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1 **Monitoring of gene expression in *Fibrobacter succinogenes* S85 under**
2 **the co-culture with non-fibrolytic ruminal bacteria**

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13 **Key words:** rumen bacteria, *Fibrobacter succinogenes*, fiber digestion, co-culture,
14 mRNA expression

Abstract

Fibrobacter succinogenes is one of the most pivotal fibrolytic bacterial species in the rumen. In a previous study, we confirmed enhancement of fiber digestion in a co-culture of *F. succinogenes* S85 with non-fibrolytic ruminal strains R-25 and/or *Selenomonas ruminantium* S137. In the present study, mRNA expression level of selected functional genes in the genome of *F. succinogenes* S85 was monitored by real-time RT-PCR. Growth profile of *F. succinogenes* S85 was similar in both the monoculture and co-cultures with non-fibrolytics. However, expression of 16S rRNA gene of *F. succinogenes* S85 in the co-culture was higher ($P < 0.01$) than that of the monoculture. This finding suggests that metabolic activity of *F. succinogenes* S85 was enhanced by coexistence with strains R-25 and/or *S. ruminantium* S137. The mRNA expression of fumarate reductase and glycoside hydrolase genes was up-regulated ($P < 0.01$) when *F. succinogenes* S85 was co-cultured with non-fibrolytics. These results indicate the enhancement of succinate production and fiber hydrolysis by *F. succinogenes* S85 in co-cultures of *S. ruminantium* and R-25 strains.

Introduction

Ruminant animals mostly depend on microbial fermentation within the rumen to acquire energy from plant fibrous materials. In the rumen microbial ecosystem, fibrolytic rumen bacteria such as *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* have been reported to be involved in ruminal fiber digestion (Flint, 1997; Krause et al., 2003). Several studies employing quantitative PCR

techniques targeting the 16S rRNA gene have revealed the predominance of *F. succinogenes* as compared to other ruminal fibrolytic species (Kobayashi et al., 2008; Mosoni et al., 2011; Lettat et al., 2012; Saro et al., 2012; Koike et al., 2014). Recent genomic studies reported that *F. succinogenes* possesses more abundant and diverse carbohydrate active enzymes, involved in polysaccharide degradation as compared to those of the other ruminococcal species (Berg Miller et al., 2009; Suen, et al., 2011a; 2011b). These findings reveal that *F. succinogenes* is the most pivotal fibrolytic bacterium in the rumen.

On the other hand, the fiber-associated bacterial community in the rumen also consists of a large number of other non-fibrolytic bacteria (Koike et al., 2003; Brulc et al., 2009) which probably play a role in ruminal fiber digestion. The mechanism of the contribution of non-fibrolytic bacteria in ruminal fiber digestion acts in an indirect manner, such as by hydrogen transfer or by cross-feeding of degradation and/or fermentation products derived from fiber (Flint, 1997). To investigate a relationship between fibrolytics and non-fibrolytics several *in vitro* co-culture studies using *F. succinogenes* and non-fibrolytic rumen bacterial species have been performed (Dehority and Scott, 1967; Kudo et al., 1987; Fondevila and Dehority, 1996). These studies revealed that fiber digestion was enhanced by coexistence of *F. succinogenes* with other non-fibrolytic strains. Based on the ecology of fiber-associated rumen bacteria, we had earlier demonstrated that non-fibrolytic group U2 and *Selenomonas ruminantium* can be a core member of the fibrolytic community in the rumen, as well as fibrolytic *F. succinogenes* (Koike et al., 2003; 2007; Koike and Kobayashi, 2009; Koike et al., 2010;

2014; Shinkai et al., 2014). Also positive interaction among fibrolytic *F. succinogenes* S85 and non-fibrolytic group U2 bacterium R-25 and/or *S. ruminantium* S137 was confirmed by *in vitro* co-culture studies, which revealed that rice straw digestibility and metabolite production were both enhanced (Sawanon et al., 2011; Fukuma et al., 2012).

