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

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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·H_2O and 5 ml of 8% Na_2CO_3.

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 (OD_{660} = 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 ×*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 ×*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 ×*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 (ø 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 ß-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 µl 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 µg) 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

147 the genes annotated as fumarate reductase was selected from the genome of *F.*
148 *succinogenes* S85, and a primer set was designed using CLC genomics workbench
149 software; version 5.0 (CLC Bio, Cambridge, MA). The copy number of 16S rRNA gene
150 (16S rDNA) and its transcript (i.e., 16S rRNA) was quantified and used as the indices of
151 cell number and metabolic activity of *F. succinogenes* S85, respectively. In order to
152 monitor the growth profile of non-fibrolytic strains, 16S rDNA copy number of *S.*
153 *ruminantium* S137 and strain R-25 was also quantified by using the specific primer sets
154 for respective strains.

155 Preparation of standard template for real-time PCR was performed as
156 described by Koike et al. (2007). The real-time PCR assay was conducted for the
157 absolute quantification of mRNA copy with the standard curve method using a dilution
158 series of standard template. In brief, each target gene was cloned using pGEM-T Easy
159 Vector Systems (Promega, Madison, WI). The concentration of the plasmid was
160 determined using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific,
161 Waltham, MA). Copy number of each standard plasmid was calculated using the
162 molecular weight of nucleic acid and the length (base pair) of the cloned standard
163 plasmid. Ten-fold dilution series ranging from 10 to 10^9 copies was prepared for each
164 target.

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

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

173 Copy number of 16S rDNA and 16S rRNA were quantified, and a ratio of
174 16S rRNA/rDNA under each culture condition of *F. succinogenes* S85 was calculated.
175 The cDNA copy number of target genes encoding fumarate reductase and GHs were
176 normalized by copy number of 16S rDNA derived from the same culture. Extent of
177 gene expression was expressed as the ratio of the copy number of each target gene per
178 10^8 copies of 16S rDNA.

179 **Statistical analysis**

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

184

185 ## Results and discussion

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

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

196 Growth profiles of *F. succinogenes* S85, strain R-25 and *S. ruminantium* S137
197 in monoculture or co-culture are shown in Fig. 1 and Table S1. Three strains showed
198 similar growth profile both in monoculture and co-culture conditions. The growth
199 profiles of *F. succinogenes* S85 and *S. ruminantium* S137 were similar, while the growth
200 rate of strain R-25 was lower than the other two strains (Fig. 1). Changes in the 16S
201 rRNA copy number and 16S rRNA/rDNA ratio of *F. succinogenes* S85 in its
202 monoculture and co-culture with non-fibrolytics are shown in Table 2. When *F.*
203 *succinogenes* S85 was incubated with non-fibrolytic bacteria, significant increase in 16S
204 rRNA/rDNA ratio was observed. As the expression of rRNA gene is correlated with
205 protein synthesis, the ratio of rRNA/rDNA has been considered as a useful index for
206 metabolic activity per single cell (Muttray and Mohn, 1999; Muttray et al., 2001;
207 Pérez-Osorio et al., 2010). In the present study, coexistence of strain R-25 did not affect
208 the metabolic activity of *F. succinogenes* S85 after 24 h of incubation, on the other hand
209 *S. ruminantium* S137 enhanced the metabolic activity of *F. succinogenes* S85 at an early
210 stage (24 h after incubation) (Table 2). These differences could be attributed to the
211 lower growth rate of strain R-25 compared to that of *S. ruminantium* S137 (Fig.1). After
212 48 h incubation, 16S rRNA/rDNA ratio in both co-cultures was significantly higher than

