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Monitoring survival of *Lactobacillus casei* ATCC 393 in probiotic yogurts using an efficient molecular tool

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ABSTRACT

The aim of the present study was to monitor the survival of the probiotic strain *Lactobacillus casei* ATCC 393 during refrigerated storage of natural regular yogurts compared with *Lactobacillus delbrueckii* ssp. *bulgaricus*. Both free and immobilized cells on supports of high industrial interest, such as fruits and oat pieces, were tested. Microbiological and strain-specific multiplex PCR analysis showed that both free and immobilized *Lb. casei* ATCC 393 were detected in the novel products at levels required to confer a probiotic effect (at least 6 log cfu/g) for longer periods than required by the dairy industry (≥30 d) during storage at 4°C. In contrast, the viable bacterial density of *Lb. delbrueckii* ssp. *bulgaricus* decreased to levels <6 log cfu/g after 14 d of cold storage. Of note, the final pH of all products was 4.2 to 4.3. Acid resistance or cold tolerance of *Lb. casei* ATCC 393 apparently allows for increased survival compared with *Lb. delbrueckii* ssp. *bulgaricus* in these yogurt formulations.
Key words: multiplex PCR, *Lactobacillus delbrueckii* ssp. *bulgaricus*, fruit, oat

INTRODUCTION

Currently, interest is increasing in developing novel foods containing probiotic microorganisms, such as bifidobacteria and lactic acid bacteria (**LAB**). Such functional foods demonstrate great potential in promoting human health. Maintenance of intestinal microbial homeostasis, prevention of pathogenic infections, stabilization of the gastrointestinal (**GI**) barrier function, and production of anticancer and antimutagenic compounds (Choi et al., 2006; Boirivant and Strober, 2007; Saulnier et al., 2009) are included among the beneficial effects of probiotic-based foods, mainly yogurt and other dairy products. Thus, a probiotic-rich diet is linked to the prevention and potential treatment of several severe digestive disorders, such as inflammatory bowel disease (Boirivant and Strober, 2007) and colorectal cancer (Fotiadis et al., 2008).

To deliver health benefits, probiotic products need to contain an adequate amount of live bacteria (at least 10^6 to 10^7 cfu/g; Oliveira et al., 2002; Boylston et al., 2004) able to survive the acidic conditions of the upper GI tract and proliferate in the intestine, a requirement that is not always fulfilled (Vinderola et al., 2002; Boylston et al., 2004). In general, the food industry has adopted the recommended level of 10^6 cfu/g of probiotic bacteria at the time of consumption. Thus, a daily intake of at least 10^8 to 10^9 viable cells, which could be achieved by consumption of at least 100 g of probiotic food, has been suggested as the minimum intake to provide a probiotic effect (Boylston et al., 2004). However, monitoring the survival of the probiotic cultures in foods is hampered by the lack of accurate, reliable, convenient, and sensitive methods of identification, with the ability to distinguish the strains of interest among other closely related microorganisms present in the products.

It has been established that cell immobilization enhances the viability of cultures (Mattila-Sandholm et al., 2002; Kourkoutas et al., 2005, 2006), probably because of the protective microenvironment formed. Many studies have focused on immobilization of probiotic bacteria in various supports, such as starch (Mattila-Sandholm et al., 2002), fruit pieces (Kourkoutas et al., 2005, 2006; Kopsahelis et al., 2007), casein (Dimitrellou et al., 2008, 2009), and wheat grains (Bosnea et al., 2009). These efforts were aimed at stabilization of cells and formulation of new types of foods fortified with immobilized health-promoting bacteria that are only released upon reaching the human gut. Cereals, such as oat pieces, as well as fruits, are expected to deliver the immobilized probiotic LAB to the human gut when used as immobilization supports (Chen et al., 2005). In addition, because they contain potential prebiotic com-

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pounds, their potential functional properties should be explored (Charalampopoulos et al., 2003).

Among LAB, *Lactobacillus casei* ATCC 393 has been extensively incorporated into food products to confer probiotic properties (Kourkoutas et al., 2005, 2006; Li et al., 2009), and *Lactobacillus delbrueckii* ssp. *bulgaricus* is widely used in yogurt production. Recently, new methods based on multiplex PCR for rapid detection and identification of *Lb. casei* ATCC 393 and *Lb. delbrueckii* ssp. *bulgaricus* in food products have been demonstrated (Karapetsas et al., 2010; Nikolaou et al., 2011).

Hence, the aim of the present study was to monitor the survival of the probiotic strain *Lb. casei* ATCC 393 during refrigerated storage of yogurts compared with *Lb. delbrueckii* ssp. *bulgaricus*, which is widely used in production of commercial yogurts. Both free and immobilized cells on supports of high industrial interest, such as fruits and oat pieces, were tested. Maintenance of probiotic cell viability is the most important factor in the characterization of a product as probiotic.

MATERIALS AND METHODS

Starter and Probiotic Cultures

Probiotic cultures of *Lb. casei* ATCC 393 (DSMZ, Braunschweig, Germany) and *Lb. delbrueckii* ssp. *bulgaricus* LB-12 (Chr. Hansen, Athens, Greece) were grown at 37°C for 72 h on de Man, Rogosa, and Sharpe (**MRS**) broth (Fluka, Buchs, Switzerland). Pressed wet weight cells (~0.5–1.0 g of dry weight) were prepared and used directly for cell immobilization and production of probiotic yogurt. A starter culture of *Streptococcus salivarius* ssp. *thermophilus* (Chr. Hansen) was grown at 37°C for 24 h in pasteurized milk.

Cell Immobilization

Cell immobilization was carried out as described in a previous study (Kourkoutas et al., 2005) with some modifications. In brief, 100 g of the immobilization support (strawberry, banana, or oat pieces cut in 1-cm^3 pieces) was introduced in 2-L cell cultures of *Lb. casei* ATCC 393 or *Lb. delbrueckii* ssp. *bulgaricus* LB-12. The mixture was allowed to ferment at 37°C for 48 h without agitation. When immobilization was complete, the fermented liquid was decanted and the immobilized biocatalyst was washed twice with pasteurized milk.

