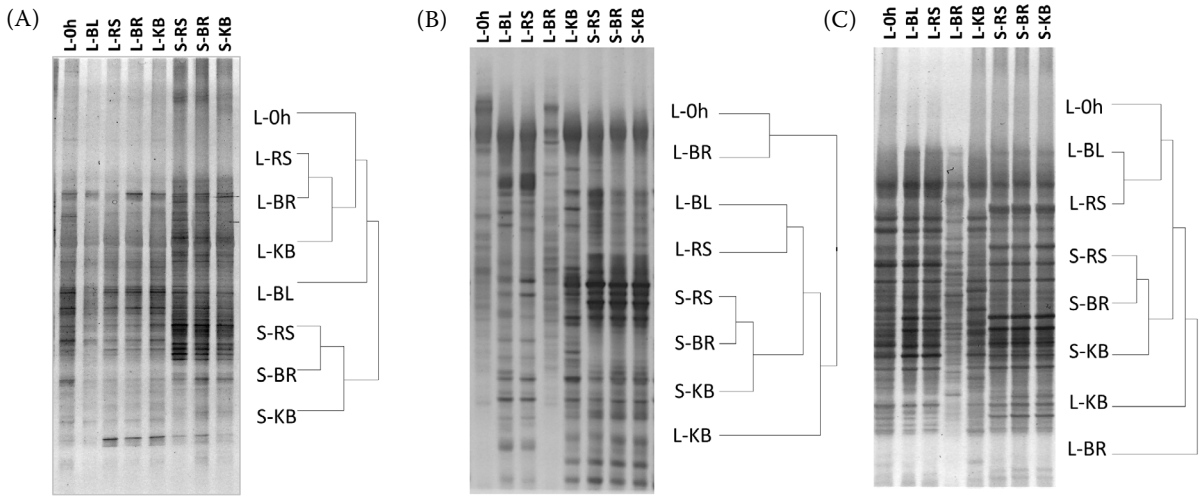

Figure 6. Liquid- and solid-associated bacterial communities determined by DGGE employing universal primers, which amplify V6–V8 region (A), group-specific primers for *Clostridium* (B) and *Bacteroides-Prevotella* (C) of the 16S rRNA genes

The first letter stands for a liquid- (L) and solid- (S) associated bacteria, the following letters express rice straw alone (RS), barley (BR), and kidney bean (KB) treatment

DISCUSSION

The amount of cumulative gas production was different between the types of rice straw after 72 h of incubation, which is related to the chemical composition of straw, particularly the fibre content (Table 1). Van Soest (1994) reported that lignin content is associated with the lower digestibility of rice straw. In the current study, lignin content varied between verities; the ADF content was lower, and hemicellulose was higher with RS-3 and RS-1. The NDF digestibility was increased by the incubation time. The NDF digestibility increases with incubation time because it is usually slowly and poorly digested (Pearce et al. 1988). However, the digestibility of the NDF depends upon the linkage between lignin and structural carbohydrates (Chesson 1988) and the phenolic component of the lignin (Jung and Varel 1988). The VFA content was increased by incubation time, and there was a difference between types of rice straw. As reported Chen et al. (2008), the higher VFA concentration may be due to an increased fermentation rate *in vitro* that was reflected by increased gas production. Doyle et al. (1986) compiled data on rice straw's chemical composition and *in vitro* organic matter digestibility (IVOMD) from many countries and reported that these parameters varied widely. This variation may be due to genetic and environmental factors and differences in the proportion of plant botanical fractions, such as leaf-to-stem ratio, in the straw (Walli et al. 1988). From the literature mentioned above, it can be concluded that the chemical composition and nutritive value of rice straw varieties are inconsistent. In our study, the results of the incubation parameters (cumulative gas production and VFA concentration) for the types of rice straw were highly correlated with the chemical composition of the straw. The structural carbohydrates such as NDF, and hemicellulose concentrations of rice straw, which the enzymatic action of microorganisms might cause (Arora et al. 2002), produced gas and VFA during *in vitro* incubation. In our study, we found that ADF content was negatively correlated to cumulative gas production and VFA concentration of rice straw, suggesting that rumen microorganisms might hardly digest ADF contents. The gradually increasing fermentation parameters with incubation time might be due to the slow digestion of rice straw. Pearce et al. (1988) also reported that NDF digestion is slow in the rumen.

