ISOLATION AND PARTIAL CHARACTERIZATION OF *RUMINOCOCCUS FLAVEFACIENS* FROM THE RUMEN OF SWAMP BUFFALO

Phoompong Boonsaen[1], Somporn Poonko[1], Jeerachai Kanjanapruetipong[1], Jintanart Wongchawalit[2], Pharima Phiriyangkul[3] and Suriya Sawanon[1,4,*]

ABSTRACT

Fibrolytic bacteria play a particularly important role in the fiber degradation in the rumen and could be the keys of improving a production performance of ruminants. The ability to utilize low-quality roughage for swamp buffalo has been reported, thus, the rumen of swamp buffalo may be a suitable source for isolation of the potent fibrolytic bacteria. Total of 165 gram-negative cocci fibrolytic bacteria were screened from the rumen contents of four swamp buffalo. Six isolates were identified as *Ruminococcus flavefaciens* and the phylogenetically grouped by type strain. Isolate OS14 showed higher fiber digestion in either mono- or cocultures with non-fibrolytic bacteria than type strains C94. Dry matter digestibility of cellulose powder in co-culture of OS14 with all strains of non-fibrolytic was increased. Moreover, the co-culture of OS14 with S137 showed the highest fiber digestion and notably increased concentration levels of acetate and propionate. These results indicate that cross-feeding relationship between *R. flavefaciens* with non-fibrolytic bacteria be able to improve fiber digestion but the amount of improvement may perhaps be based on the combination of the bacterial strains. The *in vitro* fermentation products and abundance in co-cultures need to be quantified in a further study.

Keywords: *Bubalus bubalis*, buffaloes, fibrolytic bacteria, non-fibrolytic bacteria, *R. flavefaciens*, rumen, swamp buffalo

INTRODUCTION

On planet earth, one of the most abundant biopolymers is cellulose. This structural polysaccharide is an important part of the plant cell wall structure. In tropical developing countries, agricultural crop residues such as cereal straw, bean hull, and corn husk are used as roughage feed in ruminants (Anderson, 1978; Maneerat *et al*., 2015).

[1]Department of Animal Science, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Nakhon Pathom, Thailand

[2]Department of Microbiology, Faculty of Liberal Art and Science, Kasetsart University, Nakhon Pathom, Thailand

[3]Division of Biochemistry, Department of Science, Faculty of Liberal Art and Science, Kasetsart University, Nakhon Pathom, Thailand

[4]Center for Advanced Studies for Agriculture and Food, Kasetsart University Institute for Advanced Studies, Kasetsart University, Bangkok, Thailand, *E-mail: agrsusa@ku.ac.th

Fibrous feed is always important to ruminants, as it can be digested and fermented by rumen microbes (Krause *et al*., 2003). The fibrolytic bacteria play an important role in fiber fermentation, especially the major groups found in the rumen, *Fibrobacter succinogenes, Ruminococcus flavefaciens* and *Ruminococcus albus* (Koike *et al*., 2003; Shinkai and Kobayashi, 2007; Koike and Kobayashi, 2009). *R. flavefaciens* is an anaerobic bacteria, having non-motile organisms with a coccoid shape. These bacteria obtain nutrients by breaking down the plant cell walls as the feed passes through the digestive system of the host animal. They are also capable of fermenting cellulose, cellodextrins, xylan, and cellobiose, acetate and succinate are the major products while lactate, ethanol, carbon dioxide and hydrogen as minor products, however, the reaction varies among the strains (Dehority, 2003). These bacteria species inhabit the rumen of cattle, sheep, goat, and the hindgut of horses (Puniya *et al*., 2015). They can also be found in the rumen of swamp buffalo which have a higher ability to utilize low-quality roughage than cattle (Wanapat *et al*., 2000; Wanapat and Cherdthong, 2009; Chanthakhoun *et al*., 2012). Sawanon *et al.* (2017) reported that *R. flavefaciens* showed the highest population in the rumen of swamp buffalo fed rice straw or paragrass. Therefore, cellulolytic *R. flavefaciens* are currently is the major area of this study. The objectives of this study were to isolate *R. flavefaciens* from the rumen of swamp buffalo fed rice straw, to phylogenetically and partially characterize the isolated strains and to evaluate synergism between *R. flavefaciens* and non-fibrolytic bacteria *in vitro.*

MATERIALS AND METHODS

Isolation and identification of fibrolytic bacteria

Four mature rumen fistulated swamp buffaloes (all females) were individually penned at Ruminant Research Unit, Department of Animal Science, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Nakhon Pathom, Thailand and fed *ad libitum* rice straw supplemented with a concentrate (1 kg/day, twice daily at 07:30 a.m. and 04:30 p.m.). The concentrate mixture (16% CP) consisted of 36% cassava, 30% expeller pressed palm kernel meal, 20% of solvents extracted palm kernel meal, 3.5% soybean meal, 8% molasses, 1.5% urea, 0.05% sulfur, 0.1% di-calcium phosphate, 0.5% salt, and 0.5% of premix for beef cattle. The management of the animals was approved by the Animal Usage and Ethics Committee of Kasetsart University (ID No. ACKU60 – AGK – 002).

