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Attenuated starters: an efficient means to influence cheese ripening—a review

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Abstract

Attenuated starters are lactic acid bacteria unable to grow and to produce significant levels of lactic acid but still delivering active ripening enzymes during cheese ageing. The attenuation can be performed by several methods: heating, freezing-thawing, freeze- or spray-drying, lysozyme or solvents treatment. Attenuated starters can also be obtained by the selection of lactose-negative mutants. Whatever the method used, various attenuated lactococci and lactobacilli species have been prepared and added to the milk in parallel to the primary starter. They increased in most cases proteolysis and lipolysis, reducing ripening time and improving flavour (bitterness reduction) as exhaustively presented here. Despite these promising results in cheese, the industrial use of attenuated starter is not widespread. One main reason is likely the empirical character of the attenuation methods proposed or the cost of this cell addition. Further research is needed to increase knowledge about these efficient cheese additives. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Cheese; Ripening; Starter; Attenuation; Lactic acid bacteria

1. Introduction

Cheese ripening is a time-consuming process involving complex and well-balanced reactions between glycolysis, proteolysis and lipolysis of the milk components. In most cheeses, bacterial enzymes play a major role in this process. The proteases, peptidases, esterases, and the enzymes of amino acid catabolism in the lactic acid bacteria have been extensively studied (Urbach, 1995; Gobetti, Fox & Stepaniak, 1996; Gao, Oh, Broadbent, Johnson, Weimer & Steele, 1997; Law & Haandrikman, 1997). Those of other starters, such as propionibacteria, and surface flora, have been studied less extensively. It is well known that changing the bacterial enzyme content in cheese directly affects the rate of cheese ripening and its final flavour. One of the most efficient ways to increase the bacterial enzyme pool in cheese curd, without altering the primary starter and the cheese make, is to add whole lactic acid bacteria, unable to grow and to produce

significant levels of lactic acid, but still delivering active ripening enzymes during cheese ageing. These starters are normally weakened and referred to as ‘attenuated’. Their preparation can be achieved by various treatments like heating, freezing, spray-drying, freeze-drying, fragilisation using lysozyme or solvents, or by the selection of lactose negative mutants.

Historically, the first preparation and use of attenuated starters as a cheese additive, was reported in 1975, by Pettersson and Sjöström. At this time, their main purpose was to accelerate proteolysis and shorten ripening time, which has great economic interest. Today, in most developed countries the hygienic quality of milk is so high that the problem is not only to shorten the ripening time but also to improve the flavour. This is particularly true for new kinds of cheeses such as UF- or low-fat, which commonly exhibit poor flavour development and a rubbery texture (El Soda, Chen, Riesterer & Olson, 1991). Attenuated starters, providing they are well known and reproducibly made, can improve both the rate and the quality of cheese ripening (Fox, 1988; El Soda & Pandian, 1991).

The aim of this review is to detail the various existing attenuation treatments, the bacterial species involved, the

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cheeses to which they were applied, and their effects on the ripening. The future of attenuated starter cultures is also discussed.

2. Attenuation by heat treatment

The most studied form of starter attenuation was heat treatment, which was first described by Petterson and Sjöström (1975). Table 1 shows its application to various lactic acid bacteria. This attenuation method was based on sub-lethal heat treatment of a bacteria cell suspension. The attenuated cells were added to cheese milk along with the primary starter culture. *Lactobacillus helveticus* was the bacterial species most often subjected to heat treatment. Over half of the cheese trials used *L. helveticus*, primarily because of its high peptidase activity as compared to other lactic acid bacteria. Whatever the species, strains were chosen for attenuation based on their successful use as primary starters (*L. helveticus* CNRZ 32; commercial mesophilic starter like Flora Danica) or their use as secondary starters, without causing flavour defect (like *Lactobacillus casei* L2A, Trepanier, EL Abboudi, Lee & Simard, 1992).

A critical point for heat attenuation was to define the correct temperature/time combination, so that acidification was eliminated or delayed without excessively denaturing potential ripening enzymes (e.g. proteases, peptidases, esterases, or others). Petterson and Sjöström (1975) recommended an inactivation temperature of 59°C for mesophilic starters and 69°C for thermophilic starters, such as *L. helveticus*. However, recommended heat treatments for a specific species varied, depending on the authors (Table 1). There appeared to be little agreement on which heat treatment was best. For example, parameters for *L. helveticus* varied between 67°C for 10 s and 72°C for 15 s. Also, both residual viability and enzyme activities of the attenuated cultures were not systematically reported.

The procedure for heat treatment varied. Generally, the strains were grown in milk medium under pH control, to achieve high cell densities and higher proteolytic and peptidase activities (Castaneda et al., 1990). Cells were then heat-treated directly in the milk with precise heat exchange devices (Petterson & Sjöström, 1975; Exterkate et al., 1987; Ardö & Pettersson, 1988) or by resuspending them in hot milk at a selected temperature (e.g. 72°C) for 10–15 s, followed by cooling (Bartels et al., 1987a). Exterkate et al. (1987) recommended pre-cooling cells to 5–8°C, at least 1 h before heat treatment, to ensure that acidification was then sufficiently inhibited. Cells were sometimes resuspended in phosphate buffer to be heat-treated (Castaneda et al., 1990) and this hampers any comparison with treatment in milk. Each study set forth its own set of parameters for optimised treatment to reduce acidification ability, yet retain peptidase activity.