Probiotic Yogurt Production

Yogurts were produced by inoculating pasteurized bovine milk (100 mL) with a yogurt culture consisting of 1 g (wet weight) of *Lb. delbrueckii* ssp. *bulgaricus* and 1 g (wet weight) of *Strep. salivarius* ssp. *thermophilus*. Additional adjunct cultures of immobilized *Lb. casei* ATCC 393 (10 g wet weight) or immobilized *Lb. delbrueckii* ssp. *bulgaricus* (10 g wet weight) on strawberry, banana, or oat pieces were added separately at 37 to 40°C. For comparison reasons, probiotic yogurts using free cells (1 g of wet weight) of *Lb. casei* ATCC 393 as adjunct culture were also produced. The initial cell counts for all cultures (*Lb. casei* ATCC 393, *Lb. delbrueckii* ssp. *bulgaricus*, and *Strep. salivarius* ssp. *thermophilus*) were ~9 log cfu/g. Therefore, 8 types of yogurts were prepared in total: (1) probiotic yogurt containing immobilized *Lb. casei* ATCC 393 on strawberry pieces (**y_LCSTR**), (2) probiotic yogurt containing immobilized *Lb. casei* ATCC 393 on banana pieces (**y_LCBAN**), (3) probiotic yogurt containing immobilized *Lb. casei* ATCC 393 on oat pieces (**y_LCOAT**), (4) probiotic yogurt containing free *Lb. casei* ATCC 393 (**y_LCFREE**), (5) yogurt containing immobilized *Lb. delbrueckii* ssp. *bulgaricus* on strawberry pieces (**y_LBSTR**), (6) yogurt containing immobilized *Lb. delbrueckii* ssp. *bulgaricus* on banana pieces (**y_LBBAN**), (7) yogurt containing immobilized *Lb. delbrueckii* ssp. *bulgaricus* on oat pieces (**y_LBOAT**), and (8) yogurt containing free *Lb. delbrueckii* ssp. *bulgaricus* (**y_LBFREE**).

All experiments were carried out in triplicate, and yogurts were stored at 4°C for 67 d after production. Samples from each treatment were collected at various intervals (1, 4, 7, 10, 14, 30, and 67 d) and subjected to microbiological and molecular analyses.

pH Measurement

During yogurt production, pH measurements were carried out using (pH-330i pH meter, WTW GmbH, Weilheim, Germany) pH meter.

Microbial Enumeration

Representative 10-g portions of duplicate immobilized cells or yogurt samples were blended with 90 mL of sterilized quarter-strength Ringers solution (Sigma-Aldrich, Gillingham, UK) and subjected to serial dilutions. Lactobacilli (gram-positive, catalase-negative) counts were determined on acidified MRS agar (Fluka) at 37°C for 48 h anaerobically (Anaerobic jar, Anerocult C, Merck, Darmstadt, Germany), whereas counts of *Strep. salivarius* ssp. *thermophilus* were determined on M17 agar (Fluka) at 37°C for 48 h aerobically.

Identification of Lb. casei ATCC 393 and Lb. delbrueckii ssp. bulgaricus by Multiplex PCR

To confirm the presence or absence of *Lb. casei* ATCC 393 and *Lb. delbrueckii* ssp. *bulgaricus* in yogurt

Table 1. Sequence and optimal amount of oligonucleotide primers used in this study

Primer	Primer sequence (5′ → 3′)	Amount (pmol)	Reference
p78F	GGCGACCAAGGCAGCG	10	Karapetsas et al., 2010
p156F	CTTCGGTTTCATCTTCC	50	Karapetsas et al., 2010
p189R	GGCCAACTTTTTCCATA	50	Karapetsas et al., 2010
p95F	CAGGTTGGTGTTAACTC	60	Nikolaou et al., 2011
p430F	CTGTTAAGGTTGGCGACA	20	Nikolaou et al., 2011
p169R	CTGAAAGCAAGTCTCT	60	Nikolaou et al., 2011
p630R	TCAAGATGTAGACTTGG	60	Nikolaou et al., 2011
Lac1F	AGCAGTAGGGAATCTTCCA	10	Walter et al., 2001
Lac2R	ATTYCACCGCTACACATG	10	Walter et al., 2001

samples, a methodology recently described by Sidira et al. (2010) and Saxami et al. (2012) was followed. Briefly, after growth of the cultures on MRS agar, the plates corresponding to all dilutions were washed with 1 mL of sterilized quarter-strength Ringers solution (Sigma-Aldrich) and then the cell suspension was subjected to molecular analysis based on multiplex PCR methodology (Karapetsas et al., 2010; Nikolaou et al., 2011). Genomic DNA from the lactobacilli suspensions was extracted using a DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. All multiplex PCR reactions were carried out in a total volume of 50 μL, containing 5 U of Taq DNA polymerase (HyTest Ltd., Turku, Finland), a 400 μ*M* concentration of each deoxynucleotide triphosphate (Promega, Southampton, UK), 1.5 m*M* $MgCl_2$ (HyTest Ltd.), and 100 ng of template DNA. A detailed description of all primers used, including amplicon size, optimal amount, and specificity, is presented in Tables 1 and 2. For PCR optimization, a positive (pure cultures of *Lb. casei* ATCC 393 or *Lb. delbrueckii* ssp. *bulgaricus*, respectively) and a negative control (yogurt prepared in the laboratory lacking *Lb. casei* ATCC 393 or *Lb. delbrueckii* ssp. *bulgaricus*, respectively) were also included in the assay. Amplification was carried out in a Thermal Cycler (Mastercycler Eppendorf, Hamburg, Germany) under the following conditions: for *Lb. casei* ATCC 393, 94°C (2 min), followed by 25 cycles of 94°C (15 s), 51°C (15 s), 72°C (30 s), followed by a final extension step at 72°C (1 min); for *Lb. delbrueckii* ssp. *bulgaricus*, 94°C (3 min), followed by 30 cycles of 94°C (15 s), 58°C (15 s), 72°C (1 min), followed by a final extension step at 72°C (2 min). The PCR products were separated on 1.5% (wt/vol) agarose gels, visualized under UV illumination, and photographed with a digital camera (Gel Doc EQ System, BioRad, Segrate, Italy).