The rumen contens (after 6 h of feeding) were used as the sources for rumen bacterial isolation; 0.5 g of the bacterial source was added into a 4.5 mL O_2-free dilution solution (Ogimoto and Imai 1981), then incubated on ice for 5 minutes and vortexed at maximum speed for 1 minute to detach the rumen bacterial cells from the fiber (Poonko *et al*., 2015). Subsequently, serially dilutiong with O_2-free dilution solution at 10^{-5} and 10^{-6} were consecutively inoculated into the basal medium containing 0.2% (w/v) glucose and cellobiose, and 2.0% (w/v) agar (rumen fluid glucose and cellobiose Agar; RGCA) for pure culturing by using the roll tube technique (Ogimoto and Imai, 1981), then incubated at 38 ºC for 72 h. A single colony was randomly selected and inoculated into 5 mL of the basal broth medium, containing a filter paper (Whatma® No. 1) as the carbon source (rumen fluid filter paper medium;

RF) for selecting a fiber degrading bacteria. The compositions of the experimental media are shown in Table 1. Fibrolytic bacteria were collected and screened for further identification using gram staining. The cocci pure cultures of fibrolytic bacteria were stored in an RGCA medium under -80ºC conditions. *Butyrivibrio fibrisolvens* KU-NF6, *Pseudobutyrivibrio xylanivorans* KU-NF7 and *B. fibrisolvens* KU-NF24 which were previously isolated from the rumen of swamp buffalo by our research group (Poonko *et al*., 2015) and *Selenomonas ruminantium* S137 which was isolated from the rumen of sheep (Sawanon *et al*., 2011), were used as non-fibrolytic bacteria. *R. flavefaciens* C94 type strain (ATCC19208) was used as the reference strain.

The total DNA was extracted from the bacterial cultures in the RF medium by using a Favo Prep™ Stool DNA Isolation Mini Kit (Favogen Biotech Corp. Taiwan) according to the manufacturer's instructions. Partial 16S ribosomal DNA was amplified by conventional PCR (Multi Gene Gradient Thermal Cycle, Labnetendure™, MA,USA). The universal primer sets were as follows: 27F forward (5'AGAGTTTGATCMTGGCTCAG 3') and 1492R reverse primer (5'TACGGYTACCTTGTTACGACTT 3'). PCR amplification was performed by using 20 µL in a total mixture volume containing 0.2 µL (5 U/µL) of *Taq* DNA polymerase recombinant (Thermo Scientific, USA), 2 µL of 10x *Taq* buffer with $(NH_4)_2SO_4$, 2 µL of 25 mM $MgCl_2$, 2 µL of dNTP mix at concentration of 2 mM, 1 µL of each primer, 1 µL (10 pg-1 µg) of the extracted DNA, and 10.8 µL of distilled H_2O. The thermal cycles involved pre-heating at 94ºC for 5 minutes, followed by 30 cycles each consisting of 94ºC for 30 seconds, annealing at 58ºC for 30 seconds, extension at 72ºC for 90 seconds, and a final extension at 72ºC for 10 minutes. The PCR products were purified by using a Favor Prep™ (Gel/PCR Purification Mini Kit, Favogen Biotech Corp. Taiwan.) according to the manufacturer's instructions. The DNA sequencing process was done in First Base Laboratories SDNBH (Malaysia). The sequences obtained were run through BLAST in order to determine the closest identity to the bacteria in the GenBank database website (BLAST program, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The generated sequences and reference sequences from the GenBank were aligned by using the multiple sequence alignment program Clustal X (version 1.83). The neighbor-joining method was used to perform phylogenetic analysis. The NJPLOT software were used to draw a phylogenetic tree (Perrière and Gouy, 1996). Bootstrap analysis was carried out for 1,000 replicates.

Dry matter digestibility analysis

Dry matter digestion of cellulose powder or rice straw as a carbon source in a basal medium was tested. Fibrolytic *R. flavefaciens* isolates, type strain C94 and *S. ruminantium* S137 were grown in a basal medium containing glucose and cellobiose (0.25% w/v of each), while *B. fibrisolvens* and *P. xylanivorans* strains were grown in a basal medium containing cellobiose (0.25% w/v) then incubated at 38ºC to the end of the log phase. After three culture passages, each culture was used as an inoculum (Poonko *et al*., 2015). Each inoculum (0.2 mL for monocultures and 0.1 mL for co-cultures) was inoculated into 10 mL of a basal medium containing cellulose (Sigmacell 20; Sigma-Aldrich; MO, USA) or rice straw (0.1% w/v). These cultures were incubated at 38ºC for 72 h for the initial evaluation of dry matter (DM) digestibility among the isolated strains of fibrolytic bacteria, or to be compared to selected strains and

type strain C94 in monoculture or co-cultures with non-fibrolytic bacteria. After incubation, the cultures were cooled on ice for 30 minutes to detach the bacterial cells from the fiber particles (Fukuma *et al*., 2012) and centrifuged (377 × g, 4ºC, 10 minutes). The supernatant was collected for analysis of the short chain fatty acids of mono- or co-cultures using gas chromatography (GC-14B; Shimadzu, Kyoto, Japan). The fiber residue was washed with 10 mL of 0.5M potassium phosphate buffer and re-centrifuged (2,300 × g, 4ºC, 10 minutes). The washed residue was dried at 105ºC and weighed to calculate the dry matter digestion of the isolated bacterial strains.