Lopez-Fandino and Ardö (1991) noted that the proteolytic activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* decreased with temperature at a faster rate than aminopeptidases activity. Castaneda et al. (1990) emphasised the very narrow range between adequate and excessive heat treatments. For example, heat treatment at 64°C for 18 s reduced 90% of the acidification activity in *L. helveticus*, but only 60% of the cell wall proteinase and 10% of the aminopeptidase activities. An increase of 2°C from 64 to 66°C dramatically inactivated proteinase and peptidase activities. A similar reduction in activity was also noted when *L. casei* or *Lactobacillus plantarum* was heated for 15 s at 54°C versus 50°C (Asensio, Gomez & Pelaez, 1995). Thus, preparing heat-attenuated starters properly seemed not so easy from a practical point of view. It was not apparent from these studies, whether heat treatment must be optimised for each strain, or if a particular treatment was applicable to all strains of a species.

Heat treatment destroyed, or delayed the acidification ability of the cells for several hours, so that the attenuated cultures did not significantly modify the acidification course of the curd, and subsequently the fat/moisture, and pH of the cheese. Most often, the loss of lactic acid production closely followed the loss of viability (Petterson & Sjöström, 1975). However, cell mortality measured by plate counting does not necessarily indicate cell death. Sub-lethally injured cells may have been injured to the extent that they could no longer grow. As far as we know, the mechanisms of heat inactivation of the lactic-acid-producing ability have not been fully elucidated. Attenuation may occur at different levels, such as damage to the enzymes involved in the transport of substrates through cell walls and membrane, or physical damage of the membrane itself, or denaturation of β -galactosidase as suggested by Frey, Marth, Johnson & Olson (1986). Heat-treated cells of *L. helveticus* (18 s, 64°C), observed by electron microscopy, showed no apparent damage to membrane and cell wall, but did exhibit fewer ribosomes and DNA aggregation, compared to the control. Lysed cells were not evident in micrographs of heat-treated bacteria (Castaneda et al., 1990).

Heat-attenuated starters were added to various experimental cheeses (Table 1). The original aim was to accelerate ripening, but at least three reports since 1994 have highlighted the potential use of attenuated starters for flavour development. In most experiments, the addition of attenuated starters had no effect on the pH and curd composition. The impact on proteolysis was systematically followed by measuring soluble nitrogen in phosphotungstic or trichloroacetic acids or by free amino acids or peptide analysis. Sensory evaluations were also performed.

Regardless of the bacterial species involved, or the heat-attenuation treatment used, the addition of heat-attenuated starters enhanced proteolysis and had a positive

impact on cheese flavour as shown in Table 1. Reduction of bitterness was the most frequently reported observation.

The major contribution of heat-attenuated starters to cheese making was the hydrolysis of medium-sized peptides. Indeed the absence of primary casein hydrolysis by attenuated starters was verified by several authors (Ardö & Pettersson, 1988; Vafopoulou et al., 1989). Peptidases were shown to be intracellular in lactic acid bacteria (Kunji, Mierau, Hagting, Poolman & Konings, 1996), therefore their presence in cheese would indicate that cell lysis occurred (Lortal, Valence, Bizet & Maubois, 1997). Lysis of attenuated cells was demonstrated in one case by quantifying the release of leucine aminopeptidase (Asensio et al., 1996). Bacterial autolysins would survive attenuation heat treatments since they are able to survive boiling (Valence & Lortal, 1995). Increased fat hydrolysis and the release of free fatty acids were also noted on the addition of heat-attenuated starters to cheese. Lastly, several studies reported a reduction in ripening time in the test cheeses when compared with control cheeses (Table 1).

The inoculum level of heat-attenuated starters varied among the different cheese trials. Most of the trials used between 1 and 5% (v/v). A level of 5% was not exceeded, to ensure that the attenuated starter did not affect the cheese making process. As cells were sometimes concentrated before addition (Pettersson & Sjöström, 1975), the quantity actually added to the milk is rather difficult to deduce. It was estimated, from the studies available, that 5×10^6 – 5×10^7 heat-attenuated cells were added per mL of milk in most trials. It was estimated that 10^4 – 10^6 cells mL^{-1} were still viable, taking into account a residual viability after treatment of 0.3–30%. To determine the number of attenuated cells in the curd, either dead or alive, the percent of retention must be taken into account and this was rarely done. Some authors assumed 100% retention, based on the absence of residual peptidase activity in the whey (Exterkate et al., 1987). When actually measured, cell retention was higher for rods (lactobacilli) than for cocci (Pettersson & Sjöström, 1975). This suggested that steric factors, such as size and shape, had an effect on cell retention in the curd. It was also observed that increasing the number of attenuated cells (from 2×10^8 to 2×10^9 mL^{-1} of milk), did not significantly change the number of cells trapped in the final curd (10^9 – 2×10^9 per g of cheese in all cases). It therefore appeared that the efficiency of cell incorporation decreased as the number of attenuated cells was increased. This would indicate that steric factors alone did not directly determine the number of attenuated cells retained. It is likely that only a finite number of bacteria can adsorb to particular milk components. Laloy, Vuillemand, El Soda & Simard (1996) underlined the potential role of fat globules in retaining starter cells.

