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Accelerated ripening of Ras cheese using freeze-shocked mutant strains of *Lactobacillus casei*

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Ras cheese was manufactured with Lac$^-$ and Lac$^+$ mutant strains of *Lactobacillus casei*. Cheese analysis and the organoleptic evaluation of the cheese were carried out for 6 weeks. Experimental cheeses showed higher values for soluble nitrogen, total volatile acidity and free fatty acids during cheese ripening than the controls. The organoleptic evaluation indicates that, as a general rule, the Lac$^-$ mutants obtained higher scores than the controls. Results of this work show that incorporation of frozen (Lac$^-$) strains of *Lb. casei* into Ras cheese as a source of enzymes could be useful for accelerating maturation, and could also serve to minimize the development of bitterness.

INTRODUCTION

Considerable progress has been made in the genetic analysis of lactic acid bacteria used in industrial dairy fermentations. Current research is focused on bacteriophage resistance, on mechanisms for *in vivo* inhibition of undesirable microorganisms and on several approaches to producing the desired flavour. In the area of accelerated cheese ripening the available information describes attempts to utilize strains lacking the proteinase and/or the lactose gene for this purpose. Grieve and co-workers (Grieve *et al.*, 1983; Grieve & Dulley, 1984) successfully modified starter microorganisms so that the starter culture population of cheese could be increased without increasing the rate of acid development; up to 12 weeks of advancement in flavour development was obtained. Similar observations were also made by Fedrick *et al.* (1984), while Oberg *et al.* (1986) manufactured Cheddar cheese using a proteinase-negative (Prt$^-$) mutant of *Lactococcus lactis* subsp. *cremoris* UC 73 and a commercial lactis culture blend (control). They found that cheese made with Prt$^-$ cultures graded equal to control cheese up to 90 days; it scored higher in overall texture and body but lower in cheese flavour and flavour intensity after 90 days. It also had significantly higher soluble N throughout storage. No significant differences in yields were found. When Cheddar cheese was made with 2% of a Prt$^-$ mutant of *Lc. lactis* subsp. *cremoris* UC 73 as starter and cooked at 39°C, manufacturing time increased by 40 min over control cheese. When inoculum was increased to 4% and the temperature to 42°C, manufacturing time was 3·6 h, which was 1 h less than with a 2% inoculum or the control. By providing a yeast extract carry-over of 0·0175%, it was possible to produce Cheddar cheese within a normal time-frame using only 0·1% Prt$^-$ culture. In the Netherlands, Stadhouders *et al.* (1988) also investigated the possibilities of adding either Prt$^+$ or Prt$^-$ strains to the normal starter for Gouda cheese-making. The authors concluded that cultures of exclusively Prt$^-$ variants are not useful as starters for making cheese with a good flavour. The proteinase(s) of starter lactobacilli appeared to be essential for proteolysis and flavour development. The presence of 20% Prt$^-$ variants is already sufficient to give maximal proteolysis and flavour development. Kamaly *et al.* (1989) attempted to accelerate the ripening of Cheddar cheese by adding the Lac$^-$ and Prt$^-$ mutants of *Lactococcus lactis* 25 SP and *Lc. lactis* subsp. *cremoris* KHA2 to cheese milk. Mutant-containing cheese developed higher levels of phosphotungstic acid-soluble amino nitrogen than did control cheese, although the levels of trichloroacetic acid-soluble nitrogen were similar. Mutant-containing cheeses had a slightly shorter body and more intense flavour than control cheese; a slight off-flavour, described as unclean, appeared in experimental cheese but was absent from control cheese. Bitterness did not develop in mutant-containing cheese