Enzyme assays

The isolated strains of fibrolytic bacteria were grown in 20 mL of basal media using cellulose powder (0.25% w/v) as the carbon source and incubated at 38ºC for 24 h. The bacterial cells were harvested by centrifugation (2,800×g, 4ºC, 10 minutes). Bacterial cells were washed twice with 20 mL of 50 mM potassium phosphate buffer (pH 6.8) at 4ºC, then re-suspended in 2 mL of the same buffer. The bacterial cells were disrupted by using an ultrasonic disruptor (UD-201; Tomy Seiko Co.; Tokyo, Japan) on ice for 10 minutes and centrifuged (2,800×g, 4ºC, 10 minutes) to collect the supernatant of the cell-free extracted intracellular enzymes (Kobayashi *et al*., 1998; Sawanon *et al*., 2011). Xylanase and CMCase activities were determined by measuring the reducing sugar obtained from xylan or carboxyl-methyl-cellulose (CMC) (1% for each substrate in sterilized 50 mM phosphate buffer; Sigm, Sigma-Aldrich; MO, USA) by using the 3, 5-dinitrosalicylic acid method (Miller, 1959). D-xylose or D-glucose was used as the standard substrates. One unit of xylanase or CMCase activity was defined as the amount of the enzyme that released 1 nanomole of xylose or glucose equivalent per minute from xylan or CMC. Protein concentrations were determined using Bradford protein assay (Bradford, 1976). Bovine serum albumin was used as the standard protein.

The selected *R. flavefaciens* strains were employed to determine the intra- and extra-xylanase and CMCase activities. The culture introduced into 20 mL basal media, using cellulose powder (0.25% w/v) as the carbon source (incubated at 38ºC for 24 h, and centrifuged (2,800×g, 4ºC, 10 minutes) to harvest bacterial cells for intracellular enzyme analysis. The supernatant was dialyzed against a phosphate buffer (4ºC, overnight), then concentrated with 20% polyethylene glycol (M.W. 20,000), and used for extracellular enzyme analysis. The concentration of xylanase, CMCase, and protein was determined using the same procedure as described above.

Statistical analysis

All the data (n=3 per treatment; 6 treatments for a comparison of dry matter digestibility of each isolate or 10 treatments for a comparison of dry matter digestibility of mono- and co-culture with non-fibrolytic bacteria of *R. flavefaciens* OS14 and *R. flavefaciens* C94) of dry matter digestibility were subjected to one-way analysis of variance by using R version 3.2.3 software (R Core Team, 2015). A completely randomized design was applied to evaluate multiple comparisons of culture types or fiber sources. When the effect of the culture or fiber source was significant ($P<0.05$), differences between the cultures or fiber sources were evaluated by using Duncan's new multiple range test.

RESULTS

Identification and Phylogenetic analysis of *R. flavefaciens* isolates

A total of 165 gram-negative cocci fibrolytic bacteria was gathered from the roll tubes, 6 isolates were identified based on 16S rDNA sequencing. The obtained 16S rDNA sequences were run through BLAST in order to determine the closest identity of the origin of the bacteria. The phylogenetic tree of *Ruminococcus* spp. with *Syntrophobacter wolinii* as an out group is shown in Figure 1. All isolates were closely related to *R. flavefaciens* with high sequence similarity (98 to 99%) (Table 2).

Digestibility and enzyme activity of isolated strains

Dry matter digestibility of the tested fiber sources and specific intracellular cellulase and xylanase activities of the *R. flavefaciens* isolates and C94 (type strain) are shown in Table 3. Among the tested fiber, DM digestibility of *R. flavefaciens* strains KU-F154, KU-F156, KU-F157, and KU-F158 was higher in rice straw than in cellulose powder except for OS14, OS15, and C94. DM digestibility of both the rice straw and cellulose powder of OS14 and OS15 were similar and higher than other isolates. Moreover, DM digestibility of OS14 and OS15 was shown to be significantly higher than type strains in both the tested fiber sources. Specific cellulase and xylanase activity of the *R. flavefaciens* strains ranged from 3.92 to 38.2 and 16.9 to 112.7 nmol/min/mL of culture, respectively. KU-F154, KU-F158, OS14, and OS15 showed higher specific cellulase and xylanase activity than the type strain, whereas KU-F156 and KU-F157 were lower than the other type strain. KU-F154 showed the highest specific cellulase and xylanase activity. However, OS14 and OS15 showed a higher DM digestibility as well as enzyme activities compared to the type strain C94. Even though KU-F154 showed the highest specific enzyme activity, its DM digestibility was lower than type strain C94. Therefore, OS14 and OS15 were chosen to characterize extracellular enzyme activities as shown in Table 4. The results showed that the extracellular portion of xylanase activity of OS14 and OS15 were similar to type strain C94. However, OS14 showed 1.6 and 1.1 fold-higher extracellular portion of CMCase activity than OS15 and C94, respectively. Accordingly, OS14 was the candidate for the study of synergism with non-fibrolytic bacteria.

Digestibility and fermentation of mono- or co-culture

Dry matter digestibility of the tested fiber sources of *R. flavefaciens* OS14 and type strain C94 and their combination with non-fibrolytic bacteria are shown in Table 5. Among the tested fibers, DM digestion of rice straw in the monoculture of OS14 or C94 and most of their co-cultures with non-fibrolytic bacteria were higher than the cellulose powder except in co-culture OS14 with S137 or with KU-NF6. Monoculture of OS14 exhibited higher DM digestibility of both rice straw and cellulose powder than C94. Co-culture of OS14 with all strains of non-fibrolytic bacteria could enhance the DM digestibility of cellulose powder (7 to 21%). However, only OS14 with KU-NF24 could enhance the DM digestibility of rice straw (5%). OS14 with S137 showed the highest DM digestion of cellulose powder, while OS14 with KU-NF24 showed the highest digestion of rice straw. Co-culture of C94 with non-fibrolytic bacteria enhanced the DM digestibility of cellulose powder (3 to 6%). Co-culture of C94 with KU-NF6 or KU-

NF7 enhanced the DM digestion of rice straw (4 to 5%). Co-culture of C94 with non-fibrolytic bacteria showed negligible difference compared to its monoculture. Moreover, the DM digestibility of cellulose powder or rice straw in a co-culture with non-fibrolytic bacteria, OS14 showed significantly higher than C94. Synergism in a co-culture of OS14 or C94 with non-fibrolytic bacteria occurred for cellulose powder.

