Enhancement of Functional Characteristics of Mixed Lactic Culture Producing Nisin Z and Exopolysaccharides During Continuous Prefermentation of Milk with Immobilized Cells

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ABSTRACT

Antagonistic phenomena between strains often occur in mixed cultures containing a bacteriocinogenic strain. A nisin Z producer (*Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* UL719) and 2 nisin-sensitive strains for acidification (*Lactococcus lactis* ssp. *cremoris* ATCC19257) and exopolysaccharide (EPS) production (*Lactobacillus rhamnosus* RW-9595M) were immobilized separately in gel beads and used to continuously preferment milk at different temperatures, with pH controlled at 6.0 by fresh milk addition. The process showed high volumetric productivity, with an increase from 8.0 to 12.5 L of prefermented milk per liter of reactor volume and hour as the temperature was increased from 27 to 35°C. *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* UL719 counts in prefermented and fermented (22-h batch fermentation) milks were stable during 3 wk of continuous fermentation (8.1 ± 0.1 and 8.9 ± 0.2 log cfu/mL, respectively). The *L. lactis* ssp. *cremoris* population (estimated with real-time quantitative PCR) decreased rapidly during the first week of continuous culture to approximately 4.5 log cfu/mL and remained constant afterward. *Lactobacillus rhamnosus* counts in prefermented and fermented milks significantly increased with prefermentation time, with no temperature effect. Nisin Z reached high titers in fermented milks (from 177 to 363 IU/mL), with EPS concentration in the range from 43 to 178 mg/L. Immobilization and continuous culture led to important physiological changes, with *Lb. rhamnosus* becoming much more tolerant to nisin Z, and *Lb. rhamnosus* and *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 exhibiting large increases in milk acidification capacity. Our data showed that continuous milk prefermentation with im-

mobilized cells can stimulate the acidification activity of low-acidifying strains and produce fermented milks with improved and controlled functional properties.

Key words: immobilization, exopolysaccharide mixed culture, continuous fermentation

INTRODUCTION

Lactic acid bacteria play a major role in aroma development, microbiological safety, and texture of fermented dairy products. In recent years, strains with new functional properties such as bacteriocin and exopolysaccharide (EPS) production have been studied, and some have been used for fermented milk and cheese production. Bacteriocins are defined as proteinaceous antimicrobial compounds with bacteriostatic or bactericidal activities, or both, against closely related species and a broader host spectrum for some bacteriocins, such as lantibiotics (Tagg et al., 1976; Klaenhammer, 1993). Lactic acid bacteria bacteriocins have been extensively studied to control the growth of pathogenic and spoilage microorganisms in fermented and nonfermented foods (Cleveland et al., 2001). Lactic acid bacteria can also produce and secrete EPS, which contributes to the texture, mouthfeel, taste perception, and stability of the final products (De Vuyst et al., 2001; Jolly et al., 2002). Moreover, several authors have suggested that EPS have physiological functions, such as antitumor and immunomodulating activities and improvement of probiotic bacterial growth (Ruas-Madiedo et al., 2002). These functional strains may exhibit a limited capacity to grow and produce acid in milk, as shown for EPS (Dupont et al., 2000; Grattepanche et al., 2007) and nisinogenic strains (Roberts et al., 1992; De Vuyst and Vandamme, 1994), and must usually be combined with acidifying cultures. However control of mixed cultures with bacteriocin producers is difficult, because bacteriocin production may limit the growth of bacteriocin-sensitive strains (Grattepanche et al., 2007).

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Cell immobilization has been studied for continuous starter culture and metabolite productions, and continuous milk inoculation and prefermentation, with many advantages compared with free-cell cultures: high cell density leading to high process productivity, control of strain ratios in mixed cultures, reuse of biocatalysts, enhanced tolerance to environmental stresses, retention of cell plasmids, improved resistance to contamination, stimulation of production and secretion of secondary metabolites, and physical and chemical protection of the cells (Doleyres and Lacroix, 2005; Lacroix et al., 2005).

In the present work, we studied cell immobilization and continuous culture for milk inoculation and prefermentation with a functional mixed culture containing antagonistic strains. The tested mixed culture contained 2 nisin-sensitive strains, an acidifier strain (*Lactococcus lactis* ssp. *cremoris* ATCC19257), an EPS-producing strain (*Lactobacillus rhamnosus* RW-9595M), and a high nisin Z producer (*Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* UL719). The effects of temperature and prefermentation age were examined on microbial populations in prefermented milks directly from the continuous culture and in prefermented milks that were incubated for an additional 22 h in batch, to model fresh cheese fermentation and examine metabolite production.

