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Enhancing Butyrate Production, Ruminal Fermentation and Microbial Population through Supplementation with *Clostridium saccharobutylicum*

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Butyrate is known to play a significant role in energy metabolism and regulating genomic activities that influence rumen nutrition utilization and function. Thus, this study investigated the effects of an isolated butyrate-producing bacteria, *Clostridium saccharobutylicum*, in rumen butyrate production, fermentation parameters and microbial population in Holstein-Friesian cow. An isolated butyrate-producing bacterium from the ruminal fluid of a Holstein-Friesian cow was identified and characterized as *Clostridium saccharobutylicum* RNAL841125 using 16S rRNA gene sequencing and phylogenetic analyses. The bacterium was evaluated on its effects as supplement on in vitro rumen fermentation and microbial population. Supplementation with 10^6 CFU/ml *Clostridium saccharobutylicum* increased ($p < 0.05$) microbial crude protein, butyrate and total volatile fatty acids concentration but had no significant effect on NH_3-N at 24 h incubation. Butyrate and total VFA concentrations were higher ($p < 0.05$) in supplementation with 10^6 CFU/ml *Clostridium saccharobutylicum* compared with control, with no differences observed for total gas production, NH_3-N and propionate concentration. However, as the inclusion rate (CFU/ml) of *C. saccharobutylicum* was increased, reduction of rumen fermentation values was observed. Furthermore, butyrate-producing bacteria and *Fibrobacter succinogenes* population in the rumen increased in response with supplementation of *C. saccharobutylicum*, while no differences in the population in total bacteria, protozoa and fungi were observed among treatments. Overall, our study suggests that supplementation with 10^6 CFU/ml *C. saccharobutylicum* has the potential to improve ruminal fermentation through increased concentrations of butyrate and total volatile fatty acid, and enhanced population of butyrate-producing bacteria and cellulolytic bacteria *F. succinogenes*.

Keywords: Butyric acid, *Clostridium saccharobutylicum*, Holstein cow, rumen fermentation, microbial population

Introduction

The rumen consists of a dense and diverse microbial ecosystem [1] and has the ability to convert fibrous plant material and non-protein nitrogen into important products, such as short-chain fatty acids and microbial protein [2] . In ruminants, rumen fermentation plays an important role in feed digestion and microbial production. In the last decades, attempts have been made to improve rumen productivity through manipulation of the rumen environment. Modulation of the rumen environment can enhance digestibility and nutrient utilization in the animals. One approach that is widely used is the application of natural products such as microbial additives. Microbial additives are found to be

beneficial as they promote digestion and intestinal hygiene [3], enhance animal performance and reduce the usage of antibiotics [4–6]. Microbial additives have been shown to improve ruminant animal performance in terms of live weight gain and milk production by 7–8% [7]. Thus, microbial additives have been used for many years to supplement the diets of farm animals and humans [8], and as an inoculant to improve feed quality.

Butyric acid is a short-chain fatty acid produced by anaerobic fermentation of dietary substrates in the rumen and large intestine [9]. Butyrate serves as a major energy source for epithelial cells in ruminants [10] and is significant in maintaining colonic health in both humans and animals [11]. Most importantly, butyrate stimulates epithelial cell proliferation leading to improved feed utilization by the animal. Moreover, butyrate also possesses other important functions in the intestinal epithelium, such as prevention of certain types of colitis [12]. Sodium butyrate supplementation has been reported to improve growth performance in calves [13]. Furthermore, several studies have shown that butyrate affects several other parameters, such as the mucosal barrier, feed passage, microbiota, immune system, and pathogens [14], and provides beneficial effects in improving health and performance of the host [15].

Due to the increasing demand of consumers for naturally made products, the production of butyric acid by microbial fermentation has attracted much attention. Several anaerobic bacteria can produce butyric acid as a major fermentation product by utilizing various substrates. Butyrate-producing bacteria can be found in environment- and host-associated sites, including the rumen, mouth and large intestine [16]. Butyrate-producing bacteria are associated with gastrointestinal health in humans and various animal species [17] and play an important role in the degradation of proteins, nucleic acids and structural and storage plant polysaccharides [18]. Due to its multiple beneficial effects on the host, butyrogenic bacteria are considered to have a potential use as probiotics. Thus, we aimed to enhance butyrate production by supplementing *Clostridium saccharobutylicum* in the rumen in vitro and to evaluate its effect on rumen fermentation parameters and microbial population in Holstein-Friesian cow.

Materials and Methods

Isolation, Characterization, and Molecular Identification of Butyrate-Producing Bacteria

Butyrate-producing bacteria were isolated from the rumen contents of a 48-month-old, rumen-cannulated Holstein-Friesian cow (600 ± 47 kg). The animal was fed twice daily with concentrate feed (NongHyup Co., Korea) and rice straw at a 2:8 ratio. Ruminal fluid was collected before feeding and obtained by straining the rumen contents through four layers of surgical gauze and pooled in an amber bottle with an oxygen-free headspace immediately after collection. The collected rumen fluid was sealed, maintained at 39°C, and immediately transported to the laboratory for bacterial isolation. The management of animals was approved by the Sunchon National University Committee on Animal Care (2016).

Media Preparation and Isolation of Bacteria from Rumen

One milliliter of rumen sample was placed in sterile tubes and homogenized in 9 ml of anaerobic medium containing soluble starch, glucose and cellobiose as energy sources (M2GSC) [19] pH 6 containing:10.0 g/l of casitone, 2.5 g/l of yeast extract, 4.0 g/l $NaHCO_3$, 2.0 g/l glucose, 2.0 g/l soluble starch, 2.0 g/l cellobiose, 300 ml of clarified rumen fluid, 1.0 g/l of cysteine HCl, 150 ml of Mineral Solution I (3.0 g/l of K_2HPO_4), 150 ml of Mineral Solution II (3.0 g/l KH_2PO_4, 6.0 g/l $(NH_4)_2SO_4$, 6.0 g/l NaCl, 0.6 g/l $MgSO_4 \cdot 7H_2O$, 0.6 g/l $CaCl_2$), and 1.0 g of resazurin (1% w/v). The medium was aseptically added in the bottle and flushed with CO_2 according to the anaerobic Hungate method [20]. This diluent corresponded to the first 10-fold dilution, which was then mixed by vortexing to form homogenized suspension. From this suspension, it was subsequently diluted by 10-fold serial dilutions through to a 10^{-9} dilution.