The degradation of rice straw cell walls and parenchyma tissues was increased by the incubation time. The introduction of the scanning electron microscope enabled the determination of degradable and undegradable zoons in rice straw tissues. The degree of lignification of parenchyma tissues (young and old) in Bermuda grass stems, and sheaths (Akin et al. 1977) influenced the state

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of degradation of the parenchyma tissues. Alkali treatment of rice hulls (McManus et al. 1976), and rice straw (Kawamura et al. 1973) increased degradation of predominantly soft, swollen plant residues. This result could be compared with rice straw incubated within the incubation time. The differences between types of rice straw were not clear. However, the degradable tissue zoons of the different incubation times are visible, which are in agreement with fermentation parameters and cumulative gas production.

The difference in the bacterial community in the solid and liquid fractions is due to the attachment of dominant fibrolytic bacteria to the particles. Microbial mass was larger in the solid-associated phase of the rice straw during all incubation periods, confirming that solid-associated microbes are predominant in the rumen contents and mainly contribute to fibre digestion. Craig et al. (1987) indicated that under ordinary feeding conditions, feed particle-associated bacteria are numerically predominant and occupy up to 70–80% of the total microbial population in the entire rumen contents of cows. Silva et al. (1987) reported that the rate of fibre degradation depends on the extent to which the rumen environment allows an adherent cellulolytic microbial population to develop. Cellulolytic organisms frequently attach to their substrate, and those that degrade starch and protein probably do as well (Van Soest 1994). According to DGGE bacterial diversity cluster analyses, a small difference existed between the four types of rice straw, in which the band pattern of RS-1 and RS-3 was incongruent with the RS-2 and RS-4 overall incubation. On the other hand, higher gas production and the total VFA was achieved by RS-1, followed by RS-3 during the incubation periods. Liu et al. (2002) found that gas production is an indirect measure of substrate degradation, mainly the carbohydrate fraction. Gas production is also a good predictor for the production of VFA; however, changes could occur in gas production related to microbial mass production. We found similar chemical compositions between the four types of rice straw. In conclusion, the slight changes in bacterial diversity are in agreement with the chemical composition and anatomical structure of the four types of rice straw.

By the concentrate treatment, the cumulative gas production and propionic acid concentration were increased, whereas the acetic acid concentration was not affected by the RS-BR and RS-KB treatments; this may be due to concentrates supplementation. Beever and Mould (2000) reported that acetic acid is predominant, in which cellulolytic bacteria proliferate. In contrast, amylolytic bacteria dominate on starch-enriched diets, where increased propionic acid levels are typically observed. Although some changes occurred in the fermentation parameters in our study, in general, the digestibility of rice straw was not affected by the treatments; this may be due to the low ratio of the concentrates in the diets. Finally, we conclude that the digestibility of rice straw was not improved nor inhibited by the 25% of concentrate treatment. The cumulative gas production was increased by concentrate supplementation. Gas production can be increased by providing a supplement which provides sufficient nutrients to stimulate the activity of rumen microorganisms. A study was conducted by Prasad et al. (1994); they found higher gas production when mixed concentrates were supplemented with millet straw. Failure to see the supplementation effect on the solid-associated community could result from the low proportion of supplements in the diet. Moreover, it is not surprising because the treatments did not significantly affect the fermentation parameters such as pH. Surprisingly, BR and KB treatments affected the liquid-associated bacterial community of the *Clostridium* and *Bacteroides-Prevotella* groups, and we found higher band density. The changes might be due to the presence and digestibility of protein and starch content in the diet. Because after *in vitro* digestion, the remaining materials are undigested solid particles, the amylolytic and proteolytic bacteria might be in the liquid part. Cherdthong et al. (2010) reported that a roughage: concentrates ratio of 75 : 25 could increase the cellulolytic bacteria while the amylolytic bacteria population was decreased. On the other hand, Lee et al. (2019) reported that a high-concentrate diet did not change rumen bacterial diversity, while the relative bacterial population was changed.

CONCLUSION

Within the four types of rice straw, we found that the straw's chemical composition, particularly fibre content, had a positive correlation with the VFA and cumulative gas production. Also, the bacterial community profile was slightly

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changed. Rice straw digestibility increased with incubation time; however, bacterial community structure was stable. Great bacterial diversity existed between solid and liquid fractions in rice straw alone and supplement treatment. The community structure of the *Clostridium* and *Bacteroides* groups was stable and involved in the digestion of a rice straw-based diet. However, the liquid-associated bacterial community was changed by supplements. These results indicate a rigid and stable community structure of *Clostridium* and *Bacteroides* groups involved in the digestion of rice straw-based diets in the rumen. Distinctive differences between solid and liquid fractions were described; hence, separate analyses of two fractions may greatly help understand the relationship between fermentation patterns and microbial communities in the rumen.

Conflict of interest

The authors declare no conflict of interest.

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