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Supplementing *Rhodobacter sphaeroides* in the diet of lactating Holstein cows may naturally produce coenzyme Q10-enriched milk

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Objective: To examine the effects of *Rhodobacter sphaeroides* (*R. sphaeroides*) supplementation as a direct-fed microbial (DFM) on rumen fermentation in dairy cows and on coenzyme Q10 (CoQ10) transition into milk, an *in vitro* rumen simulation batch culture and an *in vivo* dairy cow experiment were conducted.
Methods: The characteristics of *in vitro* ruminal fermentation were investigated using rumen fluids from six cannulated Holstein dairy cows at 2 h post-afternoon feeding. A control treatment was included in the experiments based on a typified total mixed ration (TMR) for lactating dairy cows, which was identical to the one used in the *in vivo* study, plus *R. sphaeroides* at 0.1%, 0.3%, and 0.5% TMR dry matter. The *in vivo* study employed six ruminally cannulated lactating Holstein cows randomly allotted to either the control TMR (C-TMR) treatment or to a diet supplemented with a 0.5% *R. sphaeroides* culture (S-TMR, dry matter basis) *ad libitum*. The presence of *R. sphaeroides* was verified using denaturing gradient gel electrophoresis (DGGE) applied to the bacterial samples obtained from the *in vivo* study. The concentration of CoQ10 in milk and in the supernatant from the *in vitro* study was determined using high performance liquid chromatography.
Results: The results of the *in vitro* batch culture and DGGE showed that the concentration of CoQ10 significantly increased after 2 h of *R. sphaeroides* supplementation above 0.1%. When supplemented to the diet of lactating cows at the level of 0.5%, *R. sphaeroides* did not present any adverse effect on dry matter intake and milk yield. However, the concentration of CoQ10 in milk dramatically increased, with treated cows producing 70.9% more CoQ10 than control cows.
Conclusion: The CoQ10 concentration in milk increased via the use of a novel DFM, and *R. sphaeroides* might be used for producing value-added milk and dairy products in the future.

Keywords: *Rhodobacter sphaeroides*; Coenzyme Q10; Dairy Cow; *In vitro*; Denaturing Gradient Gel Electrophoresis (DGGE)

INTRODUCTION

Coenzyme Q, also known as ubiquinone, is a hydrophobic lipophilic molecule synthesized by all animal tissues. It is an important component of the mitochondrial electron transport system and its reduced form (ubiquinol) works as an antioxidant [1]. The major coenzyme Q in higher plants and mammals is coenzyme Q10 (CoQ10), which contains 10 isoprenoid units [2]. Most animal-originated foods, such as meat, egg, and dairy products, are critical sources of CoQ10 [3]. Interestingly, recent studies showed that CoQ10 concentration in human maternal milk is higher in early lactation (within a couple of days post-partum) than in later lactation phases (14 days post-partum) and, more importantly, that CoQ10 concentration in human milk is highly corre-

lated with the antioxidant capacity of milk, particularly at early lactation stages [4,5]. Other studies highlighted the age-related variation of CoQ10 (i.e., it declined with age) [6,7], despite its dietary uptake and endogenous synthesis. Therefore, supplying high levels of CoQ10 to aged or young people via food intake may help to maintain or improve their health status, as CoQ10 prevents age-related functional declines in humans.

Several microorganisms belonging to the genera *Rhodobacter*, *Agrobacterium*, and *Paracoccus* have been reported as high CoQ10 producers [8]. *Rhodobacter sphaeroides* (*R. sphaeroides*), in particular, produces a higher level of ubiquinone-10 than *Agrobacterium tumefaciens* and *Paracoccus denitrificans* [9]. In addition, *R. sphaeroides* can be cultured under variable conditions, including anaerobic respiration and fermentation conditions [10]. Thus, it can be hypothesized that supplementing these high CoQ10-producing microorganisms to ruminant diets might produce CoQ10-enriched animal products (i.e., meat and milk), if such microorganisms can inhabit, or just survive for a certain period, in the gut of the ruminants and if they can be successfully supplemented (e.g., probiotics) in the diets fed to ruminants. To produce CoQ10-enriched value-added milk, the effects of *R. sphaeroides* as a feed additive upon ruminal fermentation and CoQ10 transition into milk were assessed in the present study, using *in vitro* and *in vivo* experiments.