Anaerobic M2GSC medium containing 0.75% agar was prepared in 16 × 125 mm Hungate tubes sealed with butyl septum stoppers and inoculated with 0.5 ml aliquots of appropriate serial dilutions. Roll tubes were incubated at 37°C for 24 to 48 h prior to the picking of colonies from each sample. Picked colonies were subsequently inoculated in the same medium until purified. Purified cultures were grown in broths of M2GSC at 37°C for 24 to 48 h and used for the determination of fermentation products by high-performance liquid chromatography (HPLC) and DNA extraction for molecular identification of the isolates.

Analysis of Butyric Acid Concentration

The butyric acid produced by the bacterial isolates was compared with *C. butyricum* as positive control and analyzed using HPLC. Short-chain fatty acid concentrations were analyzed using an Agilent 1200 Series HPLC System (Agilent Technologies, USA) with a UV detector set at 210 and 220 nm. Samples were eluted isocratically with 0.0085 N H_2SO_4 at a flow rate of 0.6 ml/min and a column temperature of 35°C.

16S rRNA Sequence and Phylogenetic Analysis

Isolates producing high butyric acid concentrations were identified by sequencing the 16S ribosomal RNA (16S rRNA) gene using the 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) primers [21]. The gene sequences obtained from the isolates were compared with the 16S

rRNA sequences available in GenBank using the Basic Local Alignment Search Tool (BLAST) [22] and EzBioCloud database [23]. Multiple gene sequences were aligned using ClustalW [24] within the Molecular Evolutionary Genetics Analysis (MEGA) version 6 [25] to determine the approximate phylogenetic affiliations. The phylogenetic tree was constructed using the neighbor-joining method [26] with pair-wise comparison and with evolutionary distances computed using the Kimura 2-parameter method [27]. Reliability of the tree topology was assessed with the bootstrap method using 1000 replications [28]. Only bootstrap values greater than 50% are shown on the internal nodes.

Cultivation of *C. saccharobutylicum* RNAL841125

Clostridium saccharobutylicum RNAL841125 was deposited in the Korean Culture Center of Microorganisms (KCCM). The bacterial colonies of the *C. saccharobutylicum* RNAL841125 were maintained in clostridial growth medium composed of: 2.0 g/l K_2HPO_4, 0.75 g/l KH_2PO_4, 1.5 g/l $MgSO_4 \cdot 7H_2O$, 0.017 g/l $MnSO_4 \cdot 5H_2O$, 0.01 g/l $FeSO_4 \cdot 7H_2O$, 2.0 g/l $(NH_4)_2SO_4$, 5.0 g/l NaCl, 2.0 g/l asparagine, 0.004 g/l p-aminobenzoic acid, 15.0 g/l yeast extract, and 50.0 g/l glucose. The pH of the medium was adjusted to 6.8. The headspace of the bottle was purged with N_2 gas. The medium was dispensed anaerobically under an O_2-free N_2 atmosphere and autoclaved at 121°C for 15 min. Bacterial cultures were grown anaerobically and incubated at 37°C for 24–48 h. Bacterial growth was monitored by optical density (OD) using a spectrophotometer at 600 nm.

Substrate Utilization and Enzyme Activities

Substrate utilization of RNAL841125 isolate was tested using an API 50 CH test kit (Biomérieux, France). Analyses for enzyme activities were carried out for CMCase, FPase xylanase, pectin methyl esterase, polygalactouranase and α-amylase using carboxymethyl cellulose (CMC), filter paper, pectin, xylan and starch as substrate, respectively. The enzyme activities were determined by estimating the amount of reducing sugar liberated from the enzymatic reaction from respective substrate dissolved in appropriate buffer by dinitrosalycilic acid (DNS) [29]. One unit of enzyme was defined as the amount of enzyme that released 1 μmol of glucose per min.

Rumen Fluid Collection and In Vitro Fermentation

All animal care procedures were conducted in accordance with the guidelines approved by the Sunchon National University Committee on Animal Care (SCNU IACUC-2018-01). Three rumen-cannulated Holstein cows weighing 600 ± 47 kg were fed twice daily with total mixed ration (Table 1) and Italian ryegrass (7:3 ratio). Ruminal fluid was collected before feeding and obtained by straining the rumen contents through four layers of surgical gauze and pooled in an amber bottle with an oxygen-free headspace immediately after collection. The collected rumen fluid was sealed, maintained at 39°C, and immediately transported in the laboratory.

Table 1. Feed composition used in the study.

Composition	Percentage
Soybean meal	9.27
Lupin seed	11.07
Dried distillers' grains	5.92
Rice bran	5.15
Corn	36.94
Mushroom media	18.40
Protein	11.07
Salt	0.39
Limestone	0.77
Vitamin	1.03

The buffer medium was prepared following the method described by Asanuma *et al*. [30]. The buffer was autoclaved at 121°C for 15 min, maintained in a 39°C water bath, and flushed with CO_2 gas, and the pH was adjusted to 6.9 using 10 N NaOH. The experiment was conducted under a constant flow of CO_2 gas on the rumen-buffered medium to ensure anaerobic conditions. The particle-free rumen fluid and buffer medium were mixed at a ratio of 1:3 (v/v). After mixing, 100 ml of the mixed buffered rumen fluid was anaerobically transferred into the serum bottles containing 1.0 g dry matter substrate of total mixed ration and Italian ryegrass (70:30 ratio at DM basis) and treatments were anaerobically inoculated into the serum battles under a constant flow of CO_2 gas. Treatments consisted of without inoculant (Control), supplementation with 10^6 CFU/ml, 10^7 CFU/ml, or 10^8 CFU/ml of *C. saccharobutylicum* (10^6Cs, 10^7Cs, and 10^8Cs, respectively), and supplementation with 50 mM of butyric acid (BA) (Sigma, USA). The serum bottles were capped with a butyl rubber stopper, sealed with an aluminum cap, and incubated at 39°C in a shaking incubator set at 120 rpm. Three replicates were performed for all treatments and incubation times.

