Comparative analysis of an intestinal strain of *Bifidobacterium longum* and a strain of *Bifidobacterium animalis* subspecies *lactis* in Cheddar cheese

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ABSTRACT

Bifidobacteria cultures were incorporated into Cheddar cheeses to conduct a comparative analysis between the commercially available strain *Bifidobacterium animalis* ssp. *lactis* Bb-12 and the wild-type intestinal isolate, *Bifidobacterium longum* DJO10A. They were incorporated as starter adjuncts in separate vats and as a mixed culture, and survival through manufacturing and cheese ripening was assessed. For cheese using only Bb-12, the cells may have grown during cheese manufacture as 133% of the inoculum was incorporated into the cheese, resulting in 8.00 log cfu/g. Counts remained high during ripening showing less than 1 log decrease over a 12-mo period. For cheese using a mixed culture of Bb-12 and DJO10A, both strains were incorporated at much lower levels: 3.02 and 1.11%, respectively. This resulted in cheese with 6.00 and 5.04 log cfu/g for Bb-12 and DJO10A, respectively. Bifidobacteria survival rates were low, most likely due to the moisture of the cheese being below 38%. The Bb-12 demonstrated almost 100% viability during ripening. Numbers of DJO10A started to decline after 2 mo of ripening and dropped below the level of detection (2 log cfu/g) after 4.5 mo of ripening. Neither DJO10A nor Bb-12 fortified cheeses produced detectable amounts of organic acids during ripening other than lactic acid, indicating the lack of detectable metabolic contribution from bifidobacteria during cheese production and ripening such as production of acetic acid. To determine if sublethal stresses could improve the viability of DJO10A, 2 more vats were made, 1 with DJO10A exposed to sublethal acid, cold, and centrifugation stresses, and 1 exposed to none of these stresses. Although stress-primed DJO10A survived cheese manufacture better, as 72.8% were incorporated into the cheese compared with 41.1% of the unprimed, the statistical significance of this difference is unknown. In addition, the difference in moisture levels in the cheese cannot be excluded as

influencing this difference. However, the rate of decline during ripening was similar for both. After 6 mo of ripening, cell counts in cheese were 4.68 and 4.24 log cfu/g for primed and unprimed cultures, respectively. These results suggest that whereas priming bifidobacteria with sublethal stresses before incorporation in a cheese fermentation may improve the number of viable cells that get incorporated into the cheese, it does not affect viability during cheese ripening.

Key words: bifidobacteria, Cheddar cheese, probiotic

INTRODUCTION

Bifidobacteria are gram-positive anaerobic bacteria present as branched or irregularly shaped rods that can be isolated from several sources including feces, sewage, and the oral cavity (Hartemink et al., 1996). The natural habitat of bifidobacteria is the intestines of humans, animals, and insects. In this habitat, they are considered to be beneficial to the host (O'Sullivan, 2001). Analysis of human fecal samples has shown that bifidobacteria counts can reach levels of 10^9 to 10^{10} cfu/g (Benno et al., 1989). They can be distinguished from other intestinal bacterium by the presence of fructose-6-phosphate phosphoketolase (**F6PPK**), a key enzyme of the "bifidus pathway" (Tannock, 1999).

Interest in bifidobacteria has grown significantly primarily due to numerous potential health claims, which has caused their presence to be associated with a healthy microbiota. In recent years, there has been considerable interest in incorporating these potentially health-promoting bacteria into functional food products (Rezzonico et al., 2007). Dairy-related bifidobacteria are presently used in a variety of probiotic products, including fermented milks, yogurt, ice cream, and some cheeses (Blanchette et al., 1995). Though many of the health claims have not been scientifically validated, some studies suggest that consumption of bifidobacteria may impart health benefits. To produce a beneficial effect, the cells should be viable and present in high numbers in the food product at the time of consumption. In Japan, the Fermented Milks and Lactic Acid Bacteria Association has recommended at least 10^7 viable cells

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per gram of product (Mc Brearty et al., 2001). Studies suggest that the potential health claims related to bifidobacteria are strain dependent (Roy, 2005). It also is important that the strains survive the manufacturing process and shelf life of the product.

