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Evaluation of the Thermally Dried Immobilized Cells of *Lactobacillus delbrueckii* subsp. *bulgaricus* on Apple Pieces as a Potent Starter Culture

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The aim of the present study was to evaluate the impact of thermal drying of immobilized *Lactobacillus delbrueckii* subsp. *bulgaricus* on apple pieces on the use of the derived biocatalyst in whey fermentation. The thermally dried immobilized biocatalyst was compared to wet and freeze-dried immobilized cells, in respect to maintenance of cell viability and fermentation efficiency. The thermal drying process appeared to be more efficient on survival rate as an 84% of the cells used for immobilization survived the process, while the freeze-drying process led to a 78% rate. The thermally dried immobilized biocatalyst was used in 12 repeated batch fermentations of synthetic lactose medium and whey at 37, 45, and 50 °C in order to evaluate its metabolic activity. The high number of repeated batch fermentations showed a tendency for high operational stability. Fermentations continued for up to 2 months without any significant loss of metabolic activity. SPME GC/MS analysis of aroma-related compounds revealed the distinctive character of fermented whey produced by the thermally dried immobilized bacterium cells. The effect of storage at 4–6 °C for up to 165 days of the biocatalyst, held directly after drying and after repeated batch fermentations, on fermentation activity was also studied. After storage, reactivation in whey was immediate, and the immobilized biocatalyst was able to produce up to 51.7 g/L lactic acid at 37 °C. The potential of thermally dried immobilized *L. delbrueckii* as a starter culture for food production was subsequently evaluated.

KEYWORDS: ***Lactobacillus delbrueckii* subsp. *bulgaricus*; cell immobilization; apple; whey; lactic acid; fermentation; volatiles**

INTRODUCTION

In the last 2 decades, production of cheese commodities using "nontraditional" starter cultures has attracted the interest of several laboratories (*1–6*). Such investigations have mainly been prompted by the more demanding dietary habits of consumers associated with elevated blood pressure, atherosclerosis, heart diseases, obesity, gastrointestinal diseases, and pathogenesis risk. Primary objectives constitute the reduction of salt, the production of low-fat products, the use of probiotics as key components in the final product, the improvement of sensory characteristics, and the reduction of pathogenesis risk.

In the effort of obtaining a technically and commercially feasible starter culture, focus has been given in the drying process of the starter culture. Freeze-drying (*7–11*), spray-drying (*12–16*), and fluidized bed drying (*15*) have been shown to constitute potent methods for obtaining cultures compatible with commercial needs. The observed worldwide preference of freeze-drying is due to the high preservation time that offers to the culture itself, but its industrial application usually suffers from the high investment cost of the production unit accompanied with the need to use cryoprotectants and the decrease of the cell viability. These drawbacks have however been shown to be effectively confronted by the use of immobilized cells. This technology results in enhanced survival rates and biocatalyst stability during freeze-drying, processing, and storage (*8,12,17,18*). Additionally, cell immobilization offers increased viability of probiotic bacteria in the gastrointestinal (GI) tract (*19, 20*).

Fruits and fruit juices have been extensively used as blended additives during production of a variety of foods especially in dairy industry. Apple pieces have been used as a matrix for immobilization of *Lactobacillus casei* cells (*21*), and the immobilized cells were successfully used as a probiotic additive in fermented milk production and as a starter culture in cheese production (*22, 23*). In addition, apple pieces proved to be very effective supports for the survival of the immobilized cells (*18, 21*).

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In the present study, a novel biocatalyst is evaluated. The aim was to investigate an alternative to the freeze-drying method to produce a well-preserved dry biocatalyst that will offer simplicity without the need of special equipment during the process. Immobilization of *Lactobacillus delbrueckii* subsp. *bulgaricus* on apple pieces followed by thermal drying of the immobilized biocatalyst was investigated. Data suggesting maintenance of cell viability and fermentation efficiency of the apple-supported biocatalyst after thermal drying and storage are presented, and the potential of immobilized *L. delbrueckii* on apple pieces as the starter culture is evaluated.

MATERIALS AND METHODS

Strain and Cell Immobilization. *L. delbrueckii* subsp. *bulgaricus* (ATCC 11842T) was used and grown on MRS broth (Merck, Darmstadt, Germany). Cell immobilization on apple pieces (cubes of ≈0.5 cm edge) was carried out as described previously (*21*). In brief, green apples (≈500 g) of Grand Smith variety, cut in pieces, were introduced in 1 L of *L. delbrueckii* liquid culture (≈10^9 cfu/mL) and allowed to ferment overnight at 37 °C without agitation. When immobilization was complete, the fermented liquid was decanted, and the supported biocatalysts were washed twice with whey or synthetic medium that was used for the next fermentation.