MATERIALS AND METHODS

Bacterial Strains

Lactobacillus rhamnosus RW-9595M was selected for its capacity to produce high amounts of EPS in dairy media (Dupont et al., 2000; Bergmaier et al., 2003); the structure of this EPS has been studied by Van Calsteren et al. (2002). *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* UL719 and *Pediococcus acidilactici* UL5 were used as nisin Z producer and indicator strains for bacteriocin activity assay, respectively (Meghrouh et al., 1997). All strains were obtained from the Lactic Acid Bacterial Research culture collection (Dairy Research Center STELA, University Laval, Québec, Québec, Canada). *Lactococcus lactis* ssp. *cremoris* ATCC19257 (American Type Culture Collection, Manassas, VA) was selected for its ability to acidify milk. Before use, cultures from frozen stocks (-80°C) were grown twice for 24 h in M17 broth (Difco, Detroit, MI) at 30°C for lactococci and in de Man, Rogosa, Sharpe (MRS) broth (Difco) at 30 and 37°C for *P. acidilactici* UL5 and *Lb. rhamnosus* RW-9595M, respectively.

Cell Immobilization Procedure

Each strain was immobilized separately in mixed polysaccharide gel beads by using a 2-phase dispersion

procedure, as described by Lamboley et al. (1997). The κ -carrageenan and locust bean gum polymer solution [2.75 and 0.25% (wt/vol), respectively] was inoculated at 2% (vol/vol) with an overnight pure culture of each strain. Gel beads with diameters ranging from 1 to 2 mm were selected by wet sifting with a 0.2 M KCl solution. To increase the immobilized biomass, beads with pure culture of each strain were colonized separately during 2 successive batch cultures, carried out in a 1-L bioreactor (Biogénie, Ste-Foy, Québec, Canada) with a bead inoculum of 10% (vol/vol), in M17 at 30°C for 6 h and in MRS at 37°C for 20 h, for lactococci and *Lb. rhamnosus* RW-9595M, respectively, with pH controlled at 6.0 using 6 N NaOH. The M17 and MRS media were supplemented with 50 g/L of lactose and 0.2 M KCl to avoid sugar limitation and to maintain bead integrity, respectively. After this colonization step, the immobilized biomass reached $3.16 \pm 0.24 \times 10^{10}$, $1.52 \pm 0.15 \times 10^{11}$, and $7.23 \pm 0.58 \times 10^{10}$ cfu/g of gel beads for *L. lactis* ssp. *cremoris* ATCC19257, *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719, and *Lb. rhamnosus* RW-9595M, respectively.

Continuous Prefermentation of Milk

The continuous inoculation and prefermentation of milk was performed as described by Sodini-Gallot et al. (1995), in a custom-made glass bioreactor with a useful volume of 100 mL. A commercial microfiltrated skim milk (PurFiltre, Québec, Québec, Canada), supplemented with 0.5 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.05 g/L of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ to stimulate growth of *Lb. rhamnosus* RW-9595M (Bergmaier et al., 2003) and acid production in milk (data not shown), was used for the feed medium. The system was inoculated with 25 mL of colonized gel beads from the 3 strains, with a strain ratio of 1:1:1 (by vol). The pH was continuously maintained at 6.0 by inflow of milk, and mixing was provided by a magnetic bar at 120 rpm. Before entering the bioreactor, milk at 2°C was preheated to the set temperature by immersing the tubing in a water bath. The milk feed flow rate was used to control the pH in the bioreactor (PID control software, Biogénie). The useful volume of the bioreactor was maintained by positioning the outflow tube and setting the outlet peristaltic pump at a higher flow rate value than the feeding pump. To retain the beads, a stainless-steel screen was installed at the outlet tubing and periodically purged with a gas pulse of nitrogen to prevent clogging of the mesh by the clotted milk and beads. The milk flow rate was monitored continuously by recording the power supply of the feed pump and was estimated daily by volumetric measurements by using a graduated cylinder.

Figure 1. Populations in gel beads (closed symbols) and prefermented milk (open symbols) of *Lactococcus lactis* ssp. *cremoris* ATCC19257 ($\square$, $\blacksquare$), estimated by quantitative PCR, *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* UL719 ($\circ$, $\bullet$) and *Lactobacillus rhamnosus* RW-9595M ($\diamond$, $\blacklozenge$), determined by plate counts, during continuous immobilized cell culture at different temperatures. For prefermented milk, data with more than a 1-d interval corresponded to weekend interruptions. The dashed lines correspond to the effects of age of prefermentation shown on *L. lactis* ssp. *cremoris* ATCC19257 ($\cdots$) and *Lb. rhamnosus* RW-9595M (—) cell counts predicted by the regression model for a temperature of 31°C. Bars represent standard deviations calculated from triplicate analyses.