When bifidobacteria are incorporated into dairy products as probiotics, they are subjected to manufacturing stresses, which include oxygen exposure, mechanical agitation, temperature gradients, and varying pH ranges. The survival of bifidobacteria in the finished product also depends on the strain of bifidobacteria, the storage temperature, and preservation methods (Blanchette et al., 1995).

Maintaining the viability of bifidobacteria in dairy products for extended time periods at refrigerated temperatures still remains a problem for many fermented products (Corbo et al., 2001). The production of cheese fortified with bifidobacteria has been proposed as a suitable and promising alternative to fermented milks (Stanton et al., 1998). By incorporating the organisms into cheese, the viability of bifidobacteria may be improved as the pH, lipid content, oxygen level, and storage conditions are favorable to the long-term survival of bifidobacteria during processing and ripening (Dinakar and Mistry, 1994; Stanton et al., 1998; Boylston et al., 2004). Furthermore, during ripening, the cheese matrix becomes almost oxygen free, which favors the survival of anaerobic microorganisms such as bifidobacteria (van den Tempel et al., 2002).

Bifidobacteria from human intestinal origin are frequently proposed to be more suitable for probiotic applications. However, they are sensitive to stress, and bifidobacteria from animal origins, specifically strains of *Bifidobacterium animalis*, have been reported to survive processing and storage in many dairy foods (Gomes and Malcata, 1999; Corbo et al., 2001). However, clinical studies have indicated they do not remain in the gut very long after ingestion ceases (O'Sullivan, 2006). It may be possible to develop new technologies to extend the survival of bifidobacteria in dairy foods (Ibrahim and Carr, 2006). Processing and storage conditions may activate stress-related genes, which in turn could affect the ability of the cultures to survive. It also may be possible to activate stress-related genes when growing cultures to improve the survivability of the cultures through manufacturing and product storage.

This study assessed the survival and metabolic contributions of a human intestine-adapted strain of bifidobacteria (*Bifidobacterium longum* DJO10A) and a commercially available strain (*B. animalis* ssp. *lactis* Bb-12) in Cheddar cheese. *Bifidobacterium longum* is one of the prominent species of *Bifidobacterium* in humans. The fact that it is a human-origin species has led many researchers to believe *B. longum* is a good

choice to provide potential health benefits to humans (Ishibashi et al., 1997). Results from numerous studies suggest that *B. longum* provides potential health benefits ranging from cholesterol reduction (Xiao et al., 2003) to cancer prevention (Moschner et al., 2004). *Bifidobacterium animalis* can be found in the large intestines of most mammals, which includes humans who have consumed foods containing *B. animalis* (Mitsuoka, 1969; Kim et al., 2009). It was originally isolated from animals by Mitsuoka (1969) and named *B. longum* ssp. *animalis* and distinguished from *B. longum* on the basis of melezitose and mannose fermentation. It was reclassified to the species rank as *B. animalis* based on DNA homology work performed by Scardovi et al. (1971; Mitsuoka, 1984). *Bifidobacterium animalis* ssp. *lactis* Bb-12 is frequently used as a probiotic culture in dairy food manufacturing mainly because of its reported ability to be both oxygen and acid tolerant, giving it a distinct advantage for surviving processing and storage conditions.

In this study, a comparative analysis was conducted on the performance of *B. longum* DJO10A and *B. animalis* ssp. *lactis* Bb-12 when incorporated into Cheddar cheese. The effect of sublethal stresses on the survival of *B. longum* DJO10A was also assessed.

MATERIALS AND METHODS

Bacteria, Media, and Culture Conditions

Bifidobacterium longum DJO10A (Lee et al., 2008) and *B. animalis* ssp. *lactis* Bb-12 (Chr. Hansen Inc., Horsholm, Denmark) were incorporated into Cheddar cheese for this study. The lactic acid starter culture used for cheese making was Danisco M30, a blend of *Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* (Danisco, Madison, WI). Both Bb-12 and DJO10A were cultured under anaerobic conditions in bifidobacteria low-iron media with added iron (**BLIM + Fe**) at 37°C. The BLIM + Fe medium contained 2.0% proteose peptone, 0.15% K₂HPO₄, 0.5% glucose, 0.15% MgSO₄·7H₂O, 1 mM FeCl₃, and 100 mM PIPES. The solution pH was adjusted to 7.0 with 9 M NaOH. Bifidobacteria cultures were enumerated on de Man, Rogosa, and Sharpe (**MRS**; Difco, BD, Franklin Lakes, NJ) plates for the initial viability study and on bifidobacteria iodoacetate selective medium (**BIM-25**; Muñoa and Pares, 1988) for cheese samples.