MATERIALS AND METHODS

Animal care

This study was approved by the Institutional Animal Care and Use Committee, Chung-Ang University, Seoul, Republic of Korea (NO: 2016-00105).

Preparation of *Rhodobacter sphaeroides* culture

R. sphaeroides belonging to the Korean Collection Type Culture 1434 strain (http://kctc.kribb.re.kr/English/index.aspx, Korea) were cultivated on Van Niel's medium [11], containing 1.0 g K_2HPO_4, 0.5 g $MgSO_4$, and 10 g yeast extract, at 20°C for 72 h, resulting in a final total concentration of 10^9 cfu/mL (viable units). Total bacterial counts were determined using the method of Harris and Sommers [12]. Both *in vitro* and *in vivo* experiments were conducted using these cultured microorganisms as a direct-fed microbial (DFM).

Experiment 1. *In vitro* rumen simulation experiment

To examine the effect of *R. sphaeroides* supplementation on the characteristics of ruminal fermentation, an *in vitro* batch culture experiment was conducted using the rumen contents of six ruminally cannulated Holstein dairy cows, collected 2 h post-afternoon feeding. Approximately 1 L of rumen content from each cow were filtered through four layers of muslin, pooled into a Thermos bottle, and immediately brought to the laboratory. Experimental diets consisted of four dietary treatments, including a control diet based on total mixed ration (TMR) with no supplement and three diets supplemented with *R. sphaeroides* culture at 0.1%, 0.3%, and 0.5% of TMR dry matter (DM) (v/w) in triplicate. The control diet for the *in vitro* study was identical to the one used for the *in vivo* study, which was a typical TMR diet for lactating cows. The chemical composition of the basal experimental diet is presented in Table 1. For the *in vitro* procedure, 100 mL of artificial rumen saliva [13] was placed in serum bottles containing 0.5 g of experimental diets under anaerobic techniques, in triplicate [14]. The filtered rumen fluid was injected (10%, v/v) into the serum bottles with continuously infused O_2-free CO_2 gas, and these serum bottles were crimped with butyl rubber stoppers and aluminum seals before being placed in a 39°C shaking water bath (100 rpm) and incubated for 2, 4, 8, 12, and 24 h. The volume of gas produced was measured at each time point by a pressure detector (model PSGH-28PCCA, DECO Co., Seoul, Korea) connected to a digital pressure transducer (DPT-03, Dail Information Co., Seoul, Korea). The supernatant of each

Table 1. Ingredients and chemical composition of total mixed ration (TMR) used in experiment 1 (*in vitro*) and in experiment 2 (*in vivo*)

Items	Total mixed ration (% of dry matter unless otherwise stated)
Ingredient	
Alfalfa (hay bale)	6.21
Tall fescue (straw)	9.31
Klein grass (hay)	6.21
Oats (hay)	7.76
Beet pulp	3.10
Whole cotton seed	3.10
$CaCO_3$	0.65
$NaHCO_3$	0.10
Corn (mash)	5.90
Corn silage	8.45
Molasses	2.98
Wet distiller's grain with solubles	12.84
Concentrate mix[1)]	33.39
Chemical composition	
Dry matter	76.81
Crude protein (CP)	14.60
Undegradable protein (%, CP)	35.70
Degradable protein (%, CP)	64.30
Soluble protein (%, CP)	30.30
Ether extract	4.21
Crude fiber	17.09
Neutral detergent fiber (NDF)	40.98
Acid detergent fiber (ADF)	25.88
Effective NDF (%, NDF)	73.30
Total digestible nutrients	70.00
Net energy lactation (Mcal/kg, NE_L)	1.31

[1)] Concentrate mix contained, 11.5% ground corn, 10.2% dried distiller's grains with solubles, 8.8% corn gluten feed, 7.1% corn germ meal, 7.0% palm kernel meal, 6.2% wheat bran, 6.2% rapeseed meal, 6.2% wheat flour, 5.3% wheat, 3.8% soybean meal (44% CP), 3.7% coconut meal, 2.3% full fat soya, 2.7% perilla meal, 0.4% bypass protein, 7.3% vitamin and mineral mixture.

incubation bottle was collected for pH determination and stored at –20°C for ammonia nitrogen (NH_3-N) [15], volatile fatty acids (VFAs) [16], and microbial protein synthesis [17] analyses. To determine DM digestibility, the incubated residues were transferred to a sintered glass crucible, cleaned, oven-dried, and weighed.