Although earlier co-culture studies for activation of *F. succinogenes* S85 using conventional approaches such as measurement of fiber digestibility, bacterial growth, fermentation products and enzyme activity have been reported, no direct evidence with regards to an accurate molecular evaluation has been obtained yet. On the other hand, molecular approaches enable us to monitor expression of specific genes that exist in the genome of a bacterium. Béra-Maillet *et al.* (2009) have developed a RT-qPCR method to quantify mRNA expression of functional glycoside hydrolase (GH) genes of *F. succinogenes* S85 and have succeeded in specific monitoring of GH genes expression. Thus, we aimed to obtain the molecular evidence for activation of *F. succinogenes* S85 in the co-culture with non-fibrolytic strains by quantification of mRNA expression level of functional genes in the genome of this bacterium.

Materials and Methods

Bacterial strains, medium and incubation conditions

Fibrobacter succinogenes S85 was purchased from American Type Culture Collection. Rumen bacterium R-25 and *Selenomonas ruminantium* S137 were previously isolated by our research group (Koike *et al.*, 2010; Sawanon *et al.*, 2011). Monoculture, two-member co-culture and three-member co-culture experiments were

performed as previously reported (Fukuma et al., 2012). In brief, *Fibrobacter succinogenes* S85 as a fibrolytic rumen bacterium, and rumen bacterium R-25 and *Selenomonas ruminantium* S137 as non-fibrolytic rumen bacteria were used in this study. Basal medium was prepared anaerobically which composed of (per 100 ml): 7.5 ml of mineral solutions I and II (Bryant and Burkey, 1953), 0.1 ml of 0.1% resazurin, 40 ml of clarified rumen fluid, 39 ml of distilled water, 1 ml of 5% L-cysteine-HCl·H₂O and 5 ml of 8% Na₂CO₃.

Cells were subcultured three times consecutively with the basal media containing rice straw (1.0%; w/v) or cellobiose and glucose (0.5%; w/v of each) as carbon source(s) for *F. succinogenes* S85 or non-fibrolytics, respectively. The OD was adjusted (OD₆₆₀ = 0.2) for each bacterium. This was prepared using anaerobic dilution solution (Bryant and Burkey, 1953) and used as an inoculum. The inoculum was added at a dilution of 0.1 ml to 10 ml of the basal medium containing 0.1 g of rice straw as the sole carbon source, and tubes were incubated at 39°C under anaerobic conditions. Six replicates were used for all four sets: monoculture of *F. succinogenes* S85, two-member co-culture of *F. succinogenes* S85 and strain R-25 or *S. ruminantium* S137, and three-member co-culture, out of which three tubes were used for sampling after 24 h, and the remaining three tubes were used for sampling after 48 h of incubation.

Measurement of metabolites and reducing sugars

After 24 h or 48 h incubation, the cultures of *F. succinogenes* S85 monoculture and three-member co-culture were centrifuged (16,000 ×g, 4°C, 10 min) to obtain cell-free supernatant that was used for measurement of metabolites and reducing

sugars. Short chain fatty acids were determined by gas chromatography (GC-14B, Shimadzu, Kyoto, Japan). Succinate and D-/L-lactate were measured by commercial assay kits (Megazyme, Wicklow, Ireland). Oligosaccharides derived from rice straw digestion were estimated by measuring the concentration of reducing sugar, as described by Cotta (1988).

Nucleic acid isolation

Bacterial cells adhering to rice straw in the culture were collected after 24 h or 48 h incubation using the following procedure. Cultures were centrifuged ($377 \times g$, 4°C , 10 min) to precipitate the rice straw particles, and the supernatant containing planktonic bacterial cells was removed. The residue was washed with 10 ml of 0.1 M RNase-free potassium phosphate buffer and re-centrifuged ($377 \times g$, 4°C , 10 min). RNA protect Bacterial Reagent (2 ml) (Qiagen, Hilden, Germany) was added to the washed residue. The rice-straw samples were centrifuged ($377 \times g$, 4°C , 10 min) the supernatant was removed.