Short chain fatty acid concentrations by a monoculture of *R. flavefaciens* OS14 and strain type C94 and their combination with *S. ruminantium* S137 are shown in Table 6. In cellulose powder, the concentration of acetate and propionate in a co-culture of OS14 with KU-NF6, KU-NF7, or S137 was increased, except in KU-NF24 which had an insignificant increase. Moreover, a co-culture of OS14 with S137 notably increased the acetate and propionate. However, in C94, the concentration of acetate was increased when grown with KU-NF24 or S137 and propionate was increased only when grown with S137. In rice straw, the concentrations of acetate and propionate in a co-culture of OS14 with non-fibrolytic bacteria were increased except for KU-NF6 which could not be detected by SCFA. Remarkably, in co-culture with S137, acetate and propionate were notably higher than in a co-culture with other non-fibrolytic bacteria. In contrast, in a co-culture of C94 with non-fibrolytic bacteria, acetate and propionate were decreased, except in a co-culture with S137 in which propionate was increased. Both of OS14 and C94 co-culture with KU-NF6, KU-NF7, or KU-NF24 increased butyrate either using rice straw or cellulose powder except for KU-NF6 which grew with OS14 in rice straw or in all combination with S137 in both fiber sources.

DISCUSSION

Among the predominant fibrolytic bacteria, *R. flavefaciens* has been known as one of the most abundant species in the gut of herbivorous especially ruminants (Flint, 1997; Krause *et al*., 2003). Isolation and characterization of this bacterial species from different hosts or environments have been reported (Shinkai *et al*., 2007; Shinkai *et al*., 2009; Nyonyo *et al*., 2014; Boonsaen *et al*., 2018). *R. flavefaciens* is a gram-positive cocci bacterium but some strains are gram-negative to gram-variable (Ogimoto and Imai, 1981). This bacterial species plays an important role in cellulose digestion in the rumen and they produce different combinations and proportions of the major fermentation products such as hydrogen, carbon dioxide, ethanol, acetate, formate, and lactate (Puniya *et al*., 2015). In the present study, all isolates were gram-negative cocci and produced acetate as the major fermentation product (the data not showed). The phylogenetic analysis of 16S rDNA sequences of isolated strains had high similarity (99%) to type strain C94 (Figure 1).

Based on this study, the isolated strain of *R. flavefaciens* showed various DM digestibility with different fiber sources. Interestingly, DM digestibility of either OS14 or OS15 was 1.2 to 1.4 folds-higher than type strain C94 in both cellulose and rice straw (Table 2). Moreover, they showed higher specific intracellular CMCase and xylanase activities as well as DM digestibility compared to type strain C94. Conversely, other isolated strains showed lower digestibility than this type strain even though some strains showed higher specific intracellular CMCase and xylanase activities than type strain C94. When considering extracellular enzyme activities (Table 4), OS14 showed the highest extracellular portion of total activity

and was higher than type strain C94. Moreover, higher DM digestibility was also observed. In the present study, therefore, OS14 was chosen to study synergistic activities with non-fibrolytic bacteria.

DM digestibility of rice straw was higher than cellulose powder. DM digestibility in a monoculture of OS14 was 1.1 or 1.5 fold-higher than C94 in cellulose powder or rice straw, respectively (Table 5). Although DM digestion of cellulose powder in co-cultures of either OS14 or C94 with all strains of non-fibrolytic bacteria was increased, however, each combination of OS14 with non-fibrolytic bacteria was 1.5 to 1.8 fold higher than in a combination of C94 with non-fibrolytic bacteria. Conversely, the increase of DM digestion in a combination of either OS14 or C94 with non-fibrolytic bacteria was negligible and decreased in rice straw. Therefore, OS14 seems to show higher fibrolytic potential, especially for natural forage fiber that is available in a tropical area and possibly would be an advantage of this bacterial strain to inhabit in buffalo rumen. Sawanon *et al*. (2006) reported that *R. flavefaciens* C94 showed higher DM digestion of Japanese rice straw than Avicel which is a microcrystalline cellulose similar to the report of Collings and Yokoyama (1980), *R. flavefaciens* C94 has shown higher degradation of cellulose and hemicellulose in natural fibers (manure fiber, wheat straw, Kentucky bluegrass, alfalfa and corn silage) than of filter paper. In the present study, therefore, the strain OS14 isolated from the rumen of tropical ruminant might be a specific inhabitation in animal fed low-quality forage.

Co-culture of OS14 or C94 with non-fibrolytic bacteria, synergistic can noticeably be developed only in cellulose powder. This may be possible that the synergy of these non-fibrolytic bacteria seems to depend on the fiber source. In both cellulose powder and rice straw, co-culture of OS14 or C94 with *Butyrivibrio fibrisolvens* or *Pseudobutyrivibrio xylanivorans* was increased mainly butyrate. These bacteria species can grow well in glucose and cellobiose and produce butyrate as a major fermentation product (van Gylswyk *et al*., 1996; Poonko *et al*., 2015). In the present study, they utilized the fermentation products of *R. flavefaciens* as their sole energy sources. This cross-feeding synergism might directly facilitate fiber digestion by preventing an accumulation of reducing sugar in the media which is feedback to inhibit fiber digestion enzymes (Dehority, 2003).