The form of the attenuated starters, whether liquid or freeze-dried, can also affect cell retention in curd, and

should be considered. Specific studies must be undertaken to determine the retention mechanisms involved as well as the optimal number of cells to be added to milk. This optimum could also depend on the desired objective. A de-bittering function, for example, could require lower number of heat-treated cells than flavour enhancement.

The fact that all studies using heat-treated starters led to positive results, regardless of the bacterial species or the cheese studied, underlines the great interest in such additives. Nevertheless, one should keep in mind that disappointing results were eventually neither published, nor further explored. The slightly negative effect on cheese flavour from the addition of *L. bulgaricus* or *Streptococcus thermophilus* is a case in point (Bartels et al., 1987a).

3. Attenuation by freezing–thawing

Pettersson and Sjöström (1975) first proposed freezing and thawing as an attenuation treatment for lactic acid bacteria. The first cheese trial involving freeze-shocked cells, was performed by Bartels, Johnson & Olson (1987b). This treatment submitted whole cells that were washed and concentrated tenfold, to freezing at -20°C overnight or longer and to rapid thawing at 40°C prior to milk addition. Several species were attenuated in this way (Table 2), but *L. helveticus* was again the most frequently used. In all studies, the freeze-shocked cultures did not significantly affect the moisture level and the acidity of the final cheeses. Proteolysis, however, was significantly enhanced, and the flavour of low fat and UF-cheeses was greatly improved (Table 2). When assayed, the content of volatile fatty acids was also shown to increase, but unsuitable crystal formation was described in some studies (El Soda et al., 1991). Freeze-shocked cells of *L. helveticus*, when compared to *L. casei* (Aly, 1990), *L. bulgaricus* or *S. thermophilus* (Aly, 1996), showed significantly higher proteolysis and flavour development. However, attenuated *L. helveticus* cells sometimes gave a sweet, nutty flavour, which was not normally associated with the cheese being considered (Johnson et al., 1995). Recent assays in cheese slurries confirmed that *L. helveticus* increased proteolysis and lipolysis and led to a sweet or slight sulphuric character depending on the strain (Madkor, El Soda & Tong, 1999).

The inoculum level of freeze-shocked cells varied from 0.4 to 2% (w/w) (Table 2) which corresponded to 10^7 – 10^8 attenuated cells/mL of milk. Of this number, probably 10^5 – 10^6 were still viable. In general, for a given strain, the destructive effect of freezing, on intracellular enzyme activity, was much lower than that of heat treatment (Thiboutot, Dako, El Soda, Vuillemand, Power & Simard, 1995; El Shafei, 1996).

The physical–chemical–biochemical effects of freezing are multiple and complex. Bacterial cells can freeze by

Table 1
Cheese assays with heat-treated starters^d

Aim of the work ^a	Species	Treatment and residual viability (%)	Type of cheese	Quantity added/ mL of milk	Impact on the proteolysis ^b	Other observations ^c	Sensory evaluation and ripening time	Authors
A	<i>L. helveticus</i>	69°C/15 s	Swedish semihard	5.7–8.5% (v/v) 10 ⁷ –10 ¹⁰ /mL	↗ N-TCA (+60%) ↗ N-PTA (+50% at 1 month)	—	↗ Flavour score ↘ Ripening time (1 month), no bitterness	Petterson and Sjöström (1975)
A	Mixed mesophilic starter	59°C/15 s	Swedish semihard	3–8.6% (v/v)	↗ N-TCA ↗ N-PTA (+25% at 1 month)	↗ Citrate fermentation	↗ Flavour score ↘ Ripening time, no bitterness	Petterson and Sjöström (1975)
F	<i>Streptococcus cremoris</i>	64°C/16 s	Low-fat Cheddar	10 ⁷ cfu/mL	—	↗ VFA	↗ Mature flavour No rancid flavour	Somkuti, Thompson and Flanagan (1979)
A, F	<i>L. casei</i> or <i>L. helveticus</i>	69°C/15 s	Ras	1–2% (v/w)	↗ N-soluble ↗ N-TCA ↗ N-PTA (2% > 1%) and <i>L. helveticus</i> > <i>L. casei</i>	↗ VFA (2% > 1%) <i>L. helveticus</i> > <i>L. casei</i>	↗ Flavour intensity ↗ Consistency (1 ≈ 2%), no bitterness ↘ Ripening time (1–2 months) with <i>L. helveticus</i>	Abdel Baky, El-Neshawy, Rabie and Ashour (1986)
A	Mixed lactococci strains	56.5°C/17 s 9–30%	Gouda	5% (v/v)	↗ N-PTA (20–30%)	—	↗ Flavour scores ↘ Bitterness ↘ Ripening time (25%)	Exterkate, De Veer and Stadhouers (1987)
A	<i>L. helveticus</i> or <i>S. thermophilus</i> or <i>L. bulgaricus</i>	70°C/18 s	Gouda	2–4% (w/w)	↗ N-TCA (85%) only with <i>L. helveticus</i>	—	↗ Flavour intensity (sweet) ↘ Bitterness, both only with <i>L. helveticus</i>	Bartels, Johnson and Olson (1987a)
A	<i>L. helveticus</i>	67°C/10 s 0.3%	Swedish hard, Svecia	0.7% (v/v)	↗ N-TCA ↗ N-PTA	↗ Citrate fermentation	↗ Flavour score (sweet, nutty) ↘ Bitterness	Ardö and Pettersson (1988)
A	<i>S. thermophilus</i> (25%) + <i>L. bulgaricus</i> (75%) + Lamb lipase	63°C/20 s	Feta	2% (v/v)	↗ N-soluble ↗ N-TCA ↗ N-PTA (35%)	↗ Fat hydrolysis ↗ FFA (+46%) ↗ Acetaldehyde	↗ Flavour, body, no bitterness ↘ Ripening time (50%)	Vafopoulo, Alichanidis and Zerfidis (1989)
F	<i>L. helveticus</i>	67°C/10 s	Rindless low-fat cheese	0.1–0.2% (v/v)	↗ N-soluble ↗ N-PTA ↗ Aminopeptidolytic activity	—	↗ Flavour score ↘ Bitterness	Ardö, Larsson, Lindmark Mansson and Hedenberg (1989)
A	<i>L. helveticus</i>	64°C/18 s 0.5%	St Paulin	1% (v/v)	↗ N-soluble ↗ N-PTA	No textural change	↗ Flavour, not typical for a St Paulin (sweet taste)	Castaneda, Vassal, Gripon and Rousseau (1990)

