Optimization of reuterin production in cheese by *Lactobacillus reuteri*

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Abstract Cheeses manufactured from pasteurized milk supplemented with glycerol and reuterin-producing *Lactobacillus reuteri* INIA P572 as adjunct to the commercial starter culture were analysed in order to optimize a biopreservation strategy. The highest reuterin concentration determined by a colorimetric assay was detected on day 1 in cheeses with 100–500 mM glycerol. The presence of reuterin was confirmed by a direct detection technique as HPLC. Cheeses made with *L. reuteri* and 200 or 500 mM glycerol showed a red tendency in color in comparison with control. The results with purified reuterin suggested that the development of slightly rosy colour in cheese was related to some compound produced/overproduced when higher levels of glycerol were present in cheese, but not due to reuterin. Application of *L. reuteri* INIA P572 as adjunct to the commercial starter with 100 mM glycerol led to such a reuterin concentration in cheese that could control undesirable microorganisms, avoiding the presence of color-changing compounds.

Keywords Cheese · In situ reuterin production · *Lactobacillus reuteri* · Bioprotection

Introduction

Reuterin (β -hydroxypropionaldehyde; β -HPA) is a broad spectrum antimicrobial substance produced by some strains of *Lactobacillus reuteri* during anaerobic bioconversion of glycerol (Axelsson et al. 1989). It has a high potential as food preservative. Reuterin is soluble in water, resistant to proteolytic and lipolytic enzymes, and maintains its antimicrobial activity at low pH and high NaCl concentration (Rasch et al. 2007) and at refrigeration temperatures (Arqués et al. 2008). All these properties are attractive features for its application in some dairy products. Direct addition of purified reuterin to control food-borne pathogens such as *Salmonella* spp., *Escherichia coli* O157:H7 and *Listeria monocytogenes* has been investigated in milk and dairy products (Arqués et al. 2008; El-Ziney and Debevere 1998).

The use of *L. reuteri* plus the food additive glycerol for reuterin production in dairy products is a feasible alternative for reuterin addition in foods. The application of antimicrobial compounds-producing lactic acid bacteria as starter or adjunct cultures in dairy products does not require regulatory approval or label declarations and can be used to improve the food safety through the in situ production of the antimicrobial agents (Arqués et al. 2015). Previous studies have shown that some *L. reuteri* strains were able to produce reuterin in cheese in sufficient amounts to exert an antimicrobial action against food-borne pathogens and spoilage bacteria (Angiolillo et al. 2014; Gómez-Torres et al. 2014; Langa et al. 2013). In these studies, reuterin was determined based on the dehydration into acrolein followed by a colorimetric measurement (Circle et al. 1945). In this work, the reuterin colorimetric detection in cheese was confirmed by a direct detection technique (HPLC), and the glycerol concentration for in situ reuterin production in cheese optimized in order to avoid negative side effects.

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Materials and methods

Microorganisms and reuterin

Reuterin-producing *L. reuteri* INIA P572, selected from the INIA culture collection due to its high reuterin production (Rodríguez et al. 2003), was grown in reconstituted milk supplemented with 3 g yeast extract/l before use (Langa et al. 2013). Commercial mesophilic lactic culture (CLC) MA 016 (Larbus S.A., Madrid, Spain) containing *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* was prepared as specified by the manufacturer and kept at -20°C . Purified reuterin was obtained as described by Montiel et al. (2014) and used for reuterin quantification and color analysis.

Cheese manufacture

Semi-hard cheeses were made from pasteurized cow's milk (Priégola, Madrid, Spain) in two trials carried out on different days. In each trial, milk at 33°C with 0.2 g CaCl_2 /l was distributed into five 2 l vats. Commercial starter at a rate of 10 ml/l and *L. reuteri* INIA P572 at a final concentration in milk of 10^6 cfu/ml were added to the five vats. Different concentrations (0, 50, 100, 200 and 500 mM) of glycerol (Panreac Química, Barcelona, Spain), and 0.015 g/l rennet (Maxiren, Laboratorios Arroyo, Santander, Spain) were added 20 min after inoculation with cultures. The curd was cut 40 min after rennet addition and heated at 37°C for 25 min. Whey was drained off and curd was distributed into plastic cylindrical moulds. The cheese was pressed at room temperature overnight, salted in 15% brine for 20 min, vacuum packaged in Cryovac plastic bags and ripened at 12°C for 30 days. Cheeses were sampled at days 1, 5, 10, 15, 20 and 30. Five grams of samples from two different sectors were pooled and homogenized with 90 mL of sterile sodium citrate solution (Nuñez et al. 1985). *Lactococcus lactis* counts from CLC were determined in cheese on duplicate plates of M17 (Biolife, Milano, Italy) with 5 g glucose/l (GM17 agar) and incubated at 30°C for 24 h, and *L. reuteri* counts on duplicate plates of Rogosa agar (Biolife) incubated at 37°C for 48 h under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK).