Experiment 2. *In vivo* experiment

Six Holstein lactating cows (body weight 612±27 kg, milk yield 28.4±2.3 kg/d, and parity 2nd) equipped with permanent ruminal cannulae were used in the *in vivo* experiment, to examine if supplementing diets with *R. sphaeroides* cultures affected milk production and milk composition, particularly the concentration of CoQ10 in milk. Cows were randomly allocated to one of the two dietary treatments (n = 3): control TMR (C-TMR), identical to that used in the *in vitro* study, and TMR supplemented (S-TMR) with 0.5% of *R. sphaeroides* culture (TMR dry matter basis, v/w). Cows were allowed to adapt to the experimental diets for 20 days and at the end of the adaptation to the experimental diets an aliquot of milk samples (30 mL) were then collected at the same time, over three consecutive days and pooled per cow, immediately stored at –20°C, and freeze-dried prior to the CoQ10 analysis. Milk composition was analyzed using 50 mL of milk and Milko-Scan (FOSS-4000, FOSS, Denmark).

Isolation and purification of rumen microbial DNA

For the molecular analyses, *R. sphaeroides* genomic DNA was isolated from the rumen fluid of the cows fed on C-TMR and S-TMR, using a previously described method with minor modifications [18]. Briefly, genomic DNA was extracted by bead-beating (BioSpec Products, Bartlesville, OK, USA) for 4 min at full speed (2,000 strokes/min) in the presence of zirconium beads (weight 0.7 g, diameter 0.1 mm), 282 μL buffer A (NaCl 0.2 M, Tris 0.2 M, ethylenediaminetetraacetic acid 0.02 M; pH 8), 268 μL buffer B (QIAquick 96 PCR purification kit, Qiagen, Hileden, Germany), and 200 μL phenol-chloroform-isoamyl alcohol (25:24:1, pH 8). After centrifugation (16,000×*g* for 20 min at 4°C), the supernatant was thoroughly mixed with 650 μL buffer PB (Qiagen, Germany), and DNA was purified from the sample using the Qiagen PCR purification kit following the manufacturer's protocol.

Denaturing gradient gel electrophoresis analysis

To conduct the denaturing gradient gel electrophoresis (DGGE) analysis, the photosynthetic reaction center M subunit gene, which is specific to photosynthetic bacteria such as *R. sphaeroides*, was amplified using the primers GC-clamp-557f (5′- CGC ACC TGG ACT GGA C -3′) and 750r (5′- CCC ATG GTC CAG CGC CAG AA -3′) as previously described [19]. The polymerase chain reaction (PCR) was performed in a TaKaRa Bio Ins. PCR Thermal Cycler (Kusatsu, Shiga, Japan) in a 25 μL final volume containing EmeraldAmp GT PCR MASTER mix (TaKaRa Bio Ins., Japan), 1 μL each primer (GC-cramp 557f and 750r), 2 U Taq polymerase (Ex Taq, TaKaRa Bio Inc., Japan), and 1 μL template DNA. Amplification cycles started with denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, and ended with a final extension at 72°C for 10 min. Amplification success was checked by using 2% agarose gel electrophoresis and by visualizing PCR products in a Gel Doc XR+ system (Bio-Rad, Hercules, CA, USA). Following the purification of PCR products with the QIAquick PCR purification kit (Qiagen, Germany), DGGE was conducted using a D-Code system (Bio-Rad, USA) with 8% (w/v) polyacrylamide gels containing 20% to 80% denaturant gradient in 1×TAE buffer containing a mixture of Tris base, acetic acid and ethylenediaminetetraacetic acid. Equal amounts of purified PCR products were loaded on the gel and electrophoresis was performed at 130 V for 8 h. The gel was stained in 250 mL running buffer (ethidium bromide, Bio-Rad, USA) for 20 min and stained gels were photographed under UV using the Gel Doc XR^+ documentation system (Bio-Rad, USA).