Whey. Whey was produced in the laboratory from commercial pasteurized bovine milk. Milk was heated at 37 °C, commercial rennet (0.01%) was added, and the mixture was left undisturbed for 1 h for curd formation. Subsequently, the curd was cut in squares (diameter ≈1 cm), left undisturbed for 10 min, and then cloth filtered at room temperature (18–22 °C). It contained ≈5.5% lactose.

Freeze-Drying of Immobilized *L. delbrueckii*. Biocatalysts consisting of *L. delbrueckii* immobilized cells on apple pieces were frozen to –45 °C. The frozen samples were freeze-dried overnight at 5×10^{-3} bar and at –45 °C in a freeze-dry system, Freezone 4.5 (Labconco, Kansas City, MO).

Thermal Drying Process. Thermal drying was carried out in a chamber equipped with air circulation (J. P. Selecta, Spain) at 37 °C. The drying process was monitored by determining the time needed for the biocatalyst to reach a constant weight.

Determination of Cell Viability. The number of viable cells before and after thermal drying was determined as the log of mean colony forming units (log cfu) per gram of biocatalyst. Duplicate samples, of 10 g biocatalyst each, were blended with 90 mL of sterilized 2% trisodium citrate solution and serially diluted in sterilized Ringer solution (one-fourth strength) before being subjected to thermal drying. The thermally dried samples were resuspended in sterilized 2% trisodium citrate solution for 2 h at 37 °C, decimally diluted, and subsequently plated on MRS agar plates anaerobically (Anaerocult A; Merck).

Fermentations. The thermally dried apple-supported biocatalyst (100 g wet weight) was added to either 200 mL of synthetic medium containing (% w/v) 0.5% yeast extract, 0.1% K_2HPO_4, 0.1% $(NH_4)_2SO_4$, 0.5% $MgSO_4 \cdot 7H_2O$, and 5.5% lactose in distilled water or 200 mL of whey, and a series of 12 repeated batch fermentations was carried at various temperatures (37, 45, and 50 °C). At the end of each fermentation, the fermented liquid was decanted, and the immobilized biocatalyst was washed twice (2×200 mL) with synthetic medium or whey and reused in the next fermentation run. For comparison reasons, whey fermentations were also carried out using wet immobilized and freeze-dried immobilized *L. delbrueckii* on apple pieces. After 12 repeated batch fermentations, the immobilized biocatalysts immersed in the fermented whey were stored at 4–6 °C for 165 days. After storage, the fermented whey was decanted, and reactivation was carried out in whey at 37 °C. In addition, the effect of storage for 20 and 165 days at 4–6 °C of thermally dried immobilized *L. delbrueckii* on fermentation activity was also examined. Reactivation after storage was carried out in whey at 37 °C. After storage and after the 12 repeated batch fermentations, strain identification of the immobilized cells was carried out.

All fermentations were carried out stationary, and the pH was continuously adjusted to 5.0–5.5. Fermentation time was monitored by determining the amount of residual sugar at various intervals. Fermentations were carried out until the rate of sugar metabolism was significantly reduced. At the end of each fermentation run, samples were collected and analyzed for residual sugar, lactic acid, and ethanol concentration. All treatments were carried out in triplicate, and the mean values are presented (standard deviation of metabolite levels in triplicate fermentations was about ± 5%).

Analyses. Moisture was determined according to AOAC (*24*). Residual sugar and lactic acid were determined by high-performance liquid chromatography using a Shimadzu chromatograph with a SCR-101N stainless steel column, a LC-9A pump, a CTO-10A oven at 60 °C, and a RID-6A refractive index detector. Triply distilled water was used as the mobile phase with a flow rate of 0.8 mL/min, and 1-butanol was used as an internal standard. A volume of 0.5 mL of sample and 2.5 mL of a 1% (v/v) solution of 1-butanol were diluted to 50 mL, so that the actual concentration of 1-butanol was 0.05% (v/v). Then, 40 μL of the final solution was injected directly to the column. Residual sugar and lactic acid concentrations were calculated using standard curves prepared by at least seven standard solutions by correlating the ratio of the residual sugar and lactic acid peak areas divided by the 1-butanol peak areas to residual sugar and lactic acid concentrations, respectively.

Ethanol was determined by gas chromatography using a Porapac S column. Nitrogen was used as carrier gas at 40 mL/min. The column temperature was programmed at 120–170 °C at a rate of 10 °C/min. The temperatures of the injector and FID detector were 210 and 220 °C, respectively. For the ethanol determination, a total volume of 2 μL for each sample was injected directly into the column, and the concentration of ethanol was determined using standard curves. 1-Butanol was used as internal standard at a concentration of 0.5% (v/v).

All analyses were carried out in triplicate, and the mean data are presented (standard deviation for all values was about ±5%).