Preliminary Sensory Evaluation

Yogurts produced in the laboratory were compared with commercial plain yogurts of a similar type or to yogurts containing fruit pieces and cereals for their sensory characteristics. Samples of approximately 25 g were served in random order at room temperature. Sensory evaluation was conducted in triplicate by 12 tasters (6 laboratory members and 6 volunteers) using locally approved protocols. The panel was asked to give scores on a 0 to 5 scale (0 = unacceptable, 5 = exceptional) for attributes grouped into 5 categories: appearance, viscosity, taste, aroma, and overall quality. Panelists used water to clean their palates between samples and were unaware of the identity of the samples they tasted.

Scanning Electron Microscopy

The immobilization of *Lb. casei* ATCC 393 and *Lb. delbrueckii* ssp. *bulgaricus* cells on fruit and oat pieces was monitored by scanning electron microscopy. Pieces

Table 2. Amplicon size and specificity of the primer pairs used in this study

Primer pair	Amplicon size (bp)	Strain specificity	
		Lactobacillus casei ATCC 393	*Lactobacillus delbrueckii* ssp. *bulgaricus*
p78F/p189R	144	+	−
p156F/p189R	67	+	−
p95F/p169R	107	−	+
p430F/p630R	235	−	+
p95F/p630R	569	−	+
Lac1F/Lac1R	340	+	+

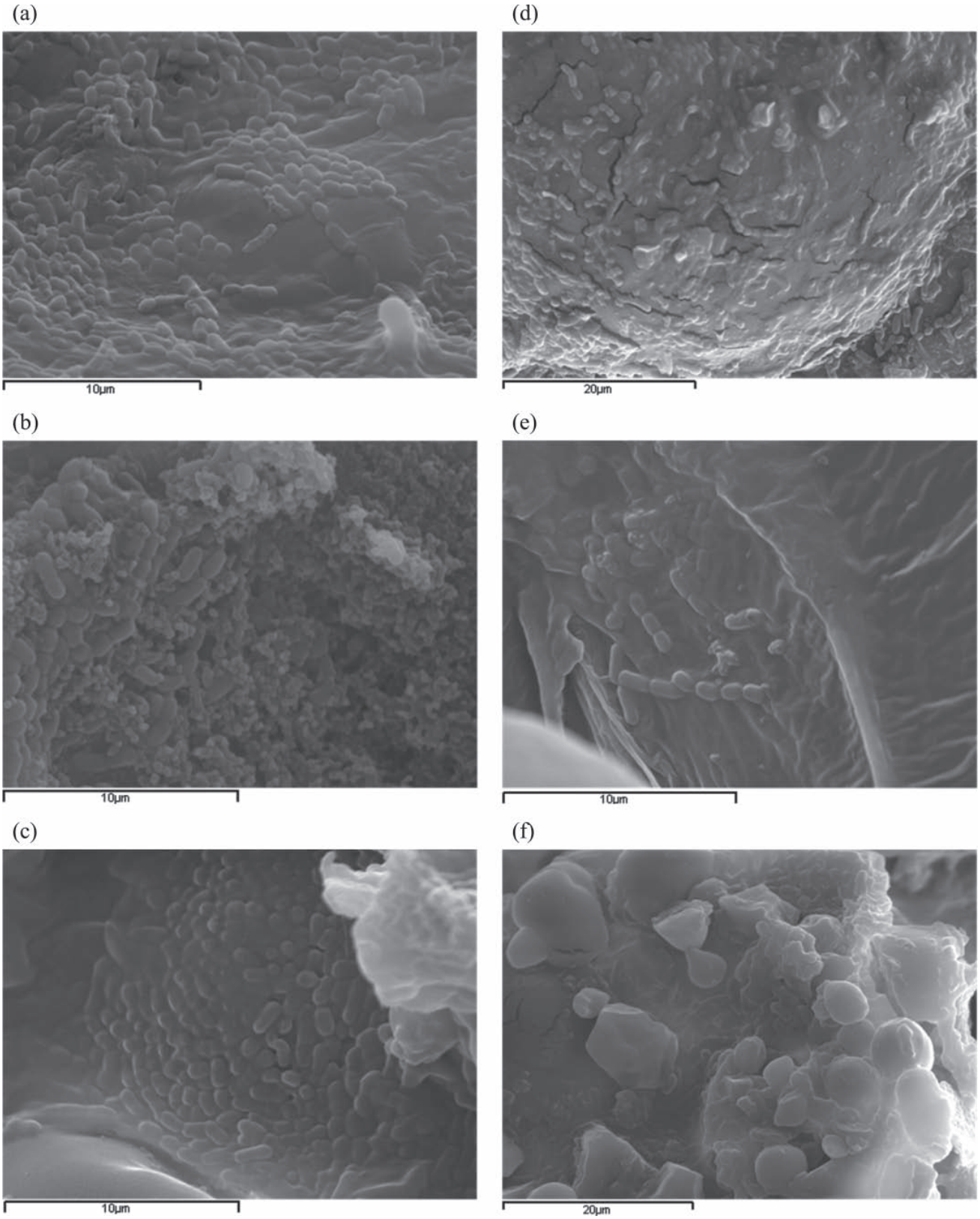

Figure 1. Electron micrographs showing immobilized cells of *Lactobacillus casei* ATCC 393 and *Lactobacillus delbrueckii* ssp. *bulgaricus* on fruit and oat pieces. Immobilized *Lb. casei* ATCC 393 on (a) strawberry pieces, (b) banana pieces, (c) oat pieces; immobilized *Lb. delbrueckii* ssp. *bulgaricus* on (d) strawberry pieces, (e) banana pieces, and (f) oat pieces.

of the immobilized cells were washed with milk and dried overnight at 30°C. The dried samples were coated with gold in a Balzers SCD 004 Sputter coater (Bal-Tec/Leica Microsystems, Wetzlar, Germany) for 2 min and examined in a JSM-6300 scanning electron microscope (Jeol, Tokyo, Japan).