A	<i>L. helveticus</i>	70°C/18 s 3%	Ras	—	↗ N-soluble	↗ Total volatile acidity ↗ FFA	↗ Flavour-, body, texture ↘ Bitterness	Ezzat and El-Shafei (1991)
F	<i>L. helveticus</i>	72°C/15 s	Low-fat Kashkaval	2% (w/w)	↗ N-soluble (+ 50%) ↗ N-TCA (+ 50%) ↗ N-PTA (+ 60%) Change in peptidic profile ↗ Free amino acids	↗ FFA	↗ Flavour, body, texture No bitterness	Aly (1994)
	Mesophilic or thermophilic starters	64°C/15 s 68°C/15 s	Cheddar slurry	1.5–10%		—	—	Farkye et al. (1995)
F	<i>L. helveticus</i>	67°C/10 s	Low-fat Gouda	0.7% (v/v)	↗ N-soluble ↗ N-PTA	↗ Volatile compounds = acetaldehyde	↗ Flavour intensity off flavour (sweet) at 28 weeks ↗ Firmness (at 12 and 28 weeks) ↘ Cohesiveness	Skeie, Narvhus, Ardö and Abrahamsen (1995)
F	<i>Lc. lactis: 1</i> <i>Lc. casei: 2</i> <i>L. plantarum: 3</i>	50°C/15 s	Low-fat goat's milk cheese	1.25% (v/v)	↗ N-TCA/NCN Change in peptidic profile	↗ Free leucine Aminopeptidase (better with 2–3)	↗ Flavour, texture ↘ Bitterness	Asensio et al. (1996)
F	<i>L. helveticus</i>	—	Rindless low-fat cheese	0.06% (w/v)	↗ N-soluble ↗ N-PTA	↗ Acetaldehyde ↗ Acetic and formic acid (at 8 weeks), pyro-glutamic acid (at 8, 16 weeks) ↘ Succinic and pyruvic acids	↗ Flavour score (at 8, 16 weeks) ↗ Firmness, number of eyes (at 16 weeks) ↘ Bitterness	Skeie, Narvhus, Ardö, Thorvaldsen and Abrahamsen (1997)
F	<i>L. bulgaricus</i> and <i>S. thermophilus</i>	—	Kareish	1.25% (w/w)	↗ SN ↗ S tyrosine ↗ S tryptophane	↗ VFA	↗ Cheese score ↘ Ripening time (33%)	Kebery, Salem, Hamed and El-Sisi (1997)
A	<i>Mesophilic starter</i>	56°C–60°C/ 17–20 s 2.5%	Lithuanian and Dutch cheeses	—	↗ N-soluble (23%)	↗ FFA ↗ Acetoin, esters	↗ Flavour, taste, consistency, holeyness ↗ Rigidity, elasticity ↘ Ripening time (25%)	Salomskiene (1998)
A	<i>Lc. lactis</i>	—	Edam (UF milk)	1%	Same SN/TN,NPN ↗ Free amino acids, (after 2 months)	Same VFA	Same organoleptic properties	El-Etriby, Al-Khamy, Zaghloul, Shahein and El-Sheikh (1998)

^a A: acceleration of ripening, F: enhancement of flavour.

^b N-TCA = TCA-soluble N: nitrogen soluble in 12% TCA (peptides + amino acids), N-PTA = PTA-soluble N: nitrogen soluble in 5% PTA (amino acids), NCN = non-casein nitrogen, NPN = non-protein nitrogen, N/NH2 = amino nitrogen fraction, SN = soluble nitrogen.

^c FFA: free fatty acids, VFA: volatile fatty acids, LDH: lactate dehydrogenase.

^d ↗ Increase, ↘ decrease.