Sequence analysis

After electrophoresis, bands of interest were carefully excised with a sterile razor blade under UV illumination and then placed in 100 μL TE buffer containing Tris and ethylenediamintetraacetic acid for 24 h at 4°C. This solution containing DNA was again amplified using the primer pairs mentioned above, purified, and sent to Genotech (Daejeon, Korea) for sequencing. The nucleotide sequences obtained were compared to that deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) using the basic local alignment search tool (BLAST) algorithm [20]. Sequence identities were determined based on the highest identity score.

Coenzyme Q10 analysis

The concentrations of CoQ10 in rumen contents and milk were determined in a high performance liquid chromatograph (920-LC, Varian Inc., Palo Alto, CA, USA) equipped with an ultraviolet detector [21] and a liquid chromatography column (Zobax Eclipse Plus C18, 4.6×100 mm, 5.0 μm packing; Agilent technologies, Santa Clara, CA, USA). Rumen contents (10 mL) from the *in vitro* study were centrifuged at 500×*g* for 15 min to remove feed particles; 1 mL of the resulting supernatant was then re-centrifuged at 16,000×*g* for 20 min to isolate rumen microbial cells. The concentration of CoQ10 in the collected cells and cow milk samples was then analyzed as previously described [22,23].

Statistical analysis

Ruminal fermentation characteristics and milk composition were subjected to analysis of variance, with diet as the main effect, using the PROC MIXED procedure in the SAS program package [24]. Where necessary, the multiple comparison was performed by Duncan's multiple range test [25]. Significance of the treatment was tested at 5% level. Orthogonal polynomial contrasts were performed to determine linear and quadratic effects of *R. sphaeroides*

supplement.

RESULTS

The effects of *R. sphaeroides* on *in vitro* ruminal fermentation are shown in Table 2. Gas production significantly increased ($p<0.05$) with increasing *R. sphaeroides* levels supplemented to the diet. Microbial protein synthesis also increased ($p<0.05$) with supplementation above 0.1% *R. sphaeroides* but NH_3-N concentration decreased ($p<0.05$) with increasing *R. sphaeroides* concentration. Final pH, DM digestibility, total VFAs concentration, and acetate/propionate (A/P) ratio were not affected by *R. sphaeroides* supplementation. The concentration of CoQ10 increased ($p<0.05$) after 2 h for *R. sphaeroides* supplementation above 0.1% and CoQ10 accumulated in culture media during *in vitro* ruminal fermentation (Figure 1). After 12 h, CoQ10 concentration obtained for 0.5% *R. sphaeroides* supplementation (115.78 μg/g) was about 10-fold greater than that of the control (12.96 μg/g).

The viability of the supplemented *R. sphaeroides* was examined using a molecular biological technique (Figure 2). As evidenced, a unique band appeared in the rumen samples obtained from cows fed on diets supplemented with 0.5% *R. sphaeroides* (Figure 2C), which was hardly perceptible for the samples obtained from cows fed with the control diet (Figure 2B). This band was very intense and migrated to the same position as the band obtained for the PCR product of pure *R. sphaeroides* cultures. Indeed, sequencing this band revealed that it was closely related to the *R. sphaeroides* (98.4%) sequences deposited in GenBank. Supplementing *R. sphaeroides* to the diet of Holstein dairy cows did not affect DM intake, 3.5% fat-corrected milk, and somatic cell count; however, it significantly increased ($p<0.05$) CoQ10 concentration in milk (Table 3) and cows supplemented with *R. sphaeroides* produced 70.9% more CoQ10 than control cows ($p<0.05$).

DISCUSSION

To the best of our knowledge, the present study is the first to demonstrate CoQ10 production in the rumen and its transition into milk, using *R. sphaeroides* as a DFM. Supplementation of *R. sphaeroides* did not show any detrimental effects on ruminal fermentation characteristics *in vitro* nor on animal performance *in vivo*, including DM intake, and milk yield and composition. However, cumulative gas production, CH_4 production and CoQ10 concentration increased and NH_3-N concentration decreased with increasing levels of *R. sphaeroides* in *in vitro* batch cultures, suggesting that *R. sphaeroides* might adapt to and inhabit the ruminal environment. Because there are limited studies available in the literature to compare with, it is difficult to explain the observed changes in ruminal fermentation characteristics when *R. sphaeroides* cultures were supplemented. Several scientists [26, 27] have reported that dietary supplementation of DFM, such