Analysis of In Vitro Rumen Fermentation Parameters

Rumen fermentation characteristics, including total gas production, pH, ammonia-nitrogen (NH_3-N), microbial crude protein (MCP), and volatile fatty acid (VFA) concentrations were examined at the end of each incubation period. Two one-milliliter samples of rumen fluid from each serum bottle were collected in microcentrifuge tubes and maintained at −80°C until further analysis of NH_3-N, VFA concentrations and microbial population.

Total gas production was measured from each serum bottle after incubation using a pressure sensor (Laurel Electronics, Inc., USA). The pH values of the rumen samples were measured immediately after opening each serum bottle using a digital pH meter (Mettler Toledo, Switzerland). For NH_3-N and VFA analyses, the ruminal fluid samples were centrifuged at 14,000 rpm for 15 min at 4°C and the supernatant was then used for the analysis. Rumen NH_3-N levels were determined according to a colorimetric

method developed by Chaney and Marbach [31] using a Libra S22 spectrophotometer (Biochrom Ltd., England) set at 630 nm. MCP was estimated following the method used by Castillo-Lopez *et al*. [32]. VFA concentrations were analyzed using an Agilent 1200 Series HPLC System (Agilent Technologies) with a UV detector set at 210 and 220 nm. Samples were eluted isocratically with 0.0085N H_2SO_4 at a flow rate of 0.6 ml/min and a column temperature of 35°C.

DNA Extraction

Microcentrifuge tubes containing ruminal fluid samples were centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was then discarded and the isolated pellets were used for the extraction of total microbial genomic DNA using a FastDNA SPIN Kit for Soil (MP Biomedicals, LLC, USA) following the manufacturer's protocol. The DNA concentration and quality were measured using an Optizen NanoQ spectrophotometer (Optizen, Korea). The DNA samples were stored at −20°C until analysis.

Quantitative Real-Time PCR Analyses

The population sizes of total bacteria, protozoa, general fungi and select bacterial species were quantified using SYBR Green-based quantitative real-time PCR (qPCR) using the Eco Real-Time PCR (Illumina, USA). The primers used for each microbial group are shown in Table 2. In addition, the butyrate kinase (*buk*) gene, which is involved in the production of butyrate, was also quantified. The reaction mixture was prepared in a total volume of 20 µl containing 10 µl of 2x QuantiSpeed SYBR No-Rox mix (PhileKorea, Korea), 0.8 µl of each 10 µM primer, and 8.4 µl template DNA at a concentration of 50 ng/µl in sterile distilled water. The qPCR reactions were performed under thermal cycle conditions of one cycle at 50°C for 2 min, and 95°C for 2 min, followed by 40 cycles at 95°C for 15 sec, 60°C for 1 min and 72°C for 30 sec. For all PCR runs, standards, negative controls (no DNA), and samples were run in triplicate.

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) using the general linear model (GLM) for a completely randomized design. All treatments were conducted in triplicate and Duncan's multiple range test was used to identify differences between specific treatments. For the in vitro rumen fermentation, the linear effects of *C. saccharobutylicum* supplementation were analyzed using orthogonal polynomial coefficients to describe the functional relationships among the control and treatment groups. Differences with p values less than 0.05 were considered statistically significant. Data analysis was carried out using Statistical Analysis Systems (SAS) version 9.3 (SAS Institute Inc., USA).

Table 2. Primers used for real-time PCR assay.

Target	Name	Sequence (5′ to 3′)
A. Microbial quantification		
General bacteria [a]	1114-F	CGGCAACGAGCGCAACCC
	1275-R	CCATTGTAGCACGTGTGTAGCC
Protozoa [b]	316-F	GCTTTCGWTGGTAGTGTATT
	539-R	CTTGCCCTCYAATCGTWCT
General anaerobic fungi [a]	GAF-F	GAGGAAGTAAAAGTCGTAACAAGGTTTC
	GAF-R	CAAATTCACAAAGGGTAGGATGATT
F. succinogenes [a]	586-F	GTTCGGAATTACTGGGCGTAAA
	706-R	CGCTGCCCCCTGAACTATC
B. Microbial crude protein (MCP)		
Bacterial crude protein (BCP) [c]	BAC338-F	ACTCCTACGGGAGGCAGCAG
	Probe	FAM/TGCCAGCAGCCGCGGTAATAC/TAMRA
	BAC805-R	GACTACCAGGGTATCTAATCC
Protozoal crude protein (PCP) [d]	F	GCTTTCGATGGTAGTGTATT
	Probe	FAM/CGGAAGGCAGCAGGCGC/TAMRA
	R	ACTTGCCCTCTAATCGTACT
C. Butyrate-producing bacteria		
butyrate kinase (*buk*) gene [e]	G_buk_F	TGCTGTWGTTGGWAGAGGYGGA
	G_buk_R	GCAACIGCYTTTTGATTTAATGCATGG

[a]Denman and McSweeney [70]; [b]Sylvester *et al*. [71]; [c]Yu *et al*. [72]; [d]Sylvester *et al*. [73]; [e]Vital *et al*. [16].