Conversion was calculated by the equation [(initial sugar concentration) – (residual sugar concentration)]/(initial sugar concentration) × 100.

Solid-Phase Microextraction (SPME) Gas Chromatography/Mass Spectrometry (GC/MS) Analysis. Fermented whey was studied for volatile byproduct composition using SPME GC/MS analysis. Ten milliliters of the sample and 2.2 g of NaCl were introduced into a 20 mL headspace vial fitted with a Teflon-lined septum sealed with an aluminum crimp seal, through which the SPME syringe needle (bearing a 2 cm fiber coated with 50/30 mm divinylbenzene/Carboxen on poly(dimethylsiloxane) bonded to a flexible fused silica core; Supelco, Bellefonte, PA) was introduced. The container was then thermostated at 60 °C for 45 min. The absorbed volatile analytes were then analyzed by GC/MS (Shimadzu GC-17A, MS QP5050, capillary column Supelco CO Wax-10 60 m, 0.32 mm i.d., 0.25 μm film thickness). Helium was used as the carrier gas at a flow rate of 1.8 mL/min. The oven temperature was set at 35 °C for 6 min, followed by a temperature gradient of 2 °C/min to 60 °C, held constant for 5 min, raised to 200 °C at 5 °C/min, and then to 250 °C at 25 °C/min with a final isothermal period of 6 min. The injector was operated in splitless mode. Injector and detector temperatures were 280 and 250 °C, respectively. The mass spectrometer was operated in the electron impact mode with the electron energy set at 70 eV and mass range *m*/*z* 29–400. The identification was effected by comparing the retention times with those of authentic compounds, by mass spectra of these authentic compounds generated in the laboratory, by mass spectra obtained from NIST107, NIST21, and SZTERP libraries, and by determining Kovats' retention indexes and comparing with those reported in the literature (*3*, *22*, *23*, *25*). Kovats' retention indexes were determined by injection of a standard mixture containing the homologous series of normal alkanes (C_8–C_{22}) in pure hexane under exactly the same experimental conditions, as described above. All authentic compounds used were obtained from Sigma-Aldrich, Poole, U.K. Quantification of volatile compounds was carried out as described recently (*3*, *22*, *26*, *27*) with some modifications. Specifically, 4-methyl-2-pentanol (Sigma-Aldrich) diluted in pure ethanol was used as an internal standard (IS) at various concentrations (1.62, 16.2, and 162 mg/L). The volatile compounds were quantified by dividing the peak areas of the compounds of interest by the peak area of the IS and multiplying this ratio by the initial concentration of

Table 1. Effect of Temperature on Kinetic Parameters during Repeated Batch Fermentations of 5.5% Lactose Synthetic Medium Using Thermally Dried Immobilized Cells of *L. delbrueckii* subsp. *bulgaricus* on Apple Pieces

fermentation temp (°C)	repeated batch fermentations	fermentation time (h)	ethanol concn (% v/v)	lactic acid concn (g/L)	residual sugar (g/L)	conversion (%)
37	1	69	0.6	43.0	0.1	99.8
	2	52	1.1	31.5	3.7	93.3
	3	46	0.4	36.5	10.7	80.5
	4	48	0.5	39.7	6.5	88.2
45	5	49	0.1	28.7	26.3	52.2
	6	72	0.2	29.3	21.0	61.8
	7	70	0.5	29.6	21.6	60.7
	8	72	0.4	30.2	19.1	65.3
50	9	74	0.1	21.2	36.6	33.5
	10	98	0.3	23.9	30.1	45.3
	11	98	0.7	21.1	28.7	47.8
	12	99	0.7	25.9	24.7	55.1

the IS (expressed as milligrams per liter). The peak areas were measured from the full scan chromatograph using total ion current (TIC). Each determination was carried out in triplicate, and the mean data are presented (standard deviation for all values was less than ±5% in most cases).

Microbiological Analysis. For the strain identification of the immobilized cells, duplicate samples, of 10 g of biocatalyst each, were blended with 90 mL of sterilized 2% trisodium citrate solution, serially diluted in sterilized Ringer solution (one-fourth strength), and plated on MRS agar plates anaerobically (Anaerocult A; Merck). The separated colonies were identified as Gram-positive, catalase-negative rods. Further identity tests included enzymatic and carbohydrate fermentation patterns using API 50 CHL galleries (bioMérieux, Marcy l'Etoile, France) and instrumental automated phenotypic identification using Vitek 2 compact (bioMérieux, Marcy l'Etoile, France) and the new colorimetric GPI card for identification of Gram-positive bacteria consisting of 43 biochemical tests. Identification tests using the API 50 CHL galleries and the Vitek 2 compact instrument were carried out according to the instruction manual.