Table 2. Effect of *Rhodobacter sphaeroides* KCTC 1434 supplementation in total mixed ration (TMR) on ruminal fermentation characteristics *in vitro*

Items	Incubation time (h)					
	0	2	4	8	12	24
pH value						
Control	7.13	7.09	6.77	6.67	6.57	6.28
0.1%	7.15	7.08	6.75	6.64	6.56	6.26
0.3%	7.16	7.08	6.74	6.65	6.54	6.26
0.5%	7.15	7.07	6.74	6.66	6.53	6.24
SEM	0.016	0.022	0.022	0.018	0.015	0.034
p value	0.6759	0.9704	0.6743	0.7368	0.3300	0.8620
Linear	0.4157	0.6459	0.2878	0.6653	0.1133	0.4506
Quadratic	0.3898	1.0000	0.9733	0.3447	0.8307	0.8487
Gas production (mL)						
Control	-	79.71^{B}	124.70^{B}	156.40^{C}	229.64^{B}	285.36^{B}
0.1%	-	79.92^{B}	123.61^{B}	169.03^{B}	240.93^{B}	287.98^{B}
0.3%	-	81.90^{AB}	129.83^{AB}	175.41^{B}	259.77^{A}	307.70^{A}
0.5%	-	83.99^{A}	135.24^{A}	187.75^{A}	271.31^{A}	319.47^{A}
SEM	-	0.819	2.371	2.905	3.541	5.180
p value	-	0.0195	0.0296	0.0004	0.0001	0.0045
Linear	-	0.0037	0.0073	0.0001	0.0001	0.0008
Quadratic	-	0.2836	0.2083	0.9623	0.9731	0.4027
Dry matter digestibility (%)						
Control	22.77	26.46	30.20	35.33	41.38	47.24
0.1%	22.87	26.60	30.23	35.45	41.43	48.01
0.3%	23.00	26.70	30.13	35.92	42.41	48.40
0.5%	23.21	27.01	30.58	35.38	41.62	49.22
SEM	0.191	0.470	0.814	0.630	0.726	0.304
p value	0.4546	0.8593	0.9637	0.9007	0.7371	0.6883
Linear	0.1321	0.4267	0.7390	0.8357	0.6121	0.0741
Quadratic	0.7803	0.8583	0.7090	0.6092	0.5789	0.9663
NH_3-N concentration (mg/100 mL)						
Control	11.81	24.60^{A}	22.12^{A}	19.35^{A}	17.05^{A}	14.72^{A}
0.1%	11.65	23.70^{AB}	21.50^{A}	17.54^{B}	15.81^{B}	12.23^{B}
0.3%	11.74	22.47^{B}	19.98^{B}	16.75^{B}	14.88^{C}	11.38^{B}
0.5%	12.01	21.97^{B}	19.47^{B}	15.37^{C}	12.71^{D}	10.06^{C}
SEM	0.263	0.542	0.423	0.263	0.198	0.262
p value	0.7982	0.0338	0.0067	0.0001	0.0001	0.0001
Linear	0.5907	0.0056	0.0010	0.0001	0.0001	0.0001
Quadratic	0.4279	0.7216	0.9058	0.4401	0.0474	0.0562
Microbial protein synthesis (mg/100 mL)						
Control	82.53^{C}	97.97^{D}	137.76^{C}	155.88^{C}	169.76^{C}	171.50^{C}
0.1%	87.15^{B}	105.33^{C}	146.84^{B}	162.33^{BC}	173.74^{BC}	175.82^{BC}
0.3%	88.52^{B}	110.04^{B}	151.71^{B}	164.75^{B}	175.68^{B}	184.50^{B}
0.5%	91.12^{A}	120.70^{A}	170.86^{A}	183.73^{A}	198.41^{A}	217.35^{A}
SEM	0.454	1.128	1.829	2.339	1.621	2.725
p value	0.0001	0.0001	0.0001	0.0004	0.0001	0.0001
Linear	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Quadratic	0.1008	0.2822	0.0249	0.0469	0.0004	0.0008
Total VFA concentration (mmol)						
Control	10.43	12.78	19.19	23.90	35.74	52.22
0.1%	10.44	12.96	19.62	24.22	36.54	52.01
0.3%	10.44	13.23	19.40	24.65	36.32	51.72
0.5%	10.40	13.10	19.55	25.14	36.09	52.25
SEM	0.171	0.208	0.245	0.453	0.318	0.538
p value	0.9974	0.5058	0.6359	0.3094	0.3870	0.5915
Linear	0.8924	0.2284	0.4621	0.0748	0.5740	0.2798
Quadratic	0.8798	0.4820	0.5924	0.8530	0.1451	0.8129
Acetate/propionate ratio						
Control	4.99	3.94	2.88	2.19	1.83	1.27
0.1%	4.81	3.99	2.97	2.09	1.77	1.27
0.3%	4.95	3.97	2.88	2.10	1.79	1.26
0.5%	4.65	3.88	3.04	2.09	1.75	1.26
SEM	0.169	0.061	0.086	0.064	0.049	0.037
p value	0.5848	0.6153	0.4987	0.6469	0.7234	0.9936
Linear	0.2327	0.5028	0.3038	0.3331	0.3630	0.8465
Quadratic	0.9315	0.2706	0.7227	0.5357	0.8683	1.0000