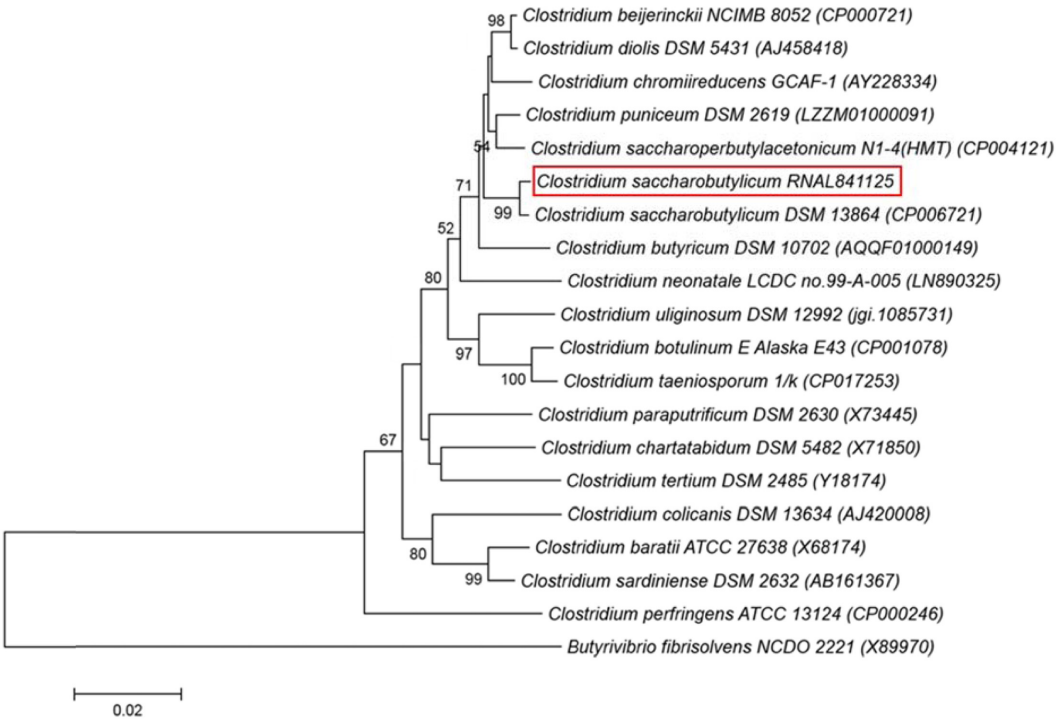

Fig. 1. Phylogenetic tree based on comparison of 16S rRNA gene sequences, indicating the taxonomic position of Clostridium saccharobutylicum RNAL841125.

The phylogenetic tree was constructed using the neighbour-joining method. Bootstrap values, expressed as 1000 replicates, are given at branching points. Only bootstrap values >50% are shown on the internal nodes. *Butyrivibrio fibrisolvens* was used as an outgroup and the bar represents 0.02 substitutions per nucleotide.

Results

Identification and Characterization of Butyrate-Producing Bacteria

A potential butyrate-producing bacteria was isolated from the rumen. The 16S rRNA gene sequencing and phylogenetic analyses demonstrated that this isolate was 99% similar to *C. saccharobutylicum* DSM 13864^T [33]. The isolate was deposited in the Korean Culture Center of Microorganisms (KCCM) as *C. saccharobutylicum* RNAL841125 (Fig. 1) with NCBI GenBank accession number MH032748. Only bootstrap values >50% were shown on the internal nodes, and *Butyrivibrio fibrisolvens* was used as an outgroup and the bar represents 0.02 substitutions per nucleotide position.

The butyrate production level of *C. saccharobutylicum* RNAL841125 was compared with the standard butyrate-producing bacteria, *C. butyricum*. Significantly higher levels ($p < 0.05$) of propionate and butyrate were produced by *C. saccharobutylicum* RNAL841125 (20.46 and 42.39 mmol/l, respectively) than *C. butyricum* (18.79 and 19.11 mmol/l, respectively) (data not shown). The substrate utilization test using the API 50 CH identification system revealed that RNAL841125 isolate could utilize 28 types of sugars as substrates. The *C. saccharobutylicum* profile revealed that the isolate metabolized D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, glucose, fructose, mannose, inozitol, α-methyl-D-mannoside, α-methyl-D-glucoside, amygdalin, salicin, maltose, lactose, melibiose, sucrose, trehalose, raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-fucose, L-fucose, D-arabitol and gluconate. Additionally, analysis of the growth of *C. saccharobutylicum* RNAL841125 on CMC, filter paper (Whatman filter paper No. 1), xylan, pectin, and starch as substrates revealed that the isolate had weak fermentation on CMC and filter paper and, no fermentation was seen in pectin (data not shown). The enzyme activities of the bacteria using CMC, filter paper (Whatman filter paper No. 1), xylan, pectin and starch are shown in Fig. 2. The results indicated that the use of CMC and filter paper as substrates produced low amounts of enzyme in comparison with xylan and starch.

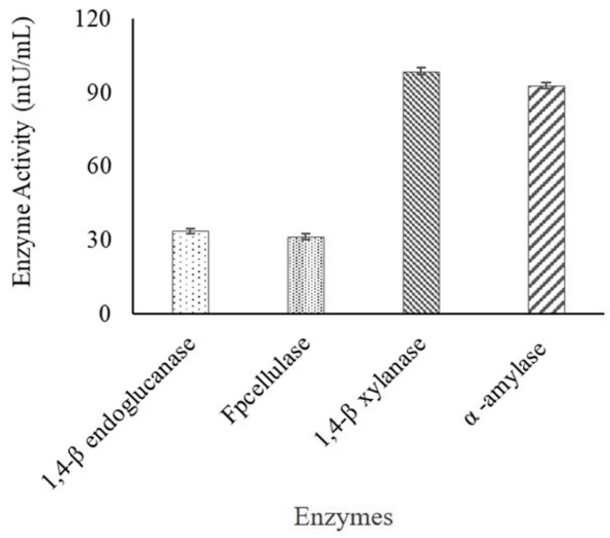

Fig. 2. Enzyme activity of *C. saccharobutylicum* RNAL841125.

