Improved viability of bifidobacteria in fermented milk by cocultivation with *Lactococcus lactis* subspecies *lactis*

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ABSTRACT

The poor survival of probiotic bacteria in commercial yogurts may limit their potential to exert health benefits in humans. The objective was to improve the survival of bifidobacteria in fermented milk. Cocultivation with some strains of *Lactococcus lactis* ssp. *lactis* improved the survival of bifidobacteria in fermented milk during refrigerated storage. Studies on one strain, *Lc. lactis* ssp. *lactis* MCC866, showed that the concentrations of dissolved oxygen were kept lower in the cocultivated fermented milk during storage compared with monocultured *Bifidobacterium longum* BB536 or samples cocultured with another noneffective *Lc. lactis* ssp. *lactis* strain. Degradation of genomic DNA was suppressed in the cocultivating system with *Lc. lactis* ssp. *lactis* MCC866. Several genes that participated in protection from active oxygen species (e.g., genes coding for alkyl hydroperoxide reductase and Fe²⁺ transport system) were expressed at higher levels during refrigerated storage in *Lc. lactis* ssp. *lactis* MCC 866 compared with another noneffective *Lc. lactis* ssp. *lactis* strain. Concentration of free iron ion was also lower in supernatants of fermented milk cocultivated with *B. longum* BB536 and *Lc. lactis* ssp. *lactis* MCC866. These results suggest that *Lc. lactis* ssp. *lactis* MCC 866 is potentially superior in reducing oxygen damage and consequently improves the survival of bifidobacteria in the cocultivating system. This cocultivation system is of industrial interest for producing fermented milk containing viable bifidobacteria with long shelf life.

Key words: *Bifidobacterium*, *Lactococcus lactis*, survival in fermented milk, dissolved oxygen

INTRODUCTION

Probiotic bacteria are living microorganisms that, when administered in adequate amounts, confer health benefits on the host (FAO/WHO, 2002). An increasing number of probiotic products is appearing in response

to current health trends. In general, high numbers of viable probiotic bacteria are desired in these products at the moment of consumption. Typically, between 5 and 8 log cfu/g of product is considered acceptable (Maragkoudakis et al., 2006). A recent meta-analysis has suggested that levels of lactobacilli of 7 to 9 log cfu/g may be necessary to elicit antidiarrheal effects (Van Niel et al., 2002). Current proposals within the European Union set the minimum level of probiotic bacteria in foods at 7 log cfu/g (EU/AGRI/38743/2003rev3).

Bifidobacteria are the major components of the intestinal microbiota of humans and animals and have attracted much attention because of their perceived contribution to health-promoting effects such as the improvement of gastrointestinal disorders and alleviation of allergy symptoms (Leahy et al., 2005; Picard et al., 2005; Turroni et al., 2009). For this reason, interest in creating food products containing these bacteria has been rapidly expanding, particularly in areas such as fermented milk and supplements. However, bifidobacteria are unable to generate sufficient acids for the manufacture of fermented foods with the appropriate aroma and flavor. They are, therefore, normally incorporated as live cultures into fermented dairy products that can serve as carrier foods, such as yogurt, by cofermentation with ordinary yogurt starters (Samona and Robinson, 1994). However, because bifidobacteria are often exposed to multiple environmental stressors such as low pH, low temperature, high osmotic pressure, nutrient starvation, and oxidation, their cell numbers and survival in yogurt are limited. Attempts to coculture bifidobacterial strains with *Lactobacillus delbrueckii* ssp. *bulgaricus* H⁺-ATPase defective mutants (Ongol et al., 2007) or *Streptococcus thermophilus* with or without other strains such as *Lactobacillus acidophilus* (Mollet, 1999) have enhanced the survival of bifidobacteria in fermented milk. Microencapsulation of bifidobacteria also increases their survival in yogurt (Adhikari et al., 2000).

We have previously reported that cocultivation with *Lactococcus lactis* ssp. *lactis* harboring cell wall-anchored proteinase (**PrtP**) stimulates the growth of bifidobacteria in fermented milk (Yonezawa et al., 2010). Oligopeptides and amino acids released from

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milk proteins by *Lc. lactis* PrtP help stimulate bifidobacterial growth during cocultivation. We also found that cocultivation with some strains of *Lc. lactis* ssp. *lactis* markedly improve the survival of bifidobacteria in fermented milk during refrigerated storage. In this report, we present results on the improved survival of bifidobacteria in fermented milk by cocultivation with *Lc. lactis* ssp. *lactis* together with some mechanistic data.

MATERIALS AND METHODS

Bacterial Strains

Type strains of bifidobacteria were purchased from the Japan Collection of Microorganisms (Riken Biore-source Center, Wako, Japan). *Bifidobacterium longum* ssp. *longum* BB536 (ATCC BAA-999) is a probiotic strain from the Morinaga Culture Collection (MCC; Morinaga Milk Industry, Zama, Japan), which was originally isolated from feces of a healthy infant. *Lactococcus lactis* ssp. *lactis* strains that originated from dairy products were obtained from MCC. Both *Lc. lactis* ssp. *lactis* strains MCC857 and MCC866 harbor PrtP and stimulate the growth of bifidobacteria in fermented milk (Yonezawa et al., 2010).

