Influence of the Temperature Gradient on the Growth of Thermophilic Lactobacilli Used as Natural Starters in Grana Cheese

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ABSTRACT

This study investigated the evolution of the *Lactobacillus* species and the microbial composition of the starter during Grana cheese making. The early stages of cheese making were studied, and both the composition of the natural whey starter and its modification in response to curd cooking were considered. The growth and distribution of the thermophilic lactobacilli in the cheese at 48 h after molding was affected by the temperature gradient between the external and internal cheese zones. Growth was maximum between 0 and 6 h in the cheese exterior and between 6 and 24 h in the core. This variation occurred because the cheese interior was around 52°C 6 h after molding, which is far from the optimum for the thermophilic lactobacilli growth. Dot-blot hybridization experiments allowed the identification of up to 280 isolates. *Lactobacillus helveticus* predominated in the natural whey starter and in the external cheese zones. Distribution of *Lactobacillus delbrueckii* and the heterofermentative lactobacilli, which slowly increased from the molding until 48 h, was more variable in the internal regions than in the external regions of the cheese. This study demonstrates that the thermophilic lactobacilli behave differently during the technological process than during experiments using laboratory models.
(**Key words**: Grana cheese, thermophilic lactobacilli, natural starter, temperature gradient)

Abbreviation key: **LAB** = lactic acid bacteria.

INTRODUCTION

Thermophilic lactobacilli are the dominant microflora of hard-cooked cheeses, such as Grana Padano, Parmigiano Reggiano, and Emmental. Grana Padano cheese, one of the most widespread traditional Italian varieties, is produced from partially skimmed raw bovine milk, which is creamed with the addition of a starter consisting of a naturally acidified cheese whey. This starter was demonstrated to be a very complex association of lactic acid bacteria (**LAB**), mainly *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus delbrueckii* ssp. *lactis*, and *Lactobacillus fermentum* (3, 10).

The variable presence of the thermophilic lactobacilli species and strains (1, 2, 14, 15) can affect both the standardized cheese-making procedure and the ripening profile. The microbiological composition of the starter may easily be affected by either the ecological relationships (6) or manufacturing parameters, such as the curd cooking temperature and the subsequent holding time at the cooking temperature before the extraction of the curd. Because the temperature gradient between the external and internal regions of a cheese wheel might affect the growth and acidification rate of LAB, depending on the zone considered, as has been previously shown for Emmenthal and Parmigiano Reggiano cheese (8, 12, 16), the evolution of the *Lactobacillus* spp. and the microbial composition of the starter during Grana cheese making were investigated. This work complements a previous study on the growth of thermophilic lactobacilli in milk under technological conditions simulating the first 24 h of Grana cheese making (11). In the present paper, the early stages of the cheese-making process were thoroughly studied, and the composition of the natural whey starter as well as its modification as the curd was cooked at about 53°C were considered. Bacterial counts and evolution of LAB throughout the cheese form are also reported, and LAB were identified reliably through the use of species-specific DNA probes in dot-blot hybridization experiments.

MATERIALS AND METHODS

Strains, Media, and Cultivation Conditions

The *Lactobacillus* strains isolated from Grana cheese, as well as *Lactobacillus acidophilus* La8 (Grana cheese isolate; Collection of Istituto

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Sperimentale Lattiero Caseario, Lodi, Italy), *L. helveticus* ATCC 15009, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, *L. delbrueckii* ssp. *lactis* Ll4 (whey starter isolate; ILC collection), and ATCC 15808 were maintained as frozen stocks at –80°C in the presence of 15% of glycerol as a cryoprotective agent. Unless otherwise specified, strains were routinely reactivated overnight at 42°C in MRS broth medium (Biokar, Beauvais, France).

Grana Cheese Manufacture

In the cheese plant of Istituto Sperimentale Lattiero Caseario, Grana cheese was manufactured from partially skimmed raw milk according to previously described technology (9). The areas delimited by zones A, B, and C were considered to be the cheese exterior, and the zones delimited by D, E, and F were considered to be the cheese interior (Figure 1a). The temperature changes that occurred throughout the cheese up to 48 h after curd molding were measured by using a Pt steel probe (model Toledo U402-M6-S7/100; Mettler Italia srl, Milan, Italy).