DNA and RNA were both co-extracted from 0.25 g of the collected rice-straw samples. Two ml stainless-steel tube (Bio medical science, Tokyo, Japan) containing the rice straw sample was flash-frozen in liquid nitrogen and the samples were ground with four pieces of stainless-steel ball ($\varnothing$ 3.2 mm; TOMY, Tokyo, Japan) for 1 min at a maximum speed using a Mini BeadBeater (BioSpec Products, Bartlesville, OK). The samples were further incubated with 100 μl of RNase-free TE buffer (pH 8.0) containing 3 mg/ml lysozyme (Thermo Fisher Scientific, Waltham, MA) for 5 min at room temperature. Crude nucleic acids containing DNA and RNA were treated with

RLT buffer (RNeasy Mini Kit, Qiagen, Hilden, Germany) and β -mercaptoethanol following the manufacturer's instruction. In order to purify DNA and RNA separately, the nucleic acids extract was divided to two aliquots of 300 μ l each. DNA was purified using the RBB+C method purification procedure (Yu and Morrison, 2004). RNA was purified using the RNeasy mini kit with the optional on-column DNase treatment step according to the manufacturer's instructions.

Concentration and purity of nucleic acids were evaluated by absorbance at A_{260} and measuring absorbance ratios at A_{260}/A_{280} and A_{260}/A_{230} using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA integrity was estimated by the band intensities of 23S and 16S rRNA on a 1% [wt/vol] agarose gel by electrophoresis.

Reverse transcription and real-time PCR

Total RNA (0.2 μ g) was reverse-transcribed into cDNA using random hexamer primers and 200 U of Superscript III Reverse Transcriptase (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer's instructions. A reverse transcriptase negative control was also included, and generated products were used in subsequent real-time PCRs.

The PCR primer sequences used in the present study are shown in Table 1. Genes encoding fumarate reductase (*frd*), cellulolytic enzymes (*cel5C*, *cel5G*, *endA_{FS}*, *cel9G* and *cel51A*) and hemicellulolytic enzymes (*xyn10D* and *xyn11C*) were selected as target genes. Primers for *frd* were newly designed. The genomic sequence of *F. succinogenes* S85 was obtained from GenBank (Accession number: CP001792). One of

the genes annotated as fumarate reductase was selected from the genome of *F. succinogenes* S85, and a primer set was designed using CLC genomics workbench software; version 5.0 (CLC Bio, Cambridge, MA). The copy number of 16S rRNA gene (16S rDNA) and its transcript (i.e., 16S rRNA) was quantified and used as the indices of cell number and metabolic activity of *F. succinogenes* S85, respectively. In order to monitor the growth profile of non-fibrolytic strains, 16S rDNA copy number of *S. ruminantium* S137 and strain R-25 was also quantified by using the specific primer sets for respective strains.

Preparation of standard template for real-time PCR was performed as described by Koike et al. (2007). The real-time PCR assay was conducted for the absolute quantification of mRNA copy with the standard curve method using a dilution series of standard template. In brief, each target gene was cloned using pGEM-T Easy Vector Systems (Promega, Madison, WI). The concentration of the plasmid was determined using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Copy number of each standard plasmid was calculated using the molecular weight of nucleic acid and the length (base pair) of the cloned standard plasmid. Ten-fold dilution series ranging from 10^{10} to 10^0 copies was prepared for each target.

Real-time PCR was performed with a KAPA SYBR FAST qPCR Kit (KAPA Biosystems, Woburn, MA) and a LightCycler 480 System (Roche Applied Science, Mannheim, Germany). Amplification conditions described by Koike et al. (2007) and Béra-Maillet et al. (2009) were used for quantification of 16S rRNA gene and GH genes,

respectively. The PCR condition for *frd* was optimized in this study. The melting curve of PCR products was monitored by heating 70°C to 95°C at 0.1°C intervals at the end of the real-time PCR to check for specific amplification. Specific amplification of the target gene was confirmed by the presence of a single peak in each melting curve.