Preparation of Fermented Milk

Seed cultures of the *Lc. lactis* ssp. *lactis* strains were grown at 30°C for 6 h in 10% (wt/wt) reconstituted skim milk (Morinaga Milk Industry) containing 0.3% (wt/wt) yeast extract (Becton Dickinson, Franklin Lakes, NJ). Seed cultures of bifidobacteria (Table 1) were prepared by growth in de Man, Rogosa, and Sharpe broth (Becton Dickinson) for 16 h at 37°C in glass test tubes. Cells were collected by centrifugation and then resuspended in sterile saline composed of 0.85% NaCl to a concentration of approximately 5×10^8 cfu/mL. The seed culture of *B. longum* BB536 was prepared by culturing at 37°C for 4 h in 11% (wt/wt) reconstituted skim milk containing 0.2% (wt/wt) yeast extract in glass test tubes. For preparation of fermented milk by a monoculture of bifidobacteria, a 1% inoculum of seed culture was added to 10% skim milk medium supplemented with an optimal concentration of yeast extract, as shown in Table 1, and incubated in glass bottles at 37°C until reaching pH 4.6. Some strains were not able to reach pH 4.6 after incubation for 16 h, even with higher concentrations of yeast extract. For preparation of fermented milk containing bifidobacteria cocultivated with *Lc. lactis* ssp. *lactis*, 10% skim milk medium was inoculated with each bifi-

dobacteria seed culture and 1% of a seed culture of *Lc. lactis* ssp. *lactis* and incubated at 37°C until the pH reached the same as that produced by monocultivation of each bifidobacteria strain in glass bottles. Fermented milk (5 mL) was dispersed into polyethylene centrifuge tubes (Eiken Kizai, Tokyo, Japan) that had an oxygen transmission rate of approximately 0.15 mL/d atm at 25°C, and stored at 10°C until analysis. To check the effect of hydrogen peroxide (H_2O_2) on the viability of bifidobacteria, catalase (Wako Pure Chemical Industry, Osaka, Japan) was supplemented in the *B. longum* BB536 monoculture medium as described previously (Shimamura et al., 1992).

Measurement of Bacterial Cell Number and Concentrations of Dissolved Oxygen, H_2O_2 and Free Iron

Viable bacteria during storage were enumerated by plating appropriate dilutions on TOS propionate agar (Eiken) at 37°C for 48 h in anaerobic condition and on BCP agar (Eiken) at 30°C for 48 h in aerobic condition for bifidobacteria and *Lc. lactis* ssp. *lactis*, respectively (Lee and Lee, 2008; Yonezawa et al., 2010). Dissolved oxygen (DO) was measured using a model FO-960S fluorescence oxygen sensor (ASR, Tokyo, Japan). Samples were kept in a cooler bath at 10°C during measurement. H_2O_2 concentrations in the supernatant (centrifuged at $5,000 \times g$ for 10 min) of fermented milk were determined using the Bioxytech H_2O_2 -560 system (Oxis International, Beverley Hills, CA). The test is based on ferrous ion oxidation in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$). Ferric iron is generated in the presence of H_2O_2 , which forms a complex with xylenol orange that absorbs at 560 nm; H_2O_2 was quantified against known amounts of H_2O_2 standards. To measure free iron concentration in fermented milk, 20 μ L of each sample was injected and directly analyzed by inductively coupled plasma mass spectrometry (Agilent 7500ce; Agilent Technologies, Santa Clara, CA) as previously reported (Yamada et al., 2006). All assays were performed in triplicate.

Monitoring of DNA Degradation

One main target of oxygen induced cellular damage is DNA (Imlay et al., 1988; Duwat et al., 1995). To examine DNA integrity, chromosomal DNA was extracted from fermented milk. To 10 mL of fermented milk, an equal volume of 2% (wt/vol) trisodium citrate was added and then centrifuged at $500 \times g$ for 5 min to remove the curd. The supernatants were centrifuged at $5,000 \times g$ for 5 min to collect pellets (bacteria). Two

milligrams of lysozyme, 1 µg of *N*-acetyl muramidase, and 0.1 mg of RNase A were added to the pellets and incubated at 37°C for 16 h, followed by further digestion with 0.5 mg of protease K. The digests were analyzed by electrophoreses with 0.8% Tris-borate EDTA agarose.