SEM, standard error of the mean; VFA, volatile fatty acids.
Control, TMR with no supplement; 0.1%, 0.3%, and 0.5% indicates TMR supplemented with 0.1%, 0.3%, and 0.5% *Rhodobacter sphaeroides*.
[A-D] Mean with different letter differ significantly between treatments ($p<0.05$).

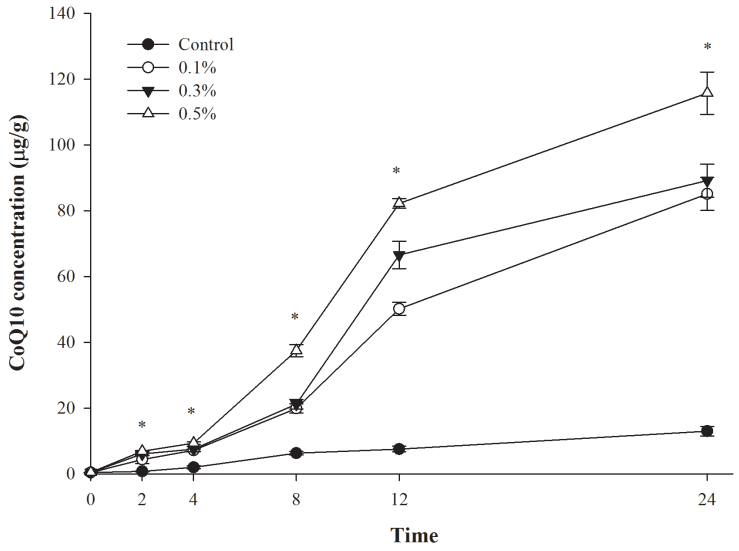

Figure 1. Effect of *Rhodobacter sphaeroides* KCTC 1434 supplementation in total mixed ration (TMR) on CoQ10 concentration *in vitro*. Control = TMR with no supplement; 0.1%, 0.3%, and 0.5% indicates TMR supplemented with 0.1%, 0.3%, and 0.5% *R. sphaeroides*). * Values differ significantly between treatments ($p<0.05$).

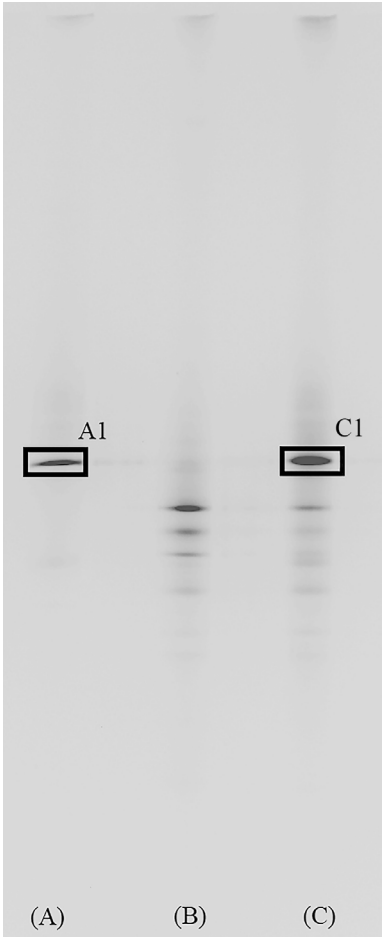

Figure 2. Denaturing gradient gel electrophoresis of rumen microbiota isolated at 12 h post feeding from *in vivo* study. (A) DNA from *Rhodobacter sphaeroides* (*R. sphaeroides*) pure culture, (B) control TMR, (C) 0.5% *R. sphaeroides* supplemented TMR. A1 (boxed) indicates the band amplified with photosynthetic reaction center M subunit (*pufM*) gene of *R. sphaeroides* and C1 (boxed) had 98.4% similarity with *R. sphaeroides*.