Copy number of 16S rDNA and 16S rRNA were quantified, and a ratio of 16S rRNA/rDNA under each culture condition of *F. succinogenes* S85 was calculated. The cDNA copy number of target genes encoding fumarate reductase and GHs were normalized by copy number of 16S rDNA derived from the same culture. Extent of gene expression was expressed as the ratio of the copy number of each target gene per 10⁸ copies of 16S rDNA.

Statistical analysis

Data were expressed as means $\pm$ standard deviation. The means for each treatment were subjected to one-way analysis of variance and Tukey's test to detect differences between treatments using GraphPad Prism (ver. 5.0d, GraphPad Software, La Jolla, CA). $P < 0.01$ was regarded as statistically significant.

Results and discussion

Although previous studies (Dehority and Scott, 1967; Kudo et al., 1987; Fondevila and Dehority, 1996; Sawanon et al., 2011; Fukuma et al., 2012) have demonstrated the enhancement of fiber digestion in mixed cultures, there was no direct evidence for activation of fibrolytic bacteria under the co-existence of non-fibrolytics. The present study is the first report of increased fibrolytic and metabolic activity of *F.*

succinogenes S85 in co-cultures with non-fibrolytics at the molecular level. In the present study, we sampled at 24 h and 48 h after incubation for the monitoring of metabolic activity of *F. succinogenes* S85, because these time points corresponded to the initial phase and middle phase of rice straw digestion by this strain, respectively (Shinkai et al., 2009).

Growth profiles of *F. succinogenes* S85, strain R-25 and *S. ruminantium* S137 in monoculture or co-culture are shown in Fig. 1 and Table S1. Three strains showed similar growth profile both in monoculture and co-culture conditions. The growth profiles of *F. succinogenes* S85 and *S. ruminantium* S137 were similar, while the growth rate of strain R-25 was lower than the other two strains (Fig. 1). Changes in the 16S rRNA copy number and 16S rRNA/rDNA ratio of *F. succinogenes* S85 in its monoculture and co-culture with non-fibrolytics are shown in Table 2. When *F. succinogenes* S85 was incubated with non-fibrolytic bacteria, significant increase in 16S rRNA/rDNA ratio was observed. As the expression of rRNA gene is correlated with protein synthesis, the ratio of rRNA/rDNA has been considered as a useful index for metabolic activity per single cell (Muttray and Mohn, 1999; Muttray et al., 2001; Pérez-Osorio et al., 2010). In the present study, coexistence of strain R-25 did not affect the metabolic activity of *F. succinogenes* S85 after 24 h of incubation, on the other hand *S. ruminantium* S137 enhanced the metabolic activity of *F. succinogenes* S85 at an early stage (24 h after incubation) (Table 2). These differences could be attributed to the lower growth rate of strain R-25 compared to that of *S. ruminantium* S137 (Fig.1). After 48 h incubation, 16S rRNA/rDNA ratio in both co-cultures was significantly higher than

that of the monoculture, indicating significant positive effects of non-fibrolytic bacteria on metabolic activity of *F. succinogenes* S85.

The ratio of 16S rRNA/rDNA at 48 h was numerically lower as compared to the 24 h condition, both in monoculture and co-culture; with the exception of the three-member coculture. However, the degree of decline from 24 h to 48 h was less in the two-member co-culture compared to those of the monoculture. These findings indicate that the metabolic activity of *F. succinogenes* S85 is shown to decline with the incubation time, but co-existing non-fibrolytic bacteria may reduce the decline of metabolic activity of this strain. Furthermore, the increased value of 16S rRNA/rDNA in the three-member co-culture after 48 h (Table 2) suggests that co-existence of both of the strains R-25 and *S. ruminantium* S137 could enhance the metabolic activity of *F. succinogenes* S85 synergistically. Reduced activity of *F. succinogenes* S85 in the monoculture could be attributed to the accumulation of metabolites (hydrogen and succinate) and/or oligosaccharides (McGavin et al., 1990; Latham and Wolin, 1977; Williams et al., 1994; Rychlik and May, 2000). Strain R-25 utilizes oligosaccharides and produces lactate, a hydrogen sink, as the main fermentation product (Fukuma et al., 2012). On the other hand, *S. ruminantium* S137 consumes lactate, succinate and oligosaccharides as growth substrates (Sawanon et al., 2011; Fukuma et al., 2012). In the present study, concentrations of oligosaccharides and succinate were significantly lower in the three-member co-culture suggesting the consumption of these metabolites by strains R-25 and *S. ruminantium* S137 (Table 3). In addition, lactate from strain R-25 served as a growth substrate for *S. ruminantium* S137 and could be converted into