At the end of each week, the continuous prefermentation was stopped. The beads were stored in a solution of 0.1% peptone, 0.2 M KCl, and 0.03 M citrate buffer at pH 5.6, and the bioreactor was washed (Sodini et al., 1997b). At the beginning of each week, the beads were transferred to the bioreactor, which was rinsed with 1 L of milk fed at high flow rate (3 L/h) to eliminate the bead storage solution. The continuous prefermentation was then stabilized for 2 d before sampling, except for the second week, for which steady state, indicated by a stable milk flow rate, was obtained after 1 d. Three temperatures were tested. To evaluate the effect of storage conditions, the same prefermentation temperature of 31°C was used before stopping and to restart the system. The 2 other temperatures (27 and 35°C) were randomly applied and repeated 4 times over the 3-wk experiment (Figure 1). The temperature was changed daily and a pseudo steady state, indicated by variations in the dilution rate of less than 0.4 h^{-1} within 4 h of continuous culture, was reached in less than 7 h. Three 200-mL samples of prefermented milk were taken during steady-state conditions, at 30-min intervals, for

chemical and microbial analyses and for batch fermentations, after a 22-h fermentation with a new condition.

Final Fermentation of the Prefermented Milk

Three 100-mL prefermented milk samples were transferred into 150-mL Erlenmeyer flasks, which were placed into a water bath set at the prefermentation temperature. The pH was recorded every minute during incubation for 22 h (Biogénie software). Acidification parameters were calculated as described previously (Grattepanche et al., 2007): maximal acidification rate ($|V_{\max}|$, milli-pH unit/min; **mupH/min**), time and pH at which $|V_{\max}|$ occurred ($t_{\max}$ and $\text{pH}_{\max}$, respectively), time during which the acidification rate was equal to or higher than $|V_{\max}|/2$ (dt_{50}), time to reach pH 5.25 ($t_{\text{pH } 5.25}$), and final pH (pH_F ; Lamboley et al., 1997). At the end of acidification, samples were collected for chemical analyses and cell enumeration.

Cell Enumeration

Cell populations in prefermented and fermented milks and in gel beads were enumerated by using the

appropriate dilutions in 0.1% peptone water (wt/vol) plated in triplicate on agar media. Enumeration of *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 and *Lb. rhamnosus* RW-9595M was performed on differential KMK (Kempler and McKay, 1980) medium (Geneq Inc., Montreal, Québec, Canada) as described previously (Grattepanche et al., 2007). *Lactococcus lactis* ssp. *cremoris* ATCC19257 had low concentrations in prefermented and fermented milks and was estimated by using a real-time PCR method (Grattepanche et al., 2005).

Dispersion of immobilized populations in gel beads was performed by using 2 methods, depending on the enumeration method used. For *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 and *Lb. rhamnosus* RW-9595M, a bead sample of approximately 1.0 g, accurately weighed, was placed in 9 mL of 0.1% peptone water (wt/vol) and treated with an UltraTurrax (Janke and Kunkel, IKA Laborte, Staufen, Germany) for 1 min at 20,000 rpm in ice. The mixture was then diluted in peptone water and plated. For *L. lactis* ssp. *cremoris* ATCC19257, approximately 0.5 g of gel beads, accurately weighed, was placed in a tube containing 9.5 mL of water and held at 90°C, with periodic vortexing until complete dissolution of the gel. After centrifugation at 10,000 × g at room temperature for 10 min, the supernatant was discarded and the cell pellet was washed twice with sterile water. The pellet was then resuspended in 400 µL of TES buffer (50 mM Tris-HCl, 1 mM EDTA, 6.7% sucrose, pH 8.0) before DNA extraction and quantification by real-time PCR. Microbiological analyses were performed in duplicate.

Quantification of Nisin Z Activity

Nisin Z in prefermented and fermented milk samples was extracted and quantified as described previously (Grattepanche et al., 2007). Analyses were performed in triplicate.

Determination of Nisin Sensitivity

Sensitivity of *Lb. rhamnosus* RW-9595M to nisin Z was measured by using the agar diffusion bioassay. Samples of prefermented milk from d 1 and 22 were diluted in peptone water to obtain a final cell concentration for *Lb. rhamnosus* RW-9595M of 10⁶ cfu/mL. A 30-mL volume of MRS containing 7.5 g/L of agar at 45°C was inoculated at 1% (vol/vol) with the bacterial suspension and poured into a Petri dish. After agar solidification, wells (6 mm in diameter) were made and filled with 100-µL pure nisin Z solutions at concentrations varying from 47 to 1,288 IU/mL. The plates were incubated at 45°C for 48 h, and inhibition zones were mea-

sured and plotted as a function of the logarithm of nisin concentration. An overnight culture of *Lb. rhamnosus* RW-9595M in MRS broth at 45°C from the frozen stock was used as a control. To test the reversibility of nisin tolerance for *Lb. rhamnosus* RW-9595M cells, prefermented milk samples at d 22 were subcultured 8 times in MRS medium with a 1% inoculum and incubated at 45°C for 18 h. Reported data are means of triplicate analyses.

Determination of EPS Concentration

The EPS concentration in fermented milk samples was determined as described previously (Grattepanche et al., 2007). Analyses were performed in triplicate.