Preparation of Bifidobacteria Cultures for Cheese

Frozen stock cultures for Bb-12 and DJO10A were inoculated into 10-mL tubes of BLIM + Fe and incubated overnight under anaerobic conditions at 37°C. The fol-

lowing day, 10-mL aliquots were aseptically transferred into separate Pyrex flasks containing 990 mL of BLIM + Fe media and incubated under anaerobic conditions at 37°C overnight. Each 1-L flask was poured into 2 separate 500-mL centrifuge tubes for centrifugation at 3,400 rpm for 20 min at 19°C in a Beckman Coulter (Brea, CA) Allegra 6KR centrifuge fitted with a GH 3–8 rotor. The pellets were transferred into sterile cups and stored under anaerobic conditions at 4°C. After 48 h, the pellets were removed from storage conditions, suspended in peptone water, enumerated on MRS agar, and delivered to cheese vats along with the lactic acid starter cultures. The unprimed cultures for the priming experiment were prepared as mentioned except no centrifugation or cold storage occurred. The stress-primed cultures were prepared as described above except the BLIM + Fe media was acidified to pH 5.75.

Cheddar Cheese Manufacture

Cheddar cheese was manufactured at the University of Minnesota's Department of Food Science and Nutrition pilot plant. Raw whole milk was HTST pasteurized at 73°C for 16 s. Cheese was prepared in separate 114-kg manually stirred vats. Each vat was filled with 114 kg of milk and inoculated with 27 mL of Danisco (Copenhagen, Denmark) M30 starter culture. Starter was added to 1 kg of steamed and cooled milk and held at 37°C for 2 h before addition to the vats. Bifidobacteria cultures were added to the vats at the same time as the starter culture. The vats were allowed to ripen for approximately 1 h before addition of 11.25 g of Chymax double-strength rennet (Chr. Hansen Inc., Milwaukee, WI). The coagulum reached the desired firmness in approximately 30 min and the curd was cut with 3/8-in wire knives. After a 10-min healing period, the temperature of the curd and whey mixture was raised from 31 to 38°C over 30 min and maintained at this temperature until the curd was cooked, approximately 30 additional min. The whey was drained in multiple draws, with the curd being continually stirred. At a pH of 5.6, the curd was salted by adding 280 g of salt to curd in the vats and manually mixing. The curd was hooped and pressed for 90 min at 40 psi. The blocks were then cut into multiple segments, which were packed in vacuum shrink bags and vacuum sealed by a Multivac (Koch, Kansas City, MO) and stored at 7.2°C.

Organic Acid Measurements in Cheese

Cheese samples were tested to determine citric, lactic, acetic, formic, succinic, and propionic acids using a Waters 2695 HPLC with an ion exchange analytical column (Aminex HPX-87H; 300 × 7.8 mm, Bio-Rad

Laboratories, Richmond, CA) fitted with a Waters 2487 dual-wavelength absorbance detector. The mobile phase was 0.009 *N* H₂SO₄ in water, and organic acid standards were used at concentrations of 0.005, 0.01, 0.03, 0.05, and 0.10% to monitor retention times. One standard was run following every 10 samples throughout. The HPLC conditions were 40°C, a flow rate of 0.6 mL/min, an injection volume of 20 µL, UV detection at 210 nm, and a run time of approximately 50 min. The samples were prepared by placing 3 g of cheese in a blender with 0.9 *N* H₂SO₄ in Milli-Q water for a total weight of 100 g. The mixture was blended for 2 min, filtered, and injected onto the column. The limit of detection for each acid was 0.17% with the variance being ±0.03.