propionate (Table 3). Therefore, hydrogen transfer and crossfeeding of metabolites/oligosaccharides in the three strains may have enhanced the removal of suppression factors for *F. succinogenes* S85, leading to further activation of the strain. When *F. succinogenes* S85 was co-cultured with strain R-25, the mRNA expression of *frd* gene was up-regulated as compared to the monocultures (Table 4). This result corresponds well to an earlier study in which enhanced succinate production of *F. succinogenes* S85 was observed with co-existence of the strain R-25 (Fukuma et al., 2012). Also, up-regulation of *frd* expression in the three-member co-culture was found (Table 4), indicating enhancement of succinate production by *F. succinogenes* S85.

Majority of GH genes in the genome of *F. succinogenes* S85, have not been characterized with regards to its functional analysis for encoding proteins (Suen et al., 2011b). In order to confirm enhancement of fibrolytic activity of *F. succinogenes* S85 under mixed cultures, genes encoding glycoside hydrolases were targeted in this study. In the monoculture of *F. succinogenes* S85, expression level of GH genes at 48 h were lower than those at 24 h with the exception of *xyn11C* (Table 5). Catabolite repression of *F. succinogenes* S85 is well known to be associated with decline of endoglucanase activity related to hydrolytic products of polysaccharides, such as cellobiose (McGavin et al., 1990). Lower expression level of GH genes in *F. succinogenes* S85 monoculture may suggest declined expression of these genes by accumulation of fiber digestion-related products. Upon co-culturing *F. succinogenes* S85 with strains R-25 or *S. ruminantium* S137, six GH genes were found to be up-regulated compared with *F. succinogenes* S85 monoculture post 48 h of incubation (Table 5). This may be attributed

to consumption of fiber digestion-related products by the non-fibrolytics, resulting in reduction of catabolite repression of *F. succinogenes* S85. Expressions of genes of *frd* and GHs were similar between monoculture and co-culture at 24 h incubation; meanwhile most of these genes were up-regulated in co-culture after 48 h incubation (Tables 4 and 5). These findings suggest that metabolic activity of *F. succinogenes* S85 is enhanced by the two non-fibrolytics between 24 h and 48 h after incubation.

Among the GH genes quantified in the present study, *endA_{FS}* and *xynIIC* showed increased expression levels compared to other GH genes at 48 h of incubation (Table 5). Béra-Maillet *et al.* (2000b) monitored GH-genes expression of *F. succinogenes* S85 grown on a cellulose filter paper and concluded that these two genes could play a major role in fiber digestion of *F. succinogenes*. Our findings in the present study suggest that the enzymes encoded by *endA_{FS}* and *xynIIC* also play a key role in digestion of less digestible natural-fiber. The importance of these enzymes has also been characterized by other functional analysis. Enzyme encoded by *endA_{FS}* gene is known to have multi-functional activity and is able to hydrolyze cellulosic and other xylanolic polysaccharides, such as oat spelt xylan (Cavicchioli and Watson, 1991). Paradis *et al.* (1997) reported that enzymes encoded by *xynIIC* showed maximum increase in xylanolytic activity for birchwood xylan among other characterized xylanase genes of *F. succinogenes* S85. Therefore, up-regulation of *endA_{FS}* and *xynIIC* expression could reasonably be explained with the enhancement of rice straw digestion of *F. succinogenes* S85 in co-culture with non-fibrolytics.