as *Saccharomyces cerevisiae*, stimulates the activities of rumen microorganisms *in vitro* and alters the rumen environment favorably. Therefore, it is speculated that *R. sphaeroides* cultures might have played a role along with ruminal microorganisms, particularly with respect to the N metabolism in the rumen, as indicated by the reduced NH_3-N concentration with increase in the levels of *R. sphaeroides* supplementation. Further investigation is warranted to examine the role of *R. sphaeroides* cultures on N metabolism in the rumen.

With CoQ10, previous studies [8,9] have reported that certain strains of *R. sphaeroides* have the ability to produce high levels of CoQ10 (2.5 mg/g of cell) in low-aeration conditions. Therefore, *R. sphaeroides* could produce CoQ10 under complex anoxic conditions such as the ruminal environment. This hypothesis was further confirmed by the results of the DGGE analysis, wherein *R. sphaeroides* specific genes were identified 12 h after their supplementation in the diet. Although further analyses, such

Table 3. Effects of supplementation of *Rhodobacter sphaeroides* KTCT 1434 on total mixed ration (TMR) on milk yield, milk composition and CoQ10 concentration in milk of lactating cow

Items	C-TMR[1)]	S-TMR[1)]	SEM	p value
Dry matter intake (kg/d)	23.5	24.0	0.57	0.5438
Milk yield (kg/d)	26.3	26.5	0.59	0.8799
Milk fat (%)	2.92	2.93	0.031	0.9430
3.5% fat corrected milk (kg/d)	23.47	23.69	0.563	0.8840
Milk protein (%)	3.28	3.27	0.013	0.7247
Somatic cell count ($\times 10^3$)	116.64	125.02	4.231	0.2339
CoQ10 concentration (μg/g)	1.79[B]	3.06[A]	0.038	0.0001

SEM, standard error of the mean.
[1)] C-TMR, TMR with no supplement; S-TMR, TMR supplemented with 0.5% *Rhodobacter sphaeroides* culture (dry matter basis).
[A,B] Mean with different letter differ significantly between treatments ($p<0.05$).

as quantitative PCR or plate-based enumeration, were not performed to confirm the number of *R. sphaeroides* in the rumen, their viability was evidenced by the increased band intensity obtained in the DGGE conducted for the genomic DNA isolated from ruminal bacteria of the experimental cows. Also noteworthy were the CoQ10 concentrations that accumulated in both control and supplemented groups over time, which suggest that CoQ10 might not be degraded by ruminal microbes and thus might have the potential to be by-passed to the lower digestive tract and be absorbed. As a lipophilic substance, CoQ10 connects to the chylomicrons of the small intestine for absorption and passes through via lymphatic vessels and glands [28]. Exogenous CoQ10 supplemented to humans and most animals is non-linearly absorbed at the small intestine [29] and can be transferred from plasma to milk in humans [4]. Thus, the post-rumen CoQ10 absorption mechanism of Holstein dairy cows seems to be similar to that of humans.

In conclusion, the present study is the first to demonstrate the effect of *R. sphaeroides* supplementation on rumen fermentation and the transference of CoQ10 into milk. Based on *in vitro* and *in vivo* results, *R. sphaeroides* might be able to adapt, survive, and produce CoQ10 in the rumen environment. The produced CoQ10 might be absorbed via rumen wall or via small intestine and then be transferred to milk, although this remains to be determined. Although the present study employed a limited number of animals to test its hypothesis, it clearly demonstrated that the concentration of CoQ10 in milk can be naturally increased by direct-fed microorganisms, and therefore, it might be possible to use this beneficial microorganism for the production of value-added milk and related dairy products in the future.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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