Microbiological Analysis

The thermophilic lactobacilli in the whey starter cultures and in cheese were counted; MRS agar (pH 6.5 ± 0.2) was used as the culture medium. Lactobacilli in the cheese form were counted for both the cheese exterior and interior at molding, 6 h after molding, 24 h after molding, and 48 h after molding (Figure 1a). After the incubation of agar plates at 44°C for 48 to 72 h under anaerobic conditions, about 300 well-separated colonies were randomly isolated from MRS agar plates and grown in MRS broth (pH 7.4 ± 0.1). After microscopic examination, 280 rod-shaped isolates were scored and further identified.

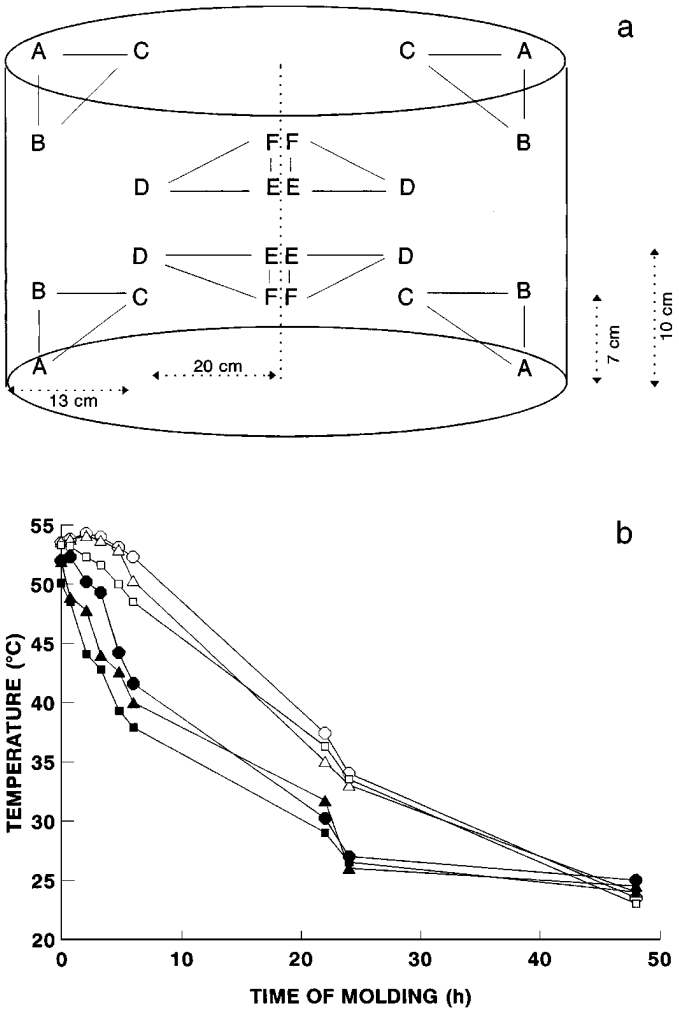

Figure 1. a. Zones of the Grana Padano cheese used for sampling for bacteriological analysis. Zones A, B, and C were in the cheese exterior, and zones F, D, and E were in the interior. b. Evolution of the temperature throughout the Grana Padano cheese form from curd molding for zones A (■), B (▲), and C (●) and for zones F (□), D (△), and E (o).

Identification of Isolated Lactobacilli

Dot-blot hybridization. The identification of isolated lactobacilli was performed by the use of species-specific DNA probes in dot-blot hybridization experiments. A Bio-Rad (Bio-Rad Laboratories Srl, Milan, Italy) dot-blot apparatus was used. Total DNA from different MRS cultures were extracted according to the method of de los Reyes-Gavilàn et al. (5). Aliquots of about 100 ng of total DNA in 0.4*N* NaOH and 10 m*M* EDTA were denatured by 10 min of heating at 95°C and then neutralized by the addition of 2 *M* ammonium acetate. Then, the mixture was spotted onto Hybond N^+ membranes (Amersham Corp., Milan, Italy), fixed with 0.4*N* NaOH, and rinsed twice with buffer (0.03 *M* trisodium citrate-0.3 *M* NaCl, pH 7.0). The filter was then ready for DNA hybridization, which was performed using the enhanced chemiluminescence direct nucleic acid labeling and detection systems (Amersham Corp.), according to instructions of the suppliers. Hybridization was carried out under stringent conditions (6 *M* urea at 42°C) using DNA probes that were specific for the identification of *L. helveticus* and *L. delbrueckii*. IS*1201*, a 1387-bp insertion sequence isolated from *L. helveticus* (5) and kindly provided by P. Tailliez (Institut National de la Recherche Agronomique, Jouy en Josas, France), was used as the DNA probe for the *L. helveticus* species. The probe was obtained from a