In conclusion, the expression of 16S rRNA, *frd* and GH genes, are associated

and indicative of metabolic and fibrolytic activity of *F. succinogenes* S85, and these were up-regulated under co-cultures with non-fibrolytic bacteria R-25 and *S. ruminantium* S137. These results validate the enhancement of succinate production and fiber digestion by *F. succinogenes* S85 under the co-existence with non-fibrolytics at the molecular level.

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Table 1. List of targeted genes and specific PCR primer sets used in this study.

Target strain	Target genes* [Fisuc Locus**]	GH family	Function (Reference for protein characterization)		Sequence (5'-3')	Annealing temp. (°C)	Product size (bp)	Reference for primer set
<i>F. succinogenes</i> S85	16S rRNA	-	Ribosome RNA small subunit	Fw	GGTATGGGATGAGCTTGC	60	446	Tajima et al. 2001
				Rv	GCCTGCCCCCTGAACTATC			
	<i>frd</i> [Fisuc_2493]	-	Fumarate reductase	Fw	GTTTCCTTCAACCAGAACCTC	62	194	This study
				Rv	CTTGTAATCCCAAGCACCGA			
	<i>cel5C (cedA)</i> [Fisuc_1584]	5	Cellodextrinase (Huang and Forsberg, 1987)	Fw	GGGTCACGATTTCCACCTC	62	200	Béra-Maillet et al., 2009
				Rv	CCCAGAAGATTTTCGTCCTTG			
	<i>cel5G (cel3)</i> [Fisuc_2230]	5	Endo-glucanase (McGavin et al., 1989)	Fw	AGCGATGGTAAGGTCACCTG	62	240	Béra-Maillet et al., 2009
				Rv	GTGGATGGTGGCGTAGTCC			
	<i>endA_{FS}</i> [Fisuc_2362]	9	Endo-glucanase (Cavicchioli & Watson 1991)	Fw	GGTCCGAACCTGGATCTTGG	62	200	Béra-Maillet et al., 2009
				Rv	TCGCCAGTGTAGAGGTCGTA			
	<i>cel9G (endB)</i> [Fisuc_0057]	9	Endo-glucanase (Béra-Maillet et al., 2000)	Fw	TTACCAACGGAGCGGTGT	62	206	Béra-Maillet et al., 2009
				Rv	AGCCGAGCATCAAAGTCG			
	<i>cel51A (celF)</i> [Fisuc_3111]	51	Endo-glucanase (Malburg et al., 1997)	Fw	CAAGAACGGTGGCGAATC	62	186	Béra-Maillet et al., 2009
				Rv	CGGGTGTTGTCCAGTAGAG			
	<i>xyn10D</i> [Fisuc_1791]	10	Endo-xylanase (Jun et al., 2003)	Fw	GGCAAGAACGATGTGACCTT	62	200	Béra-Maillet et al., 2009
				Rv	TGTCCTTGCGGTAGTCACTG			
	<i>xyn11C</i> [Fisuc_0362]	11	Endo-xylanase (Paradis et al., 1993)	Fw	GCTGAAGTATTGCGGGAAGG	62	193	Béra-Maillet et al., 2009
				Rv	CTATGGCTGGACGGTGGAT			
Strain R-25	16S rRNA		Ribosome RNA small subunit	Fw	CTAGGTGTAGGGGGTATC	60	440	Koike et al., 2010
				Rv	GCTGCCCTCTGTCGTTG			
<i>S. ruminantium</i> S137	16S rRNA		Ribosome RNA small subunit	Fw	TGCTAATACCGAATGTTG	57	513	Tajima et al. 2001
				Rv	TCCTGCACTCAAGAAAGA			

* Former name of the gene was written in the parentheses.