Table 2
Cheese assays with freeze-shocked starters^d

Aim of the work ^a	Species	Treatment and residual viability (%)	Type of cheese	Quantity added/mL of milk	Impact on the proteolysis ^b	Other observations ^c	Sensory evaluation and ripening time	Authors
A	<i>L. helveticus</i>	–24°C/24 h 3%	Gouda	2% (w/w) 10 ⁸ –10 ⁹ /mL	↗ N-TCA (50–60%) ↗ N-PTA	FFA ↔ Change in peptidic profile	↗ Flavour intensity and quality ↘ Bitterness ↗ Firmness	Bartels et al. (1987b)
F	<i>L. helveticus</i> + liposome-entrapped proteinase	–24°C/24 h	Gouda (with UF milk)		↗ N-TCA ↗ N-PTA		↗ Flavour intensity ↘ Bitterness	Spangler, El-Soda, Johnson, Olson, Amundson and Hill (1989)
A, F	<i>L. helveticus</i> or <i>L. casei</i>	–20°C/24 h (0.8%) –20°C/24 h (0.9%)	RAS	1 and 2% (v/v)	↗ SN ↗ N-TCA ↗ N-PTA	↗ VFA	↗ Flavour intensity ↗ Body and texture ↘ Ripening time (50%)	Aly (1990)
A	<i>L. casei</i> or <i>Pediococcus</i>	–24°C/24 h	Low-fat Cheddar	—	↗ N-TCA (52% L.c, 33% P.) ↗ N-PTA	—	↗ Flavour intensity ↘ Bitterness (but internal calcium lactate crystals for P.)	El Soda et al. (1991)
A	<i>L. helveticus</i>	–20°C/24 h 3%	Ras	—	↗ SN	↗ VFA ↗ FFA	↗ Flavour ↘ Bitterness (weak)	Ezzat and El-Shafei (1991)
A	<i>L. helveticus</i>	–15°C/96 h	Gouda	0.4%	↗ N-TCA ↗ N-PTA (70%)	Change in peptidic profile ↗ Small peptides	↘ Bitterness	Kim, Kim and Olson (1994a, b)
F	<i>L. helveticus</i>	–20°C/24 h	Low-fat Kashkaval	2% (w/w)	↗ SN ↗ N-TCA ↗ N-PTA	↗ FFA	↗ Flavour intensity ↗ Body, texture No bitterness	Aly (1994)
A, F	<i>L. helveticus</i>	–20°C/2 to 4.5 week 54%	Low-fat Cheddar	—	↗ N-TCA ↗ N-PTA	—	↗ Flavour intensity not typical for a Cheddar cheese (sweet, nutty) off-flavors at 1.5 month	Johnson, Etzel, Chen and Johnson (1995)
A, F	<i>L. helveticus</i> or <i>L. bulgaricus</i> or <i>S. thermophilus</i>	–20°C or –100°C/ 1 week	Domiat	2% (w/w)	↗ SN ↗ N-TCA ↗ N-PTA	↗ VFA (L.h > L.b > S.t)	↗ Flavour intensity ↗ Body, texture (L.h > L.b > S.t) No bitterness ↘ Ripening time with L.h (75%)	Aly (1996)
F	<i>L. bulgaricus</i> or <i>S. thermophilus</i>		Kareish	1.25% (w/w)	↗ SN ↗ S tyrosine ↗ S tryptophan	↗ VFA	↗ Cheese score ↘ Ripening time (33%)	Keব্য et al. (1997)

^aA: acceleration of ripening, F: enhancement of flavour.

^bN-TCA = TCA-soluble N: nitrogen soluble in 12% TCA (peptides + amino acids), N-PTA = PTA-soluble N: nitrogen soluble in 5% PTA (amino acids), NCN = Non-casein nitrogen, NPN = non-protein nitrogen, N.NH2 = amino nitrogen fraction, SN = soluble nitrogen.

^cFFA: free fatty acids, VFA: volatile fatty acids, LDH: lactate dehydrogenase.

^d↗ Increase, ↘ decrease.

intracellular ice formation or by dehydration, freezing removes water from both the external and internal environments and increases the concentration of solutes both inside and outside the cells (Ray & Speck, 1973). Ice crystals can cause damage to the cell membrane. After freezing and thawing treatment, the authors noted that bacterial cells lose many micro- and macromolecular cellular components in the suspending media. Many theories have been advanced to explain the death of bacterial cells by freezing. Several factors, e.g. lowered temperature, both extra- and intracellular ice formation, both extra- and intracellular solute concentration, either singly or in combination, can damage frozen bacterial cells. Cell wall damage has also been observed in *Lactobacillus acidophilus* due to freezing (Johnson, Ray & Speck, 1984).

The addition of freeze-shocked cells to cheese significantly increased soluble nitrogen in phosphotungstic acid. This again suggests that cell lysis had occurred and that intracellular peptidases were released into the cheese. The practice of freezing cells to increase subsequent cell lysis was reported by several researchers (Bie & Sjöström, 1975; Thiboutot et al., 1995). The attenuation by freezing is certainly easier to achieve than heat attenuation. However, no studies applied both treatments to the same starter strains, therefore, their respective impacts on cheese ripening are difficult to compare.

4. Other methods of attenuation

4.1. Lysozyme treatment

Law, Castanon and Sharpe (1976) treated a suspension of lactococci cells in deionised water for 15 min at 37°C with 0.1–2 mg mL⁻¹ of lysozyme (optimum at 0.5 mg mL⁻¹). These lysozyme-sensitive cells (LSC) did not produce acid in skim milk over a 6 h period. The cells were not lysed by the treatment. Proteolytic activity, however, was reduced by 36%, which can be explained by a partial loss of cell wall material, to which proteases were associated. In Cheddar cheese experiments, LSC lysed in the curd during brining, which was shown by the release of active dipeptidase and DNA. The addition of 3×10^9 LSC g⁻¹ of cheese led to a 1.8 increase in free amino acids, compared to the control without LSC. A seven-fold increase (2×10^{10} LSC g⁻¹) of cells led to a 2.5 increase in free amino acids, which is significant but not proportional. The lysis markers (dipeptidase, DNA) were also only two times higher. This led to the conclusion that the incorporation efficiency decreased as the number of cells increased as previously suggested by Petterson and Sjöström (1975). Unfortunately, the loss of LSC into the whey was not checked to confirm this hypothesis.