pH and reuterin determination

Cheese pH was obtained in duplicate with a Crison penetration electrode (model 52-3.2; Crison Instruments S.A., Barcelona, Spain) by means of a Crison GPL 22 pH meter (Crison). For reuterin determination, 2 g cheese was mixed with 4 ml Milli-Q water (Millipore Corporation, Bedford, MA, USA) and homogenized by an Ultra-Turrax T8

homogenizer (IKA, Labortechnik, Staufen, Germany). Mixture was kept in constant agitation for 20 min and centrifuged at 12,000 g for 5 min. Supernatants were filtered ($0.22\text{ }\mu\text{m}$; Merck Chemicals and Life Science, Madrid, Spain) and used for quantifying reuterin on triplicate by a colorimetric assay (Circle et al. 1945) and by HPLC (Talarico et al. 1988). Standard curves for both techniques were obtained by addition of purified reuterin ($0\text{--}30\text{ }\mu\text{mol/g}$) to cheeses elaborated without *L. reuteri* and glycerol.

Color determination

Color changes (L^* = lightness; a^* = green to red; b^* = blue to yellow) on cheese surface were measured in quadruplicate using a CM-700d spectrophotometer (Konica Minolta Inc., Osaka, Japan) and analyzed with Spectramagic NX VA.9 software (Konica Minolta Inc.) as described by Gómez-Torres et al. (2014). Effect of the addition of different concentrations of purified reuterin ($0\text{--}20\text{ }\mu\text{mol/g}$) on the color of homogenates from cheeses elaborated without *L. reuteri* and glycerol were monitored during 30 days of storage at 12°C .

Statistical analysis

Data were subjected to ANOVA with the SPSS program 12.0 for Windows (SPSS Inc., Chicago, IL, USA). Significant differences were assessed by Tukey's test at $P < 0.01$ using the same program.

Results and discussion

Cheese weight was between 187.3 and 198.2 g on day 1. The pH values were in the range of 5.08–5.19 on day 1 and of 4.87–5.16 on day 30. Differences in pH values of cheeses without glycerol and cheeses with *L. reuteri* plus glycerol were lower than 0.30 at any time of the ripening (data not shown).

CLC counts (Table 1) in cheese without glycerol were 9.51 log cfu/g on day 1 and decreased by 0.78 units at the end of the ripening period. In cheeses with 50 mM glycerol, differences in CLC counts with respect to cheese without glycerol throughout the ripening period were not significant. On the contrary, in cheeses with 100–500 mM glycerol counts decreased significantly ($P < 0.01$) throughout ripening. After 30 days, CLC values in cheeses made with 100–500 mM glycerol were between 5.23 and 5.66 log units lower than those in cheese without glycerol (Table 1). In this regard, lower lactic acid bacteria counts found in ewes milk cheeses made with *L. reuteri* INIA P572 and glycerol (Gómez-Torres et al. 2016) did not affect odor and aroma overall quality.

Table 1 Counts (log cfu/g) of commercial lactic culture (CLC) and *L. reuteri* INIA P572 in cheese made with different concentrations of glycerol

	Glycerol (mM)	Days					
		1	5	10	15	20	30
CLC	0	9.51 ^{aA}	9.59 ^{aA}	9.71 ^{aA}	9.63 ^{aA}	9.42 ^{aA}	8.73 ^{aB}
	50	9.46 ^{aA}	9.43 ^{aA}	9.55 ^{aA}	9.34 ^{aA}	9.30 ^{aA}	8.79 ^{aB}
	100	9.40 ^{aA}	7.37 ^{bB}	7.15 ^{cB}	6.16 ^{bC}	4.60 ^{bD}	3.50 ^{bE}
	200	9.40 ^{aA}	7.70 ^{bB}	7.61 ^{bB}	4.78 ^{cC}	3.22 ^{cD}	3.07 ^{bD}
	500	9.42 ^{aA}	7.87 ^{bB}	7.22 ^{cC}	3.76 ^{dD}	3.69 ^{bcD}	3.45 ^{bE}
<i>Lactobacillus reuteri</i>	0	6.15 ^{aA}	6.13 ^{aA}	5.97 ^{aA}	5.90 ^{aA}	6.30 ^{aA}	6.34 ^{aA}
	50	6.30 ^{aA}	5.55 ^{aA}	5.51 ^{abA}	5.48 ^{abA}	5.86 ^{abA}	5.17 ^{abA}
	100	6.43 ^{aA}	5.38 ^{aB}	5.20 ^{abB}	5.38 ^{abB}	5.01 ^{abcB}	4.44 ^{bC}
	200	6.33 ^{aA}	4.99 ^{aAB}	4.63 ^{bB}	5.01 ^{bAB}	4.69 ^{bcAB}	4.09 ^{bB}
	500	6.47 ^{aA}	5.14 ^{aB}	4.85 ^{bB}	5.06 ^{bB}	4.40 ^{cB}	4.42 ^{bB}

Values with different superscripts indicate statistically significant ($P < 0.01$) differences for a given organism and time (^{a–d}) or a given organism and cheese (^{A–E})

L. INIA P572 counts (Table 1) in control cheese without glycerol remained at 6.34 log cfu/g at the end of the ripening period. However, values in cheeses with glycerol showed a gradual decrease of *L. reuteri* INIA P572 during ripening, with counts at 30 days below 4.50 log cfu/g when 100–500 mM glycerol was added.