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during 6 months. Results of this work suggest that the incorporation of Lac$^-$ and Prt$^-$ mutants of lactococci into cheese milk, as a source of enzymes, could be useful in accelerating cheese ripening. In Ireland, Farkye *et al.* (1990) followed proteolysis and flavour developments in Cheddar cheese made with Prt$^+$ *Lc. lactis* subsp. *cremoris* UC-317 or its Prt$^-$ variant UC-041. The conclusion reached in this work was to some extent different from previous reports. The levels of water-soluble N in Prt$^+$ and Prt$^-$ cheeses of the same age were not significantly different, but the former had a significantly higher level of amino N throughout ripening. Prt$^+$ cheeses received slightly higher scores for flavour, body and texture than Prt$^-$ cheese of the same age. Nevertheless, the overall quality of all the cheeses was good, suggesting that starter peptidase activity may be more important than starter proteinases in flavour development in cheese during ripening. The previously described work was accomplished using genetically modified strains of *Lactococcus*. On the other hand, no information is available as far as the *Lactobacillus* species are concerned.

The aim of the present investigation was, therefore, to study the technological characteristics of different *Lb. casei* strains as accelerated-ripening agents and to evaluate Lac$^-$ and Lac$^+$ mutants of *Lb. casei*.

MATERIALS AND METHODS

Microorganisms

The strains used were obtained from the following sources: *Lb. casei* 20012, from Dr G. Fitzgerald, University of Cork, Ireland; *Lb. casei* Lac$^-$ mutants and wild types *Lb. casei* ATCC 4646 and *Lb. casei* 11578, from Dr Bruce Chassy, National Institute of Health, Maryland, USA. *Lb. casei* MS_1, MS_2, MS_3 and MS_4 are commercial strains obtained from different sources.

Culture preparation

The strains were sub-cultured for 12 h at 32°C in reconstituted non-fat dry milk (12·5% TS) at least twice before use. The inoculum for cheese was prepared by growing the cells in MRS medium (De Man *et al.*, 1960) at 32°C. The pH was maintained at 5·85 ± 0·2, using a pH controller (BIOLAFITTE Fermentor BL. 02.1). A 10N NaOH solution was added as needed, with a peristaltic pump, to maintain the pH at the set values. Growth was stopped at the early stationary phase, after approximately 8 h. The culture was then centrifuged at 10000 *g* (4°C). The pellets were washed three times with 0·01M K_2HPO_4 buffer and then frozen for 36 h at −20°C.

Table 1. Different strains of *Lactobacillus casei* used during the study

	Strain	Number[a] of cells × 10^5	Vat[b]
Experiment I	*Lb. casei* MS_1	2·23	B
	Lb. casei MS_2	2·06	C
	Lb. casei MS_3	1·96	D
Experiment II	*Lb. casei* 20012	1·12	B
	Lb. casei 4646 Lac$^-$	4·53	C
	Lb. casei 4646 Lac$^+$	3·33	D
Experiment III	*Lb. casei* MS_4	1·96	B
	Lb. casei 11578 Lac$^-$	2·24	C
	Lb. casei 11578 Lac$^+$	1·91	D

[a]Cells ml^{-1} milk (after being freeze-shocked).
[b]In the three experiments, Vat A was considered as a control.

Cheese manufacture

Ras cheese was manufactured in the dairy plant of the University of Alexandria, as described by Abdel-Tawab (1963), with the following modifications: cow's milk standardized to 3% fat was used; 1% of an active lactic culture (MD 088–1, Eurozyme, Paris, France) was used as a starter. The frozen pellets of *Lb. casei* strains were thawed in water at 30°C and added individually, just prior to renneting. Three sets of experiments were run in duplicate, according to Table 1, which also indicates the number of cells ml^{-1} milk in each case.

Cheese analysis

Composition of the cheeses
The pH was measured by probing (directly) the cheese homogenate with a glass electrode. Total solids were determined by oven-drying at 105°C. Fat was determined by the Gerber method. Determination of total nitrogen was by the Kjeldahl method (Kosikowski, 1977).

Ripening indices
Soluble nitrogen at pH 4·6 was determined as described by Gripon *et al.* (1977). The procedure of Osteux *et al.* (1958), as modified by El-Nemer (1982), was used to determine the total volatile acidity in 15 g of cheese, while the free fatty acids in cheese were measured using the procedure described by Godinho and Fox (1981).