Statistical Analysis

All experiments were carried out in triplicate. Significance was established at $P < 0.05$. Results were analyzed for statistical significance with ANOVA, and Duncan's multiple range test was used to determine significant differences among results; coefficients, ANOVA tables and significance ($P < 0.05$) were computed using Statistica version 5.0 (StatSoft Inc., Tulsa, OK). Differences between means were evaluated using the Student's *t*-test (data were computed using the Excel 2007 software; Microsoft Corp., Redmond, WA).

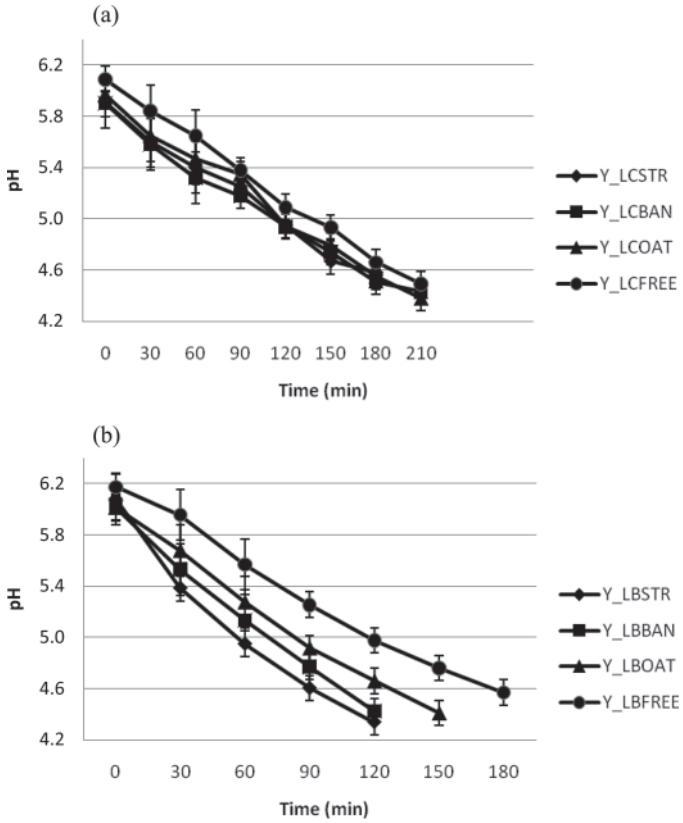

Figure 2. Fermentation kinetics during probiotic yogurt production using free and immobilized cells of (a) *Lactobacillus casei* ATCC 393 and (b) *Lactobacillus delbrueckii* ssp. *bulgaricus* on fruit and oat pieces. y_LCSTR = probiotic yogurt containing immobilized *Lb. casei* ATCC 393 on strawberry pieces, y_LCBAN = probiotic yogurt containing immobilized *Lb. casei* ATCC 393 on banana pieces, y_LCOAT = probiotic yogurt containing immobilized *Lb. casei* ATCC 393 on oat pieces, and y_LCFREE = probiotic yogurt containing free *Lb. casei* ATCC 393; y_LBSTR = probiotic yogurt containing immobilized *Lb. delbrueckii* ssp. *bulgaricus* on strawberry pieces, y_LBBAN = probiotic yogurt containing immobilized *Lb. delbrueckii* ssp. *bulgaricus* on banana pieces, y_LBOAT = probiotic yogurt containing immobilized *Lb. delbrueckii* ssp. *bulgaricus* on oat pieces, and y_LBFREE = probiotic yogurt containing free *Lb. delbrueckii* ssp. *bulgaricus*. Error bars indicate standard deviations (n = 3).

RESULTS

Cell Immobilization and Probiotic Yogurt Production

The number of immobilized *Lb. casei* ATCC 393 or *Lb. delbrueckii* ssp. *bulgaricus* cells ranged from 8 to 9 log cfu/g in all cases. Cell immobilization was also studied by electron microscopy (Figure 1).

Free or immobilized cells of *Lb. casei* ATCC 393 were used as adjunct cultures in probiotic yogurts, and yogurts containing free or immobilized *Lb. delbrueckii* ssp. *bulgaricus* were also produced for comparison. In all cases, milk coagulation was achieved by a yogurt culture consisting of *Lb. delbrueckii* ssp. *bulgaricus* and *Strep. salivarius* ssp. *thermophilus*. Fermentation kinetics are shown in Figure 2. Faster fermentation rates were observed in immobilized cells, as expected.

Monitoring of Probiotic Cell Survival

Lactobacilli counts in yogurt samples during refrigerated storage are presented in Figure 3. A significant reduction was observed ($P < 0.05$) only in yogurts containing free or immobilized *Lb. delbrueckii* ssp. *bulgaricus* (samples y_LBSTR, y_LBBAN, y_LBOAT, and y_LBFREE).

After cell enumeration, the presence or absence of *Lb. casei* ATCC 393 and *Lb. delbrueckii* ssp. *bulgaricus* in Petri dishes corresponding to all dilutions was confirmed by multiplex PCR. The results demonstrated that both free and immobilized *Lb. casei* ATCC 393 were detected in probiotic yogurts at levels required to confer a probiotic effect (≥6 log cfu/g) after 30 d of storage at 4°C (Figure 4a), but not after 67 d of refrigerated storage (Figure 4b). In contrast, both free and immobilized *Lb. delbrueckii* ssp. *bulgaricus* were detected in yogurts at levels ≥6 log cfu/g only after 10 d of storage at 4°C (Figures 3 and 5). Cell viability decreased to lower levels (<6 log cfu/g) during further storage (>10 d) despite the protection provided by immobilization (Figure 3). Of note, the same results concerning the viability of *Lb. delbrueckii* ssp. *bulgaricus* were observed in probiotic yogurts containing both *Lactobacillus* strains (samples y_LCSTR, y_LCBAN, y_LCOAT, and y_LCFREE; data not shown). At 67 d, extended spoilage was observed in all samples due to growth of yeasts and molds, which may have resulted in further decrease of bacterial viability (data not shown).

