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Behavior and viability of spontaneous oxidative stress-resistant *Lactococcus lactis* mutants in experimental fermented milk processing

M.N. Oliveira[1], K.E. Almeida[1], M.R. Damin[1], T. Rochat[2], J.-J. Gratadoux[2], A. Miyoshi[3], P. Langella[2] and V. Azevedo[3]

[1]Departamento de Tecnologia Farmacêutica e Bioquímica, Universidade de São Paulo, São Paulo, SP, Brasil
[2]Unité d'Ecologie et Physiologie du Système Digestif, Institut National de la Recherche Agronomique, Domaine de Vilvert, France
[3]Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

Corresponding author: M.N. Oliveira
E-mail: monolive@usp.br

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ABSTRACT. Previously, we isolated two strains of spontaneous oxidative (SpOx2 and SpOx3) stress mutants of *Lactococcus lactis* subsp *cremoris*. Herein, we compared these mutants to a parental wild-type strain (J60011) and a commercial starter in experimental fermented milk production. Total solid contents of milk and fermentation temperature both affected the acidification profile of the spontaneous oxidative stress-resistant *L. lactis* mutants during fermented milk production. Fermentation times to pH 4.7 ranged from 6.40 h (J60011) to 9.36 h (SpOx2); V_{max} values were inversely proportional to fermentation time. Bacterial counts increased to above 8.50 $\log_{10}$ cfu/mL. The counts of viable SpOx3 mutants were higher than those

of the parental wild strain in all treatments. All fermented milk products showed post-fermentation acidification after 24 h of storage at 4°C; they remained stable after one week of storage.

Key words: *Lactococcus lactis*; Acidification kinetics; Oxidative stress; Spontaneous mutants

INTRODUCTION

Lactococcus lactis is a mesophilic (optimal growth temperature around 30°C) and microaerophilic fermenting lactic acid bacteria, widely used in the dairy industry for the production of cheeses and fermented milk products (Duwat et al., 2000). During industrial processes, *L. lactis* can be exposed to low and high temperature and pH, high osmotic pressure, nutrient starvation, and oxygen, resulting in stress that can affect viability and reproducibility. Among these stress factors, oxidation can be considered one of the most deleterious to bacteria, causing cellular damage at both the molecular and metabolic level, spontaneous mutation, and bacteriostatic and bactericidal effects (Berlett and Stadtman, 1997; Fridowich, 1998; Miyoshi et al., 2003). The kinetics of acidification of the stressed bacteria can be affected, as well as sensory properties of the fermented product (Damin et al., 2008).

The main characteristic of *L. lactis* is acid production; growth and acidification can be monitored by measuring lactic acid production or pH (Walstra et al., 2006). pH can be measured during productions, which is very advantageous for monitoring and controlling the acidification process (Tamime et al., 2001; Almeida et al., 2008). Quantifying the acidifying activity of lactic acid bacteria allows the comparison of different combinations of starters in several substrates in order to define the better trial.

Rochat et al. (2005) developed a strategy to isolate oxidative stress-resistant *L. lactis* MG1363 derivatives. They evaluated the resistance of spontaneous oxidative stress (SpOx) mutants against other oxidative stresses, acidic conditions and bile salts. Initially, it was found that three SpOx mutants selected on H_2O_2 were not resistant to other stress conditions. However, two SpOx mutants (SpOx2 and SpOx3) were more resistant to such conditions, after exposure to H_2O_2, than was the parental dairy strain. In the present paper, we examined the behavior of these strains during milk fermentation, investigating the effects of milk total solid contents and fermentation temperature on the kinetics of acidification parameters, viability and post-acidification development of fermented milk.

MATERIAL AND METHODS

Strains and starter culture

We used three strains of *L. lactis* subsp *cremoris* (J60011, SpOx2 and SpOx3) from the *Unité de Recherches Laitières et de Génétique Appliquée*, *Institut National de la Recherche Agronomique* - UR-LGA, INRA (Jouy-en-Josa, France) collection. Dairy-derived strains of *L. lactis* MG1363 (lac+ and prot+) assigned J60011, constructed by conjugation, were used as controls. Two robust SpOx *L. lactis* mutants (SpOx2 and SpOx3) were chosen (Rochat et al., 2005). Bacteria were stored at -40°C in M17 medium (Oxoid, Basingstoke,

England) with 0.5% lactose added. When required, they were grown overnight at 30°C in whole milk that had been sterilized at 121°C for 10 min, and the culture was used immediately to inoculate milk.