Fructose-6-Phosphate Phosphoketolase Assay

Cells were grown in MRS broth at 37°C for 12 h, and 1.5 mL was centrifuged for 5 min at 1,350 rpm in a Hermle Labnet (Edison, NJ) Z 233 MK centrifuge with rotor model C-0230–2A to pellet the cells. Cells were washed twice with 1.0 mL of 0.05 *M* phosphate buffer (pH 6.5) containing L-cysteine·HCl (500 µg/mL). Pellets were resuspended in 50 µL of the same solution. Acid-washed glass beads were added at 50% of the volume before cell disruption by beadbeating for 30 s in a Biospec Products (Bartlesville, OK) Mini-Beadbeater and samples were placed on ice immediately after treatment. Aliquots (50 µL) of NaF 6 mg/mL containing K idoacetate (10 mg/mL) and 50 µL of fructose-6-phosphate (80 mg/mL) were added to the cells. The cell mixture was then incubated at 37°C for 30 min. After incubation, 300 µL of hydroxylamine·HCl (13.9 g/100 mL) was added to the cell mixture, which was then incubated at room temperature for 10 min. After room-temperature incubation, 200 µL of 15% TCA, 200 µL of 4 *M* HCl, and 200 µL of FeCl₃·6H₂O (5% wt/vol in 0.1 *M* HCl) was added to the cell mixture. A red to violet color was indicative of F6PPK activity (Bezkorovainy and Miller-Catchpole, 1989).

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was carried out with a Bio-Rad CHEF-DR III series PFGE machine, and all solutions were prepared by Bio-Rad for PFGE. Samples were prepared by cultivating bifidobacteria in BLIM + Fe media and incubating under anaerobic conditions at 37°C until an optical density at 600 nm reached 0.84. Two percent CleanCut agarose solution (Bio-Rad) was melted by heating to 80°C in an incubator for 30 min. One milliliter of bifidobacteria culture was transferred to a 1.5-mL tube for each cul-

Figure 1. Survival of *Bifidobacterium animalis* ssp. *lactis* Bb-12 after 48 h of storage at different temperatures; prestorage counts were 9.43 log cfu/mL. The black columns represent the prestorage counts and the white columns represent the counts after storage. The standard deviation between duplicate measurements is included.

ture tested and centrifuged at max speed for 5 min at 4°C. Pellets were resuspended with 150 μ L of Bio-Rad cell suspension buffer and heated to 50°C before mixing with 150 μ L of 2% CleanCut agarose. The mixture was kept at 50°C and transferred to Bio-Rad plug molds for each culture tested. Plugs were then solidified by cooling at 4°C for 15 min. The solidified agarose plugs were placed into 15-mL Falcon tubes containing 1 mL of lysozyme solution and incubated at 37°C for 2.5 h with gentle agitation. Lysozyme solution was removed and the plugs were washed with molecular-grade water before addition of proteinase K solution and incubated at 37°C overnight without agitation. Proteinase K solution was poured off through a screen cap and the plugs were washed 4 times using 1 $\times$ wash buffer diluted with molecular water for 1 h per washing at room temperature with gentle agitation. To inactivate proteinase K, the plugs were washed with 1 $\times$ wash buffer containing 1 mM phenylmethylsulfonyl fluoride during the second washing. The plugs were then stored at 4°C in 1 $\times$ wash buffer. The restriction enzyme *Xba*I (New England Biolabs Inc., Ipswich, MA) was then added (50 U per 100- μ L plug) and samples were incubated overnight at 37°C. After overnight digestion, the buffer was removed and samples were incubated in 1 mL of 1 $\times$ wash buffer for 30 min with gentle agitation at room temperature. The 1 $\times$ wash buffer was decanted and samples were equilibrated in fresh 0.5 $\times$ Tris-borate-EDTA buffer. The plugs were then added to the wells of the CHEF-DR III series PFGE machine. The plugs were loaded directly into the wells of a 1% PFGE certified agarose gel and sealed with melted 1% agarose. Approximately

1/3 of each sample plug was loaded into separate wells, along with a lambda DNA size marker. The PFGE conditions were set to 6 V/cm, included angle of 120°, initial switch time of 1.0, final switch time 15.0, run time of 18 h, and 0.5 $\times$ Tris-borate-EDTA running buffer at 14°C. Gels were stained using molecular water containing 1 μ g/mL of ethidium bromide for 30 min. Gels were destained with molecular-grade water for 30 min. Photographs of the gel were taken using the UVP GelDoc-It imaging system (BioImaging System, Upland, CA).