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

215 The ratio of 16S rRNA/rDNA at 48 h was numerically lower as compared to
216 the 24 h condition, both in monoculture and co-culture; with the exception of the
217 three-member coculture. However, the degree of decline from 24 h to 48 h was less in
218 the two-member co-culture compared to those of the monoculture. These findings
219 indicate that the metabolic activity of *F. succinogenes* S85 is shown to decline with the
220 incubation time, but co-existing non-fibrolytic bacteria may reduce the decline of
221 metabolic activity of this strain. Furthermore, the increased value of 16S rRNA/rDNA
222 in the three-member co-culture after 48 h (Table 2) suggests that co-existence of both of
223 the strains R-25 and *S. ruminantium* S137 could enhance the metabolic activity of *F.*
224 *succinogenes* S85 synergistically. Reduced activity of *F. succinogenes* S85 in the
225 monoculture could be attributed to the accumulation of metabolites (hydrogen and
226 succinate) and/or oligosaccharides (McGavin et al., 1990; Latham and Wolin, 1977;
227 Williams et al., 1994; Rychlik and May, 2000). Strain R-25 utilizes oligosaccharides
228 and produces lactate, a hydrogen sink, as the main fermentation product (Fukuma et al.,
229 2012). On the other hand, *S. ruminantium* S137 consumes lactate, succinate and
230 oligosaccharides as growth substrates (Sawanon et al., 2011; Fukuma et al., 2012). In
231 the present study, concentrations of oligosaccharides and succinate were significantly
232 lower in the three-member co-culture suggesting the consumption of these metabolites
233 by strains R-25 and *S. ruminantium* S137 (Table 3). In addition, lactate from strain R-25
234 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 *xyn11C* 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 *xyn11C* 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 xylanosic polysaccharides, such as oat spelt xylan (Cavicchioli and Watson, 1991). Paradis *et al.* (1997) reported that enzymes encoded by *xyn11C* 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 *xyn11C* 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
F. succinogenes S85	16S rRNA	-	Ribosome RNA small subunit	Fw	GGTATGGGATGAGCTTGC	60	446	Tajima et al. 2001
				Rv	GCCTGCCCCTGAACTATC			
	frd	-	Fumarate reductase	Fw	GTTCCTTCAACCAGAACCTC	62	194	This study
	[Fisuc_2493]			Rv	CTTGTATTCCCAAGCACCGA			
	cel5C (cedA)	5	Cellodextrinase	Fw	GGGTCACGATTTCCACCTC	62	200	Béra-Maillet et al., 2009
	[Fisuc_1584]		(Huang and Forsberg, 1987)	Rv	CCCAGAAGATTTCGTCCTTG			
	cel5G (cel3)	5	Endo-glucanase	Fw	AGCGATGGTAAGGTCACTGC	62	240	Béra-Maillet et al., 2009
	[Fisuc_2230]		(McGavin et al., 1989)	Rv	GTGGATGGTGGCGTAGTCC			
	$endA_{FS}$	9	Endo-glucanase	Fw	GGTCCGAACTGGATCTTGG	62	200	Béra-Maillet et al., 2009
	[Fisuc_2362]		(Cavicchioli & Watson 1991)	Rv	TCGCCAGTGTAGAGGTCGTA			
	cel9G (endB)	9	Endo-glucanase	Fw	TTACCAACGGAGCGGTGT	62	206	Béra-Maillet et al., 2009
	[Fisuc_0057]		(Béra-Maillet et al., 2000)	Rv	AGCCGAGCATCAAAGTCG			
	cel51A (celF)	51	Endo-glucanase	Fw	CAAGAACGGTGGCGAATC	62	186	Béra-Maillet et al., 2009
	[Fisuc_3111]		(Malburg et al., 1997)	Rv	CGGGTGTTGTCCCAGTAGAG			
	xyn10D	10	Endo-xylanase	Fw	GGCAAGAACGATGTGACCTT	62	200	Béra-Maillet et al., 2009
	[Fisuc_1791]		(Jun et al., 2003)	Rv	TGTCCTTGCGGTAGTCACTG			
	xyn11C	11	Endo-xylanase	Fw	GCTGAAGTATTGCGGGAAGG	62	193	Béra-Maillet et al., 2009
	[Fisuc_0362]		(Paradis et al., 1993)	Rv	CTATGGCTGGACGGTGGAT			
Strain R-25	16S rRNA		Ribosome RNA small subunit	Fw	CTAGGTGTAGGGGGTATC	60	440	Koike et al., 2010
				Rv	GCTGCCCTCTGTCGTTG			
S. ruminantium 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}
P-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}
P-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.