*Bss*HII-digested pBluescript plasmid that had been cloned in *Escherichia coli* CNRZ 1814 (Station de Recherches Laitières, Institut National de la Recherche Agronomique) as described by Tailliez et al. (17). The *Eco*RI fragment of the pY85 plasmid (4), kindly provided by B. Mollet (Nestlé Research Centre, Vevey, Switzerland), was obtained after transformation of *E. coli* HB101 as described by Pilloud and Mollet (13) and was used as DNA probe for *L. delbrueckii*. Dot-blot experiments using freshly prepared total DNA were repeated for those isolates that showed weak or no hybridization signals with the two probes.

Phenotypic and biochemical tests. Isolated lactobacilli that did not show hybridization with DNA probes that were specific for *L. helveticus* or *L. delbrueckii* were identified by phenotypic and biochemical tests. These tests included either the growth recorded at 10 and 45°C or gas production from glucose at 37°C and the sugar fermentation pattern. These latter two were evaluated by using the API 50 CHL gallery (API-BioMérieux, Montalieu-Vercieu, France), according to the instructions of the manufacturer.

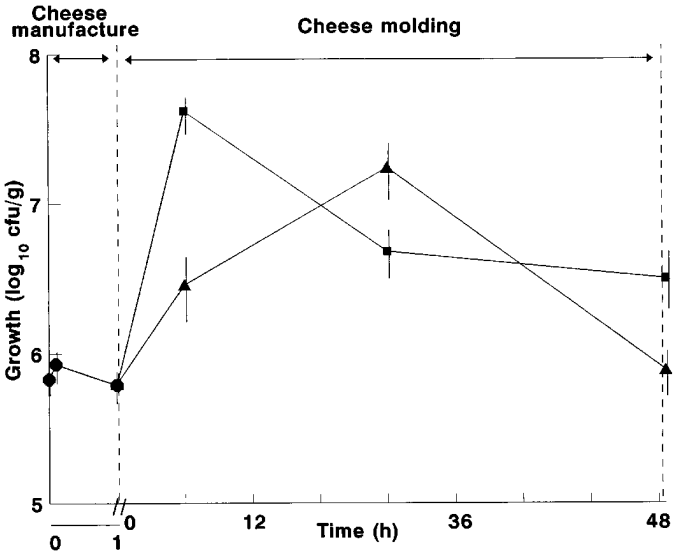

Figure 2. Growth of the thermophilic lactobacilli in Grana Padano during cheese making in the cheese curd and during cheese molding (●), in the cheese exterior (mean values for zones A, B, and C) (■), and in the cheese interior (mean values for zones F, D, and E) (▲). Bars indicate standard errors of the means.

RESULTS

A temperature gradient between the cheese exterior, which was in contact with air, and the central zones of the cheese form was recorded during molding; this result was consistent with previous findings (9). The pattern of the temperature gradient that was observed in our cheese-making trials is shown in Figure 1b.

The evolution of thermophilic rod LAB within the first 49 h of cheese making is shown in Figure 2. In the scalded curd, the LAB count was about the same as in the milk with the added whey starter. Six hours after molding, the LAB count significantly changed depending on zone location. The highest growth, a difference from the central cheese zones of more than 1 decimal log, was observed in the external zones of the cheese. After 24 h, the LAB count was maximum in the cheese interior, but in the exterior was in the stationary growth phase (Figure 2). After 48 h, the LAB count dropped to a lower level than that recorded after 6 h in all the cheese regions, but the LAB distribution throughout the form was similar to that observed after 6 h of molding.