However, in contrast to heat-attenuated starters, addition of LSC did not change the quality or the intensity of cheese flavour. This finding was surprising, since lysozyme treatment was more likely to preserve starter enzymes activity than would heat treatment. Another explanation could be that lactococci intracellular enzymes played a role in secondary proteolysis, but not a direct role on Cheddar flavour development. The use of lysozyme-treated cells should be further explored to definitively assess their effect on cheese flavour development, trying other species and/or other cheese types.

Goudedranche, Ducruet, Vachot, Pannetier and Maubois (1986) improved the texture and the flavour of UF-St Paulin and Camembert cheeses by the addition of lysozyme directly in the retentate (0.5–1.0 g kg⁻¹). Lysozyme can complex with the caseins. The mechanism involved is the subject of several studies (Thapon & Brulé, 1986; De Roos, Walstra & Geurts, 1998). The formation of such enzyme–protein complexes has the advantage of limiting loss of lysozyme into the serum. Nevertheless, the direct addition of lysozyme to milk for large-scale cheese making may not be economically viable compared to LSC, unless a cheaper supply of enzyme becomes available.

4.2. Solvents

Starter attenuation by treatment with various solvents, such as *n*-butanol, altered the lipid structure of the cell membrane (Jain, Gleeson, Upreti & Upreti, 1978), and resulted in cells unable to produce lactic acid. Exterkate (1984) reported increases in peptidase activity. The addition of butanol-treated lactococci to milk accelerated Gouda cheese ripening only slightly, but significantly reduced bitterness, compared to control cheeses (Stadhouders, Hup, Exterkate & Visser, 1983). Even if solvents are found to give a desired attenuation effect, this approach may be impractical because of cost, health hazards, and legal barriers.

4.3. Spray- and freeze-drying

Johnson and Etzel (1995) compared the attenuation of *L. helveticus* CNRZ 32 by spray drying and freeze drying to freezing. They noted that frozen starters occupy large storage volumes, and result in higher storage and shipping costs. Dried cultures, in contrast, offer an economical and practical alternative.

Table 3 shows the respective attenuation effects, as assessed by cell survival, residual β -galactosidase and aminopeptidase activities, and lactic acid production, compared to the control. Regardless of the treatment used, proteolysis (soluble nitrogen in TCA and PTA) increased 1.6–2.8 fold. Cheese flavour intensity was enhanced in all cases; however, cheeses displayed sweet, nutty flavours, which were uncharacteristic of Cheddar

Table 3
Effect of various treatments of attenuation on *L. helveticus* CNRZ 32 and impact of the attenuated cells on the proteolysis of low-fat experimental Cheddar (from Johnson & Etzel, 1995 and Johnson et al., 1995)^a

	Control	SDLT	SDHT	FD	FR (–20°C)
% Survival	100	15	0.08	48	54
% Residual activity					
β-galactosidase	100	17	0	0	2
aminopeptidase	100	85	0	0	15
Lactic acid production	No lag period	No lag period	5 h lag period	No lag period	No lag period
(pH after 8 h)	3.8	4.0	4.8	4.25	4.5
Increase in proteolysis					
N-PTA	1	2.8	1.6	2.5	2.9
N-TCA	1	2.1	1.3	1.9	2.1

^aSDLT (spray-drying low temperature), SDHT (spray-drying high temperature), FD (freeze-drying), FR (freezing).

cheese. Moreover, undesirable off-flavours were noted at 1.5 months in cheeses made with frozen or freeze-dried cells. Various attenuation treatments were performed on a single batch of *L. helveticus* CNRZ 32. The effects of attenuation were, by decreasing effect, spray-drying at high temperature > freeze-drying > freezing > spray-drying at low temperature (Johnson et al., 1995). Sensory results did not directly relate to residual activity in the cells after attenuation.

5. Lactose-negative mutants as attenuated starters

A completely novel approach to increasing starter enzymes without modifying acidification was proposed by Dulley, Brooks and Grieve (1978). They isolated variants of *Streptococcus lactis* C2 that spontaneously lost their ability to ferment lactose (Lac[–]). These variants could be classified as attenuated starters, even though they were not derived by physical treatment, because they no longer significantly affected milk acidification.

Most of these lactose-negative variants were also proteinase negative, (Prt[–]). Both of these properties are plasmid encoded. Selection, stability (lack of reversion), and resistance to freezing was first demonstrated for one of these variants by Grieve, Lockie and Dulley (1983). Frozen concentrate of this Lac[–] variant was added to milk, along with primary starter (Table 4). Slight differences were found in the levels of TCA nitrogen. This was expected, given the proteinase-deficient nature of the variant. All trials, however, exhibited higher levels of free amino acids (PTA nitrogen) than control cheeses. Except for a reduction in bitterness, the effect on the cheese flavour scores was not significant. In a similar study, Kamaly et al. (1989) added Lac[–] Prt⁺ variants of *S. lactis* or *Streptococcus cremoris* to milk at a level of 1.8×10^8 cfu ml^{–1}, along with primary starter. The Cheddar cheeses produced from these starters were identical to

the control (pH, moisture, etc.). This showed that the attenuated starter did not interfere with cheese manufacture. As shown in Table 4, PTA nitrogen increased substantially and free fatty acids increased slightly. Nakajima et al. (1991) used nitrosoguanidine to chemically derive Lac[–] Prt⁺ mutants of a strain of *Lactococcus lactis* subsp. *cremoris*. Cells were then added to milk at a concentration of 2.5×10^8 cfu ml^{–1}, which was approximately eight times higher than the control. Proteolysis increased in the cheese, but sensory data were not given in the study, with the exception of reduced bitterness.