Acidification Capacity of *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 and *Lb. rhamnosus* RW-9595M

Additional acidification tests were carried out with prefermented milk harvested at d 22 and milk inoculated with pure cultures (controls) of *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 and *Lb. rhamnosus* RW-9595M, under conditions selecting for the growth of each strain, to compare their acidifying capacity in the 2 types of samples. Cell concentrations of pure culture inocula were adjusted to those in prefermented milk samples (9.2×10^7 and 2.9×10^6 cfu/mL for *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 and *Lb. rhamnosus* RW-9595M, respectively). Incubation was performed at 30°C for 48 h to preferentially grow *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 and at 45°C for *Lb. rhamnosus* RW-9595M. The pH was monitored and acidification parameters were calculated. Experiments were performed in duplicate.

Statistical Analyses

Multiple regression (JMP In Software, version 4, SAS Institute Inc., Cary, NC) was used to fit bacterial populations in prefermented and fermented milk, acidification parameters, and metabolite responses as first- and second-order functions of temperature (T) and age of prefermentation (A). The regression model equation for temperature, between 27 and 35°C, and age of prefermentation, between 2 and 22 d, was in the form:

$$Y(T, A) = \alpha_{00} + \alpha_{10}T + \alpha_{20}(T - \bar{x}_T)^2 + \alpha_{01}A + \alpha_{02}(A - \bar{x}_A)^2 + \varepsilon,$$

where $\bar{x}_T$ and $\bar{x}_A$ were means of temperature (31°C) and age of prefermentation (11.6 d), respectively. Regression coefficients α_{ij} were interpreted as the intercept (α_{00}), the linear and quadratic effect of temperature (α_{10}

Table 1. Regression coefficients for models for bacterial populations in prefermented and fermented milks as a function of temperature (T) and age of the prefermentation

Milk	Item	<i>Lactococcus lactis</i> ssp. <i>cremoris</i> ATCC19257 ¹			<i>Lactococcus lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i> UL719			<i>Lactobacillus rhamnosus</i> RW-9595M		
		Estimate	SE	P-value	Estimate	SE	P-value	Estimate	SE	P-value
Prefermented milk	Intercept	6.3726	1.8861	0.0077	8.5139	0.3000	<0.0001	3.3668	1.4105	0.0408
	T	-0.0128	1.8650	0.8308	-0.0123	0.0094	0.2214	0.07990	0.0440	0.1029
	T ²	0.0249	0.0582	0.2824	0.0042	0.0035	0.2622	0.0103	0.0164	0.5462
	Age	-0.0990	0.0218	0.0043	-0.0035	0.0042	0.4328	0.0815	0.0198	0.0026
	Age ²	0.0047	0.0048	0.3542	<0.0001	0.0008	0.9743	-0.0095	0.0036	0.0275
	Model	R ² = 0.65, P = 0.0339			R ² = 0.33, P = 0.4166			R ² = 0.76, P = 0.0070		
Fermented milk	Intercept	13.2750	2.4822	0.0005	8.3314	0.4667	<0.0001	2.3658	1.8593	0.2351
	T	-0.2035	0.0775	0.0275	0.0193	0.0146	0.2180	0.1221	0.0380	0.0447
	T ²	-0.0700	0.0290	0.0387	-0.0019	0.0054	0.7341	0.0344	0.0217	0.1472
	Age	-0.2106	0.0348	0.0002	-0.0057	0.0065	0.4100	0.1341	0.0261	0.0006
	Age ²	-0.0006	0.0067	0.9306	-0.0009	0.0012	0.4769	-0.0144	0.0048	0.0148
	Model	R ² = 0.85, P = 0.0008			R ² = 0.29, P = 0.4952			R ² = 0.83, P = 0.0017		

¹Estimated by the quantitative PCR method.

and α_{20} , respectively), and the linear and quadratic effect of age of the prefermentation (α_{01} and α_{02} , respectively), and ε was the residual. Significance of the overall model was tested with the Fisher test. Student's *t*-test was used to test the significance of model coefficients. *P*-values of less than 0.05 for a 95% confidence interval indicate a statistically significant nonzero coefficient.

RESULTS

Milk Dilution Rate

The dilution rate of prefermented milk increased from 8.0 ± 1.1 to 12.5 ± 1.5 h⁻¹ as the temperature increased from 27 to 35°C. The prediction model for dilution rate explained 80% of the total variation and was highly significant (*P* < 0.01). Analyses of variance of the effects showed that only the temperature factor was significant (*P* = 0.0011) on the milk dilution rate.

Microbial Populations in Prefermented and Fermented Milks

The immobilized population of *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 remained stable at 10.72 ± 0.04 log cfu/g of gel beads over the 3 wk of continuous fermentation (Figure 1). Populations of *L. lactis* ssp. *cremoris* ATCC19257 decreased by 1 log after 2 wk and remained constant afterward. A decrease in immobilized cell population of *Lb. rhamnosus* RW-9595M from 10.44 ± 0.04 to 9.28 ± 0.11 log cfu/g was observed for the first week (Figure 1).