By using the dot-blot hybridization technique, almost all of the 280 thermophilic lactobacilli isolates were identified as *L. helveticus* and *L. delbrueckii*. Two application examples of the dot-blot hybridization are shown in Figure 3. No hybridization took place between the *L. helveticus* probe and *L. acidophilus* La8 (row F, spot 1, Figure 3a), *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 (row H, spot 11, Figure 3a), *L. delbrueckii* ssp. *lactis* ATCC 15808 (row A, spot 2, Figure 3a), and *L. delbrueckii* ssp. *lactis* Ll4 (row F, spot 10, Figure 3a). Similarly, no hybridization signals were recorded between the *L. delbrueckii* probe and *L. helveticus* ATCC 15009 (row D, spot 1, Figure 3b). In these two examples, isolates F, 20C48, 20D48, and 18T were shown to be heterofermentative lactobacilli by phenotypic tests (data not shown); isolates 9E, 16E, 7D48, 20E, A5, and 3G24 were identified as *L. delbrueckii* (Figure 3b), and all other isolates were identified as *L. helveticus* (Figure 3a).

In this manner, 204 isolates (about 73% of the isolates) were classified as *L. helveticus*, and 56 (20%) were classified as *L. delbrueckii*. The remaining 20 isolates (7%) were not able to be identified by dot-blot hybridization and were classified by the classic phenotypic tests mostly into heterofermentative lactobacilli (Table 1), except for isolates 25S, 12B, and 15B, which were typed or identified as *L. helveticus*. The heterofermentative lactobacilli were mainly *L. fermentum* and, to a lesser extent, *Lactobacillus brevis* and *Lactobacillus buchneri* (data not shown).

Results indicated that the *L. helveticus* species predominate in the natural whey starter; *L. helveticus* was identified in about 93% of the isolates, but *L.*