Viable Colony Counts of Bifidobacteria in Cheddar Cheese

Samples were enumerated by serial dilution and viable plate counts. Viable counts of bifidobacteria for cheese samples were determined using BIM-25 selective plating. Cheese (10 g) was diluted in 90 mL of peptone water (0.1%) in sterile sample bags. The samples were then macerated in a stomacher for 5 min at high speed to obtain a slurry for the first dilution, and subsequent serial dilutions were made in 0.1% peptone water solution before spread plating on BIM-25 plates. Plates were incubated under anaerobic conditions for 48 to 72 h at 37°C using gas pack anaerobic systems (Becton-Dickinson Microbiology Systems, Cockeysville, MD).

RESULTS

Optimization of Bifidobacteria Culture Preparation for Cheese Production

To facilitate Cheddar cheese production containing bifidobacteria, the cultures needed to be grown from stock, separated from broth and stored before trans-

Figure 2. Survival of *Bifidobacterium longum* DJO10A after 48 h of storage at different temperatures; prestorage counts were 8.91 log cfu/mL. The black columns represent the prestorage counts and the white columns represent the counts after storage. The standard deviation between duplicate measurements is included.

Figure 3. Survival of *Bifidobacterium animalis* ssp. *lactis* Bb-12 during Cheddar cheese ripening. Cheese milk was inoculated with Bb-12 to obtain an initial concentration of 6.95 log cfu/mL. The standard deviation between duplicate measurements is included.

porting to the pilot plant facility. An initial study was conducted to evaluate storage temperatures on viability. Both Bb-12 and DJO10A were cultured and harvested by centrifugation as described in the material and methods. Enumerations before centrifugation and storage yielded 9.43 and 8.91 log cfu/mL for Bb-12 and DJO10A, respectively. The pellets were transferred in sterile cups and stored under anaerobic conditions at -20 , 4 , 21 , and 37°C for 48 h.

The pellets were then resuspended in peptone water (0.1%) and enumerated on MRS agar plates to determine which conditions yielded the greatest amount of viable cultures. Cell enumerations for Bb-12 after

centrifugation and storage yielded 8.98, 9.37, 9.32, and 7.95 log cfu/mL at storage conditions of -20 , 4 , 21 , and 37°C , respectively (Figure 1). Enumerations for DJO10A yielded 7.11, 8.19, 8.13, and 6.45 log cfu/mL at storage conditions of -20 , 4 , 21 , and 37°C , respectively (Figure 2). Given 4 and 21°C maintained the most viable cells, 4°C was chosen as the storage temperature for cultures used in subsequent cheese making.

Production of Cheddar Cheese Including Bb-12

One control vat with only the starter culture (as a control cheese) and 1 vat containing Bb-12 were manufactured. The vat containing Bb-12 was inoculated with 1,000 mL of Bb-12 and resulted in 6.95 log cfu/mL present in the cheese milk. Survival of Bb-12 was monitored throughout ripening. The Bb-12 counts on selective agar plates under anaerobic conditions show that Bb-12 was successfully incorporated into cheese in high numbers (Figure 3). The initial counts for Bb-12 determined shortly after cheese-making were 8.0 log cfu/g of cheese. During ripening, Bb-12 numbers decreased less than 1 log over a 12-mo period.

Production of Cheddar Cheese Including a Mixture of Bb-12 and DJO10A

A control vat with only the starter culture and 1 vat containing a blend of Bb-12 and DJO10A were manufactured. Both Bb-12 and DJO10A were incorpo-

Figure 4. Bifidobacteria colonies isolated from Cheddar cheese. The photograph shows a mixture of *Bifidobacterium longum* DJO10A and *Bifidobacterium animalis* ssp. *lactis* Bb-12 colonies on BIM-25 selective media. (A) Larger (white) colonies with DNA fingerprints that match DJO10A. (B) Smaller (purple) colonies with DNA fingerprints that match Bb-12. Color version available in the online PDF.