Scanning Electron Microscopy (SEM). Pieces of apple-immobilized biocatalyst were washed with one-fourth sterilized Ringer solution and dried overnight at 37 °C. The dried samples were coated with gold in a Balzers SCD 004 Sputter coater for 2 min and examined in a JSM-6300 scanning electron microscope.

Experimental Design and Statistical Analysis. All treatments were carried out in triplicate, and the mean values are presented (standard deviation for all values was about ±5% in most cases). The experiments were designed and analyzed statistically by ANOVA. 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 v.5.0 (StatSoft, Inc., Tulsa)].

RESULTS AND DISCUSSION

Biocatalyst Production. *L. delbrueckii* cells were routinely immobilized on apple pieces, exhibiting 9.69 log cfu/g viable cells after immobilization. Cell immobilization was also studied by electron microscopy (data not shown). The immobilized biocatalyst was successfully thermally dried (almost total moisture removal) in ≈20 h, while retaining a survival rate of 84 ± 9%. Off note, the more popular method of freeze-drying exhibited a survival rate of 78 ± 7%, a result that may be originating by the extreme conditions to which the cells are imposed during freeze-drying. Subsequently, metabolic activity of the thermally dried immobilized biocatalyst was monitored by synthetic medium and whey fermentations.

Repeated Batch Fermentations. In a preliminary attempt to evaluate the metabolic activity and operational stability of the thermally dried immobilized biocatalyst, fermentations of synthetic lactose medium and whey were subsequently set up (**Tables 1** and **2**) and compared with whey fermentations set up with wet as well as freeze-dried immobilized cells (**Table 2**). Although the optimum growth temperature of *L. delbrueckii* is 37–43 °C, fermentations were carried out at temperatures up to 50 °C in order to evaluate the potential of the thermally dried immobilized biocatalyst as a starter culture in production of various foods.

Fermentation time was significantly ($p < 0.01$) increased with the rise of fermentation temperature. On the other hand, lactic acid was statistically ($p < 0.01$) lower in fermentation temperatures greater than 37 °C, but still ranged in high levels (21.1–45.9 g/L). Whey fermentations were associated with higher concentrations of lactic acid ($p < 0.01$) compared to fermentations with lactose synthetic medium. Although no significant ($p > 0.05$) differences in ethanol concentration of whey fermentations using wet, freeze-dried, or thermally dried immobilized biocatalysts were observed, Duncan's multiple range test clearly showed that the highest ethanol content ($p < 0.01$) was recorded in synthetic medium fermentations at 37 °C using thermally dried immobilized *L. delbrueckii*. However, ethanol production remained at very low levels in all cases. Fermentation temperature affected significantly ($p < 0.01$) both residual sugar and conversion. The higher residual sugar and the lowest ($p < 0.05$) conversion values were reported in whey fermentations at 50 °C using thermally dried immobilized *L. delbrueckii* cells.

Variations observed in concentrations of residual sugar, ethanol, and lactic acid in batch fermentations could be attributed to the fact that fermentations were stopped deliberately in order to retain cell activity and obtain high values of productivity. When it was impossible for practical reasons to cease fermentation on time, residual sugar ranged in very low levels, proving thus the biocatalyst's suitability for complete fermentation. The low lactic acid content and high residual sugar in fermentations carried out at 50 °C could be attributed to the high temperature (greater than optimum temperature for growth and metabolism of *L. delbrueckii*) (*28, 29*).

The high number of repeated batch fermentations showed a tendency for high operational stability of the thermally dried *L. delbrueckii* apple-supported biocatalyst. Fermentations continued for up to 2 months without any significant loss of metabolic activity. In all cases, no reduction of the fermentative activity of the thermally dried immobilized *L. delbrueckii* cells was observed during the repeated batch fermentations (**Tables 1** and **2**).

Although *L. delbrueckii* subsp. *bulgaricus* belongs to the group of obligatory homofermentative lactic acid bacteria, ethanol was detected as a byproduct in fermentations. It is well-known that, depending on the carbohydrate and on the growth conditions (pH, nutrient density, number of bacterial cells in