A commercial starter culture (MA016, Rodhia Food, ZA des Engenières, France) containing *L. lactis* subsp *lactis* and *L. lactis* subsp *cremoris* was used for comparison with the mutant strains. Spray-dried inocula (10 mg) were diluted in 50 mL sterilized skim milk reconstituted at 9% of total solids 5 min before use.

Comparative behavior of SpOx mutants during milk fermentation

Pasteurized milk Top Paulista B type (Paulista, São Paulo, Brazil), which contained 12.2 ± 0.0 g/100 g of total solids (TS), was used. Skim milk powder was added to obtain 15% TS (Gloria, Itaperuna, Brazil). After analysis, this milk was found to contain 14.9 ± 0.3 g/100 g TS.

Subsequently, the milk (either 12% TS or 15% TS) was poured into 250-mL flasks, which had been sterilized at 121°C for 15 min and stored for 24 h at 4°C before use. The flasks were warmed and inoculated at the fermentation temperature of 30° or 35°C with 0.25 per 100 mL of the commercial starter, J60011 (control), SpOx2, or SpOx3 inoculum.

After inoculation, the milk was mixed with a magnetic stirrer (Fisatom, São Paulo, Brazil) in a water-bath for 5 min until stabilization of temperature at 30° or 35°C, and connected to a CINAC system equipment (Ysebaert, Frépillon, France) (Corrieu et al., 1988), which allows continuous recording of the pH and computes the acidification rate during the incubation period. The kinetics of acidification was followed until pH 4.7 was reached, which corresponded to the final fermentation time. Four kinetic parameters were considered: a) V_{max} (maximum acidification rate, measured in pH units per min (UpH/min); b) t_{Vmax} (time to reach the maximum acidification rate), c) pH corresponding to V_{max}, and d) $t_{pH\ 4.7}$ (time in h to reach pH 4.7). Cooling the product in an ice bath interrupted the fermentation when pH 4.7 was reached. The product was then manually stirred with a stainless steel perforated disk with up and down movements for ~2 min, followed by dispensing the product into 100-mL cups. These cups were heat-sealed using a thermal sealing machine (Selopar, BrasHolanda, Pinhais, Brazil). All fermented milk samples were stored at 4°C for one week.

Experimental design and statistical analyses

The trials were conducted according to a factorial design with two variables (total solid content of milk and fermentation temperature) and two levels for each variable: (T1) milk 12% TS/30°C; (T2) milk 12% TS/35°C; (T3) milk 15% TS/30°C, and (T4) milk 15% TS/35°C. A total of four batches were run per bacterial strain, each carried out in two replicates. Results were examined by analysis of variance (ANOVA) using Statistica 6.0, Statsoft (Tulsa, USA). Mean values were compared using the Tukey test, with significance set at $P < 0.05$.

Counts of viable cells and post-fermentation acidification

Microbiological analysis of the fermented milk was performed after 24 h (d1) and seven days (d7) of storage of the products at 4°C. Samples (1.0 mL) were added to 9.0 mL sterile tryptone diluent (0.1%, w/v); appropriate dilutions were made, and subsequently, plated

onto M17 medium (Oxoid, Basingstoke, England) supplemented with 0.5% lactose, and incubated at 30°C for 48 h.

Post-fermentation acidification was determined by pH measurements at d1 and d7 using a pH meter (Quimis model Q-400M1, São Paulo, Brazil).