rated at 6.60 and 6.08 log cfu/mL, respectively. When enumerating cheese samples on BIM-25 selective media plates, 2 distinct colony morphologies developed. Numerous smaller colonies that were purple in color developed on the selective plates, as did larger whiter colonies with pink centers (Figure 4). Multiple colonies exhibiting each type of morphology were confirmed as bifidobacteria by using the F6PPK enzyme testing. Analysis using PFGE to compare DNA fingerprints of these colonies with those from frozen stock cultures confirmed the purple isolates as Bb-12 and the larger whiter colonies as DJO10A (Figure 5). Cheese was enumerated over time with the initial counts for Bb-12 being 6.00 log cfu/g and 5.04 log cfu/g for DJO10A. During ripening the DJO10A colonies decreased below the limit of detection at approximately 4.5 mo. The viable count of Bb-12 cultures remained steady through the testing period (Figure 6).

Metabolic Contribution of Bifidobacteria

The change in organic acid development was determined using HPLC. Samples were tested early in the shelf life and then tested again 90 d later to determine if significant production of organic acids occurred during storage (Table 1). The HPLC analysis showed no detectable production of acetic, succinic, formic, or propionic acids for any of the samples tested. The control 1 and Bb-12 cheeses revealed the level of citric acid increasing over 90 d ripening from 0.18 to 0.30%, and 0.18 to 0.23%, respectively. Lactic acid was initially 1.60% and increased to 1.74% over 90 d for the control cheese, but held steady for the Bb-12 cheese.

Although the control 2 cheese did not show any significant increase in citric acid over 90 d of ripening, the Bb-12/DJO10A cheese may have increased slightly from 0.24 to 0.31%. Lactic acid increased over 90 d for control 2, from 1.49 to 1.65%, but showed no increase for the Bb-12/DJO10A cheese.

Effect of Priming DJO10A with Sublethal Stresses on Its Survival During Cheese Manufacture and Storage

Two vats of Cheddar cheese were made incorporating DJO10A grown under stress-primed and unprimed conditions. The unprimed vat contained DJO10A cultures grown in buffered medium at pH 7.0 and exposed to no centrifugation or cold shock stresses. The primed vat contained DJO10A cultures that were grown in BLIM + Fe media at pH 5.75 and harvested by centrifugation with pellets stored at 4°C until cheese making. On the morning of cheese making, the anaerobic jars containing DJO10A primed and DJO10A unprimed cultures were transported to the University of Minnesota pilot plant.

Figure 5. Pulsed-field gel electrophoresis-generated DNA fingerprints of *Bifidobacterium animalis* ssp. *lactis* Bb-12 and *Bifidobacterium longum* DJO10A. Lane 1 = DNA fingerprint of the Bb-12 frozen stock culture; lane 2 = DNA fingerprint of culture from the smaller purple colony from the mixed strain inoculation; lane 3 = DNA fingerprint of the DJO10A frozen stock culture; lane 4 = DNA fingerprint of culture from the larger white colony with pink center.

The primed pellet was removed from the anaerobic jar and resuspended in 1,000 mL of peptone water. The cultures were enumerated before inoculating the cheese vats to determine if viability was affected by centrifugation or storage or a combination of centrifugation and storage at refrigerated conditions.

Cultures were poured directly into the cheese-making vats at the same time as the starter cultures. The unprimed vat was inoculated at 5.40 log cfu/mL of cheese milk. Milk was concentrated from 114 kg to yield 13.6 kg of cheese. If all of the added bifidobacteria went into the cheese, 6.30 log cfu/g should be detectable in the finished cheese. However, just 5.93 log cfu/g was

Figure 6. Survival of *Bifidobacterium longum* DJO10A and *Bifidobacterium animalis* ssp. *lactis* Bb-12 cultures during Cheddar cheese ripening. Cheese milk was coinoculated with Bb-12 and DJO10A to obtain an initial concentration of 6.60 and 6.08 log cfu/mL, respectively: Bb-12 (■); and DJO10A (▲). The standard deviation between duplicate measurements is included.