** Locus tags refer to the ORF call in the genome sequence of *F. succinogenes* S85 in GenBank (accession no. CP001792).

Table 2. Changes in 16S rRNA copy numbers and 16S rRNA/rDNA ratio of *Fibrobacter succinogenes* S85 in monoculture and in co-cultures with non-fibrolytic strains.

Incubation time	16S rRNA Log copy number (g of rice straw) ⁻¹	16S rRNA/rDNA
24 h		
Monoculture of S85	12.18 ± 0.39	1098 ± 110 ^b
Coculture with R-25	12.10 ± 0.28	1018 ± 46 ^b
Coculture with S137	12.14 ± 0.15	1713 ± 194 ^a
Coculture with R-25 and S137	11.97 ± 0.21	1710 ± 45 ^a
<i>P</i> -value	0.7354	< 0.0001
48 h		
Monoculture of S85	11.46 ± 0.10	416 ± 68 ^d
Coculture with R-25	11.98 ± 0.48	986 ± 111 ^c
Coculture with S137	11.87 ± 0.03	1478 ± 139 ^b
Coculture with R-25 and S137	11.76 ± 0.14	2677 ± 300 ^a
<i>P</i> -value	0.0749	< 0.0001

Different letters represent significant difference within a column at a given time point ($P < 0.01$).

Table 3. Concentration of organic acids and reducing sugars in the culture of *Fibrobacter succinogenes* S85 monoculture and three-member coculture.

	$\mu\text{mol (ml of culture)}^{-1}$	
	24 h	48 h
Acetate		
Monoculture of S85	0.23 ± 0.10	2.25 ± 0.04^b
S85 + R-25 + S137	1.19 ± 0.25	3.02 ± 0.10^a
<i>P</i> -value	0.0365	0.0084
Propionate		
Monoculture of S85	nd	nd
S85 + R-25 + S137	1.61	2.94
<i>P</i> -value	-	-
D-Lacate		
Monoculture of S85	nd	nd
S85 + R-25 + S137	nd	0.10
<i>P</i> -value	-	-
Succinate		
Monoculture of S85	1.17 ± 0.23	4.87 ± 0.75^a
S85 + R-25 + S137	1.04 ± 0.25	0.42 ± 0.29^b
<i>P</i> -value	0.5762	< 0.0001
Reducing sugars		
Monoculture of S85	1.01 ± 0.02^a	4.89 ± 0.18^a
S85 + R-25 + S137	0.24 ± 0.12^b	0.38 ± 0.05^b
<i>P</i> -value	< 0.0001	< 0.0001

Different letters represent significant difference within an item at a given time point ($P < 0.01$).

nd, not detected.

Table 4. Expression of fumarate reductase (*frd*) of *Fibrobacter succinogenes* S85 in monoculture and co-cultures with non-fibrolytic strains.

	Incubation time	
	24 h	48 h
	Log copy number of transcripts (10 ⁸ copies of 16S rDNA) ⁻¹	
Monoculture of S85	7.30 ± 0.02	7.19 ± 0.03 ^c
Coculture with R-25	7.50 ± 0.19	7.47 ± 0.04 ^{ab}
Coculture with S137	7.61 ± 0.26	7.39 ± 0.09 ^{bc}
Coculture with R-25 and S137	7.40 ± 0.33	7.64 ± 0.18 ^a
<i>P</i> -value	0.3129	0.0004

Different letters represent significant difference within a column at a given time point ($P < 0.01$).