Table 2. Effect of Temperature on Kinetic Parameters during Repeated Batch Fermentations of Whey before and after Storage at 4–6 °C for 165 Days Using Wet, Freeze-Dried, and Thermally Dried Cells of *L. delbrueckii* subsp. *bulgaricus* Immobilized on Apple Pieces

immobilized biocatalyst	storage	fermentation temp (°C)	repeated batch fermentations	fermentation time (h)	ethanol concn (% v/v)	lactic acid concn (g/L)	residual sugar (g/L)	conversion (%)
wet	no storage	37	1	52	0.9	39.2	tr[a]	100.0
			2	62	0.1	41.9	3.8	93.1
			3	48	0.1	42.4	2.1	96.2
			4	46	0.1	47.3	3.3	94.0
		45	5	49	0.1	45.1	8.9	83.8
			6	69	0.1	43.0	10.3	81.3
			7	73	0.2	40.4	13.9	74.7
			8	72	0.1	42.8	9.9	82.0
		50	9	74	0.7	21.2	31.8	42.2
			10	99	0.5	29.0	19.9	63.8
			11	98	0.2	30.7	17.3	68.5
			12	99	0.2	31.8	17.4	68.4
	after storage	37	13	52	0.4	37.2	0.4	99.3
freeze-dried		37	1	70	0.5	44.1	0.2	99.6
			2	48	0.1	43.2	0.5	99.1
			3	46	0.1	50.2	tr	100.0
			4	46	0.1	45.0	0.3	99.5
		45	5	47	0.1	41.6	5.2	90.5
			6	69	0.2	42.6	9.4	82.9
			7	71	0.3	36.5	18.6	66.2
			8	72	0.1	42.1	10.0	81.8
		50	9	74	0.2	23.8	30.1	45.3
			10	99	0.2	24.3	21.7	60.5
			11	98	0.1	27.6	19.4	64.7
			12	99	0.1	29.7	17.9	67.5
	after storage	37	13	77	0.4	39.6	0.3	99.5
thermally dried		37	1	67	0.3	40.6	tr	100.0
			2	46	0.1	45.9	tr	100.0
			3	46	0.1	45.0	0.1	99.8
			4	46	0.1	44.7	0.2	99.6
		45	5	49	tr	45.9	9.4	82.9
			6	69	0.3	35.2	17.4	68.4
			7	73	0.5	30.0	21.9	60.2
			8	72	0.4	34.3	12.1	78.0
		50	9	74	tr	21.9	36.4	33.8
			10	98	0.2	20.5	34.2	37.8
			11	98	0.1	29.6	29.9	45.6
			12	99	0.1	30.0	24.7	55.1
	after storage	37	13	71	0.5	33.8	3.6	93.5

[a] tr: trace.

the inoculum), homofermentative lactic acid bacteria may change their metabolic pathway to produce end products of the fermentation of mixed acids (*30*, *31*), such as formate, acetate, ethanol, and CO_2.

Despite the fact that browning of apples during thermal drying occurred, the results suggested that it did not affect viability and activity of *L. delbrueckii* cells. Browning could be limited if thermal drying was performed in an oxygen-free atmosphere.

Effect of Storage on Metabolic Activity. After 12 repeated batch fermentations, the immobilized biocatalysts immersed in the fermented whey were stored at 4–6 °C for 165 days. Reactivation was carried out in whey at 37 °C (**Table 2**). Storage and the drying method affected significantly ($p < 0.01$ and $p < 0.05$, respectively) the fermentation time. A strong interaction was also observed ($p < 0.01$). Ethanol content was only affected by storage ($p < 0.05$), while lactic acid concentration was affected by both factors ($p < 0.01$). The drying method had a significant effect ($p < 0.01$) on sugar and conversion, and a strong interaction between storage and the drying method was evident ($p < 0.01$).

Fermentation time was significantly increased after storage in fermentations using freeze-dried and thermally dried immobilized biocatalysts. Storage resulted in statistically lower lactic acid concentrations. Ethanol and residual sugar ranged in very low levels, while conversion ranged in high values (**Table 2**). Similar results on reactivation of immobilized cells on apple pieces after long-term storage were recently reported (*18*, *21*, *23*).

In order to investigate the possibility of storage of thermally dried immobilized *L. delbrueckii* bacterium cells, the immobilized biocatalyst produced after thermal drying was stored at 4–6 °C for 20 and 165 days. After storage, the thermally dried immobilized biocatalysts were used in whey fermentations, and reactivation was almost immediate.

Storage affected significantly fermentation time, lactic acid, residual sugar, and conversion ($p < 0.01$), while no effect was observed on ethanol concentration ($p > 0.05$). Using thermally dried immobilized cells stored for 20 and 165 days, lactic acid reached concentrations of 50.9 and 51.7 g/L, respectively, while the corresponding conversion values were 99.1% and 92.3%. Ethanol content was less than 1% (v/v) in both cases.

Strain identification of the immobilized cells after storage and after the repeated batch fermentations revealed the presence of only *L. delbrueckii* subsp. *bulgaricus* (99% positive identification). Thus, the presence of any subdominant lactic acid bacteria or contaminant psychrotrophic microorganisms that may participate to the fermentations should be excluded, and hence, treatments, such as sterilization of apple prior to immobilization or sterilization of whey, could be avoided, reducing operating costs on the industrial scale.