	μmol (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}
P-value	0.0365	0.0084
Propionate		
Monoculture of S85	nd	nd
S85 + R-25 + S137	1.61	2.94
P-value	-	-
D-Lacate		
Monoculture of S85	nd	nd
S85 + R-25 + S137	nd	0.10
P-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}
P-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}
P-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}
P-value	0.3129	0.0004

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

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

Incubation time	Log copy number of transcripts (10^8 copies of 16S rDNA)$^{-1}$						
	cel5C	*cel5G*	*endA*$_{FS}$	*cel9G*	*cel51A*	*xyn10D*	*xyn11C*
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
P-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}
P-value	< 0.0001	0.1062	< 0.0001	0.0004	0.0003	< 0.0001	0.0018

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)

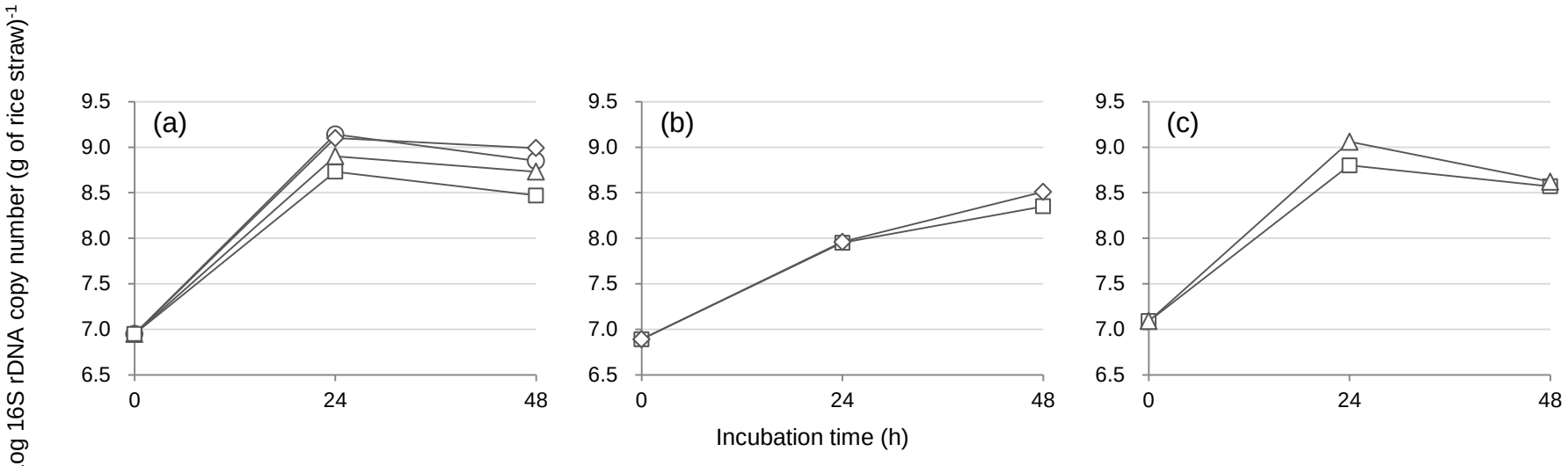
Log 16S rDNA copy number (g of rice straw)-1
(a)
(b)
(c)
9.5
9.0
8.5
8.0
7.5
7.0
6.5
0
24
48
Incubation time (h)

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
P-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
P-value	0.0154	0.6102	0.9197

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