incorporated in the cheese (Figure 7), indicating that 41.1% of the inoculum ended up in the finished cheese. The primed vat was inoculated at 5.80 log cfu/mL of milk. If all of the bifidobacteria went into the cheese, final counts should have been 6.72 log cfu/g of cheese. Enumerations on primed cheese directly after cheese-making yielded 6.59 cfu/g of cheese indicating that 72.8% of the inoculum ended up in the finished cheese. After 6 mo of ripening, cell counts were 4.24 and 4.68 log cfu/g for unprimed DJO10A and primed DJO10A, respectively. The decline in counts during cheese ripening for both unprimed and primed cultures showed a similar rate of decline. Unprimed DJO10A declined approximately 1.65 logs, whereas the primed DJO10A declined approximately 1.95 logs during ripening through 24 wk. The DJO10A unprimed and DJO10A primed cheeses both showed drops in citric acid levels to below the level of detection after 90 d of ripening (Table 1). However, lactic acid increased during storage, from 0.80 to 1.15%, and from 0.71 to 1.37%, respectively.

DISCUSSION

Bifidobacterium animalis ssp. *lactis* Bb-12 was robust and survived Cheddar cheese manufacture and storage well. The colony count remained high throughout the storage period, dropping less than 1 log during 1 yr of ripening. These results are consistent with previous studies showing good survival of bifidobacteria in cheese (Dinakar and Mistry, 1994; McBrearty et al., 2001).

Organic acid analysis for all cheeses during ripening revealed minimal production of any organic acids ex-

cept lactic acid (Table 1). The control cheese and Bb-12 cheese showed slight increases in the level of citric acid after 90 d of storage. The reasons for the increases in citric acid for these treatments are not known. Concentrations of citric acid during ripening can be affected by Ca, P, and lactose levels in the cheese (Upreti et al., 2006). Citric acid also may be formed by fermentation of glucose with the aid of certain molds.

Levels for all other organic acids did not reach 0.17%, which was the minimum detectable level. The amounts of organic acid produced by the cultures during storage, especially acetic acid, could have major implications on finished product acceptance. Perceptible amounts of acetic acid could give the cheese a sour off flavor, uncharacteristic of Cheddar cheese. Previous research has indicated that detectable amounts of acetic acid may be produced during aging and storage when certain species of bifidobacteria are incorporated into cheese (Daigle et al., 1999). Acid levels for bifidobacteria-fortified cheese were comparable to those of control cheese during testing. The data from our study suggest that a mixed strain inoculation using DJO10A and Bb-12 resulted in no production of acetic acid during cheese manufacture or during ripening (Table 1). This suggests that bifidobacteria are not metabolically active during cheese manufacture and storage. These cultures may be acceptable for fortifying cheese without adversely affecting the sensorial properties.

During ripening of the mixed-strain vat, DJO10A-viable cells dropped below the limit of detection after 15 wk of storage. However Bb-12 cultures held steady through the testing period with the final count being

Table 1. Organic acid profiles of the different Cheddar cheeses at 2 different time points expressed as a percentage^{1,2}

Cheese ³	Age, d	Citric acid	Succinic acid	Lactic acid	Formic acid	Acetic acid	Propionic acid
First point							
Control 1	15	0.18	<0.17	1.60	<0.17	<0.17	<0.17
Bb-12	15	0.18	<0.17	1.62	<0.17	<0.17	<0.17
Control 2	90	0.20	<0.17	1.49	<0.17	<0.17	<0.17
Bb-12 + DJO10A	90	0.24	<0.17	1.44	<0.17	<0.17	<0.17
DJO10A control	4	0.20	<0.17	0.80	<0.17	<0.17	<0.17
DJO10A primed	4	0.19	<0.17	0.71	<0.17	<0.17	<0.17
Second point							
Control 1	105	0.30	<0.17	1.74	<0.17	<0.17	<0.17
Bb-12	105	0.23	<0.17	1.62	<0.17	<0.17	<0.17
Control 2	180	0.23	<0.17	1.65	<0.17	<0.17	<0.17
Bb-12 + DJO10A	180	0.31	<0.17	1.43	<0.17	<0.17	<0.17
DJO10A control	94	<0.17	<0.17	1.15	<0.17	<0.17	<0.17
DJO10A primed	94	<0.17	<0.17	1.37	<0.17	<0.17	<0.17

¹Calculated on a weight basis.²All values contain a variance of $\pm 0.3\%$.³DJO10A = *Bifidobacterium longum* DJO10A; Bb-12 = *Bifidobacterium animalis* ssp. *lactis* Bb-12.

approximately 6 log cfu/g. After 12 wk of ripening small segments of cheese for analysis were taken out of vacuum-packed bags and then the bags were quickly resealed. Prior to 12-wk ripening, all cheese to be enumerated was acquired from unopened vacuum pouches. This may account for the rapid decline of DJO10A cultures after 16 wk of storage as intestinal bifidobacteria strains are less aero-tolerant than are strains of *B. animalis* ssp. *lactis* such as Bb-12.