Table 3. SPME GC/MS Analysis of Aroma-Related Compounds Extracted from Fermented Whey Using Wet, Freeze-Dried, and Thermally Dried Cells of *L. delbrueckii* subsp. *bulgaricus* Immobilized on Apple Pieces

			immobilized cells (mg/L)		
	compound	identification method[a]	wet	freeze-dried	thermally dried
esters	ethyl acetate	RT, KI, MS	1.8	2.6	1.1
	methyl propanoate	MS	0.1>	0.1>	0.1>
	ethyl propanoate	RT, MS	nd	1.0	0.8
	propyl acetate	MS	nd	1.1	0.2
	methyl butanoate	MS	3.9	nd	0.2
	butyl formate	MS	nd	nd	0.1
	ethyl butanoate	RT, KI, MS	9.9	0.1	1.3
	ethyl ethoxyacetate	MS	nd	nd	0.1>
	propyl propanoate	MS	0.2	nd	0.1>
	ethyl 2-methylbutanoate	RT, MS	0.1>	0.1>	0.1>
	ethyl 3-methylbutanoate	RT, MS	0.1>	0.1>	0.1>
	butyl acetate	MS	0.3	nd	0.1>
	2-methylpropyl propanoate	MS	0.1>	0.1>	0.1>
	2-methylpropyl 2-methylpropanoate	MS	nd	0.1>	nd
	butyl isobutyrate	MS	nd	nd	0.1>
	isoamyl acetate	RT, MS	2.0	2.7	1.2
	ethyl pentanoate	MS	0.1>	nd	0.1>
	butyl propanoate	RT, MS	0.1	nd	nd
	isobutyl butanoate	MS	0.4	nd	0.1
	2-methylbutyl 2-methylbutanoate	MS	nd	0.1>	nd
	butyl butanoate	MS	0.7	nd	0.1>
	ethyl hexanoate	RT, KI, MS	0.4	nd	0.1
	ethyl orthoformate	MS	0.1>	nd	nd
	hexyl acetate	RT, KI, MS	0.1>	0.1>	0.1>
	methyl 6-heptanoate	MS	nd	nd	0.1>
	propyl hexanoate	RT, MS	0.1>	0.1>	0.1>
	ethyl heptanoate	RT, MS	0.1>	nd	0.1>
	2-methylpropyl hexanoate	RT, MS	0.1>	0.1>	0.1>
	ethyl 2-hydoxypropanoate	RT, MS	0.4	0.2	0.1
	methyl octanoate	RT, MS	0.1	0.2	0.1
	butyl hexanoate	MS	0.1>	nd	nd
	hexyl butanoate	RT, MS	0.1>	nd	0.1>
	ethyl octanoate	RT, KI, MS	0.1	1.2	0.7
	methyl nonanoate	MS	nd	0.1>	nd
	ethyl-2,4-hexadienoate	RT, MS	0.1>	0.1>	0.1>
	propyl octanoate	MS	0.1>	0.1>	nd
	ethyl nonanoate	RT, MS	0.1>	0.1>	0.1>
	methyl decanoate	RT, MS	0.1	0.1	nd
	ethyl decanoate	RT, KI, MS	0.3	0.2	0.2
	diethyl succinate	RT, MS	1.8	0.2	0.7
	(*Z*)-ethyl-4-decenoate	RT, KI, MS	nd	0.3	nd
	2-phenylethyl acetate	RT, KI, MS	0.2	0.4	0.3
	ethyl dodecanoate	KI, MS	0.1	0.1>	0.1>
	isopropyl myristate	MS	0.1>	nd	nd
	ethyl tetradecanoate	KI, MS	0.1	nd	0.1
	isopropyl palmitate	MS	0.1	0.1	0.1
	ethyl 9-hexadecenoate	MS	0.2	0.2	0.1
organic acids	pentanoic acid	MS	nd	nd	2.7
	2-methylbutanoic acid	KI, MS	nd	0.7	0.9
	octanoic acid	RT, KI, MS	1.8	1.5	1.8
	nonanoic acid	RT, KI, MS	0.1	nd	0.1
	n-decanoic acid	RT, KI, MS	0.1	0.7	0.1
alcohols	ethanol	RT, KI, MS	>1000	>1000	>1000
	2-butanol	RT, MS	0.1>	0.1>	0.1>
	3,3-dimethyl-2-butanol	MS	nd	nd	0.1>
	1-butanol	RT, MS	0.3	nd	0.1>
	2-methyl-1-butanol	RT, MS	0.8	2.0	1.0
	3-methyl-1-butanol	RT, MS	0.1>	nd	nd
	2-heptanol	RT, MS	0.2	0.6	0.5
	1-hexanol	RT, KI, MS	0.2	0.2	0.1
	1-octen-3-ol	RT, KI, MS	0.1>	nd	0.1>
	1-heptanol	RT, KI, MS	0.1>	0.1>	0.1>
	2-ethyl-1-hexanol	RT, KI, MS	0.1>	0.1>	0.1>
	2-nonanol	RT, MS	nd	0.1	0.1
	1-octanol	RT, KI, MS	0.1	0.1	0.1
	2,3-butanediol	RT, KI, MS	0.1	0.2	0.2
	1-nonanol	RT, MS	nd	0.2	nd
	1-decanol	RT, MS	0.2	0.2	0.2
	3,7-dimethyl-1,6-octadien-3-ol	MS	0.1	0.1	0.1
	benzyl alcohol	RT, KI, MS	0.1	0.1	0.1
	phenylethyl alcohol	RT, KI, MS	4.1	6.2	5.5