Effects of *C. saccharobutylicum* Supplementation on In Vitro Rumen Fermentation Parameters

The effects of supplementation with *C. saccharobutylicum* on rumen fermentation parameters are shown in Table 3. In response to supplementation with 10^6 CFU/ml *C. saccharobutylicum*, total gas production at 6 and 12 h were higher ($p < 0.05$) compared to the control and other treatments. Meanwhile, treatment with 10^6 CFU/ml *C. saccharobutylicum* and control has similar gas production and significantly higher ($p < 0.05$) value than the other treatments at 24 h. The rumen pH values after 6 and 24 h of incubation were lower ($p < 0.05$) in treatments supplemented with *C. saccharobutylicum* compared to the control and treatment with 50 mM butyric acid. Ruminal NH_3-N concentrations at 6 h were significantly higher ($p < 0.05$) in treatments supplemented with *C. saccharobutylicum* and 50 mM butyric acid compared with the control. However, after 24 h incubation, no differences in the NH_3-N concentration was observed among the treatments. On the other hand, results for microbial crude protein (MCP) are shown in Fig. 3. Microbial crude protein and bacterial crude protein were significantly higher ($p < 0.05$) following treatment with 10^6 CFU/ml *C. saccharobutylicum* compared to other treatments. Meanwhile, no differences in the protozoal crude protein were observed among treatments. The effect of supplementation with *C. saccharobutylicum* on VFA concentrations are shown in Table 4. Higher concentrations ($p < 0.05$) of acetate were obtained in treatment with 10^6 CFU/ml *C. saccharobutylicum* and 50 mM butyric acid after 12 h incubation compared with other treatments. No differences were observed in the propionate concentrations between the treatment supplemented with 10^6 and 10^7 CFU/ml *C. saccharobutylicum* and control. Moreover, after 6 and 24 h of incubation, higher ($p < 0.05$) contents of butyrate and total volatile fatty acid were obtained following treatment with 10^6 CFU/ml *C. saccharobutylicum* compared to control and other

Table 3. Effect of treatments on total gas production, pH, and NH_3-N during *in vitro* rumen fermentation at 0, 6, 12, and 24 h.

Parameters	Time (h)	Treatments[1]					SEM[2]	*p* value	
		Control	10^6Cs	10^7Cs	10^8Cs	BA		All	Linear [3]
Total gas (ml)	6	59.00^b	62.33^a	61.00^{ab}	60.67^{ab}	56.00^c	0.741	0.0015	0.3350
	12	72.67^b	76.67^a	75.33^{ab}	73.33^b	67.00^c	0.941	0.0004	0.8674
	24	84.67^a	85.00^a	83.00^b	81.67^b	78.00^c	0.480	<.0001	0.0008
pH	0	6.60	6.59	6.58	6.57	6.60	0.007	0.0560	0.0319
	6	5.69^a	5.62^b	5.61^b	5.61^b	5.67^a	0.010	0.0017	0.0024
	12	5.59	5.55	5.54	5.53	5.58	0.014	0.0974	0.0013
	24	5.46^a	5.43^b	5.42^b	5.42^b	5.49^a	0.007	0.0001	0.0021
NH_3-N (mg/dL)	0	11.65	13.60	13.52	12.74	13.15	0.391	0.1219	0.2471
	6	19.82^b	21.46^a	21.41^a	21.36^a	21.41^a	0.293	0.0358	0.0073
	12	22.80^a	23.46^a	23.10^a	21.49^b	22.93^a	0.248	0.0065	0.0053
	24	23.07	23.63	23.41	23.00	23.31	0.122	0.0980	0.5454

[a-c]Means with different superscripts within a row are significantly different ($p < 0.05$).

[1] Treatments: Control – no inoculant; 10^6Cs – 10^6 CFU/ml *C. saccharobutylicum*; 10^7Cs – 10^7 CFU/ml *C. saccharobutylicum*; 10^8Cs – 10^8 CFU/ml *C. saccharobutylicum*; BA – 50 mM Butyric acid.

[2] SEM, standard error of mean.

[3] Linear effect (Control × *C. saccharobutylicum* supplementation).

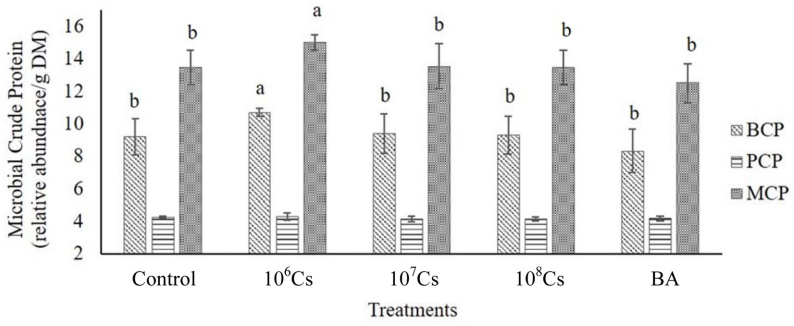

Fig. 3. Effect of *C. saccharobutylicum* supplementation on microbial crude protein (MCP) at 24 h.

Treatments are as follows: Control – no inoculant; 10^6Cs – 10^6 CFU/ml *C. saccharobutylicum*; 10^7Cs – 10^7 CFU/ml *C. saccharobutylicum*; 10^8Cs – 10^8 CFU/ml *C. saccharobutylicum*; BA – 50 mM Butyric acid. MCP = microbial crude protein; BCP = bacterial crude protein; PCP = protozoal crude protein. MCP = BCP + PCP.

treatments. Meanwhile, increasing the *C. saccharobutylicum* inoculum supplemented in the samples showed linear effects ($p < 0.05$) on total gas production, pH, acetate, propionate, acetate to propionate ratio and total VFA. At 24 h incubation, the values decreased as the inclusion rate of *C. saccharobutylicum* supplemented was increased.

Table 4. Effect of treatments on VFA production (mmol/l) during in vitro rumen fermentation at 0, 6, 12, and 24 h.