Lactococcal strains, chosen for their high rate of lysis, were treated with ethidium bromide to obtain two Lac[–] Prt⁺ mutants (Birkeland et al., 1992). When incorporated into a Norwegian Gouda cheese, both strains produced a significant reduction in ripening time. They lysed extensively during the first three weeks of ripening as demonstrated by the release of intracellular enzymes (proline iminopeptidase, lactate dehydrogenase) into the cheese matrix. Proteolysis was faster and more extensive, and overall cheese quality was improved.

Requena and Mc Kay (1993), used plasmid curing to derive a Lac[–] Prt⁺ *Lactococcus* strain. It was used along with the parental strain in the production of a low-fat semi-hard cheese (Rodriguez et al., 1996). Addition of the variant increased proteolysis and reduced the ripening time by half. In a further study (Rodriguez et al., 1997), total free amino acid content was found to be 50% higher at the end of ripening, and GC-MS analysis showed a doubling of volatiles (sum of 30 quantified peaks).

Since only Lac[–] variants or mutants of lactococci were assessed, and only in Cheddar cheese, results could not be easily compared to heat- or freeze-shocked cells. However, mutants provide an interesting direction for further investigation. There are no legal barriers to the use of spontaneous variants, or mutants derived by classical mutagenesis, in cheese. Similar mutants can be easily obtained for other genera and species (e.g. lactobacilli).

Table 4
Cheese assays with mutant starters^d

Aim of the work ^a	Species	Kind of mutant	Type of cheese	Quantity added/mL of milk	Impact on the proteolysis ^b	Other observations ^c	Sensory evaluation and ripening time	Authors
A, F	<i>S. lactis</i>	Lac ⁻ (Prt ⁻)	Cheddar	10 ⁸ cfu or 8.5 × 10 ⁸ cfu	↗ N-PTA Not significant for N-TCA	—	↗ Flavour score ↘ Bitterness ↘ Ripening time (1–8 weeks after 3 months to 4–12 weeks after 6 months)	Grieve and Dulle (1983)
A, F	<i>S. lactis</i>	Lac ⁻ (Prt ⁻)	Cheddar	0.38 to 2.25% w/v (7 × 10 ⁹ cfu/g of cheese)	↗ N-PTA (after 3 months) ↗ N-TCA (less significant)	—	↗ Total flavour score ↗ Estimated age ↘ Characteristic flavour of Cheddar due to an atypical flavour (undesirable)	Aston, Grieve, Durward and Dulle (1983)
A	<i>S. lactis</i>	Lac ⁻ (Prt ⁻)	Cheddar	1.5 × 10 ⁹ cfu	—	—	↘ Ripening time (50%) early slightly fruity flavour	Fedrick, Cromie and Dulle (1986)
A, F	<i>S. lactis</i> or <i>S. cremoris</i>	Lac ⁻ (Prt ⁻)	Cheddar	1.8 × 10 ⁸ cfu	↗ N-PTA ↗ N-TCA (after 2 months)	↗ FFA (C10:0 to C18:1) Similar lower FFA (C4:0 to C8:0)	↗ Flavour intensity No bitterness slight off flavour (unclean)	Kamaly, Johnson and Marth (1989)
A	<i>L. lactis</i> ssp. <i>cremoris</i>	Lac ⁻ (Prt ⁻)	Gouda	2.5 × 10 ⁸ cfu	↗ N-TCA	—	No bitterness	Nakajima, Toyoda, Kitamura and Ahiko (1991)
A	<i>L. lactis</i> ssp. <i>lactis</i> or <i>L. lactis</i> ssp. <i>cremoris</i> (2 strains)	Lac ⁻	Norwegian Gouda-type cheese	3 × 10 ⁷ cfu or 6 × 10 ⁷ cfu	↗ N-TCA ↗ Amino acids (TNBS) ↗ Number of peptides (FPLC)	↗ Autolysis (↗ LDH and proline iminopeptidase)	↘ Ripening time ↗ Texture after 15 weeks ↗ Total score No statistical difference between control and experimental cheese in relation to flavour	Birkeland, Abrahamson and Langsrud (1992)
A, F	<i>L. lactis</i> ssp. <i>lactis</i> added to a selected starter	Lac ⁻ (Prt ⁻)	Low-fat semi-hard UF cheese	10 ⁷ cfu/g retentate	↗ N-TCA (↗ NCN, ↗ NPN after 60 and 90 days) ↗ N-PTA (↗ N:NH ₂ after 15 days, × 2 at 90 days) ↗ Free aa (+59% at 3 months)	↗ total volatile components (× 2) ↗ FFA	↘ Ripening time (50%) No bitterness	Rodriguez, Requena, Goudedranche, Maubois and Juarez (1996); Rodriguez, Requena, Valero, Martinez-Castro, Lopez-Fandino and Juarez (1997)
A	<i>L. lactis</i>	Lac ⁻	Cheddar	—	↗ N-TCA ↗ Tyrosine	↗ VFA ↗ FFA	↘ Ripening time (75%) ↗ Flavour	Ramasamy, Narasimhan, Khan, Dhanalakshmi, Palani Dorai and Vijayalakshmi (1997)