Table 3. Continued

			immobilized cells (mg/L)		
	compound	identification method[a]	wet	freeze-dried	thermally dried
alcohols	1-dodecanol	MS	0.1	0.1>	nd
	phenol	RT, MS	nd	nd	0.1>
	4-methylphenol	KI, MS	0.1	0.1	0.1
	2-ethylphenol	KI, MS	nd	0.1	0.1
	2,4-bis[1,1-dimethylethyl]phenol	MS	0.1	0.1	0.1
carbonyl compounds	2-heptanone	RT, MS	0.1>	0.1>	0.1>
	5-methyl-3-heptanone	MS	nd	nd	0.1>
	2-heptenal	RT, KI, MS	nd	0.1>	0.1>
	(*E*)-2-octenal	RT, MS	0.1>	0.1>	0.1>
	furfural	RT, KI, MS	0.2	0.1	0.1>
	decanal	RT, KI, MS	0.1>	0.1>	0.1>
	benzaldehyde	RT, KI, MS	0.1	0.1>	0.1>
	5-methyl-2-furancarboxaldehyde	MS	0.1>	nd	nd
	caprolactam	MS	0.1	0.1	0.1
hydrocarbons	decane	RT, KI, MS	nd	nd	0.1
	α-farnesene	KI, MS	0.4	0.1	0.1

[a] Key: RT, positive identification by retention times that agree with authentic compounds and by mass spectra of authentic compounds generated in the laboratory; KI, tentative identification by Kovats' retention index; MS, tentative identification by mass spectra obtained from NIST107, NIST21, and SZTERP libraries; nd, not detected.

SPME GC/MS Analysis. In order to evaluate the aromatic profile, data obtained with the SPME GC/MS technique from fermented whey using thermally dried immobilized *L. delbrueckii* on apple pieces were compared to whey fermented with freeze-dried and wet immobilized cells. Quantitative results of the volatile compounds are presented in **Table 3** In total, 87 compounds were detected, 73 in fermented whey using thermally dried immobilized cells, 59 in fermented whey using freeze-dried immobilized cells, and 67 in fermented whey using wet immobilized cells. The most important compounds identified were esters, organic acids, alcohols, and carbonyl compounds.

The majority of the identified compounds were esters. Esters, such as ethyl acetate, ethyl butanoate, propyl butanoate, ethyl octanoate, and 2-phenylethyl acetate have been associated with fruity and floral notes (*32*–*34*). They may derive from milk (*35*) and/or by the esterification of alcohols by bacterial enzymes (*36*). The higher number of esters identified in whey fermented by thermally dried immobilized cells compared to whey fermented by freeze-dried immobilized cells was considered as positive for the successful application of the thermally dried immobilized biocatalyst in food production, showing a potential positive effect in the flavor character.

Organic acids detected were pentanoic, 2-methylbutanoic, octanoic, nonanoic, and decanoic acid. Organic acids are important flavor compounds, although they have a relatively high odor threshold. They mainly originate from the action of bacterial lipase activity on the milk lipids (*40*). Many acids including nonanoic and decanoic acids have been isolated from skim milk powder and were responsible for the characteristic sweet, fatty, and buttery-like odors. Octanoic, nonanoic, and decanoic acids were also previously detected in milk whey concentrate (*36*).

Many metabolic pathways are involved in the biosynthesis of alcohols, such as lactose metabolism, methyl ketone reduction, amino acid metabolism, and degradation of linoleic and linolenic acids (*37*, *38*). For example, ethanol that provides an alcoholic, mild flavor note occurs in fresh milk (*35*) and may derive from lactose metabolism. Aliphatic primary alcohols such as 1-hexanol may impact a fruity, nutty note to the flavor (*39*). The presence of branched-chain primary alcohols indicated the reduction of aldehydes, (e.g., 3-methyl-1-butanol derives from reduction of the aldehyde produced by leucine). Secondary alcohols, such as 2-butanol, 2-heptanol, and 2-nonanol, are formed by enzymatic reduction of the corresponding methyl ketones, which themselves are derived from fatty acids by β-oxidation or from β-keto acids (*38*). 1-Octen-3-ol, present in fermented whey using wet and thermally dried immobilized *L. delbrueckii* cells, is known for a distinctive mushroom flavor and has been detected in ultrahigh temperature sterile milk and in oxidized dairy products (*36*). Phenylethanol present in all cases belongs to the most odorous aromatic alcohols, presenting rose flower notes, and may derive from phenylalanine (*40*). 4-Methylphenol (*p*-cresol) originates from tyrosine (*41*).