In prefermented milk, the population of *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 of 8.11 ± 0.12 log cfu/mL was not significantly changed by age of prefermentation and temperature (Figure 1 and Table 1). The

populations of *L. lactis* ssp. *cremoris* ATCC19257 and *Lb. rhamnosus* RW-9595M in milk effluent decreased and increased, respectively, with age of prefermentation time, whereas temperature showed no significant effect (*P* > 0.005; Figure 1 and Table 1). The model for these 2 strains was significant and explained 65 and 76% of the total variation for *L. lactis* ssp. *cremoris* ATCC19257 and *Lb. rhamnosus* RW-9595M cell counts, respectively (Table 1). Figure 1 shows the effect of covariate age at 31°C on *L. lactis* ssp. *cremoris* ATCC19257 and *Lb. rhamnosus* RW-9595M populations in prefermented milk. The covariate models for these 2 populations indicate a maximum increase of approximately 1.8 log and a decrease of approximately 1.9 log for *Lb. rhamnosus* RW-9595M and *L. lactis* ssp. *cremoris* ATCC19257, respectively, with age of prefermentation. The highest cell count measured for *Lb. rhamnosus* RW-9595M (7.85 ± 0.03 log cfu/mL) was reached at d 20 at 35°C. The population of *L. lactis* ssp. *cremoris* ATCC19257 decreased by more than 3 log cfu/mL over a 3-wk continuous fermentation, as shown by counts measured on d 2 and 22, both at 31°C (Figure 1).

For fermented milks, age of prefermentation and temperature had no significant effects on *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 counts, which were higher (plus 0.67 ± 0.16 log cfu/mL) than in prefermented milks (Table 1 and Figure 2). Analyses of variance showed that age of prefermentation had highly significant linear effects on *L. lactis* ssp. *cremoris* ATCC19257 and *Lb. rhamnosus* RW-9595M populations, and the models explained 85 and 83% of total variations (Table 1). The effect of age of prefermentation on cell counts was also greater for these 2 strains in fermented than in prefermented milk, with *L. lactis* ssp. *cremoris* ATCC19257 counts decreasing by approximately 4.2 log during a 22-d culture and *Lb. rhamnosus*

Figure 2. Populations of *Lactococcus lactis* ssp. *cremoris* ATCC19257 (□), estimated by quantitative PCR, *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* UL719 (○), and *Lactobacillus rhamnosus* RW-9595M (◇), determined by plate counts, in fermented milks after a 22-h incubation of milks pre-fermented at different temperatures. Data with more than a 1-d interval corresponded to weekend interruptions. The lines correspond to the effects of age of prefermentation on *L. lactis* ssp. *cremoris* ATCC19257 (dotted line) and *Lb. rhamnosus* RW-9595M (dashed line) cell counts predicted by the regression model for a temperature of 31°C. Bars represent the standard deviations calculated from triplicate analyses.

RW-9595M increasing by approximately 2.84 log between d 2 and 14 and decreasing slightly thereafter (Figure 1 and 2). Cell counts for *L. lactis* ssp. *cremoris* ATCC19257 and *Lb. rhamnosus* RW-9595M were also significantly affected by temperature (Table 1). In fermented milk, the highest population of *Lb. rhamnosus* RW-9595M (9.13 ± 0.03 log cfu/mL) was reached at d 20 for 35°C. After 20 d, the population of *L. lactis* ssp. *cremoris* ATCC19257 in fermented milk was less than 2 log cfu/mL for all 3 temperatures. A sharp decrease in cell counts for this strain was observed on d 11, 13, and 20 when fermentation was conducted at 35°C.

Batch Acidification of Prefermented Milk

Figure 3 shows the changes in pH during a 22-h batch incubation of prefermented milks produced at different temperatures. The maximal acidification rate ($|V_{\max}|$), time to reach pH 5.25 ($t_{\text{pH } 5.25}$), and final pH (pH_F) values were all significantly affected by incubation temperature and age of prefermentation (Table 2). The lowest pH (4.02 ± 0.01) and $t_{\text{pH } 5.25}$ (243 ± 28 min) were mea-

sured on d 20 for the highest temperature of 35°C (Figure 3). The $|V_{\max}|$ decreased significantly with both temperature and age of the prefermentation (Table 2). The highest value of $|V_{\max}|$ (2.95 ± 0.10 mupH/min) was measured on d 20 (35°C), whereas the lowest $|V_{\max}|$ (0.81 ± 0.03 mupH/min) was tested on d 3 (27°C). However, $t_{\max}$ (145 ± 19 min) and $\text{pH}_{\max}$ (5.77 ± 0.03), corresponding to the time and pH at which $|V_{\max}|$ occurred, were not significantly changed by temperature and age of prefermentation (data not shown). For d 21 (27°C) and 22 (31°C), 2 $|V_{\max}|$ were recorded (Figure 3). The second $|V_{\max}|$ values of 2.08 ± 0.11 and 2.77 ± 0.14 mupH/min for d 21 and 22 were recorded for $\text{pH}_{\max}$ of 4.99 ± 0.07 and $t_{\max}$ of 957 ± 10 and 585 ± 12 min, respectively.