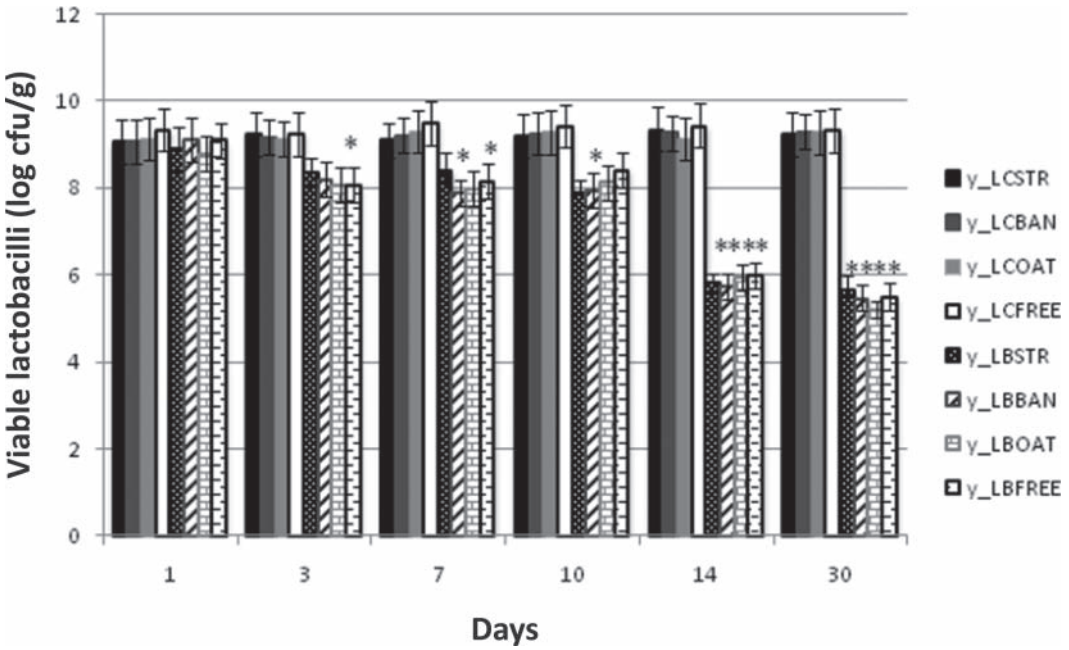

Figure 3. Lactobacilli counts in yogurt samples during refrigerated storage. On d 67, extended spoilage was observed and no lactobacilli were detected during the microbiological analysis. y_LCSTR = probiotic yogurt containing immobilized *Lb. casei* ATCC 393 on strawberry pieces, y_LCBAN = probiotic yogurt containing immobilized *Lb. casei* ATCC 393 on banana pieces, y_LCOAT = probiotic yogurt containing immobilized *Lb. casei* ATCC 393 on oat pieces, and y_LCFREE = probiotic yogurt containing free *Lb. casei* ATCC 393; y_LBSTR = probiotic yogurt containing immobilized *Lb. delbrueckii* ssp. *bulgaricus* on strawberry pieces, y_LBBAN = probiotic yogurt containing immobilized *Lb. delbrueckii* ssp. *bulgaricus* on banana pieces, y_LBOAT = probiotic yogurt containing immobilized *Lb. delbrueckii* ssp. *bulgaricus* on oat pieces, and y_LBFREE = probiotic yogurt containing free *Lb. delbrueckii* ssp. *bulgaricus*. Error bars indicate standard deviations (n = 3). Significant differences were determined by Student's *t*-test (*$P < 0.05$ vs. d 1).

Preliminary Sensory Evaluation

The sensory characteristics of the novel products were compared with similar types of commercial yogurts. The preliminary sensory evaluation ascertained the fine taste and the overall high quality of the experimental yogurts containing immobilized *Lb. casei* ATCC 393 or *Lb. delbrueckii* ssp. *bulgaricus*, although the tasters showed a preference ($P < 0.05$) for the commercial products (data not shown), probably because of small differences in laboratory-scale production compared with commercial production. Of note, no significant differences ($P > 0.05$) were observed between commercial yogurts with no fruits and yogurts containing free cells for all attributes except viscosity. All yogurts produced in the laboratory had lower ($P < 0.05$) values for viscosity, because no filtration was carried out during production. However, the new probiotic yogurts were approved and accepted by the panel (data not shown).

DISCUSSION

Probiotic starter cultures in food production should be designed to meet health-promoting claims and technological effectiveness, as well as economic feasibility criteria (Ammor and Mayo, 2007). *Lactobacillus casei* ATCC 393 has been proposed previously for the production of probiotic fermented milk and cheese because of its excellent technological properties (Kourkoutas et al., 2005, 2006), and the use of cell immobilization has shown significantly increased survival rates over the use of free cells during food manufacture and storage (Kourkoutas et al., 2005, 2006). Recently, the probiotic properties of free and immobilized *Lb. casei* ATCC 393 were assessed by documenting survival after transit through the GI tract, adhesion at the large intestine, and regulation of the intestinal microbial flora in rats (Sidira et al., 2010; Saxami et al., 2012). Although the probiotic properties of *Lb. delbrueckii* ssp. *bulgaricus* are not fully characterized (Guarner et al., 2005; Mater et al., 2005; Guglielmotti et al., 2007), this strain is extensively used in yogurt production. Thus, the scope of the present study was to investigate the survival of free and immobilized *Lb. casei* ATCC 393 on supports widely used in commercial products, such as strawberry, banana, and oat pieces, compared with free or immobilized *Lb. delbrueckii* ssp. *bulgaricus*, which were used as controls. Studies reporting the survival of adjunct probiotic strains in food products in adequate levels for conferring the health benefits are scarce in the