RESULTS AND DISCUSSION

Behavior of SpOx mutants during the fermentation process

Using commercial culture, it was observed that V_{max} T1< V_{max} T2 = V_{max} T4 < V_{max} T3 ($P \leq 0.05$; Table 1). Fermented milk prepared with J60011 resulted in a V_{max} that was significantly lower in T1 than in the other treatments. In T2 (12% TS and 35°C), V_{max} was the highest observed. Lower values of V_{max} were observed with SpOx2, irrespective of treatment, especially at T4. However, with SpOx3, V_{max} varied significantly ($P \leq 0.05$; Table 1). The effect of total solid contents of milk and temperature on V_{max} was strain-dependent. V_{max} increased with total solid content and temperature in commercial, J60011 and SpOx3 cultures. The maximum acidification rate of SpOx2 decreased with increasing temperature in 15% TS milk. Since the variations in the acidification activity of different strains are related to their specific ability to assimilate the nutritive compounds in the medium (Badis et al., 2004), this fact could explain the behavior of SpOx2 under the conditions studied.

Table 1. Effects of total solid (TS) content of milk (12 and 15%) and fermentation temperature (30° and 35°C) on acidification characteristics, viable cell count, and post-fermentation acidification of milk fermented to pH 4.7 by commercial (Com), J60011 (control), SpOx2 and SpOx3 (mutants) starter cultures of *Lactococcus lactis* added at 0.25%.

Treatment	Culture	TS (%)	Temperature (°C)	V_{max} (UpH/min)	pH corresponding to V_{max}	Counts (log_{10} cfu/mL)		Post-fermentation acidification	
						d1	d7	d1	d7
T1	Com	12	30	9.27^{abcd}	5.44^{ab}	9.35^{cfg}	9.75^{hi}	4.49^{ab}	4.46^{ab}
T2	Com	12	35	10.40^{efg}	5.83^{d}	8.93^{bcd}	9.10^{cdefg}	4.48^{a}	4.44^{a}
T3	Com	15	30	10.54^{fg}	5.5^{bc}	9.59^{g}	9.48^{gh}	4.55^{cd}	4.54^{cde}
T4	Com	15	35	10.49^{efg}	5.84^{d}	9.25^{def}	8.64^{a}	4.52^{bc}	4.50^{bc}
T1	J60011	12	30	8.96^{abc}	5.38^{ab}	9.19^{cde}	9.09^{bcdef}	4.48^{a}	4.44^{a}
T2	J60011	12	35	11.82^{h}	5.77^{d}	8.57^{a}	8.61^{a}	4.48^{a}	4.44^{a}
T3	J60011	15	30	10.07^{defg}	5.39^{ab}	9.40^{efg}	9.23^{efg}	4.56^{cd}	4.52^{cd}
T4	J60011	15	35	10.41^{efg}	5.84^{d}	8.64^{ab}	8.73^{abc}	4.57^{d}	4.52^{cd}
T1	SpOx2	12	30	8.81^{ab}	5.35^{a}	9.55^{fg}	9.45^{fgh}	4.48^{a}	4.46^{a}
T2	SpOx2	12	35	9.55^{bcdef}	5.70^{cd}	8.92^{bc}	9.04^{bcde}	4.51^{ab}	4.47^{ab}
T3	SpOx2	15	30	9.83^{cdef}	5.45^{ab}	9.57^{fg}	8.71^{ab}	4.56^{cd}	4.56^{c}
T4	SpOx2	15	35	8.29^{a}	5.80^{d}	9.38^{cfg}	9.17^{defg}	4.63^{e}	4.63^{f}
T1	SpOx3	12	30	8.63^{ab}	5.35^{a}	9.31^{cfg}	9.03^{bcde}	4.50^{ab}	4.47^{ab}
T2	SpOx3	12	35	10.45^{efg}	5.71^{cd}	9.40^{efg}	9.91^{i}	4.48^{a}	4.45^{a}
T3	SpOx3	15	30	9.51^{bcd}	5.53^{abc}	9.58^{fg}	9.62^{hi}	4.56^{cd}	4.55^{de}
T4	SpOx3	15	35	10.94^{gh}	5.76^{d}	9.17^{cde}	8.83^{abcd}	4.57^{d}	4.53^{cde}

Different superscript letters in the same column indicate significant differences. Tukey test, $\alpha = 0.05$. V_{max} = maximum acidification rate (measured in pH units per min); d1, d7: fermented milk stored at 4°C for 24 h and seven days, respectively.