Nisin and EPS Productions

Nisin Z was not detected in prefermented milk produced during continuous culture with a detection limit of the method of 40 IU/mL. Nisin Z concentration in fermented milks after a 22-h incubation varied from 177 ± 38 to 363 ± 30 IU/mL (Figure 4). However, no

Figure 3. Change of pH during a 22-h batch incubation of prefermented milks produced at different temperatures and different ages of fermentation. Bars represent the standard deviations calculated from 3 repetitions.

significant effects were observed for age of prefermentation and temperature of fermentation, and the regression model was not significant (Table 3).

Concentrations of EPS in fermented milks ranged from 43 ± 7 to 178 ± 29 mg/L (Figure 5). The regression model for EPS production in fermented milk was significant and explained 74% of the total variation (Table

3). Both linear and quadratic effects of age of prefermentation and a linear effect of temperature were significant (Table 3). Concentrations of EPS predicted by the covariate model increased with age of prefermentation from 67 to 145 mg/L for 27 or 31°C, and from 104 to 182 mg/L at d 14 for 35°C (Figure 5). The highest amount of EPS (178 ± 29 mg/L) was measured on d 11 and at 35°C (Figure 5). A significant correlation was also calculated between cell counts of *Lb. rhamnosus* RW-9595M and EPS concentrations ($R^2 = 0.73$ and $P < 0.0001$).

Nisin Sensitivity

Figure 6 shows nisin Z sensitivities for *Lb. rhamnosus* RW-9595M produced in different culture conditions. Cells in prefermented milks (d 1 and 22) were less sensitive to nisin Z, tested at different concentrations, than cells from control-batch cultures. Moreover, cells in prefermented milk on d 22 were much more tolerant toward nisin Z than on d 1, especially for high nisin concentrations. Nisin tolerance of cells produced during subcultures of cells in prefermented milk on d 22 decreased progressively with the number of subcultures and reached a sensitivity level similar to that of cells in prefermented milk on d 1 after 8 subcultures (Figure 6).

Acidification Capacity of *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 and *Lb. rhamnosus* RW-9595M

During incubation at 30°C, milk acidification in prefermented milk started later than in the control (Figure 7). Nevertheless, after a 48-h incubation, the final pH was significantly different (Table 4). For both cultures, $[V_{\max}]$ and $t_{\text{pH } 5.25}$ were not significantly different (Table 4). However, dt_{50} , the time during which the acidification rate was equal to or higher than $[V_{\max}]/2$, was approximately 1.6 higher for prefermented milk ($1,137 \pm 58$ min) than the control (721 ± 33 min). Cell counts of *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 in experimental and control fermented milks were not significantly different (Table 4), whereas counts of *Lb. rhamnosus* RW-9595M in prefermented milk increased from 6.46 to 7.27 ± 0.01 log cfu/mL.

At 45°C, only *Lb. rhamnosus* RW-9595M grew during incubation of prefermented milk, and *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 was not detected after a 48-h incubation (Table 4). Milk acidification was delayed compared with *Lb. rhamnosus* RW-9595M control cultures (Figure 7), but $[V_{\max}]$ was approximately 2-fold higher and $t_{\text{pH } 5.25}$ was 45% less (Table 4). After a 48-h incubation, pH_F reached 3.86 ± 0.06 and 5.06 ± 0.03 for prefermented milk and the control, respectively, whereas final cell counts were identical (Table 4).

Table 3. Regression coefficients of the models for nisin Z and EPS productions in fermented milks after a 22-h incubation

Model parameter ¹	Nisin Z			EPS		
	Estimate	SE	P-value	Estimate	SE	P-value
Intercept	532.6157	151.4999	0.0066	-35.8780	69.6184	0.6187
T	-7.5755	4.7278	0.1435	4.6023	1.1726	0.0432
T ²	-2.9998	1.7672	0.1238	1.0938	0.8121	0.2110
Age	-0.6864	2.1244	0.7540	2.9868	0.9762	0.0136
Age ²	0.1304	0.3903	0.7460	-0.4909	0.1793	0.0229
Model	R ² = 0.41, P = 0.2696			R ² = 0.74, P = 0.0105		

¹T = incubation temperature during continuous milk prefermentation and final batch incubation.

cheese production operated with similar conditions. Several factors could explain this difference in dilution rate, including different growth and acidifying capacities of lactic cultures in milk, different milk compositions, and lower cell loads in beads. However, the inoculation of milk with 1 to 2×10^8 cfu/mL was very similar in both studies.