Real-Time PCR

Bacteria were collected from fermented milk as described above. Extraction and reverse transcription of RNA were performed using a RNeasy kit (Qiagen, Valencia, CA) and transcriptase (Applied Biosystems, Foster City, CA), respectively. The RNA levels of genes related to active oxygen species protection (Condon, 1987; Shimamura et al., 1992; Miyoshi et al., 2003) were determined by real-time PCR using an ABI Prism 7500 Fast Real Time PCR system (Applied Biosystems). All primer pairs were designed from genome sequences of *Lc. lactis* IL1403 and *B. longum* NCC2705 (<http://www.ncbi.nlm.nih.gov>) by Primer3 (<http://primer3.sourceforge.net/>; Supplementary Table 1, available online: <http://www.journalofdairyscience.org/>). The amplification program consisted of 94°C for 10 s, followed by 40 cycles of 94°C for 5 s and 60°C for 30 s. Each PCR product was sequenced by using an ABI 3100-Avant apparatus (Applied Biosystems) and the sequences were analyzed by a BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The PCR products were judged to have been amplified precisely if the BLAST search produced the top hits with the targeted genes. Melting curves were obtained by heating from 60°C to 95°C in increments of 0.2°C/s, with continuous fluorescence collection. All values indicated averages of relative expression levels of each gene at different time points against those after 8 h of fermentation with *Lc. lactis* MCC857 in fermented milk cocultivated with *B. longum* BB536 for *Lc. lactis* or against those after 8 h of *B. longum* BB536 monoculture for bifidobacteria (mean ± SD, triplicate assays)

Statistical Analyses

All data were acquired in triplicate assays and are expressed as mean ± SD. Intergroup differences in the survival rate of bifidobacterial strains during refrigerated storage and those in gene expressions of *Lc. lactis* ssp. *lactis* were analyzed using the Student's *t*-test. Intergroup differences in gene expressions of *B. longum* BB536 and iron concentration were analyzed using the non-repeated-measures ANOVA with Bonferroni correction, respectively. Values of *P* < 0.05 were considered statistically significant.

Table 1. Effect of cocultivation with *Lactococcus lactis* ssp. *lactis* MCC866 on the survival of *Bifidobacterium* strains¹

Strain	Target	Count of bifidobacteria (log cfu/mL)		Survival rate at 14 d (%)	pH	
		0 d	14 d		0 d	14 d
<i>B. longum</i> ssp. <i>longum</i> JCM 1217 ^T	Coculture with MCC866 + 0.3% yeast extract	7.88 ± 0.03	5.78 ± 0.11	0.82 ± 0.22*	4.56 ± 0.03	4.40 ± 0.02
<i>B. longum</i> ssp. <i>infantis</i> JCM 1222 ^T	Coculture with MCC866 + 0.3% yeast extract	9.12 ± 0.02	5.83 ± 0.01	0.05 ± 0.00	4.56 ± 0.01	4.55 ± 0.03
<i>B. breve</i> JCM 1192 ^T	Coculture with MCC866 + 0.3% yeast extract	8.85 ± 0.03	5.28 ± 0.07	0.03 ± 0.01*	4.60 ± 0.02	4.40 ± 0.02
<i>B. pseudocatenulatum</i> JCM 1200 ^T	Coculture with MCC866 + 0.3% yeast extract	9.05 ± 0.03	4.48 ± 0.10	<0.01	4.63 ± 0.00	4.69 ± 0.02
<i>B. bifidum</i> JCM 1254 ^T	Coculture with MCC866 + 1.0% yeast extract	8.56 ± 0.04	4.77 ± 0.03	0.02 ± 0.01	4.66 ± 0.02	4.44 ± 0.04
<i>B. adolescentis</i> JCM 1275 ^T	Coculture with MCC866 + 1.0% yeast extract	9.67 ± 0.01	6.15 ± 0.18	0.03 ± 0.01	4.66 ± 0.01	4.66 ± 0.08
<i>B. longum</i> ssp. <i>longum</i> BB536	Coculture with MCC866 + 0.3% yeast extract	7.04 ± 0.08	5.06 ± 0.06	1.07 ± 0.06*	4.83 ± 0.07	4.43 ± 0.06
		9.00 ± 0.02	6.37 ± 0.11	0.24 ± 0.05	4.84 ± 0.05	4.82 ± 0.03
		7.78 ± 0.06	5.33 ± 0.06	0.36 ± 0.09*	4.69 ± 0.02	4.44 ± 0.11
		8.44 ± 0.08	5.16 ± 0.07	0.05 ± 0.01	4.74 ± 0.04	4.74 ± 0.09
		6.32 ± 0.10	5.51 ± 0.10	16.02 ± 5.57*	5.02 ± 0.01	4.45 ± 0.04
		8.93 ± 0.01	7.77 ± 0.01	6.86 ± 0.14	5.14 ± 0.09	5.12 ± 0.13
		8.97 ± 0.07	8.57 ± 0.11	40.14 ± 3.94*	4.53 ± 0.01	4.40 ± 0.03
		8.82 ± 0.03	7.08 ± 0.22	2.01 ± 1.07	4.54 ± 0.01	4.53 ± 0.06

¹Type strains of bifidobacteria were purchased from the Japan Collection of Microorganisms (Wako, Japan).

**P* < 0.05, significant intergroup difference.

RESULTS

Effect of Cocultivation with *Lc. lactis* ssp. *lactis* on the Survival of Bifidobacteria

Some strains of *Lc. lactis* ssp. *lactis* stimulate the growth of bifidobacteria (Yonezawa et al., 2010) and others also improve the survival of bifidobacteria during refrigerated storage (our unpublished data). *Lactococcus lactis* ssp. *lactis* MCC866, which efficiently improved the growth and survival of *B. longum* BB536 (Table 1), was used to test the effect on the survival of different species of bifidobacteria in a cocultivation system. Type strains of *Bifidobacterium pseudocatenuatum* and *Bifidobacterium adolescentis* barely grew in the skim milk medium when cocultivated with *Lc. lactis* ssp. *lactis* MCC866. Furthermore, although the pH after fermentation for each strain during monocultivation or cocultivation was adjusted to the same levels, dissociation was observed after 14 d of storage for almost all strains. However, although it was strain-dependent, the survival rates after 14 d of storage in cocultivation were significantly higher compared with monoculture, with the exception of *Bifidobacterium breve* JCM 1192^T (Table 1).