Parameters	Time (h)	Treatments [1]					SEM [2]	*p* value	
		Control	10^6Cs	10^7Cs	10^8Cs	BA		All	Linear [3]
Acetate	0	25.40	25.90	25.80	25.73	25.42	0.319	0.4727	0.4596
	6	33.48^{bc}	34.92^{ab}	32.89^{bc}	31.98^{c}	36.06^{a}	0.415	0.0077	0.0739
	12	36.07^{ab}	37.78^{a}	34.59^{bc}	33.49^{c}	37.38^{a}	0.576	0.0071	0.0084
	24	37.32^{bc}	38.08^{ab}	36.78^{bc}	36.10^{c}	38.89^{a}	0.346	0.0045	<.0001
Propionate	0	5.54^{a}	5.14^{b}	4.90^{c}	4.79^{c}	4.98^{bc}	0.041	<.0001	<.0001
	6	10.38	10.21	10.13	10.01	9.77	0.122	0.0828	0.0478
	12	11.32^{a}	11.28^{a}	11.03^{ab}	10.63^{b}	10.54^{b}	0.145	0.0459	0.0347
	24	13.16^{a}	13.04^{a}	12.93^{a}	11.26^{b}	11.43^{b}	0.271	0.0016	0.0008
Butyrate	0	5.39^{b}	6.71^{a}	6.60^{a}	6.46^{a}	5.72^{b}	0.151	0.0005	0.0038
	6	12.00^{d}	15.37^{a}	14.49^{ab}	13.94^{bc}	13.21^{c}	0.296	0.0009	0.0039
	12	15.17	16.09	15.24	15.13	13.67	0.549	0.1915	0.7699
	24	16.68^{b}	19.37^{a}	17.86^{ab}	16.67^{b}	16.73^{b}	0.437	0.0128	0.5277
A/P ratio [4]	0	4.59^{c}	5.03^{b}	5.27^{ab}	5.38^{a}	5.11^{ab}	0.066	0.0008	0.0003
	6	3.23^{b}	3.42^{b}	3.25^{b}	3.19^{b}	3.70^{a}	0.065	0.0029	0.4135
	12	3.19^{b}	3.35^{ab}	3.14^{b}	3.16^{b}	3.55^{a}	0.076	0.0687	0.5833
	24	2.84^{c}	2.92^{c}	2.85^{c}	3.21^{b}	3.41^{a}	0.052	0.0001	0.0037
Total VFA	0	36.33^{c}	37.76^{a}	37.29^{ab}	36.98^{ab}	36.12^{a}	0.152	0.0001	0.0868
	6	55.85^{c}	60.50^{a}	57.51^{bc}	55.93^{c}	59.04^{ab}	0.644	0.0044	0.4361
	12	62.56	65.15	60.86	62.36	61.59	0.939	0.1739	0.4037
	24	67.16^{b}	70.49^{a}	67.57^{b}	64.03^{c}	67.06^{b}	0.492	0.0003	0.0004

[a-d] Means with different superscripts within a row are significantly different ($p < 0.05$).

[1] Treatments: Control – no inoculant; 10^6Cs – 10^6 CFU/ml *C. saccharobutylicum*; 10^7Cs – 10^7 CFU/ml *C. saccharobutylicum*; 10^8Cs – 10^8 CFU/ml *C. saccharobutylicum*; BA – 50 mM Butyric acid.

[2] SEM, standard error of mean.

[3] Linear effect (Control × *C. saccharobutylicum* supplementation).

[4] A/P = acetate to propionate ratio.

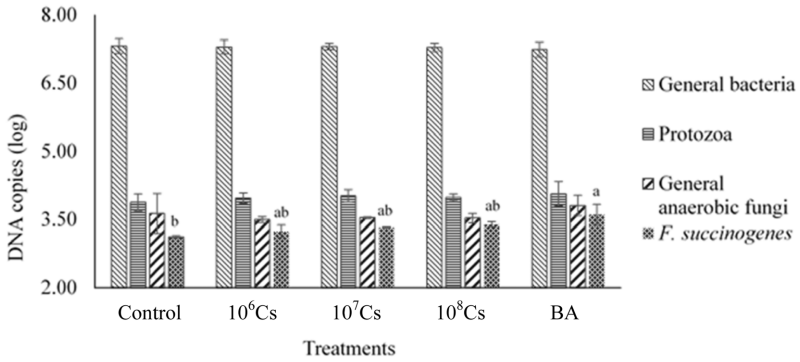

Fig. 4. Quantification of total bacteria, total fungi, protozoa, and *Fibrobacter succinogenes* by real-time PCR at 24 h.
Treatments are as follows: Control – no inoculant; 10^6Cs – 10^6 CFU/ml *C. saccharobutylicum*; 10^7Cs – 10^7 CFU/ml *C. saccharobutylicum*; 10^8Cs – 10^8 CFU/ml *C. saccharobutylicum*; BA – 50 mM Butyric acid.

Ruminal Microbial Population Abundances

Supplementation effect of *C. saccharobutylicum* on microbial population in the rumen after 24 h incubation is shown in Fig. 4. No differences in the abundances of general bacteria, protozoa and general anaerobic fungi were observed among treatments. Meanwhile, there was an increase in the population of *Fibrobacter succinogenes* when *C. saccharobutylicum* was supplemented compared with that of the control. Moreover, analysis of the butyrate kinase (*buk*) gene, which is associated with butyrate producers, showed that supplementation with 10^6 and 10^7 CFU/ml of *C. saccharobutylicum* enhanced the population of butyrate-producing bacteria in the rumen (Fig. 5). However, the butyrate-producing bacteria population tended to decrease, as the inclusion rate of *C. saccharobutylicum* was increased. Furthermore, this result is in concordance with the butyrate production observed on the in vitro rumen fermentation experiment, wherein the butyrate production decreased as a higher number of *C. saccharobutylicum* was supplemented.