Co-culture of OS14 with S137 showed a higher total SCFA, acetate and propionate concentration than C94 with S137 in both cellulose and rice straw. The combination of *R. flavefaciens* with *S. ruminantium* in cellulose powder showed a higher total SCFA, acetate and propionate concentration. Moreover, higher DM digestibility of cellulose powder was also observed. *S. ruminantium* are succinate and lactate-utilizing bacteria. This bacterial species has been shown to have synergism with other fibrolytic bacteria. *S. ruminantium* S137 is involved in fiber digestion by co-operating with *F. succinogenes* S85 (Sawanon *et al*., 2011). Sawanon and Kobayashi (2006) reported that cross-feeding between *R. flavefaciens* C94 and *S. ruminantium* S137 could enhance fiber digestion by the consumption of succinate or lactate produced by *R. flavefaciens* C94 and converted into propionate. In this study, co-culture of OS14 or C94 with S137 enhanced DM digestibility with asimultaneously increasing of propionate concentration, this might be due to a reduction of succinate and lactate accumulation by S137. This has been indicated by Fukuma *et al*. (2012 and 2015) that the coexistence of S137 increases DM digestion with a concomitant increase

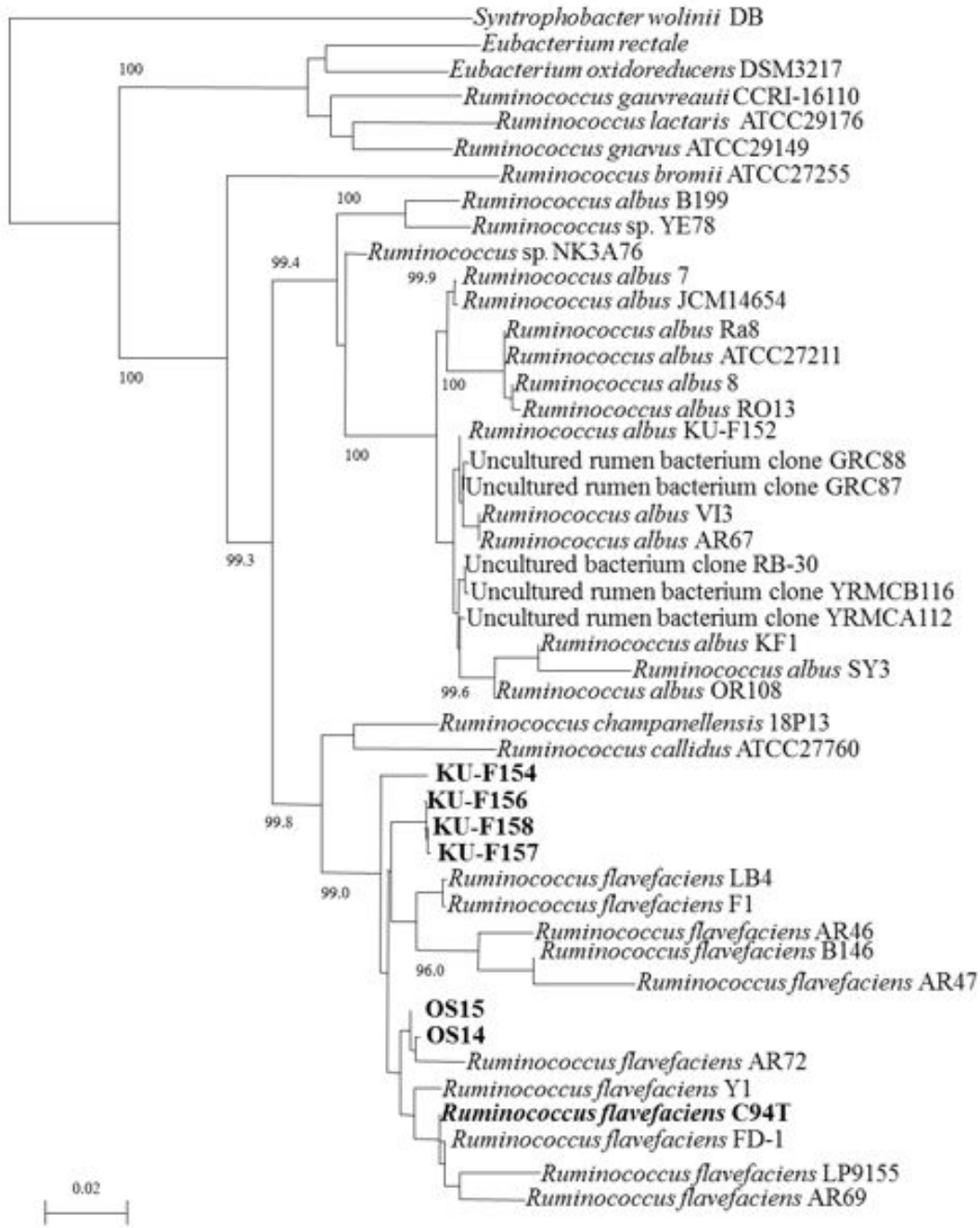

Figure 1. Phylogenetic tree of *Ruminococcus flavefaciens* isolated from buffalo rumen. The isolated strains and type strains are shown in boldface. The tree was constructed using neighbor-joining analysis of a distance matrix obtained from a multiple-sequence alignment and boot-strapped, with 1,000 iterations. *Syntrophobacter wolinii* DB was used as an out-group sequence.

Table 1. Composition of experimental medium in 100 mL of total volume.