Changes of pH, Survival of Bacteria, and Concentrations of DO and H₂O₂ in Fermented Milk Containing *B. longum* BB536 During Refrigerated Storage

Viable bacterial numbers of monocultivated *B. longum* BB536 decreased by approximately 2 logs at 14 d, decreased by a further 2 logs during the next 7 d, and exhibited approximately 5 log-fold lower numbers after 4 wk of storage compared with those after fermentation (Figure 1A). A similar temporal decrease of bifidobacteria was observed during cocultivation with *Lc. lactis* ssp. *lactis* MCC857 (Figure 1A). However, the survival rates were markedly improved by cocultivation with *Lc. lactis* ssp. *lactis* MCC866; viable bacterial numbers of *B. longum* BB536 were maintained until 21 d and were maintained at over 2 logs higher than those of monocultivated bacteria after 4 wk of storage (Figure 1A). Survival of *Lc. lactis* ssp. *lactis* MCC857 and MCC866 was stable until 21 d, without marked difference between monocultivation and cocultivation with *B. longum* BB536 (Figure 1B). The pH for fermented milk containing *Lc. lactis* ssp. *lactis* decreased about 0.2 in the first week for both monocultivation and cocultivation with *B. longum* BB536, and maintained at a steady level in the later stages of storage (Figure 1C). No marked change of pH was found for the samples of monocultivated *B. longum* BB536 during storage

(Figure 1C). Concentrations of DO in fermented milk containing bifidobacteria monocultivated or cocultivated with *Lc. lactis* ssp. *lactis* MCC857 started increasing from 7 d and leveled to 6 ppm or higher at 14 d during storage. However, the DO concentrations were low in the fermented milk cocultivated with *Lc. lactis* ssp. *lactis* MCC866 until 14 d and were maintained at the lower levels compared with monocultivation or cocultivation with *Lc. lactis* ssp. *lactis* MCC857 at 21 d (Figure 1D). The DO levels in samples with *Lc. lactis* ssp. *lactis* MCC866 were lower compared with those with MCC857 in monocultivation during storage (Figure 1D). The concentrations of H₂O₂, an intermediary metabolite of scavenged oxygen, were maintained at lower levels in fermented milk except for those of cocultivation of *B. longum* BB536 and *Lc. lactis* ssp. *lactis* MCC866, in which the concentrations were higher after fermentation compared with the other samples, increased further at 7 d and 14 d, and then, along with the increased concentrations of DO, decreased at 21 and 28 d to levels similar to those of the other samples (Figure 1E). Furthermore, supplementation of catalase to *B. longum* BB536 monoculture did not affect the viability of *B. longum* BB536 or the concentrations of DO, although H₂O₂ was lowered during storage (Figures 1 A, D, and E).

DNA Degradation During Refrigerated Storage

Because the electrophoresis patterns obtained from samples at 7 and 14 d were similar to that at 0 d (data not shown), DNA was predicted to be stable until 14 d. However, degradations were observed in fermented milk containing *B. longum* BB536 in monoculture or in coculture with *Lc. lactis* ssp. *lactis* MCC857 after 21 d of storage, but no marked degradation was found for those cocultured with *Lc. lactis* ssp. *lactis* MCC866, even after 28 d of storage (Figure 2).

Gene Expressions of Bacteria in Fermented Milk

Table 2 shows the relative expression levels of major genes that participated in oxygen utilization and protection from active oxygen species examined by reverse transcription-quantitative PCR. For *Lc. lactis* ssp. *lactis*, the genes *noxC*, *noxD*, *noxE*, *poxL*, *glpD*, *ahpC*, and *feoB* were expressed at the highest levels after fermentation compared with during the refrigerated stage (Table 2). Significantly higher expression levels during fermentation were found for all genes except *noxD*, *ahpF*, and *feoB* in *Lc. lactis* ssp. *lactis* MCC857 compared with *Lc. lactis* ssp. *lactis* MCC 866. Notably, some genes were expressed at higher levels during storage for 7 and 14 d in *Lc. lactis* ssp. *lactis*

Figure 1. Changes of survival of bifidobacteria and circumferential conditions in fermented milk during refrigerated storage. A) Cell numbers of *Bifidobacterium* (cfu/mL); B) cell numbers of *Lactococcus lactis* (cfu/mL); C) pH; D) dissolved oxygen (ppm); E) H_2O_2 concentration (μM). Experiments were performed 4 times with comparable results. Results of representative experiments are shown; all data represent triplicate assays.