Flavour evaluation
Flavour intensity and description were evaluated using, as general guide, the flavour scores and criticism proposed by Kosikowski and Iwasaki (1975), with 0 indicating flavour defects and 5 indicating strong flavour.

RESULTS AND DISCUSSION

Gross composition

Addition of frozen concentrates of lactobacilli did not affect the gross composition of the cheese. The lactobacilli-treated cheeses showed values for pH, dry matter, fat (%) and protein (%) very close to those for the control. It is, for instance, notable that, at the end of ripening period (6 weeks), the control cheese in the three experiments showed pH 5·8, dry matter 75·4%, fat 36·0% and protein 27·6%, corresponding to values for the cheese treated with *Lb. casei*. In Experiment I these were 5·8, 81·0%, 38·0% and 33·7%, while they were 5·4, 76·7%, 39·0% and 30·2% in Experiment II and 5·6, 71·0%, 32·0% and 31·9% in Experiment III. Kamaly *et al.* (1989) also found no changes in the composition of cheese made with Lac$^-$ mutants compared with the control.

Proteolysis during cheese ripening

The lactobacilli-treated cheeses showed higher rates of proteolysis than the control. The per cent increase in proteolysis compared with the control after 6 weeks of ripening was 65, 34 and 19 for *Lb. casei* MS_1, MS_2 and MS_3, respectively (Fig. 1); a similar trend was found in the other experiments. Among the *Lb. casei* strains tested, the cheese containing *Lb. casei* MS_4 showed the highest level of protein breakdown, followed by 11578 Lac$^+$ and 11578 Lac$^-$. Our findings are in agreement with the work of Kamaly *et al.* (1989) who also concluded that Cheddar cheese containing Lac$^-$ mutant developed higher levels of soluble amino nitrogen than did the control cheese.

The higher levels of proteolysis obtained early during the maturation of Ras cheese with lactobacilli compared with the control can be explained by the existence of a membrane interface aminopeptidase as well as cell-wall-associated proteinase in lactic acid bacteria, in addition to the highly active intracellular peptide hydrolase system (Desmazeaud, 1983; Marshall & Law, 1984; Khalid *et al.*, 1990).

The cheese manufactured with the two Lac$^-$ strains used in this investigation, *Lb. casei* ATCC 4646 Lac$^-$ and 11578 Lac$^-$, showed lower levels of proteolysis compared with the wild Lac$^+$ strains. Relative increases in levels of soluble nitrogen (Fig. 1) of about 16% and 15% were measured in the case of *Lb. casei* 4646 Lac$^+$ and 11578 Lac$^+$, respectively. Similar observations were also reported by Abu-Tarboush *et al.* (1989). In fact these authors showed that, among 14 strains of lactococci tested, four lactose-negative mutants were significantly less proteolytic. This is probably due to the difference in the levels of cell-wall proteinase activities in the Lac$^+$ and Lac$^-$ mutants since it was shown that the levels of intracellular peptidases between the Lac$^-$ and Lac$^+$ strains of *Lb. casei* 4646 and 11578 are identical (El-Soda *et al.*, 1989).