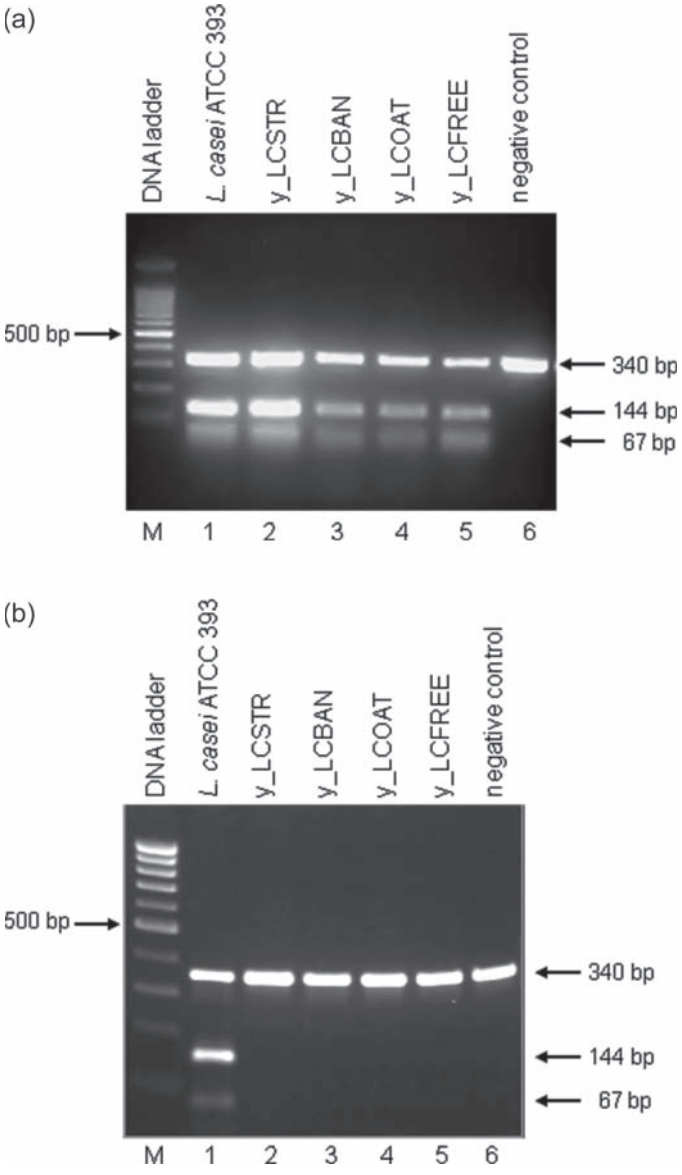

Figure 4. Molecular identification of *Lactobacillus casei* ATCC 393 in yogurts. After lactobacilli enumeration on de Man, Rogosa, and Sharpe agar plates, the presence or absence of *Lb. casei* ATCC 393 at levels ≥6 log cfu/g in yogurts after (a) 30 and (b) 67 d of storage at 4°C was confirmed by strain-specific multiplex PCR. y_LCSTR = probiotic yogurt containing immobilized *Lb. casei* ATCC 393 on strawberry pieces (lane 2), y_LCBAN = probiotic yogurt containing immobilized *Lb. casei* ATCC 393 on banana pieces (lane 3), y_LCOAT = probiotic yogurt containing immobilized *Lb. casei* ATCC 393 on oat pieces (lane 4), and y_LCFREE = probiotic yogurt containing free *Lb. casei* ATCC 393 (lane 5). For PCR optimization, a positive (pure culture of *Lb. casei* ATCC 393; lane 1) and a negative control (yogurt prepared in the laboratory lacking *Lb. casei* ATCC 393; lane 6) were also included. The PCR products of 67 and 144 bp are unique to *Lb. casei* ATCC 393, whereas the PCR product of 340 bp is universal for lactobacilli.

literature because of the lack of an accurate, reliable, convenient, and sensitive method of identification.

The high survival rates of *Lb. casei* ATCC 393 observed in the present study could be attributed to the acid-resistant nature of the strain (Kourkoutas et al., 2005; Sidira et al., 2010; Zhang et al., 2012). In contrast, the reduction in viable cell numbers of *Lb. delbrueckii* ssp. *bulgaricus* was probably due to the high acid sensitivity of the strain (Dave and Shah, 1997). However, the fact that *Lb. delbrueckii* ssp. *bulgaricus* cells may not be recoverable (although still viable) because of cold or acid-stress cannot be excluded. Similar results reporting survival of free and encapsulated probiotic cultures in yogurt after refrigerated storage for long periods have been published (Sultana et al., 2000; Vinderola et al., 2000; Picot and Lacroix, 2004; Capela et al., 2006; Maragkoudakis et al., 2006; Donkor et al., 2007), although a significant decline in viable counts was observed in many cases, resulting in levels <6 log cfu/g (Vinderola et al., 2000; Picot and Lacroix, 2004). However, in these studies, cell survival was based on assays unable to identify the probiotic among other LAB strains; hence, the probiotic characterization of the products was dubious. To the best of our knowledge, this is the first report concerning detection and identification of the adjunct probiotic strain at the required concentration for conferring a probiotic effect in yogurts after cold storage.

Production of probiotic foods containing viable probiotic strains at necessary levels for improving human health throughout their shelf-life is a technological challenge. Our data suggested that both free and immobilized *Lb. casei* ATCC 393 cells survived for longer periods during refrigerated storage than required by the commercial sector at essential levels for providing the probiotic character (≥6 log cfu/g), taking into account that the usual maximum shelf-life of yogurts is 25 d. The higher survival rates of *Lb. casei* ATCC 393 compared with *Lb. delbrueckii* ssp. *bulgaricus* observed in this study could be attributed to possible differences in the acid-resistant nature of the 2 strains. Recently, the acid-resistant properties of *Lb. casei* Zhang were investigated (Zhang et al., 2012; Wu et al., 2012a,b). An acid-resistant mutant of *Lb. casei* Zhang, which showed a 318-fold higher survival rate during acid stress compared with the parental strain, was selected by adaptive evolution (Zhang et al., 2012). A comparison study of the 2 strains by an elegant proteomics analysis demonstrated that adaptation to low pH is associated with changes in critical regulatory cellular pathways, including DNA replication and protein synthesis (Wu et al., 2012a). Moreover, specific physiological alterations of the acid-resistant mutant's cell membrane were recorded to maintain functionality in response to acid-