The time to reach V_{max} (t_{Vmax}) varied significantly, ranging from 3.03 to 6.57 h (Figure 1). The effect of temperature and total solid content was observed with all cultures. The lowest values of t_{Vmax} were obtained with the higher temperature and total solid content. When the commercial culture was used, t_{Vmax} at 35°C was 3.01 h on average, and at 30°C, t_{Vmax} was 5.93 h on average, almost doubling. The same behavior was observed when J60011, SpOx2 and SpOx3 were employed, but with minor differences between the treatments. Nevertheless, a higher t_{Vmax} was obtained with SpOx2 in T1 (6.57 h). In all cases, the *L. lactis* cultures reached V_{max} in less time at 35°C than at 30°C.

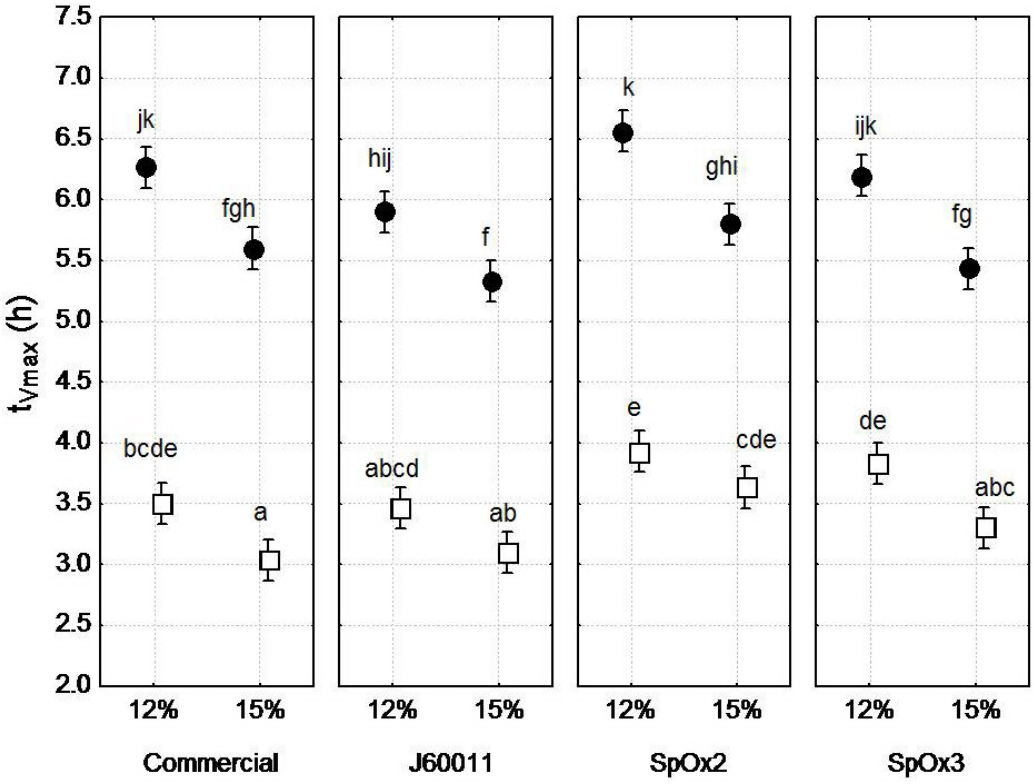

Figure 1. Effect of total solid contents of milk (12 and 15%) and fermentation temperature (30° and 35°C) on time to reach V_{max} (t_{Vmax}) of milk fermented to pH 4.7 by commercial, J60011 (control), SpOx2 and SpOx3 (mutants) cultures of *Lactococcus lactis*. Strains were added at 1% (circles, 30°C and squares, 35°C). Values with different letters are significantly different ($P < 0.05$).