The results (Figs 2 and 3) illustrating the breakdown

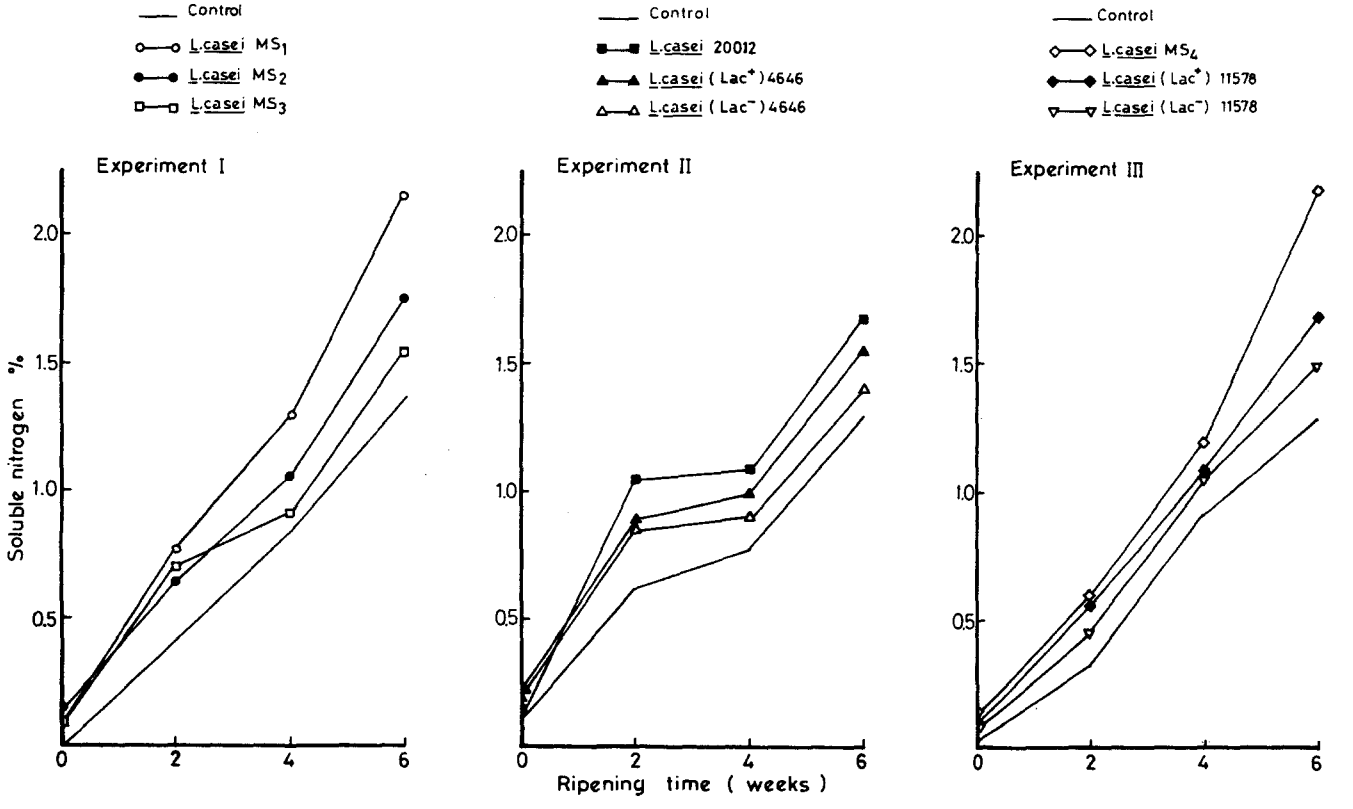

Fig. 1. Evaluation of soluble nitrogen during cheese ripening.