The high productivity of the continuous milk prefermentation is explained by the high viable population in beads, which did not vary significantly during 3 wk, but was approximately 40% lower than those reported

by Sodini-Gallot et al. (1995) and Sodini et al. (1997a, 1998) during continuous milk prefermentation with 3 lactococci and one leuconostoc. After 2 wk of continuous prefermentation, *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 accounted for almost 90% of the total immobilized cell population, with the other 2 strains comprising 5% each. This dominance of *L. lactis* ssp. *lactis* biovar. *diacetylactis* strains in mixed fermentations was observed previously by Sodini et al. (1997b) and Lambole et al. (2001) during continuous fermentation of milk and whey medium with immobilized cells,

Figure 5. Exopolysaccharide (EPS) concentration in fermented milks after a 22-h incubation of prefermented milk produced at different ages and temperatures. The dashed lines show the EPS concentration in fermented milks predicted by the regression model for different ages of prefermentation and temperature. Bars represent the standard deviations calculated from analysis of 3 samples.

Figure 6. Sensitivity to nisin Z of *Lactobacillus rhamnosus* RW-9595M produced during control free-cell batch culture in de Man, Rogosa, Sharpe broth (□), in prefermented milks harvested at d 1 (△) and d 22 (◇; S0) and after 3, 6, 7, and 8 subcultures of cells from prefermented milk harvested at d 22 (◇; dashed lines, S3, S6, S7, and S8). Bars represent standard deviations calculated from 3 repetitions.

and was tentatively explained by their ability to use citrate. In addition, the high sensitivity of *L. lactis* ssp. *cremoris* ATCC19257 and *Lb. rhamnosus* RW-9595M to nisin Z (MIC of 5 and 11 IU/mL) shown by Grattepan-

che et al. (2005) may explain their low populations in beads, even though nisin activity was not detected in gel beads or prefermented milks at the respective detection limits of 70 and 40 IU/mL. *Lactobacillus rhamnosus*

Figure 7. Acidifying capacity of prefermented milk samples harvested at d 22, at a temperature incubation of 30 (▲) and 45°C (◆) and control cultures of *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* UL719 at 30°C (●) and *Lactobacillus rhamnosus* RW-9595M at 45°C (■). Error bars represent duplicate experiments.

Table 4. Acidification parameters and cell counts, after a 48-h incubation at 30 and 45°C, of prefermented milk samples harvested at d 22 and control pure cultures of *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* UL719 and *Lactobacillus rhamnosus* RW-9595M¹

Temperature	Culture	Cell count, log cfu/mL		Acidification parameter		
		<i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i> UL719	<i>Lb. rhamnosus</i> RW-9595M	pH _F	t _{pH 5.25} , min	V _{max} mupH/min
Incubation at 30°C	Control	8.12 ± 0.04 ^a	NA	4.79 ± 0.04 ^d	1305 ± 136 ^h	-1.55 ± 0.13 ^j
	Sample	8.03 ± 0.04 ^a	7.27 ± 0.01 ^b	4.47 ± 0.08 ^e	1310 ± 46 ^h	-1.51 ± 0.14 ^j
Incubation at 45°C	Control	NA	8.80 ± 0.05 ^c	5.06 ± 0.03 ^f	2624 ± 191 ⁱ	-0.87 ± 0.08 ^k
	Sample	ND	8.77 ± 0.09 ^c	3.86 ± 0.06 ^g	1426 ± 97 ^h	-1.82 ± 0.17 ^l

^{a-l}Data without a common letter are different, using a Student's *t*-test at *P* = 0.05.

¹Reported data are means and standard deviations of duplicate experiments. NA = control was performed with pure culture; ND = not detected at the detection limit of 100 cfu/mL for the enumeration method.

RW-9595M had a limited capacity to grow in milk in pure culture with free cells, presumably explained by a deficient proteolytic system (Grattepanche et al., 2007), which could contribute to low populations in beads.

In fermented milks, *L. lactis* ssp. *cremoris* ATCC19257 counts decreased with age of prefermentation and were lowest at 35°C. These data could be explained by both the high nisin Z titer in fermented milks and a suboptimal temperature for growth of *L. lactis* ssp. *cremoris* (Mundt, 1986). It should be emphasized that real-time PCR methods that use DNA as a target do not distinguish between live and dead cells. However, in a continuous fermentation operated with conditions favoring growth, as in this study, we expect low counts of dead cells. In contrast to immobilized cells, *Lb. rhamnosus* RW-9595M counts in both prefermented and fermented milks increased with age of prefermentation and accounted for 27% of the total population in prefermented milk on d 20 for a temperature of 35°C. These data could be due to physiological changes of the strain developing with time, as discussed below.

The increase in acidifying capacity of prefermented milks with age of prefermentation and temperature can be partly explained by the increase of *Lb. rhamnosus* RW-9595M cell counts in prefermented milks with time. In addition, the acidification activities of both *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 and *Lb. rhamnosus* RW-9595M in prefermented milks were shown to be greatly enhanced after 22 d of continuous culture. For samples taken during the last 2 d (d 21 at 27°C, and d 22 at 31°C), 2 |V_{max}| values were measured for pH_{max}, 5.80 ± 0.04 and 4.95 ± 0.08, corresponding to values recorded for pure cultures of *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 and *Lb. rhamnosus* RW-9595M at the same temperatures (Grattepanche et al., 2007).