Temperature also influenced the pH at which V_{max} was reached (Table 1). The pH corresponding to V_{max} ranged from 5.35 (SpOx2 in T1) in 5.84 (commercial and J60011 in T4). When fermentations were carried out at 35°C, pH at V_{max} increased to ~5.80. At the higher temperature, V_{max} was reached at higher pH values, which means that an increase in 5°C allowed favorable conditions that the acidification rates of the *L. lactis* strains studied could be reached in shorter times at 30°C (Table 1). According to Kristo et al. (2003), lower total solid

contents mean lower buffering capacity, which in turn mean a greater decrease in pH for the same amount of acid produced and vice versa.

The time to reach pH 4.7 varied significantly, from 6.40 h (J60011 in T2) to 9.36 h (SpOx2 in T4) in fermented milk batches (Figure 2). The commercial strain reached pH 4.7 in ~8.65 h at 30°C, while at 35°C, the time decreased to 7.44 h. The culture J60011 was faster than the commercial culture in all treatments (Figure 2), especially in T2, when the difference was significant. This treatment gave the highest V_{max} (Table 1), which affected fermentation time.

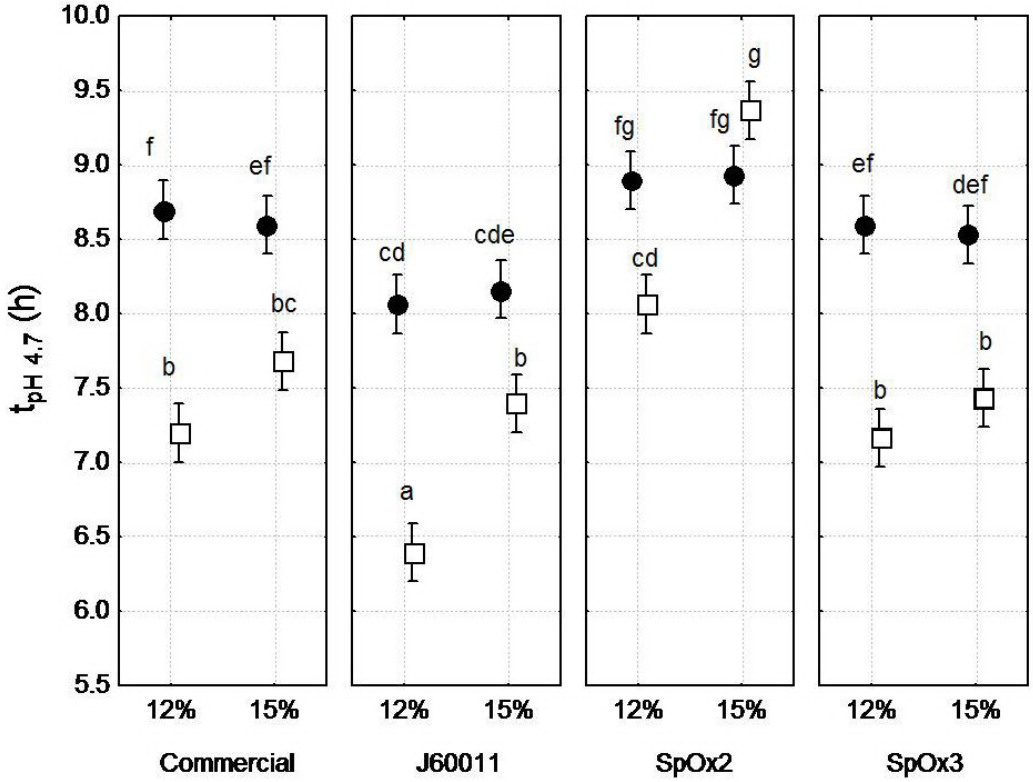

Figure 2. Effect of total solid contents of milk (12 and 15%) and fermentation temperature (30° and 35°C) on fermentation time ($t_{pH\ 4.7}$) of milk fermented to pH 4.7 by commercial, J60011 (control), SpOx2 and SpOx3 (mutants) cultures of *Lactococcus lactis*. Strains were added at 1% (circles, 30°C and squares, 35°C). Values with different letters are significantly different ($P < 0.05$).