MCC866 compared with *Lc. lactis* ssp. *lactis* MCC857: 2 examples are alkyl hydroperoxide reductase (*ahpC*: 2.26-fold, *ahpF*: 5.86-fold), which form 2-component enzymes to convert H_2O_2 to H_2O ; and Fe^{2+} transport system (*feoB*, 11.31-fold at 7 d and 14.69-fold at 14 d). In contrast to results for *Lc. lactis* ssp. *lactis*, the expression levels of genes encoding alkyl hydroperoxide reductase and H_2O -forming NADH oxidase were lower after fermentation (0 d) than during the exponential phase in *B. longum* BB536 (Table 3). Furthermore, the expression levels of these genes were enhanced markedly during refrigerated storage (Table 3). Notably, the expression level of *ahpC* was more than 500-fold higher in *B. longum* BB536 monocultivation at 14 d of storage compared with those after fermentation, which was significantly higher compared with those cocultivated with *Lc. lactis* ssp. *lactis*. The expression levels of *ahpC* were significantly lower during fermentation or storage when cocultivated with *Lc. lactis* ssp. *lactis* MCC866 compared with those with *Lc. lactis* ssp. *lactis* MCC857 (0 d: 0.19-fold; 7 d: 0.24-fold, and 14 d: 0.63-fold).

Free Iron Concentration in Fermented Milk Containing *B. longum* BB536

The free iron level was significantly lower in fermented milk cocultivated with *Lc. lactis* ssp. *lactis* MCC866 (57.2 ± 14.1 ng/g) at 0 d compared with monocultivation (93.9 ± 11.7 ng/g) or cocultivation with *Lc. lactis* ssp. *lactis* MCC857 (93.7 ± 11.3 ng/g).

DISCUSSION

During refrigerated storage of fermented milk, microorganisms are often exposed to multiple environmental stresses such as low pH, low temperature, high osmotic pressure, nutrient starvation, and oxidation, which lead to the loss of viability. However, among the reasons responsible for the loss of survival of bifidobacteria in fermented milk, low pH and oxidation are considered the most important factors (Shah and Jellen, 1990; Talwalkar and Kailasapathy, 2003; Jayamanne and Adams, 2006).

Figure 2. Degradation of DNA in fermented milk during refrigerated storage. A gel representative of 3 experiments is shown. BB536 = *Bifidobacterium longum*; MCC857 = *Lactococcus lactis* MCC857; MCC866 = *Lactococcus lactis* MCC866.

Attempts to provide a mildly acidic environment by stopping fermentation at a higher pH (Gilliland et al., 2002) or cocultivating bifidobacterial strains with *Lb. delbrueckii* ssp. *bulgaricus* H⁺-ATPase defective mutants (Ongol et al., 2007) enhanced the survival of bifidobacteria in fermented milk. In the present study, the concentration of DO was kept at a low level in fermented milk cocultivated with *Lc. lactis* ssp. *lactis* MCC866 during storage, even though the pH was slightly lowered compared with BB536 monocultivation. These results suggest that the level of DO, rather than the moderate postacidification, affected the survival of *B. longum* BB536 during storage, and *Lc. lactis* ssp. *lactis* strain MCC866 improved the survival of bifidobacteria during refrigerated storage, probably by protecting cells from oxidative stress. We also observed survival improvement of other bifidobacteria in cocultivation with *Lc. lactis* ssp. *lactis* MCC866. This was likely due to the difference in ability to grow in skim milk medium by each bifidobacterial strain: *B. longum* BB536 grew well in skim milk medium and so might derive a great benefit from *Lc. lactis* ssp. *lactis* MCC866.

Environmental oxygen is generally scavenged to H₂O via H₂O₂ in an organism without a respiratory chain. However, H₂O₂ is one of the major reactive oxygen species and eventually inhibits bacterial growth (Marty-Teyssset et al., 2000; van de Guchte et al., 2001). To protect from oxidative damage, H₂O₂ is sequentially degraded by enzymes such as catalase or peroxidase in some microorganisms. Contrary to our expectation, the concentration of H₂O₂ was higher in the fermented milk containing bifidobacteria when cocultivated with the *Lc. lactis* ssp. *lactis* MCC866 at 14 d of storage compared with those of monocultivation. Furthermore, H₂O₂ levels subsequently decreased along with increased levels of DO after 21 d of storage in the cocultivating system containing bifidobacteria and *Lc. lactis* ssp. *lactis* MCC866. These results suggest that the fate of environmental oxygen was conversion to H₂O₂. Because accumulation of H₂O₂ only occurred in the cocultivating system but not in fermented milk monocultivated with *Lc. lactis* ssp. *lactis* MCC866, it can be suggested that the imbalance of H₂O₂ and H₂O was caused by the coexistence of *B. longum* BB536 and *Lc. lactis* ssp.