Item	Basal medium	RGCA	RF
Mineral solution I[a]; mL	7.5	7.5	7.5
Mineral solution II[b]; mL	7.5	7.5	7.5
Resazurin, 0.1%; mL	0.1	0.1	0.1
Rumen fluid [c]; mL	30.0	30.0	30.0
Distilled water; mL	50.0	50.0	50.0
Bactopeptone; g	0.2	0.2	0.2
Yeast extract; g	0.12	0.12	0.12
L-Cysteine-HCL.H_2O; g	0.05	0.05	0.05
Glucose; g	-	0.25	-
Cellobiose; g	-	0.25	-
Filter paper; 0.5×1.0 cm^2	-	-	2 pieces/5 mL
Na_2CO_3, 8%; mL	5.0	5.0	5.0
Agar; g	-	2.0	-

[a]Contains 0.6 g of K_2HPO_4. per 100 mL

[b]Contains 0.6 g of KH_2PO_4, 1.2 g $(NH_4)_2SO_4$, 1.2 g NaCl, 0.25 g $MgSO_4$ and 0.12 g $CaCl_2 \cdot 2H_2O$ per 100 mL (Ogimoto and Imai, 1981)

[c]Fresh ruminal fluid was filtered by 4 layers of cheesecloth and autoclaved at 121ºC for 10 minutes, then centrifuged at 2,800 × g for 15 minutes at 4ºC and stored at -20ºC.

RGCA = Rumen fluid glucose and cellobiose agar, RF = Rumen fluid filter paper medium

Table 2. 16S rDNA sequence identity of ruminal bacteria isolated from swamp buffalo rumen.

Isolate	Nearest relative analysis		
	Accession No.	Assignment	Identity(%)
KU-F154	AM915269	*R. flavefaciens* C94T	98.0
KU-F156	AM915269	*R. flavefaciens* C94T	98.0
KU-F157	AM915269	*R. flavefaciens* C94T	98.0
KU-F158	AM915269	*R. flavefaciens* C94T	98.0
OS14	AM915269	*R. flavefaciens* C94T	99.0
OS15	AM915269	*R. flavefaciens* C94T	99.0

Table 3. Dry matter digestibility and specific intracellular fibrolytic enzyme activity of *Ruminococcus flavefaciens* isolated from buffalo rumen.

Isolate	Digestibility (%)		Specific enzyme activity (nmol/min/mg protein)	
	Cellulose	Rice straw	Cellulase	Xylanase
KU-F154	$17.5\pm1.9^{c, A}$	$4.7\pm0.4^{c, B}$	38.2	112.7
KU-F156	$16.3\pm2.1^{c, A}$	$7.3\pm2.1^{c, B}$	3.9	33.0
KU-F157	$15.6\pm3.1^{c, A}$	$7.3\pm2.1^{c, B}$	9.9	16.9
KU-F158	$15.7\pm1.9^{d, A}$	$7.5\pm4.2^{c, B}$	20.7	95.8
OS14	$32.5\pm1.5^{a, A}$	$33.0\pm0.4^{a, A}$	26.1	53.3
OS15	$29.5\pm1.5^{a, A}$	$31.6\pm1.1^{a,A}$	27.5	77.1
C94	$22.8\pm1.8^{b, A}$	$27.1\pm2.7^{b, A}$	17.0	48.3

^{abcd}Values in the same column with different superscripts differ ($P<0.05$).

^{AB} Value in the same row with different superscripts differ ($P<0.05$).

Table 4. Extracellular CMCase and xylanase activities of *Ruminococcus flavefaciens* OS14, *R. flavefaciens* OS15 and *R. flavefaciens* C94 (nmol/min/mg protein).

Isolate	Specific enzyme activity						Extracellular portion of total activity (%)	
	Intracellular		Extracellular		Total			
	CMC.	Xyl.	CMC.	Xyl.	CMC.	Xyl.	CMC.	Xyl.
OS14	8.0	16.2	12.8	42.3	20.8	58.5	61.5	72.3
OS15	8.1	22.9	5.3	56.7	13.5	79.6	39.4	71.2
C94	8.5	24.0	10.1	62.1	18.6	86.1	54.3	72.1

CMC. = CMCase, Xyl. = Xylanase

Table 5. Dry matter digestibility by monoculture of *Ruminococcus flavefaciens* OS14, *R. flavefaciens* C94 and their combination with *Butyrivibrio fibrisolvens* KU-NF6, *Pseudobutyrivibrio xylanivorans* KU-NF7, *B. fibrisolvens* KU-NF24 and *Selenomonas ruminantium* S137 after incubated at 38ºC for 72 h in basal media containing rice straw or cellulose powder.

Bacterial strain		**Dry matter digestibility (%)**	
		Cellulose powder	**Rice straw**
Monoculture	OS14	$21.6\pm1.8^{d, B}$	$39.7\pm1.5^{b, A}$
Co-culture	OS14+KU-NF6	37.2 ± 2.9^{b}	39.6 ± 0.5^{b}
	OS14+KU-NF7	$29.1\pm2.5^{c, B}$	$41.0\pm1.4^{b, A}$
	OS14+KU-NF24	$30.9\pm2.2^{c, B}$	$44.2\pm1.1^{a, A}$
	OS14+S137	$43.4\pm1.1^{a, A}$	$39.0\pm0.6^{b, B}$
Monoculture	C94	$18.8\pm2.5^{e, B}$	$25.8\pm1.1^{e, A}$
Co-culture	C94+KU-NF6	$21.4\pm3.4^{d, B}$	$29.7\pm1.9^{cd, A}$
	C94+KU-NF7	$22.5\pm1.2^{d, B}$	$31.1\pm0.4^{c, A}$
	C94+KU-NF24	$20.3\pm1.7^{d, B}$	$26.9\pm0.1^{e, A}$
	C94+S137	24.0 ± 3.1^{d}	28.1 ± 1.6^{de}

^{abcde}Values in the same column with different superscripts differ ($P<0.05$)

^{AB} Value in the same row with different superscripts differ ($P<0.05$)

Table 6. Short chain fatty acid concentration by monoculture of *Ruminococcus flavefaciens* OS14, *R. flavefaciens* C94 and their combination with *Butyrivibrio fibrisolvens* KU-NF6, *Pseudobutyrivibrio xylanivorans* KU-NF7, *B. fibrisolvens* KU-NF24 and *Selenomonas ruminantium* S137 after incubated at 38ºC for 72 h in basal media containing rice straw or cellulose powder.