The SpOx2 culture gave the longest fermentation times, irrespective of treatment. Unlike with the other strains, fermentation time at 35°C and 15% TS (T4) was longest (9.36 h), which gave the lowest V_{max} (8.29 x 10^{-3} UpH/min, Table 1). However, in general, SpOx2 also showed lower V_{max} when compared to the other strains studied, which could characterize it as a bacterial strain with low-acidifying activity.

With SpOx3, fermentation times at 30°C were ~8.57 h, while with the temperature increased to 35°C, $t_{pH\ 4.7}$ was ~7.25 h. Figure 2 shows that all cultures gave shorter fermentation times at 35°C except for SpOx2 at 15% TS ($P \leq 0.05$).

The behavior of SpOx mutants during milk fermentation varied with percentage of milk solids and temperature and was strain-dependent. Adamberg et al. (2003) observed that when temperature was increased from 25 to 38°C, the specific growth rate and the specific lactate production rate of *L. lactis* also increased. Similar observations were made when *L. lactis* ATCC19435 was grown in whole wheat flour from temperatures of 30 to 40°C. Lactic acid production was optimal between 33 and 35°C, while D-lactic acid and by-product formation increased at temperatures above 30°C (Akerberg et al., 1998). SpOx mutants showed higher acidifying capacity at 30°C than did *L. lactis* ATCC19257 at the same temperature, as reported by Grattepanche et al. (2007).

Counts of viable SpOx mutants in fermented milk

Bacterial counts were done after one and seven days of storage of the fermented milk products at 4°C. The *L. lactis* counts 24 h after the end of fermentation (d1), varied from 8.57 $\log_{10}$ to 9.59 $\log_{10}$ cfu/mL. Commercial, J60011 and SpOx2 counts in T2 were lower than in other treatments, while T3 (at 15% and 30°C) gave the highest counts. There was no significant difference in SpOx3 counts in T1, T2 and T3. However, at 15% TS, the increase in fermentation temperature resulted in lower counts of SpOx3 ($P \leq 0.05$). The counts of SpOx3 was higher than that of J60011 in all treatments at d1, confirming the results obtained by Rochat et al. (2005).

After seven days in storage, bacterial counts ranged from 8.64 $\log_{10}$ (commercial in T4) to 9.91 $\log_{10}$ cfu/mL (SpOx3 in T2). Commercial and J60011 counts were higher at 30°C. SpOx2 and SpOx3 did not have the same behavior and SpOx3 gave the highest counts (Table 1).

Our results are similar to those obtained by Gadaga et al. (2001), who studied *L. lactis* in single culture; they found that viability was reduced during storage of fermented milk. In our study, in general, bacterial counts were higher for all cultures at 35°C.

Post-fermentation acidification

All samples showed post-fermentation acidification after 24 h of storage at 4°C (Table 1). On average, using commercial, J60011, SpOx2 and SpOx3 cultures, pH decreased at d1 0.19, 0.18, 0.15, and 0.17 pH units, respectively. Fermented milk prepared with commercial culture showed a pH at d1 ranging from 4.48 to 4.55, with significant differences mainly in T2 and T3. After seven days of storage, a similar situation was observed, i.e., the largest differences were observed in T2 and T3. At d1 and d7, using J60011, SpOx2 and SpOx3 cultures in T1 and T2, there were no significant differences in pH. Values were higher with milk with 15% solids. The lowest degree of post-fermentation acidification was observed with the SpOx2 culture in T4 (0.07 pH units). The pH values of the fermented milk remained stable during one week of storage in all treatments (Table 1).

CONCLUSIONS

Total milk solids and fermentation temperature both affected the acidification profile of spontaneous oxidative stress resistant *L. lactis* mutants in fermenting milk. Fermentation times ranged from 6.40 h (J60011) to 9.36 h (SpOx2), while V_{max} values were inversely proportional to fermentation time. Counts surpassed 8.50 $\log_{10}$ cfu/mL. The counts of viable SpOx3

were higher than those of J60011 in all treatments. All fermented milk products showed post-acidification after 24 h of storage at 4°C, and remained stable for another six days.

Thus, we suggest that along with their resistance to oxidative stress, SpOx3 mutant shows characteristics that allow it to be used as a starter in the dairy industry. More research is required regarding sensory properties of the fermented product.

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