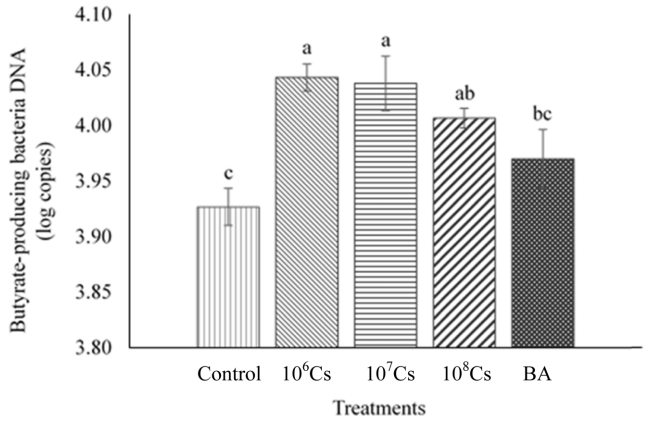

Fig. 5. DNA copies of *buk* gene of butyrate-producing bacteria during in vitro rumen fermentation at 24 h.
Treatments are as follows: Control – no inoculant; 10^6Cs – 10^6 CFU/ml *C. saccharobutylicum*; 10^7Cs – 10^7 CFU/ml *C. saccharobutylicum*; 10^8Cs – 10^8 CFU/ml *C. saccharobutylicum*; BA – 50 mM Butyric acid.

Discussion

Butyrate is a major product of microbial fermentation in the rumen of ruminants and contributes about 70% of the daily metabolizable energy requirement for ruminants [34]. Several studies have illustrated the beneficial effect of butyrate in gut health as it plays a significant role in modulating bacterial energy metabolism in the gut ecosystem. Moreover, its effect depends on the diets, microbes and their abundance in the gut ecosystem, and gut transit time [35, 36]. Butyrate and its derivatives generally exhibit a significant effect on animal production, which includes the enhancement of gut development, control of enteric pathogens, reduction of inflammation, improvement of growth performance, and modulation of gut microbiota [37]. It was also reported that due to their antimicrobial activity, SCFA, including butyrate, have been used as feed additives to control pathogenic bacteria [38, 39]. In ruminants, persistent increase of butyrate level in the GI tract have positive effects on nutrient utilization efficiency [36].

In this study, *Clostridium saccharobutylicum* RNAL841125 was isolated from the rumen of Holstein-Friesian cow. This bacterium is a Gram-positive, obligately-anaerobic, spore-forming bacterium belonging to *Clostridum* cluster XIVa and is one of the four distinct species of solvent-producing

clostridia, along with *C. acetobutylicum*, *C. beijerinckii*, and *C. saccharoperbutylacetonicum* [33, 40, 41]. This bacterium is able to utilize several carbohydrates as the sole source of carbon and energy, converting them into products including acetic and butyric acids, acetone, butanol, ethanol, CO_2, and H_2 [33, 41]. In this study, the bacterium utilized a wide range of carbohydrates, including the polymers starch and xylan and saccharides such as glucose, arabinose, xylose, and cellobiose, which is consistent with the findings of Johnson *et al*. [41]. Weak fermentation was observed in *C. saccharobutylicum* RNAL841125 on CMC and filter paper; however, no fermentation was observed in pectin as substrate, suggesting that these substrates are not suitable for the growth of this bacterium. Moreover, the only complex polysaccharide not utilized by *C. saccharobutylicum* is pectin, which consequently, distinguishes this species from *C. acetobutylicum*, *C. saccharoperbutylacetonicum* and *C. beijerincki* [33]. In a study conducted by Meesukanun and Satirapipathkul [42], *C. saccharobutylicum* BAA 117 was able to utilize the hydrolysate of Cassava rhizome, which consists mainly of cellulose, hemicellulose and lignin, as substrate for fermentation in batch culture. We also found that lower concentrations of enzyme were produced using CMC and filter paper as substrates compared with xylan and starch; these findings may be related to the weak fermentation of the bacterium on these substrates.

The in vitro gas production technique has been used widely to study feed degradation [43]; it can provide important information on the kinetics of feed digestion in the rumen and predicts the substrate utilization efficiency [44]. In this study, our results showed that gas production at 6 and 12 h has significantly higher value following treatment with 10^6 CFU/ml *C. saccharobutylicum*. This increase in gas production might be attributed to the bacterium's high growth activity; thus, microbial fermentation during the incubation period is high. However, after 24 h incubation, similar gas production was observed between the control and treatment with 10^6 CFU/ml *C. saccharobutylicum*. Our results were consistent with the results reported by Doto and Liu [45], where addition of a butyrate-producing bacteria, *C. butyricum*, seemingly had no influence on the rate of gas production at 24 h incubation.

pH is the main index reflecting internal homeostasis of the rumen environment; maintaining a relatively stable ruminal pH is important to ensure efficient rumen fermentation. Our results illustrated a decrease in rumen pH upon supplementation with *C. saccharobutylicum* which indicates that the decrease in pH resulted from the active fermentation of carbohydrates. Moreover, it was reported that mildly acidic pH values seemed to promote butyrate formation [46]. Furthermore, an in vitro fermentor study with a fecal inoculum [47] demonstrated that two major butyrate-producing bacterial groups, *Roseburia/E. rectale* species and *F. prausnitzii*, were found to have thrived at pH 5.5, whereas their population decreased at pH 6.5. In accordance with these population changes, it was found that butyrate was the main product of fermentation at pH 5.5, while acetate and propionate were the main products at pH 6.5. Thus, they suggested that changes in the pH likely affects the microbial community structure and activity.