Table 2. Gene expression of *Lactococcus lactis* MCC857 and MCC866 during an 8-h fermentation and refrigerated storage in fermented milk cocultivated with bifidobacteria¹

Gene	Annotation	Target	Fermentation ²	Storage at 10°C		
				0 d	7 d	14 d
<i>noxC</i>	H ₂ O ₂ -forming NADH oxidase	MCC857	1.00 ± 0.07	9.00 ± 1.58	0.92 ± 0.11	0.65 ± 0.10
		MCC866	0.42 ± 0.05**	4.77 ± 0.87*	1.09 ± 0.41	0.78 ± 0.12
<i>noxD</i>	H ₂ O ₂ -forming NADH oxidase	MCC857	1.00 ± 0.40	7.10 ± 1.07	0.03 ± 0.01	0.05 ± 0.01
		MCC866	0.74 ± 0.45	1.74 ± 0.43**	0.12 ± 0.03**	0.05 ± 0.01
<i>noxE</i>	H ₂ O-forming NADH oxidase	MCC857	1.00 ± 0.05	3.41 ± 0.23	1.74 ± 0.02	1.13 ± 0.03
		MCC866	0.10 ± 0.01**	2.29 ± 0.10**	1.59 ± 0.11	1.08 ± 0.08
<i>poxL</i>	Pyruvate oxidase	MCC857	1.00 ± 0.09	51.76 ± 1.78	0.67 ± 0.13	0.85 ± 0.13
		MCC866	0.25 ± 0.01**	12.36 ± 0.88**	0.39 ± 0.09*	0.31 ± 0.05**
<i>glpD</i>	Glycerol-3-phosphate dehydrogenase	MCC857	1.00 ± 0.16	72.67 ± 1.13	0.45 ± 0.12	0.35 ± 0.06
		MCC866	0.32 ± 0.02**	16.02 ± 0.73**	0.72 ± 0.16	0.31 ± 0.07
<i>ahpC</i>	Alkyl hydroperoxide reductase	MCC857	1.00 ± 0.12	1.08 ± 0.22	0.54 ± 0.04	0.34 ± 0.04
		MCC866	0.57 ± 0.06**	1.04 ± 0.09	0.65 ± 0.16	0.77 ± 0.05**
<i>ahpF</i>	Alkyl hydroperoxide reductase	MCC857	1.00 ± 0.06	1.57 ± 0.48	0.8 ± 0.12	0.37 ± 0.05
		MCC866	1.07 ± 0.18	1.69 ± 0.01	1.25 ± 0.20	2.17 ± 0.24**
<i>sodA</i>	Superoxide dismutase	MCC857	1.00 ± 0.12	0.59 ± 0.03	0.71 ± 0.17	0.51 ± 0.14
		MCC866	0.16 ± 0.02**	0.66 ± 0.06	0.54 ± 0.05	0.40 ± 0.06
<i>dpsA</i>	Non-heme iron-binding ferritin	MCC857	1.00 ± 0.12	4.01 ± 0.86	5.14 ± 0.14	4.01 ± 0.17
		MCC866	0.39 ± 0.03**	3.91 ± 0.53	2.66 ± 0.26**	1.29 ± 0.05**
<i>feoB</i>	Fe ²⁺ transport system protein B	MCC857	1.00 ± 0.14	3.94 ± 0.29	0.39 ± 0.03	0.36 ± 0.00
		MCC866	0.95 ± 0.11	6.00 ± 0.43**	4.41 ± 0.38**	5.29 ± 0.70**

¹All values indicate averages of relative expression levels of each gene at different time points against those at 8 h of *Lactococcus lactis* MCC857 in fermented milk cocultivated with *Bifidobacterium longum* BB536 (mean ± SD, triplicate assays).

²Skim milk was fermented for 16 h until reaching pH approximately 4.6. The 8-h fermentation corresponds to an exponential phase with pH approximately 5.0.

* $P < 0.05$, ** $P < 0.01$: significant intergroup difference between *Lc. lactis* ssp. *lactis* MCC857 and *Lc. lactis* ssp. *lactis* MCC866 at each time point.

lactis MCC866. The mechanism is uncertain; however, one possibility is that the activity of H₂O₂ reducer such as alkyl hydroperoxide reductase of *Lc. lactis* ssp. *lactis* MCC866 was suppressed in the presence of *B. longum* BB536, without affecting the activity of NADH oxidase. The activity of NADH peroxidase is generally one-tenth to one-thirtieth of that of NADH oxidase (Miyoshi et al., 2003); therefore, it is expected that even a minor

change in the activity of NADH peroxidase might affect the fate of H₂O₂ in fermented milk. A several-millimolar lethal dose of for H₂O₂ is thought to be needed to affect the bacterial growth dose (Duwat et al., 1995, 1999). However, the concentrations in the cocultivating system of bifidobacteria and *Lc. lactis* ssp. *lactis* MCC866 during storage were dozens of orders of magnitude lower compared with the suggested lethal dose. Doleyres et

Table 3. Gene expression of *Bifidobacterium longum* BB536 in fermented milk with or without *Lactococcus lactis* MCC857 or MCC866 during 8-h fermentation and storage¹