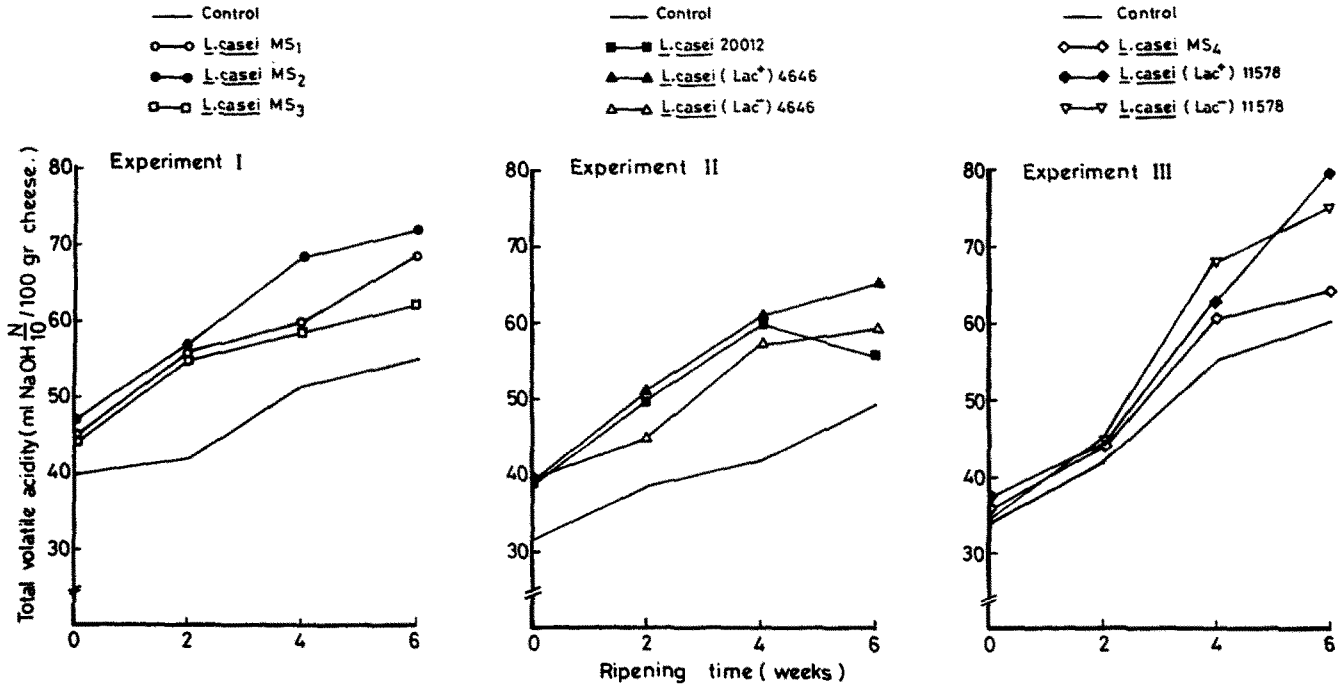

Fig. 2. Evaluation of total volatile acidity during cheese ripening.