Ammonia nitrogen is the vital source of nitrogen for microbial protein synthesis in the rumen [48]. Satter and Slyter (1974) [49] suggested that the lowest concentration of NH_3-N in rumen liquor should not be less than 5 mg/dL to maintain higher bacterial growth rate. Moreover, deficit amount of NH_3-N in the rumen restricts microbial protein synthesis, whereas high concentrations of NH_3-N also inhibit microbial utilization of this compound [50]. Our results showed that the concentrations of NH_3-N following treatment with different concentrations of *C. saccharobutylicum* ranged from 23.0 to 23.67 mg/dL, suggesting that growth and protein synthesis of microorganisms were not constrained. Moreover, increased NH_3-N concentrations indicates a greater catabolism of protein and nonprotein nitrogen [51] and higher nitrogen concentrations are available for microbial utilization and protein synthesis [52, 53]. The study showed that at 6 h incubation, treatments had higher NH_3-N concentration compared to control, while at 12 h, treatment supplemented with 10^8 CFU/ml *C. saccharobutylicum* had lower NH_3-N concentration. However, after 24 h incubation, no differences in the NH_3-N concentration were observed among treatments. Also, several studies have reported that between a control and probiotics, probiotics have seemingly no effects on the NH_3-N concentration in the rumen [54, 55]. Furthermore, Kowalski *et al*. [57] demonstrated that supplementation of 2% of microencapsulated sodium butyrate had no effect on NH_3-N concentration in dairy cows. Other studies also revealed that probiotics comprised of *Lactobacillus plantarum*, *Enterococcus faecium* and *Clostridium butyricum* [57] and *C. butyricum* alone [45] did not influence NH_3-N concentration.

Microbial crude protein (MCP) is the major source of metabolizable protein for ruminant animals because of its quantity and excellent amino acid profile [58]. In this study, the highest amount of MCP was obtained following treatment with 10^6 CFU/ml *C. saccharobutylicum*. According

to Block [59], increased MCP production in the rumen improves the efficiency of feed utilization in cattle and results in the supply of a more ideal protein source. In addition, bacterial crude protein (BCP) was also increased following supplementation with *C. saccharobutylicum.* The numerical increase of BCP might be due to the microbial efficiency during fermentation. According to Castillo-Lopez *et al*. [60], readily available N in the form of urea and high starch may have supported more microbial growth leading to more BCP synthesis.

Volatile fatty acids, including butyrate, are considered the most important end products of rumen fermentation providing cows with the majority of energy precursors for metabolic processes [61] and have been established to be significant factors in the postnatal development of the ruminal epithelium [62]. The rumen epithelium is responsible for many important physiological functions including absorption, transport, and SCFA metabolism [37, 63]. In this study, we found that higher concentrations of butyrate was produced following treatment with 10^6 CFU/ml *C. saccharobutylicum*, indicating an increase in the carbon and energy sources for fatty acid synthesis [64]. Several studies had demonstrated that treatment with butyrate-producing bacteria resulted in enhanced butyrate production. In the study by Gorka *et al*. [65], sodium butyrate supplementation in calves demonstrated that butyrate and total VFA were higher compared with their control treatment, suggesting enhanced rumen fermentation. Moreover, Li *et al*. [36] illustrated that butyrate infusion affected rapid changes in the rumen VFA concentrations, resulting in an increase in butyrate concentration, while both acetate and propionate concentrations were reduced. Additionally, they showed that butyrate concentration continued to increase and peaked at 168 h infusion, however, within 24 h after withdrawal of butyrate infusion, there was a reduction in the ruminal butyrate concentration. In contrast with their report on the decrease of acetate concentration, our study illustrated a minute increase in acetate concentration in treatment with 10^6 CFU/ml *C. saccharobutylicum* until 24 h. This is consistent with the results by Kowalski *et al*. [56] where the supplementation of 2% of microencapsulated sodium butyrate increased the acetate concentration. Direct relationship of rumen pH and VFA is well known, therefore, lower ruminal pH in treatments supplemented with *C. saccharobutylicum* is related to the increase in ruminal VFA [66].

The addition of microorganisms in the rumen can alter the microbial community in the rumen, and the microbial community changes in response to changes in feed and feed levels in the rumen [67]. The microbial population was not significantly affected by the addition of *C. saccharobutylicum*; however, cellulolytic bacteria, *F. succinogenes* increased its population with addition of *C. saccharobutylicum*, which might suggest that addition of *C. saccharobutylicum* enhances *F. succinogenes* population. *C. saccharobutylicum* had exhibited hemicellulolytic activity which enables the microorganism to convert a range of agricultural substrates and monomeric sugars of hemicellulose to solvents and acids. The supplementation with *C. saccharobutylicum* might have stimulated the growth of *F. succinogenes* leading to the increase in population of this microbe. A study [39] illustrated that butyrate infusion impacted the relative abundance of as many as 6 phyla, including Bacteroidetes, Firmicutes, Fibrobacteres, Synergistetes, Planctomycetes, and Verrucomicrobia. Meanwhile, butyrate-producing bacteria are important to gut homeostasis [68], thus, increasing the population is beneficial in the animal. In this study, the population of butyrate-producing bacteria in the rumen was found to be higher when supplemented with *C. saccharobutylicum* in comparison with that of the control. Our results were consistent with the results of a study by Li *et al*. [39], who found that an increased butyrate levels in the rumen had stimulatory effects on butyrate-producing bacteria populations. Additionally, they reported that introducing butyrate-producing bacteria into the gut ecosystem is a possible means to treat and prevent colon cancer and enterocolitis, including inflammatory bowel diseases. Meanwhile, the type of diet can also affect the population of butyrate-producing bacteria. In cattle, consumption of high-fiber diets increased the population of major butyrate-producing bacteria, *Butyrivibrio,* resulting in an elevated butyric acid concentration in the rumen [18]. Furthermore, studies demonstrated that elevated butyrate production in the rumen has a direct stimulating effect on the butyrate-producing bacteria population, or indirect effects, such as metabolic cross-feeding of fermentation products from other bacterial groups [39, 71].

Our study demonstrated that supplementation with 10^6 CFU/ml *C. saccharobutylicum* RNAL841125 has the potential to improve the in vitro rumen fermentation parameters through increased concentration of butyric and total volatile fatty acids, and microbial crude protein. In addition, supplementation with 10^6 CFU/ml of *C. saccharobutylicum* enhanced the population of butyrate-producing bacteria and *F. succinogenes*.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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