Gene	Annotation	Target	Fermentation ²	Storage at 10°C		
				0 d	7 d	14 d
<i>ahpC</i>	Alkyl hydroperoxide reductase	BB536	1.00 ± 0.17 ^a	1.76 ± 0.37 ^a	76.24 ± 29.9 ^a	501.15 ± 101.46 ^a
		+MCC857	0.20 ± 0.02 ^b	0.95 ± 0.14 ^a	35.71 ± 1.13 ^a	61.66 ± 3.75 ^b
		+MCC866	0.28 ± 0.07 ^b	0.18 ± 0.05 ^b	8.52 ± 0.92 ^b	38.51 ± 1.29 ^c
<i>ahpF</i>	Alkyl hydroperoxide reductase	BB536	1.00 ± 0.60 ^a	0.48 ± 0.14 ^a	1.94 ± 0.18 ^a	11.3 ± 1.85 ^a
		+MCC857	3.58 ± 0.50 ^b	0.22 ± 0.04 ^a	11.22 ± 0.81 ^b	9.53 ± 0.70 ^a
		+MCC866	1.51 ± 0.23 ^a	1.81 ± 0.19 ^b	5.48 ± 1.02 ^c	9.50 ± 0.58 ^a
<i>nox</i>	H ₂ O-forming NADH oxidase	BB536	1.00 ± 0.32 ^a	0.81 ± 0.40 ^a	2.53 ± 0.47 ^a	14.19 ± 0.30 ^a
		+MCC857	3.99 ± 0.55 ^b	0.58 ± 0.31 ^a	18.09 ± 0.68 ^b	14.95 ± 0.98 ^{ab}
		+MCC866	2.75 ± 0.37 ^b	3.61 ± 0.68 ^b	9.31 ± 0.15 ^c	17.09 ± 0.90 ^b

^{a-c}Values with different superscripts indicate significant difference between cultures at each time point for each enzyme ($P < 0.01$).

¹All values indicate averages of relative expression levels of each gene at different time points against those at 8 h of *Bifidobacterium longum* BB536 in fermented milk (mean ± SD, triplicate assays). All expression values indicate relative score defined average score of fermented milk using *B. longum* BB536 at 8 hr as 1.00 (mean ± SD, triplicate assays).

²Skim milk was fermented for 16 h until decreasing pH approximately 4.6. Period of 8 h is an exponential phase at pH approximately 5.0.

al. (2004) reported that type strain of *B. longum* could almost survive in a H_2O_2 concentration of 2,000 ppm (58.82 μM). In the present study, it was found that the viability of *B. longum* BB536 was not affected in the presence of hydrogen peroxide at concentrations up to 100 μM , a level higher than those detected in the media of coculture of *B. longum* BB536 with *Lc. lactis* ssp. *lactis* MCC866 (data not shown). Therefore, we suggest that the differences of H_2O_2 concentration observed in the present study would not affect the survival of bifidobacteria.

One of the main targets for reactive oxygen species is DNA. Presently, DNA degradation was evident in fermented milk containing *B. longum* BB536 in monoculture or coculture with *Lc. lactis* ssp. *lactis* MCC857, but not in those cocultivated with *Lc. lactis* ssp. *lactis* MCC866 after 21 d of storage. Rezaïki et al. (2004) reported the degradation of genomic DNA of *Lc. lactis* ssp. *lactis* after aeration for 48 h. In the present study, the degradation of genomic DNA was observed after 21 d of storage and approximately 1 wk later after the DO was elevated to a near-saturating level in fermented milk containing *B. longum* BB536 in monoculture or coculture with *Lc. lactis* ssp. *lactis* MCC857. Degradation was not associated with cell lysis, as whole microorganisms were collected by centrifugation. Whether the degradation of genomic DNA was the cause or the consequence of the lost viability of bifidobacterial cells in the fermented milk is uncertain, because the partial loss of viability was observed 1 wk earlier than the detection of degradation of genomic DNA for each sample.

Both *Lc. lactis* and *B. longum* are devoid of catalase, a key enzyme for scavenging H_2O_2 , and have to rely on enzymes such as NADH oxidase and NADH peroxidase to scavenge environmental oxygen. Indeed, NADH oxidase and NADH peroxidase function harmonically in preventing bacterial cells from oxidation; NADH oxidase functions in conversion of oxygen to H_2O_2 , and NADH peroxidase is involved in scavenging H_2O_2 (Shimamura et al., 1992). Alkyl hydroperoxide reductase (*ahpC*), which forms 2-component enzymes to convert oxygen to H_2O via H_2O_2 , was expressed at higher levels during storage in *Lc. lactis* ssp. *lactis* MCC 866 compared with *Lc. lactis* ssp. *lactis* MCC857. On the other hand, a significant difference was observed in the level of expression of *ahpC* by *B. longum* BB536 between each cultivating system after 14 d storage: monoculture > coculture with *Lc. lactis* ssp. *lactis* MCC857 > coculture with *Lc. lactis* ssp. *lactis* MCC866. The different levels of *ahpC* expression by *B. longum* BB536 and *Lc. lactis* ssp. *lactis* may indicate that, from the need to scavenge H_2O_2 , *B. longum* BB536 activates its own enzymes such as *ahpC* in monocultivating systems, while

BB536 does not activate them in the *Lc. lactis* ssp. *lactis* cocultivating systems. However, further studies are needed to confirm the results as well as to evaluate the mechanisms. Taken together, these results suggest a key role of *ahpC* in maintaining low DO levels in fermented milk during refrigerated storage.