A shift from homo- to heterofermentative metabolism resulting in a decrease of lactic acid yields has been reported during repeated-batch fermentation of a chemically semidefined medium for lactic acid production by

an immobilized culture of *Lb. plantarum* (Krishnan et al., 2001). Lambole et al. (1999) also showed a slight but significant increase in acidifying activity of an immobilized mixed mesophilic lactic starter as a function of fermentation time, which was tentatively related to an increase in the immobilized population of *L. lactis* ssp. *lactis* biovar. *diacetylactis* and a change in culture composition with time. In the present study, cell immobilization and continuous fermentation were clearly shown to enhance the acidification properties of *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 and *Lb. rhamnosus* RW-9595M.

Nisin Z activities tested in fermented milk (177 to 363 IU/mL) did not change with prefermentation time or temperature and were in the active range for controlling spoilage microorganisms (Roberts and Zottola, 1993). The lack of detection of nisin Z in prefermented milks can be explained by the high detection limit of the activity test and the very short mean residence time of milk in the continuous reactor. Similarly, Desjardins et al. (2001) showed that nisin production in steady-state conditions of continuous cultures was limited by some biosynthesis steps of the mature peptide, such as posttranslational reactions, transport, or maturation.

We previously showed that the EPS of *Lb. rhamnosus* RW-9595M improved the structural stability of milk gel at a low concentration of 30 mg/L (Grattepanche et al., 2007). Furthermore, an increase in EPS content from 30.0 to 47.8 mg/L increased the initial apparent viscosity of milk gel. In addition, Doleyres et al. (2005) reported that addition of EPS from *Lb. rhamnosus* RW-9595M at 125 to 500 mg/L resulted in yogurts with lower yield stress and viscoelastic moduli compared with control yogurts without EPS. Therefore, we can expect that EPS concentration in the fermented milk at 35°C (165 ± 26 mg/L) significantly improved the rheology of the milk gel. The EPS production of *Lb. rhamnosus* RW-9595M was previously shown to correlate with cell counts for batch and continuous cultures in supplemented whey permeate (Bergmaier et al., 2005).

The amount of EPS tested in milks fermented was maximal at 35°C and similar to that reported by Dupont et al. (2000) during milk batch acidification with *Lb. rhamnosus* RW-9595M at 37°C for 21 h, although *Lb. rhamnosus* RW-9595M cell counts were approximately 10-fold lower in fermented milks from continuous prefermentation. However, they were 3.5-fold higher than for milk inoculated with mixed culture (10^8 cfu/mL of each strain) and batch fermented at 34°C because of nisin Z production by *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719 and high nisin sensitivity (Grattepanche et al., 2007). In this work, *Lb. rhamnosus* RW-9595M cell counts in fermented milk largely increased with age of prefermentation, even though high nisin Z titers (>177 IU/mL) were produced. These data may be explained by the large increase in nisin Z tolerance shown for this strain (particularly for high bacteriocin concentrations), which was reversible during 8 successive subcultures.

Doleyres et al. (2004) previously reported that cell immobilization and continuous fermentation increased the tolerance of *Bifidobacterium longum* and *L. lactis* ssp. *lactis* biovar. *diacetylactis* strains to different environmental stresses, including nisin, antibiotics, H₂O₂, and simulated gastric and intestinal juices. These authors suggested that conditions occurring in gel beads, such as limited diffusion of substrate and inhibitory products, leading to a steep pH gradient and quorum sensing, can induce a nonspecific stress adaptation of immobilized cells. Proteomic and transcriptomic studies have shown that cell immobilization modified expression of genes implicated at all cellular levels: membrane, metabolism, DNA replication, transcription, translation and elongation factors, motility, adaptation, protection, protein folding, and nucleotide biosynthesis (Donlan, 2002; Junter and Jouenne, 2004). Additional work is needed to elucidate the stress adaptation mechanisms of immobilization.

To conclude, continuous prefermentation with immobilized cells allowed high milk inoculation and volumetric productivity that could likely be further improved by selecting a nisin-tolerant acidifying strain. *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* UL719 cell counts and nisin production were high in fermented milk throughout the 22-d continuous fermentation. Moreover, it was possible to control populations of *Lb. rhamnosus* RW-9595M in fermented milk by changing the incubation temperature. A high concentration of nisin-sensitive strain *Lb. rhamnosus* RW-9595M was measured in fermented milks, leading to an enhanced amount of EPS in fermented milk compared with traditional inoculation of milk. We showed that cell immobilization and continuous fermentation resulted in large improvements in nisin tolerance for this strain and

acidification parameters for both *Lb. rhamnosus* RW-9595M and *L. lactis* ssp. *lactis* biovar. *diacetylactis* UL719. This technology permitted the production of fermented milks with EPS and nisin Z concentrations, which are relevant for their effects on texture and the control of bacterial contaminants.

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