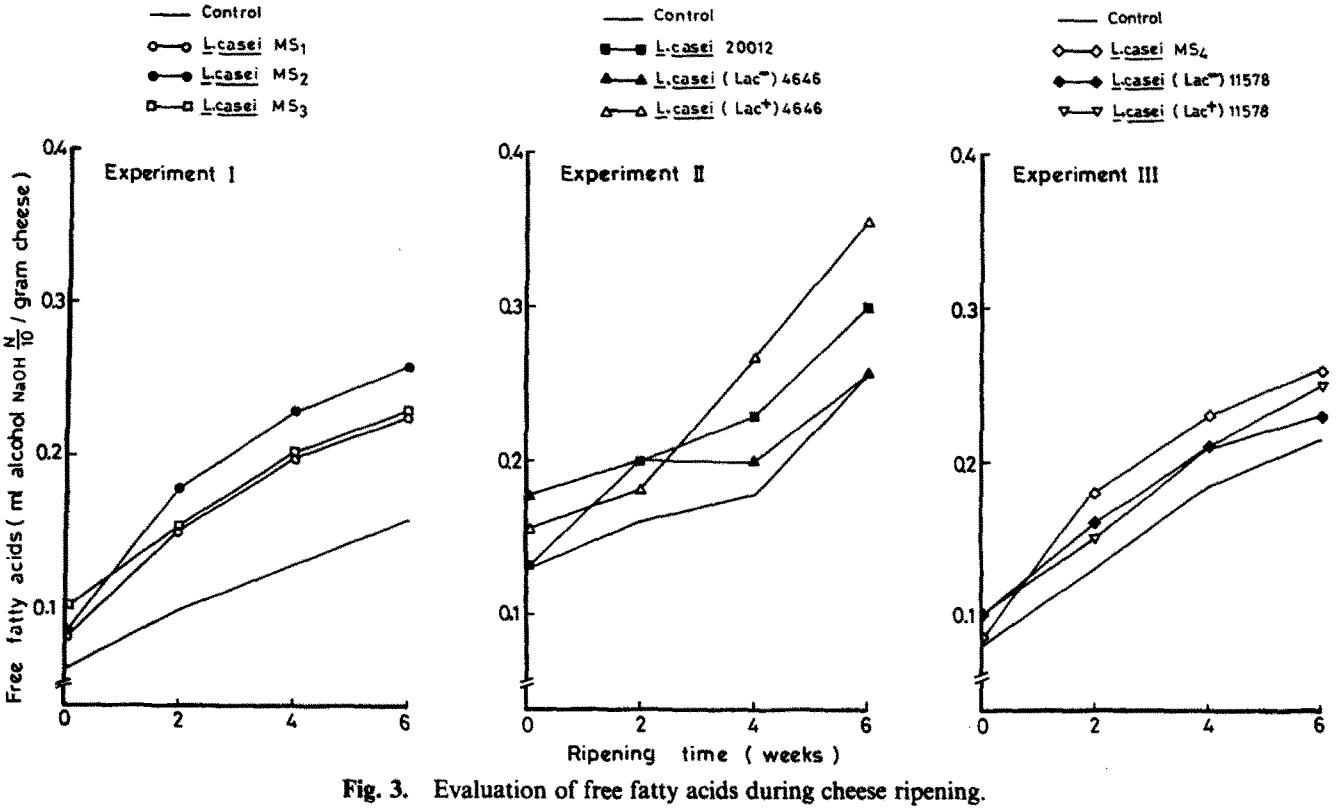

Fig. 3. Evaluation of free fatty acids during cheese ripening.

of fat during cheese ripening indicate higher rates of total volatile acidity (TVA) formation and free fatty acid (FFA) liberation in lactobacilli-containing cheese. The wild strains always exhibited higher lipolytic activities than the Lac$^-$ mutants. The values for FFA and TVA for the *Lb. casei* 4646 Lac$^+$ were 41% and 32%, respectively, compared with the control, while they were only 25% and 12% for the Lac$^-$ mutant. This is probably due to the loss of a cell-wall lipolytic enzyme with the loss of the lactose plasmid, since it was previously demonstrated that the esterolytic activities in the mutant and wild strains of *Lb. casei* ATCC 4646 and *Lb. casei* 11578 are identical (El-Soda *et al.*, 1989).

Table 2. Organoleptic properties of Ras cheese after 6 weeks

		Flavour (2·5)	Body & texture (2·5)	Total (5·0)
Experiment I	Control	2·0	2·0	4·0
	Lb. casei MS_1	2·0	2·0	4·0
	Lb. casei MS_2	2·0	2·5	4·5
	Lb. casei MS_3	2·0	2·0	4·0
Experiment II	Control	1·5	2·0	3·5
	Lb. casei 20012	2·0	2·0	4·0
	Lb. casei 4646 Lac^-	2·5	2·0	4·5
	Lb. casei 4646 Lac^+	1·0	1·5	2·5
Experiment III	Control	1·5	2·0	3·5
	Lb. casei MS_4	2·5	1·5	4·0
	Lb. casei 11578 Lac^-	2·0	2·0	4·0
	Lb. casei 11578 Lac^+	1·0	1·5	2·5

Organoleptic evaluations

Organoleptic evaluations of the different cheeses (Table 2) indicate that the cheese containing Lac^- mutants obtained higher scores than the control. Most of the *Lb. casei*-treated cheese showed a pronounced acid flavour defect not detected in Lac^- mutants, which indicates that, although the rate of protein and fat breakdown in the wild strains was higher than in the Lac^- mutants, the overall preference was for the cheese made with the strains lacking the lactose plasmid. Bitter flavour was not observed in any of the *Lactobacillus*-treated cheese, as was the case in previous experiments (El-Soda *et al.*, 1981) where a crude cell-free extract of *Lb. casei* was used. This is probably because of improper spatial arrangements between enzymes and substrates in the fortified cheese.

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