As shown in Figure 1, supplementation of catalase to *B. longum* BB536 monocultures did not affect the viability of *B. longum* BB536 or the concentration of DO, although the concentration of H_2O_2 was decreased during storage. These results indicated that scavenging of O_2 by *Lc. lactis* ssp. *lactis* MCC866 principally contributed to the improved viability of *B. longum* BB536. We observed no expressional difference of the gene encoding H_2O_2 -forming NADH oxidase between *Lc. lactis* ssp. *lactis* MCC866 and MCC857; however, some differences in activities of their proteins or of other H_2O_2 -producing enzymes might occur.

Previous reports have indicated an influence of dissolved oxygen in yogurt on the viability of bifidobacteria (Talwalkar and Kailasapathy, 2003; Jayamanne and Adams, 2006). Attempts to provide a low concentration of DO environment by cocultivating with *Streptococcus thermophilus* STH450, a strain having a high oxygen uptake ability, have also been reported to enhance the survival of bifidobacteria in fermented milk (Teraguchi et al., 1987). These findings are similar to our findings; however, the advantage of our coculture system is that *Lc. lactis* can grow at low temperatures, which allows this system to be potentially activated even under refrigerated storage conditions.

Concerning iron metabolism, Herve-Jimenez et al. (2009) reported that, in a coculture system of *S. thermophilus* with *Lb. delbrueckii* ssp. *bulgaricus*, the expression of genes potentially encoding iron transporters of *S. thermophilus* decreased, whereas that of iron-chelating dpr (*dps*, DNA binding protein-starved cells, like the peroxide resistance gene) as well as that of the fur (ferric transport regulator protein, *perR*) regulator genes increased. The authors proposed that *S. thermophilus* sets up an adaptive response to H_2O_2 released from *Lb. bulgaricus* by lowering the intracellular iron concentration in the coculture system.

In our study, as compared with *Lc. lactis* ssp. *lactis* MCC866, the expression of the gene encoding Fe^{2+} transport system in *Lc. lactis* ssp. *lactis* MCC857 when cocultivated with *B. longum* BB536 was markedly lower at 14 d, whereas that of *dpsA*, which codes for the iron chelator non-heme iron-binding ferritin, was higher. Because *B. longum* BB536 produces H_2O_2 during its growth in milk (data not shown), we propose that an adaptive H_2O_2 response may occur in *Lc. lactis* ssp. *lactis* MCC857 in response to H_2O_2 generated in the cocultivating system. However, the gene expression

pattern for *Lc. lactis* ssp. *lactis* MCC866 was different from that of *Lc. lactis* ssp. *lactis* MCC857 and was somewhat inconsistent with that found by Herve-Jimenez et al. (2009). The difference of mechanism to reduce H₂O₂ between *Lc. lactis* ssp. *lactis* strains is not clear and needs further study. However, one possibility is that *Lc. lactis* ssp. *lactis* MCC866 has a different H₂O₂ response system because this strain has stronger activity of H₂O₂ scavengers such as alkyl hydroperoxide reductase. Otherwise, taking into account the facts that MCC866 seems to be more active in uptake of free iron and the expression of *dpsA* progressively lessened during storage (Table 2), the intracellular concentration of free ferrous iron might be lower as a result of efficient biological utilization in the bacterial cells.

The extracellular free ferrous ion levels were lower in the fermented milk containing *B. longum* BB536 cocultivated with *Lc. lactis* ssp. *lactis* MCC866 compared with those of monoculture or coculture with *Lc. lactis* ssp. *lactis* MCC857. Ferrous ion plays an important role as a coenzyme of oxidoreductase in vivo. Furthermore, numerous studies have revealed the importance of free intracellular iron in exacerbating oxidative stress, by participating in the Fenton reaction (Storz and Imlay, 1999). The low concentration of free ferrous ion in coculture with *Lc. lactis* ssp. *lactis* MCC866 might consequently protect bifidobacterial cells from strong oxidants such as hydroxyl radical. *Lactococcus lactis* ssp. *lactis* has been reported to have respiratory-chain harboring genes coding for iron-binding proteins (Pedersen et al., 2008). We also observed that expression of genes coding respiratory-chain components such as cytochrome bd were significantly higher in *Lc. lactis* ssp. *lactis* MCC866 compared with MCC857 during refrigerated storage (Supplementary Table 2, available online: <http://www.journalofdairyscience.org>).

In conclusion, the data from *Lc. lactis* ssp. *lactis* MCC866 suggest that bifidobacterial cells are protected from oxygen damage from lowered DO levels in a coculture system. Several genes that participate in protecting cells from active oxygen species and that are expressed at higher levels by this strain during refrigerated storage are thought to be related to the improved survival of bifidobacteria in the cocultivated fermented milk. Other mechanisms may also contribute to the improved viability of bifidobacteria; an improved acid-tolerance of *B. longum* BB536 was observed after fermentation in coculture with *Lc. lactis* ssp. *lactis* MCC866 compared with *B. longum* BB536 monoculture (data not shown). Further studies are needed to reveal the mechanisms such as assessing the activities of enzymes that participated in protection from active oxygen species in the fermented milk.

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