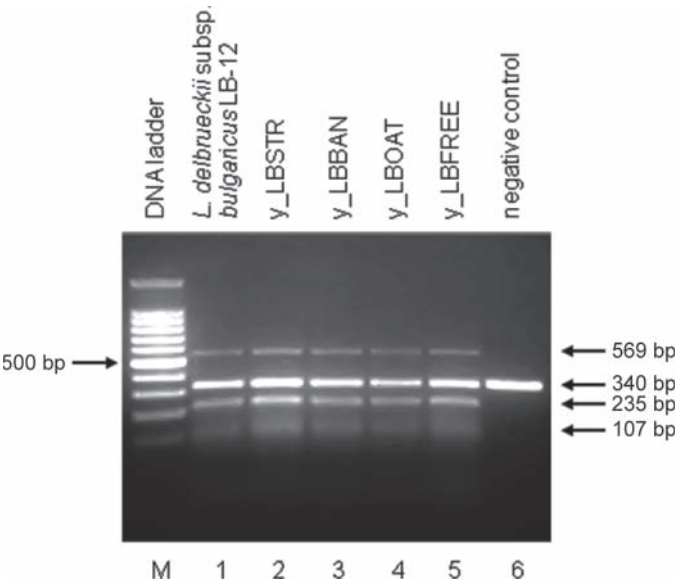

Figure 5. Molecular identification of *Lactobacillus delbrueckii* ssp. *bulgaricus* in yogurts. After lactobacilli enumeration on de Man, Rogosa, and Sharpe agar plates, the presence of *Lb. delbrueckii* ssp. *bulgaricus* at levels ≥6 log cfu/g in yogurts after 10 d of storage at 4°C was confirmed by species-specific multiplex PCR. y_LBSTR = yogurt containing immobilized *Lb. delbrueckii* ssp. *bulgaricus* on strawberry pieces (lane 2), y_LBBAN = yogurt containing immobilized *Lb. delbrueckii* ssp. *bulgaricus* on banana pieces (lane 3), y_LBOAT = yogurt containing immobilized *Lb. delbrueckii* ssp. *bulgaricus* on oat pieces (lane 4), and y_LBFREE = yogurt containing free *Lb. delbrueckii* ssp. *bulgaricus*. For PCR optimization, a positive (pure culture of *Lb. delbrueckii* ssp. *bulgaricus*; lane 1) and a negative control (yogurt prepared in the laboratory lacking *Lb. delbrueckii* ssp. *bulgaricus*; lane 6) were also included in the reaction. The PCR products of 107, 235, and 569 bp are unique for *Lb. delbrueckii* ssp. *bulgaricus*, whereas the PCR product of 340 bp is universal for lactobacilli.

stress, such as increased membrane fluidity and lower membrane permeability (Wu et al., 2012b). Distinct changes in both cytosolic proteins and membrane structure have also been demonstrated for *Lb. bulgaricus* CFL1 (Streit et al., 2008). Undoubtedly, it would be of great interest to investigate whether similar alterations apply to *Lb. casei* ATCC 393 and *Lb. delbrueckii* ssp. *bulgaricus* during acid and cold adaptation.

Prebiotic supports, such as fruits and oat pieces, may constitute a useful vehicle for the transfer of probiotic cells in the GI tract (Sidira et al., 2010; Saxami et al., 2012). It is strongly believed that future clinical tests will ensure the beneficial effects of immobilized probiotics on prebiotic supports (unpublished data).

CONCLUSIONS

Both free and immobilized *Lb. casei* ATCC 393 were detected at the concentration necessary to confer a probiotic effect during refrigerated storage for longer periods than are required by the commercial sector, using a combined microbiological and molecular approach. Because consumption of probiotic products is associated with beneficial effects, future clinical trials will give more insight into the role of immobilized probiotic microorganisms on prebiotic supports, such as fruits and oat pieces, on promotion of human health.

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REFERENCES

Ammor, M. S., and B. Mayo. 2007. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. Meat Sci. 76:138–146.

Boirivant, M., and W. Strober. 2007. The mechanism of action of probiotics. Curr. Opin. Gastroenterol. 23:679–692.

Bosnea, L., Y. Kourkoutas, N. Albantaki, C. Tzia, A. A. Koutinas, and M. Kanellaki. 2009. Functionality of freeze-dried *Lb. casei* cells immobilized on wheat grains. Lebenson. Wiss. Technol. 42:1696–1702.

Boylston, T. D., C. G. Vinderola, H. B. Ghoddusi, and J. A. Reinheimer. 2004. Incorporation of bifidobacteria into cheeses: Challenges and rewards. Int. Dairy J. 19:315–387.

Capela, P., T. K. C. Hay, and N. P. Shah. 2006. Effect of cryoprotectants, prebiotics and microencapsulation on survival of probiotic organisms in yoghurt and freeze-dried yoghurt. Food Res. Int. 39:203–211.

Charalampopoulos, D., S. S. Pandiella, and C. Webb. 2003. Evaluation of the effect of malt, wheat and barley extracts on the viability of potentially probiotic lactic acid bacteria under acidic conditions. Int. J. Food Microbiol. 82:133–141.

Chen, K. N., M. J. Chen, J. R. Liu, C. W. Lin, and H. Y. Chiu. 2005. Optimization of incorporated prebiotics as coating materials for probiotic microencapsulation. J. Food Sci. 70:M260–M266.

Choi, S. S., Y. Kim, K. S. Han, S. You, S. Oh, and S. H. Kim. 2006. Effects of *Lactobacillus* strains on cancer cell proliferation and oxidative stress *in vitro*. Lett. Appl. Microbiol. 42:452–458.

Dave, R. I., and N. P. Shah. 1997. Viability of yoghurt and probiotic bacteria in yoghurts made from commercial starter cultures. Int. Dairy J. 7:31–41.

Dimitrellou, D., Y. Kourkoutas, A. A. Koutinas, and M. Kanellaki. 2009. Thermally-dried immobilized kefir on casein as starter culture in dried whey cheese production. Food Microbiol. 26:809–820.

Dimitrellou, D., K. Tsaousi, Y. Kourkoutas, P. Panas, M. Kanellaki, and A. A. Koutinas. 2008. Fermentation efficiency of thermally-dried immobilized kefir on casein as starter culture. Process Biochem. 43:1323–1329.

Donkor, O. N., S. L. I. Nilmini, P. Stolic, T. Vasiljevic, and N. P. Shah. 2007. Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage. Int. Dairy J. 17:657–665.

Fotiadis, C. I., N. Stoidis, G. Spyropoulos, and D. Zografos. 2008. Role of probiotics, prebiotics and synbiotics in chemoprevention for colorectal cancer. World J. Gastroenterol. 14:6453–6457.