479 **Table 5.** Expression of glycoside hydrolase genes of *Fibrobacter succinogenes* S85 in monoculture and co-cultures with non-fibrolytic
480 strains.
481

Incubation time	Log copy number of transcripts (10^8 copies of 16S rDNA) ⁻¹						
	<i>cel5C</i>	<i>cel5G</i>	<i>endA_{FS}</i>	<i>cel9G</i>	<i>cel51A</i>	<i>xyn10D</i>	<i>xyn11C</i>
24 h							
Monoculture of S85	7.10 ± 0.06	7.10 ± 0.06 ^a	7.44 ± 0.06 ^a	6.91 ± 0.04	6.94 ± 0.07	7.14 ± 0.13	7.19 ± 0.12
Coculture with R-25	7.11 ± 0.10	7.00 ± 0.09 ^a	7.35 ± 0.04 ^{ab}	6.83 ± 0.05	7.15 ± 0.19	7.23 ± 0.17	7.22 ± 0.12
Coculture with S137	7.14 ± 0.14	6.80 ± 0.20 ^a	7.32 ± 0.03 ^{bc}	6.90 ± 0.18	7.04 ± 0.27	7.14 ± 0.29	7.13 ± 0.14
Coculture with R-25 and S137	7.07 ± 0.22	6.29 ± 0.33 ^b	7.25 ± 0.03 ^c	6.68 ± 0.25	6.99 ± 0.32	7.00 ± 0.35	6.98 ± 0.33
<i>P</i> -value	0.9053	0.0004	0.0003	0.1665	0.6400	0.6234	0.3573
48 h							
Monoculture of S85	6.79 ± 0.07 ^c	6.90 ± 0.06	7.25 ± 0.01 ^d	6.44 ± 0.16 ^c	6.74 ± 0.03 ^c	6.93 ± 0.02 ^c	7.23 ± 0.02 ^b
Coculture with R-25	7.05 ± 0.03 ^b	6.64 ± 0.05	7.43 ± 0.02 ^c	6.60 ± 0.04 ^{bc}	7.01 ± 0.07 ^{ab}	7.08 ± 0.04 ^b	7.48 ± 0.06 ^{ab}
Coculture with S137	7.05 ± 0.09 ^b	6.78 ± 0.02	7.62 ± 0.09 ^b	6.78 ± 0.07 ^{ab}	6.94 ± 0.13 ^b	7.04 ± 0.10 ^{bc}	7.51 ± 0.10 ^{ab}
Coculture with R-25 and S137	7.26 ± 0.11 ^a	6.91 ± 0.31	7.81 ± 0.03 ^a	6.90 ± 0.13 ^a	7.17 ± 0.13 ^a	7.29 ± 0.06 ^a	7.75 ± 0.25 ^a
<i>P</i> -value	< 0.0001	0.1062	< 0.0001	0.0004	0.0003	< 0.0001	0.0018

482

483 Different letters represent significant difference within a column at a given time point ($P < 0.01$).

Figure legend

Fig. 1 Growth profiles of *Fibrobacter succinogenes* S85 (a), strain R-25 (b) and *Selenomonas ruminantium* S137 (c) in *F. succinogenes* S85 monoculture (circle), co-culture with strain R-25 (diamond), co-culture with *S. ruminantium* S137 (triangle) and three-member co-culture (square)

Log 16S rDNA copy number (g of rice straw)⁻¹

Fig. 1

Table S1. Changes in 16S rDNA copy numbers of *Fibrobacter succinogenes* S85, strain R-25 and *Selenomonas ruminantium* S137 in monoculture of S85 and in co-cultures with non-fibrolytic strains.

Incubation time	Log copy number (g of rice straw) ⁻¹		
	S85	R-25	S137
24 h			
Monoculture of S85	9.14 ± 0.36	-	-
S85 + R-25	9.10 ± 0.27	7.96 ± 0.15	-
S85 + S137	8.90 ± 0.18	-	9.06 ± 0.01
S85 + R-25 + S137	8.73 ± 0.22	7.95 ± 0.08	8.80 ± 0.21
<i>P</i> -value	0.1814	0.9761	0.2064
48 h			
Monoculture of S85	8.85 ± 0.03	-	-
S85 + R-25	8.99 ± 0.44	8.51 ± 0.25	-
S85 + S137	8.73 ± 0.05	-	8.62 ± 0.54
S85 + R-25 + S137	8.47 ± 0.22	8.35 ± 0.27	8.57 ± 0.68
<i>P</i> -value	0.0154	0.6102	0.9197

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