Carbonyl compounds identified included mainly aldehydes and ketones. Methyl ketones are formed in a metabolic pathway that is connected to the β-oxidation pathway. They represent, particularly 2-heptanone, a group of flavor compounds strongly associated with stale, cardboard, or metallic flavors in many spray-dried dairy products (*42*). Benzaldehyde identified in all cases and usually present in milk whey concentrate (*36*) is described as having an aromatic note of bitter almond (*38*) and may originate from an α-oxidation of phenylacetaldehyde or from a β-oxidation of cinnamic acid (*43*).

The hydrocarbons detected may not have a major contribution to flavor and aroma, although these compounds may serve as precursors for the formation of other aromatic compounds (*44*).

Technological Considerations. Immobilized *L. casei* cells on apple pieces have been recently used as adjunct in cheese making (*23*). Although viability of the probiotic microorganizm was shown to encourage the application of such technology in the dairy industry, its limitations associated with the commercial needs of continuous supplies of preserved and marketable ready-to-use immobilized lactic acid bacteria cells are yet to be confronted. Industrialization of such cultures should satisfy criteria of increased productivity, reduced cost, ease of handling and transportation, and improved quality of the final products. In addition, dried cultures are considered advantageous in industrial practice compared to wet cultures, which are incompatible with the commercial needs due to their physical status.

It becomes clear from our findings that thermal drying led to high cell viability and fermentative activity, which offers economic advantages over other drying methods, such as freeze-drying, due to reduced energy demand and no requirement of cryoprotectants. Lack of cryoprotectants, which are widely used during freeze-drying, implies significant reduction of cost in

industrial applications but, more importantly, no risk of cryoprotectant residues in the final product that may result in quality deterioration. The applicability of thermally dried immobilized *L. delbrueckii* cells on apple pieces as a biocatalyst in the food industry is further supported by the relatively long preservation time that was observed in our study while retaining a high survival rate. Maintenance of high survival rates during drying and storage was clearly demonstrated in our study. However, the use of apple pieces as a cell immobilization support may also play a positive role in cell survival. In addition to that, apple pieces can constitute a useful vehicle for the transfer of probiotic cells in the gastrointestinal tract due to the containing cellulose, which can remain stable, as it is not digested. This property, along with the high metabolic activity of the biocatalyst at high temperatures observed in our study, may allow probiotic bacteria to survive in the large intestine and reach the colon, as well as during high-temperature food processing. Although the potential probiotic role of *L. delbrueckii* strains is still a controversial issue (*28*, *45*, *46*), the technology could be applied to a high number of probiotic lactic acid bacteria.

Production of probiotic foods containing viable probiotic strains at suitable levels for beneficial effects throughout their self-life is a technological challenge. Our data suggested that apple pieces can provide a suitable matrix for immobilization and along with the thermal drying process can lead to the production of a biocatalyst that may have implications in the production of a variety of probiotic foods, such as cheese, yogurt, fermented milk, and other foodstuffs. It is strongly believed that future clinical tests will ensure the beneficial effects of fruit-based probiotics. The improved aroma of the fermented whey using thermally dried immobilized *L. delbrueckii* on apple pieces encourages further research on this subject. The immobilized biocatalyst can be produced using whey, which is a liquid waste of negligible cost. It is possible that the thermally dried immobilized biocatalyst can be added to various solid foods (e.g., breakfast cereals) to provide probiotic properties. A "multistage fixed bed tower" (MFBT) bioreactor (*47*) can be used in industrial fermentations in order to achieve support division, while cell immobilization can be performed in the bioreactor. Experiments concerning long-term storage of the immobilized biocatalysts (*21*, *23*) were very encouraging, since preparation of a new biocatalyst and emptying and filling of the bioreactor can be avoided when industrial production is halted.

As a final consideration, it is concluded that thermally dried immobilized *L. delbrueckii* on apple pieces offers the possibility for the development of a marketable process for preservation of lactic acid bacteria, in order to be supplied in a series of food industrial applications. The thermally dried immobilized biocatalyst appears to have a great potential as a starter culture for food production. However, more research is needed on this field to investigate its use with other species, as well as with other fermentation substrates.

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