^aA: acceleration of ripening, F: enhancement of flavour.^bN-TCA = TCA-soluble N: nitrogen soluble in 12% TCA (peptides + amino acids), N-PTA = PTA-soluble N: nitrogen soluble in 5% PTA (amino acids), NCN = non-casien nitrogen, NPN = non-protein nitrogen, N:NH₂ = amino nitrogen fraction, SN = soluble nitrogen.^cFFA: free fatty acids, VFA: volatile fatty acids, LDH: lactate dehydrogenase.^d↗ Increase, ↘ decrease.

No further delicate preparation (heat treatment) is needed. Moreover, if genetically modified starters are someday allowed in cheese, a much larger selection of attenuated starter would result. Directed mutagenesis could be used to inactivate lactic acid production, and enhance cell lysis or enzyme activity leading to greater flavour production.

6. Patents related to the use of attenuated starters in cheese

Few patents claimed the use of attenuated starters, alone or in combination with free enzymes, to enhance cheese flavour and reduce ripening time. Lagarde (1987) patented the inoculation of milk with bacterial protoplasts. They were obtained from various species, including lactic acid bacteria, by a lysozyme treatment, and thus could be classified in LSC as described above. 10^7 – 10^8 cells mL^{-1} of lactococci were added per mL of milk for soft cheese making, improving both proteolysis and flavour. Simard, Lee and Trepanier (1993) patented a method to accelerate the maturation of Cheddar cheese by the addition of live and heat-shocked *L. casei* (67°C, 22 s) in combination with neutrase. Cheese assays revealed a good correlation between the % increase in flavour and the quantity of heat-shocked cells.

7. Conclusion

It is still questionable as to what constitutes a good attenuated starter. As shown in this review, attenuated cells clearly represent a tool to enhance cheese ripening. Indeed, a large majority of the cheese experiments led to promising results. However, industrial use of attenuated starters is not widespread today, as far as we know. This may be due to several reasons:

- Most of the methods proposed are empirical; the mechanism of attenuation is rarely known or described.
- The extent of attenuation is often strain dependent and the selection of a given strain or species is frequently arbitrary.
- Each attenuation method has advantages and limits. Unfortunately, no systematic comparison of the various methods was performed using one species, strain or cheese variety, thus preventing any general conclusion from being made.
- No consensus exists on the minimum cell concentration needed to have any impact on cheese ripening, as well as the correct dead-to-live cells ratio. Do attenuated starters mainly act after lysis or is there an optimum balance between live and lysed cells? The intracellular location of peptidases would suggest that

attenuated starters must lyse in cheese or they must at least become permeable (Niven & Mulholland, 1998) to increase secondary proteolysis. However, the enzymes involved in amino acid catabolism, which lead to flavour compounds, require regenerated cofactors to be active, implying that live cells may be needed (Crow, Coolbear, Gopal, Martley, McKay & Riepe, 1995).

- Approximate costs for the use of attenuated starters are hard to ascertain, thus limiting a potential development of the concept. At a direct vat inoculum, the level of organisms per mL of milk varies from 10^6 to 5×10^6 . Most attenuated organisms are added to milk at higher levels (5×10^6 – 5×10^7 mL) than normal starters. In order to cover this additional cost, storage costs would need to be reduced accordingly. As shown in Tables 1–4, reductions of ripening time were achieved in some trials but this was not systematic. Thus, it would appear that the use of attenuated starters to reduce ripening time may be economically questionable. This is particularly true for cheeses requiring shorter ripening times, like soft or fresh cheeses (same cost but less gain in storage). Moreover, all the assays carried out with more than 5×10^7 attenuated cells mL^{-1} of milk can be regarded as unrealistic because of prohibitive additional costs. Nevertheless, the fact that attenuated starters do not appear so effective in shortening ripening time should not mask the fact that they can have a major impact by eliminating defects like bitterness, by enhancing/modifying the flavour or by improving the texture as observed in several assays. This is particularly true for low-fat cheeses or UF-cheeses. Such a positive effect is difficult to 'quantify' in terms of gain.
- Lastly, attenuated starters were shown to have no effect on the growth of the acidifying starter but their impact toward the rest of the ecosystem, like non-starter lactic acid bacteria, is not described. This should be investigated because the growth of NSLAB can have a significant influence on the ripening and depends on various environmental factors (Lane, Fox, Walsh, Folkerstma & McSweeney, 1997).

In the end, a 'good' attenuated starter can be defined as follows: a strain (or mixture of strains) that is easy to produce and to attenuate in a repeatable and cost-effective way, that is able to positively and significantly influence the ripening process (reduce time and/or increase flavour) at the lowest inoculation level in milk. This implies good retention of the starter in cheese curd, a significant enzyme content, and adequate lysis of the attenuated starter. No doubt, the answer to these various requirements will allow the development of various, efficient and safe cheese additives.

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