Bacterial strain		SCFA (µmol/mL of culture)			
		C2	C3	C4	Total
Cellulose powder					
Monoculture	OS14	2.3	0.2	ND	2.5
Co-culture	OS14+ KU-NF6	3.4	0.4	2.7	7.1
	OS14+ KU-NF7	2.7	0.1	1.5	4.3
	OS14+ KU-NF24	0.1	ND	1.7	1.8
	OS14+S137	17.0	19.1	ND	36.1
Monoculture	C94	7.7	0.3	ND	8.0
Co-culture	C94+ KU-NF6	4.3	0.1	1.9	6.5
	C94+ KU-NF7	6.1	0.2	1.5	7.8
	C94+ KU-NF24	8.4	0.2	0.8	9.4
	C94+S137	9.1	6.3	ND	15.4
Rice straw					
Monoculture	OS14	1.4	0.1	ND	1.5
Co-culture	OS14+ KU-NF6	ND	ND	ND	ND
	OS14+ KU-NF7	4.8	0.7	4.5	10.1
	OS14+ KU-NF24	1.5	0.2	4.1	5.9
	OS14+S137	9.3	8.2	ND	17.5
Monoculture	C94	8.2	3.6	ND	11.8
Co-culture	C94+ KU-NF6	4.7	2.2	4.3	11.3
	C94+ KU-NF7	5.0	0.6	3.0	8.7
	C94+ KU-NF24	5.7	0.4	2.8	9.0
	C94+S137	7.3	5.1	ND	12.4

ND = Not detected

in propionate, produced from the conversion of D-lactate and succinate and could increase the metabolite activity of fibrolytic bacteria.

In conclusion, we isolated 6 strains of fibrolytic bacteria closely related to *R. flavefaciens*. Strain OS14 showed the highest fiber digestion alone or in combination with non-fibrolytic bacteria and higher than the type strain. In a combination of OS14 with *B. fibrisolvens* or *P. xylanivorans* could increase fiber digestion and the concentration of butyrate. Interestingly, OS14 with S137 showed the highest fiber digestion and notably increased of acetate and propionate. These results demonstrated the synergism between fibrolytic bacteria and non-fibrolytic bacteria. The cross-feeding of interspecies obviously occurred between *R. flavefaciens* OS14 and *S. ruminantium* S137 as improvement of fiber digestion and an increase of propionate concentration. The other *in vitro* fermentation products, i.e., lactate, succinate as well as their abundance in co-cultures needs to be quantified in further studies to understand the interaction of these bacteria on fiber digestion. OS14 and S137 might be a target for *in vivo* experiments to evaluate the efficiency of rumen fermentation enhancement.

ACKNOWLEDGEMENTS

This research was supported by the Kasetsart University Research and Development Institute (KURDI) and the Center for Advanced Studies for Agriculture and Food (CASAF), Kasetsart University Institute for Advanced Studies, Kasetsart University, Thailand.

The authors would like to express thanks to Professor Y. Kobayashi, Laboratory of Animal Function and Nutrition, Graduate School of Agriculture, Hokkaido University, Japan, for supporting of laboratory analysis of samples.

REFERENCES

Anderson, D.C. 1978. Use of cereal residues in beef cattle production systems. *J. Anim. Sci.*, **46**(3): 849-861.

BLAST program. National Center for Biotechnology Information, U.S. National Library of Medicine 8600 Rockville Pike, Bethesda MD, 20894 USA. Available on https://blast.ncbi.nlm.nih.gov/Blast.cgi

Boonsaen, P., M. Kinjo, S. Sawanon, Y. Suzuki, S. Koike and Y. Kobayashi. 2018. Partial characterization of phylogeny, ecology and function of the fibrolytic bacterium *Ruminococcus flavefaciens* OS14, newly isolated from the rumen of swamp buffalo. *Anim. Sci. J.*, **89**: 377-385.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*., **72**(1-2): 248-254.

Chanthakhoun, V., M. Wanapat, P. Kongmun and A. Cherdthong. 2012. Comparison of ruminal fermentation characteristics and microbial population in swamp buffalo and cattle. *Livest. Sci*., **143**: 172-176.

Collings, G.F. and M.T. Yokoyama. 1980. Gas-liquid chromatography for evaluating polysaccharide degradation by *Ruminococcus flavefaciens* C94 and *Bacteroides succinogenes* S85. *Appld Environ. Microbiol*., **39**(3): 566-571.

Dehority, B.A. 2003. *Rumen Microbiology.* Nottingham University Pres., Nottingham,

UK.

Flint, H.J. 1997. The rumen microbial ecosystem-some recent developments. *Trends Microbiol*., **5**(12): 483-488.

Fukuma, N., S. Koie and Y. Kobayashi. 2012. Involvement of recently cultured group U2 bacterium in ruminal fiber digestion revealed by co-culture with *Fibrobacter succinogenes* S85. *FEMS Microbiol. Lett*., **336**(1): 17-25.