Guarner, F., G. Perdigon, G. Corthier, S. Salminen, B. Koletzko, and L. Morelli. 2005. Should yoghurt cultures be considered probiotic? Br. J. Nutr. 93:783–786.

Guglielmotti, D. M., M. B. Marcó, M. Golowczyc, J. A. Reinheimer, and A. del L. Quiberoni. 2007. Probiotic potential of *Lactobacillus delbrueckii* strains and their phage resistant mutants. Int. Dairy J. 17:916–925.

Karapetsas, A., E. Vavoulidis, A. Galanis, R. Sandaltzopoulos, and Y. Kourkoutas. 2010. Rapid detection and identification of probiotic *Lactobacillus casei* ATCC 393 by multiplex PCR. J. Mol. Microbiol. Biotechnol. 18:156–161.

Kopsahelis, N., P. Panas, Y. Kourkoutas, and A. A. Koutinas. 2007. Evaluation of thermally-dried immobilized cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* on apple pieces as a potent starter culture. J. Agric. Food Chem. 55:9829–9836.

Kourkoutas, Y., L. Bosnea, S. Taboukos, C. Baras, D. Lambrou, and M. Kanellaki. 2006. Probiotic cheese production using *Lactobacillus casei* cells immobilized on fruit pieces. J. Dairy Sci. 89:1439–1451.

Kourkoutas, Y., V. Ksolis, M. Kallis, E. Bezirtzoglou, and M. Kanellaki. 2005. *Lactobacillus casei* immobilization on fruit pieces for probiotic additive, fermented milk and lactic acid production. Process Biochem. 40:411–416.

Li, X. Y., X. G. Chen, D. S. Cha, H. J. Park, and C. S. Liu. 2009. Microencapsulation of a probiotic bacteria with alginate-gelatin and its properties. J. Microencapsul. 26:315–324.

Maragkoudakis, P. A., C. Maris, P. Rojez, N. Manalis, F. Magkanari, G. Kalantzopoulos, and E. Tsakalidou. 2006. Production of traditional Greek yoghurt using *Lactobacillus* strains with probiotic potential as starter adjuncts. Int. Dairy J. 16:52–60.

Mater, D. D. G., L. Bretigny, O. Firmesse, M.-J. Flores, A. Mogenet, J.-L. Bresson, and G. Corthier. 2005. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* survive gastrointestinal transit of healthy volunteers consuming yogurt. FEMS Microbiol. Lett. 250:185–187.

Mattila-Sandholm, T., P. Myllarinen, R. Crittenden, G. Mogensen, R. Fonden, and M. Saarela. 2002. Technological challenges for future probiotic foods. Int. Dairy J. 12:173–182.

Nikolaou, A., G. Saxami, Y. Kourkoutas, and A. Galanis. 2011. A new methodology for rapid detection of *Lactobacillus delbrueckii* ssp. *bulgaricus* based on multiplex PCR. J. Microbiol. Methods 84:362–364.

Oliveira, M. N., I. Sodini, F. Remeuf, J. P. Tissier, and G. Corrieu. 2002. Manufacture of fermented lactic beverages containing probiotic cultures. J. Food Sci. 67:2336–2341.

Picot, A., and C. Lacroix. 2004. Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. Int. Dairy J. 14:505–515.

Saulnier, D., J. Spinler, G. Gibson, and J. Versalovic. 2009. Mechanisms of probiosis and prebiosis: Considerations for enhanced functional foods. Curr. Opin. Biotechnol. 20:135–141. http://dx.doi.org/10.1016/j.copbio.2009.01.002.

Saxami, G., P. Ypsilantis, M. Sidira, C. Simopoulos, Y. Kourkoutas, and A. Galanis. 2012. Distinct adhesion of probiotic strain *Lactobacillus casei* ATCC 393 to rat intestinal mucosa. Anaerobe 18:417–420.

Sidira, M., A. Galanis, P. Ypsilantis, A. Karapetsas, Z. Progaki, C. Simopoulo, and Y. Kourkoutas. 2010. Effect of probiotic-fermented milk administration on gastrointestinal survival of *Lactobacillus casei* ATCC 393 and modulation of intestinal microbial flora. J. Mol. Microbiol. Biotechnol. 19:224–230.

Streit, F., J. Delettre, G. Corrieu, and C. Beal. 2008. Acid adaptation of *Lactobacillus delbrueckii* ssp. *bulgaricus* induces physiological responses at membrane and cytosolic levels that improves cryotolerance. J. Appl. Microbiol. 105:1071–1080.

Sultana, K., G. Godward, N. Reynolds, R. Arumugaswamy, P. Peiris, and K. Kailasapathy. 2000. Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. Int. J. Food Microbiol. 62:47–55.

Vinderola, C. G., N. Bailo, and J. Reinheimer. 2000. Survival of probiotic microflora in Argentinian yoghurts during refrigerated storage. Food Res. Int. 33:97–102.

Vinderola, C. G., G. A. Costa, S. Regenhardt, and J. A. Reinheimer. 2002. Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria. Int. Dairy J. 12:579–589.

Walter, J., C. Hertel, G. W. Tannock, C. M. Lis, K. Munro, and W. P. Hammes. 2001. Detection of *Lactobacillus, Pediococcus, Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 67:2578–2585.

Wu, C., J. Zhang, W. Chen, M. Wang, G. Du, and J. Chen. 2012a. A combined physiological and proteomic approach to reveal lactic-acid-induced alterations in *Lactobacillus casei* Zhang and its mutant with enhanced lactic acid tolerance. Appl. Microbiol. Biotechnol. 93:707–722.

Wu, C., J. Zhang, M. Wang, G. Du, and J. Chen. 2012b. *Lactobacillus casei* combats acid stress by maintaining cell membrane functionality. J. Ind. Microbiol. Biotechnol. 39:1031–1039.

Zhang, J., C. Wu, G. Du, and J. Chen. 2012. Enhanced acid tolerance in *Lactobacillus casei* by adaptive evolution and compared stress response during acid stress. Biotechnol. Bioproc. Eng. 17:283–289.