Reuterin concentrations in cheeses during ripening determined by the colorimetric method is shown in Table 2. This antimicrobial compound was not detected in cheese without glycerol, whereas it was produced in all cheeses made with *L. reuteri* INIA P572 and glycerol. The highest concentration of reuterin was observed on day 1 in cheeses made with 100–500 mM glycerol, reaching levels between 4.42 and 4.71 $\mu\text{mol/g}$. However, in cheeses made with 50 mM glycerol, maximum reuterin production was 1.05 $\mu\text{mol/g}$. On day 30, reuterin concentration decreased in all cheeses to values below 0.6 $\mu\text{mol/g}$. HPLC quantification of reuterin in 1-day cheeses made with 100–500 mM glycerol confirmed the colorimetric measurements since values between 4.12 and 5.28 $\mu\text{mol/g}$ were observed by this chromatographic technique (data not

shown). Reuterin was not detected by HPLC in cheese made with 50 mM glycerol or in any of the other cheeses from day 5 onwards, since levels in these cheeses were below the HPLC detection limit (2.6 $\mu\text{mol/g}$). Maximum reuterin concentrations observed in the present work were around 4.5 $\mu\text{mol/g}$ in 1-day cheeses made with glycerol at values equal or higher than 100 mM. Reuterin concentrations of between 0.95 and 0.98 $\mu\text{mol/g}$ have been previously reported in 1-day cheeses made with *L. reuteri* and 50 or 100 mM glycerol (Gómez-Torres et al. 2014). These differences might be attributed to the use of a *L. reuteri* supernatant instead of purified reuterin for quantification.

On day 30, the lowest values for L^* were 79.89 and 78.15 in cheeses with *L. reuteri* and 200 or 500 mM glycerol, respectively, whereas in control cheese L^* was 85.04. An increase of a^* throughout the ripening period was observed, with negative or under 0.65 values in all cases except in cheeses with *L. reuteri* and 200 or 500 mM glycerol, that showed a remarkable increase in the red direction from day 10 onwards, with values from 1.17 and 1.45 to 5.16 and 5.87 after 30 days, respectively. All b^* measurements were positive and increased in the yellow direction with the ripening time, reaching values between 14.35 and 16.12 on day 30 (data not shown). Gómez-Torres et al. (2014) also found a color defect in cheeses manufactured with *L. reuteri* and glycerol, which was attributed to the reaction of reuterin, or some of its derivatives, with cheese components. On the other hand, application of an active coating enriched with *L. reuteri* and glycerol to Fior di Latte cheese did not affect the sensory quality of the product, including color (Angiolillo et al. 2014). In the present work, no changes in color parameters were recorded in cheese homogenates with increasing concentrations (0–20 $\mu\text{mol/g}$) of purified reuterin and stored 30 days at 12 °C, with negative values for a^* parameter in all cases (data not shown). These results strongly suggest that the

Table 2 Reuterin production ($\mu\text{mol/g}$) in cheese made with *L. reuteri* INIA P572 as adjunct to the commercial lactic starter at different concentrations of glycerol

Glycerol (mM)	Days					
	1	5	10	15	20	30
0	ND	ND	ND	ND	ND	ND
50	1.05 ^{bA}	0.15 ^{dD}	0.31 ^{cC}	0.48 ^{bB}	0.29 ^{cC}	0.45 ^{bB}
100	4.42 ^{aA}	2.40 ^{aB}	1.85 ^{bC}	0.94 ^{aD}	0.90 ^{bD}	0.58 ^{aE}
200	4.71 ^{aA}	2.11 ^{cB}	2.33 ^{aB}	0.96 ^{aD}	1.57 ^{aC}	0.55 ^{aE}
500	4.60 ^{aA}	2.29 ^{bB}	2.41 ^{aB}	1.16 ^{aD}	1.63 ^{aC}	0.53 ^{aE}

Values with different superscripts indicate statistically significant ($P < 0.01$) differences for a given time (^{a–d}) or a given cheese (^{A–E})

development of the slight rosy coloration in cheeses made with the higher glycerol concentrations (200 and 500 mM) cannot be attributed to the reaction of the reuterin, or some of its derivatives, with cheese components, but rather to other compounds that might be produced at high glycerol concentrations.

Conclusion

According to colorimetric and HPLC measurements in cheese, application of reuterin-producing *L. reuteri* plus glycerol reached the highest reuterin levels in 1-day cheeses made with 100–500 mM glycerol. However, addition of concentrations higher than 100 mM glycerol could cause a red tendency in cheese color. *L. reuteri* used as adjunct to the starter culture with 100 mM glycerol resulted in reuterin levels in cheese higher than 4 $\mu\text{mol/g}$. Thus, the application of this strategy led to reuterin concentrations that might control the growth of spoilage and pathogenic contaminants potentially present during the manufacture and ripening of cheese, avoiding the production of color-changing compounds.

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