Fukuma, N.M., S. Koie and Y. Kobayashi. 2015. Monitoring of gene expression in *Fibrobacter succinogenes* S85 under the co-culture with non-fibrolytic ruminal bacteria. *Arch. Microbiol*., **197**(2): 269-276.

Kobayashi, Y., N. Okuda, M. Matsumoto, K. Inoue, M. Wakia and S. Hoshino. 1998. Constitutive expression of a heterologous *Eubacterium ruminantium* xylanase gene (xynA) in *Butyrivibrio fibrisolvens*. *FEMS Microbiol. Lett*., **163**(1): 11-17.

Koike, S. and Y. Kobayashi. 2009. Fibrolytic rumen bacteria: their ecology and functions. *Asian Austral. J. Anim*., **22**: 131-138.

Koike, S., J. Pan, Y. Kobayasi and K. Tanaka. 2003. Kinetics of *in sacco* fiber-attachment of representative ruminal cellulolytic bacteria monitored by competitive PCR. *J. Dairy Sci*., **86**: 1429-1435.

Krause, D.O., S.E. Denman, R.I. Mackie, M. Morrison, A.L. Rae, G.T. Attwod and C.S. McSweeney. 2003. Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol. Lett*., **27**: 663-693.

Maneerat, W., S. Prasanpanich, S. Tumwasorn, V. Laudado and V. Tufarelli. 2015. Evaluating agro-industrial by-products as dietary roughage source on growth performance of fattening steers. *Saudi J. Biol. Sci*., **22**(5): 580-584.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem*., **31**(3): 426-428.

Nyonyo, T., T. Shinkai and M. Mitsumori. 2014. Improved culturability of cellulolytic rumen bacteria and phylogenetic diversity of culturable cellulolytic and xylanolytic bacteria newly isolated from the bovine rumen. *FEMS Microbiol. Ecol*., **88**: 528-537.

Ogimoto, K. and S. Imai. 1981. *Atlas of Rumen Microbiology*. Japan Scientific Societies Press, Tokyo, Japan.

Perrière, G. and M. Gouy. 1996. WWW-Query: An on-line retrieval system for biological sequence banks. *Biochimie*, **78**(5): 364-369.

Poonko, S., P. Boonsaen and S. Sawanon. 2015. Fibrolytic bacterium isolated from buffalo rumen phylogenetically closely related to Butyrivibrios and Pseudobutyrivibrios. *Kasetsart Journal (Natural Science)*, **49**: 547-559.

Puniya, A.K., R. Sinh and D.N. Kamra. 2015. *Rumen Microbiology: From Evolution to Revolution*. Springer, New Delhi, India.

R. Core Team. 2015. *R: A Llanguage and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Sawanon, S. and Y. Kobayashi. 2006. Synergistic fibrolysis in the rumen by cellulolytic *Ruminococcus flavefaciens* and non-cellulolytic *Selenomonas ruminantium*: Evidence in defined cultures. *Anim. Sci. J*., **77**(2): 208-214.

Sawanon, S., S. Intawiang, P. Boonsan and P. Phiriyangkul. 2017. Microbial diversity

and rumen ecology of swamp buffalo fed rice straw or fresh paragrass. *Journal of Agricultul.*, **33**(1): 109-120.

Sawanon, S., S. Koie and Y. Kobayashi. 2011. Evidence for the possible involvement of *Selenomonas ruminantium* in rumen fiber digestion. *FEMS Microbiol. Lett.*, **325**(2): 170-179.

Shinkai, T. and Y. Kobayashi. 2007. Localization of ruminal cellulolytic bacteria on plant fibrous materials as determined by fluorescence in situ hybridization and real-time PCR. *Appl. Environ. Microbiol.*, **73**: 1646-1652.

Shinkai, T., N. Matsumoto and Y. Kobayashi. 2007. Ecological characterization of three different phylogenetic groups belonging to the cellulolytic bacterial species *Fibrobacter succinogenes* in the rumen. *Anim. Sci. J.*, **78**: 503-511.

Shinkai, T., R. Ohji, N. Matsumoto and Y. Kobayashi. 2009. Fibrolytic capabilities of ruminal bacterium *Fibrobacter succinogenes* in relation to its phylogenetic grouping. *FEMS Microbiol. Lett*, **294**: 183-190.

van Gylswyk, N.O., H. Hippe and F.A. Rainey. 1996. *Pseudobutyrivibrio ruminis* gen. nov., sp. nov., a butyrate-producing bacterium from the rumen that closely resembles *Butyrivibrio fibrisolvens* in phenotype. *Int. J. Syst. Evol. Miol.*, **46**(2): 559-563.

Wanapat, M. and A. Cherdthong. 2009. Use of real-time PCR technique in studying rumen cellulolytic bacteria population as affected by level of roughage in swamp buffalo. *Curr. Microbiol.*, **58**: 294-299.

Wanapat, M., A. Ngarmsang, S. Korkhuntot, N. Nontaso, C. Wachirapakorn, G. Beakes and P. Rowlison. 2000. A comparative study on the rumen microbial population of cattle and swamp buffalo raised under traditional village conditions in the northeast of Thailand. *Asian Austral. J. Anim.*, **13**: 918-921.

Wanapat, M., P. Kongmun, V. Chanthakhoun and R. Pilajun. 2009. A comparative study of predominant cellulolytic bacteria of swamp buffalo and beef cattle using real-time PCR, p. 112-114. *In The Agriculture Annual Conference*, Khon Kaen University, Khon Kaen, Thailand.