Both DJO10A unprimed and DJO10A primed cultures were successfully incorporated into Cheddar cheese (Figure 7). However, only 41.1% of the unprimed cultures added to the vat were incorporated into cheese, whereas 72.8% of the primed cultures added to the vat were incorporated into cheese. Assuming the loss of cells in the whey was similar for both vats, the data suggest that the primed cultures survived cheese manufacturing in greater numbers. The DJO10A cells have been cultured in BLIM + Fe media and would not be able to

adapt to growth in milk before the lactic acid starters produce lactic acid at significant rates and drop the pH quickly. The DJO10A cells most likely expend their metabolic capabilities while coping with the stress from acid shock. Unprimed cultures declined 1.65 log, and primed cultures declined 1.95 log, indicating not much difference occurred in the rate of decline in cell numbers during ripening between the 2 treatments. The Code of Federal Regulations 133.113 states the legal maximum for moisture in Cheddar cheese is 39%. Composition analysis for the primed cheese revealed that moisture was 41.67%, which is quite high for Cheddar cheese, compared with 38.18% for the unprimed cheese. After the whey had been drained for the unprimed cheese, the drop in pH was rapid and only 30 min passed from whey drain to salting (Table 2). The pH for salting was reached without having sufficient time for the whey to be expelled from the curd. The increased moisture in the primed cheese may be a contributing factor for the

Table 2. Composition of Cheddar cheeses¹

Cheese ²	pH	Moisture ³	Salt ⁴	Fat ⁵	Protein ⁶
Control	5.15	40.69	0.92	31.52	23.31
Bb-12	5.20	40.39	1.03	31.59	23.31
Control	5.35	37.73	1.15	34.39	22.87
Bb-12 + DJO10A	5.37	37.73	1.20	34.39	22.87
DJO10A control	5.34	38.18	1.44	31.43	24.97
DJO10A primed	5.38	41.67	1.49	29.54	23.37

¹Expressed as a weight percentage.²DJO10A = *Bifidobacterium longum* DJO10A; Bb-12 = *Bifidobacterium animalis* ssp. *lactis* Bb-12.³Moisture values contain a variance of $\pm 0.3\%$.⁴Salts values contain a variance of $\pm 0.1\%$.⁵Fat values contain a variance of $\pm 0.5\%$.⁶Protein values contain a variance $\pm 0.5\%$.

Figure 7. Survival of *Bifidobacterium longum* DJO10A unprimed and DJO10A primed cultures during Cheddar cheese ripening. Cheese milk in separate vats was inoculated with DJO10A unprimed and DJO10A primed cultures to obtain an initial concentration of 5.40 and 5.80 log cfu/mL, respectively: DJO10A unprimed (■); and DJO10A primed (▲). The standard deviation between duplicate measurements is included.

higher incorporation levels of bifidobacteria into the cheese.

Adding bifidobacteria cultures at the same time as starter cultures appears to be an effective method of incorporating bifidobacteria into Cheddar cheese. Commercial Bb-12 probiotic cultures can be included into cheese with lactic acid starters and remain viable in high numbers for more than 1 yr. The DJO10A wild-type strain can be added with starter cultures and remain viable through at least 6 mo of storage. Priming with sublethal stresses may have influenced the ability of bifidobacteria to survive cheese manufacture in 1 cheese-making experiment, but had no detectable effect on survival during ripening. Although a larger percentage of the inoculum for DJO10A-primed cells was incorporated into cheese when compared with unprimed cells, it cannot be concluded that this difference was solely due to the priming because a difference in moisture levels was observed between both batches of cheese. The survival rate of DJO10A cells during ripening was consistent with and without priming. More repetitions of the priming experiment would help determine if the increased survival of primed cultures during cheese manufacture was a real difference or just due to variations in the moisture levels of the cheese.

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