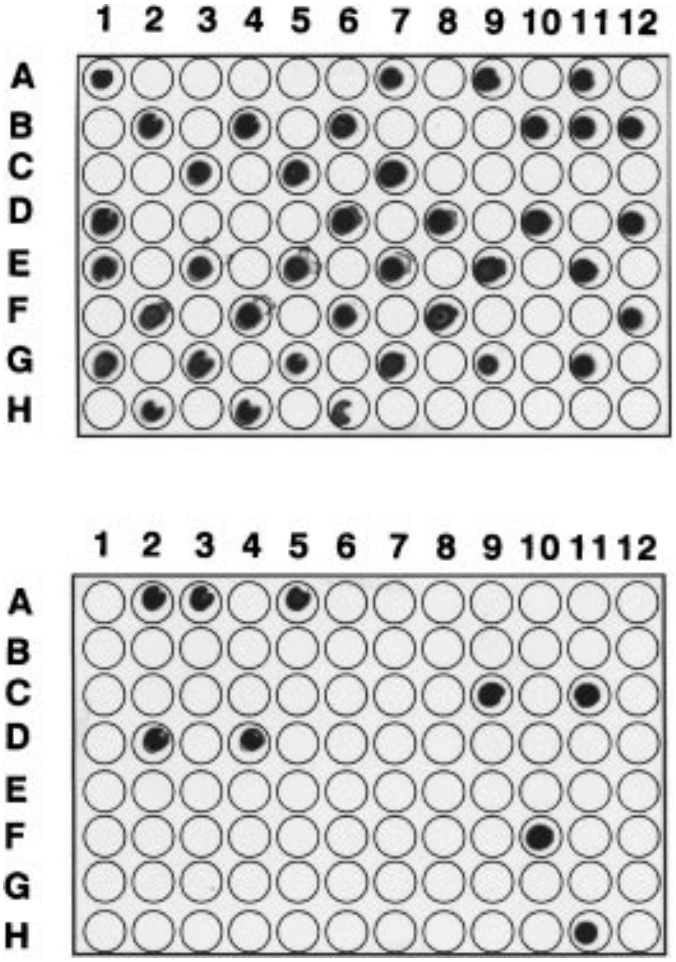

Figure 3. Dot-blot hybridization of different lactobacilli isolates. Top half: the insertion sequence IS*1201*, which is specific for *Lactobacillus helveticus* (5, 17), was used as a probe. Bottom half: the *Eco*RI fragment of pY85, which is specific for *Lactobacillus delbrueckii* (4), was used as a probe. Isolates are, from left to right, row A: 13S, *L. delbrueckii* ssp. *lactis* ATCC 15808, 9E, place empty, 16E, empty, 9S, empty, 17S, empty, 17A48, empty; row B: empty, 7B48, empty, 6A48, empty, 20I, empty, F, empty, 26S, 17B, 3S; row C: 20C48, empty, 10G, empty, 4T, empty, 27S, empty, 7D48, empty, 20E, empty; row D: *L. helveticus* ATCC 15009, A5, empty, 3G24, empty, 14I, empty, 19C, empty, 38S, empty, 4G48; row E: 11B, empty, 20G, empty, 1C, empty, 14A48, empty, 8A, empty, 12S, empty; row F: *L. acidophilus* La8, 6T, empty, 18A48, empty, 3F, empty, 5B, empty, *L. delbrueckii* ssp. *lactis* L14, empty, 13G48; row G: 6S, empty, 32S, empty, 1D, empty, 20S, empty, 25T, empty, 19H, empty; row H: empty, 7A48, empty, 2T, empty, 22T, empty, 20D48, empty, 18T, and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, λDNA (negative control).

delbrueckii was identified in only 6.9%. In the curd, *L. helveticus* progressively decreased until 48 h after molding, but *L. delbrueckii* significantly increased from 6 to 48 h after molding as did the heterofementative lactobacilli (Table 1).

The distribution of the bacterial species changed according to the cheese zone. In particular, *L. helveticus* was always predominant in external zones A, B, and C, and *L. delbrueckii* was predominant at 48 h from molding in internal zones D, E, and F (Table 1).

DISCUSSION

The incubation of milk with individual strains of *L. helveticus*, *L. delbrueckii* ssp. *bulgaricus*, and *L. delbrueckii* ssp. *lactis* under a temperature gradient simulating the typical thermal cycle of Grana cheese was demonstrated to determine either a decrease, although of variable intensity, of the acidification rate or a lag (11). The distribution of the thermophilic lactobacilli species in the cheese from 48 h after molding was affected by the temperature gradient between the external and internal cheese zones; in our experiments, this gradient ranged from 7 to 15°C after 4 to 7 h to 8 to 10°C after 24 h. Only after the 48-h molding time was the temperature fairly uniform throughout the cheese form. During this period, the population of acidifying bacteria was affected selectively. The growth of thermophilic lactobacilli was maximum between 0 and 6 h in the cheese exterior and between 6 and 24 h in the core of the form. This result was consistent with the fact that, in the external zones of the cheese form, the temperature after 6 h dropped to the optimal growth temperature range (37 to 42°C) faster than it did in the core. The temperature in the core was still around 52°C at 6 h after molding (Figure 1b), which was far from the optimum for the growth of thermophilic LAB. Because of the negative effect of high temperature on the bacteria development, the growth of these microrganisms was therefore delayed, but not stopped, in the Grana cheese core. This result is in agreement with theory that the "thermophilic" lactobacilli may have an upper limit of growth of 55°C (7). The results of the present study are in contrast to those (11) showing that the thermophilic *Lactobacillus* spp. strains grown in a medium such as the liquid milk, under a temperature gradient typical of the Grana cheese, were unable to grow or promote acidification. A lack of acidification was similarly observed during the incubation of a natural whey starter in skimmed milk under a temperature gradient that was similar to that of Grana cheese (G. Mucchetti, 1996, unpublished data). The capacity of LAB to develop differently in milk than in curd might explain this apparent discrepancy.

The DNA-DNA hybridization experiments, using probes that were specific for both *L. helveticus* and *L. delbrueckii*, allowed the definitive identification of most thermophilic rod LAB. Only 3 of 204 *L. helveticus* isolates were not identified by the dot blot, thus

TABLE 1. Distribution of the thermophilic *Lactobacillus* species in whey starter and within a Grana Padano cheese form at molding and at 6 and 48 h after molding.

	Lactobacillus helveticus		*Lactobacillus delbrueckii*		Heterofermentative lactobacilli	
	(%)					
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
Whey starter	93.1		6.9		...	
Whole cheese (mean of all zones)						
Molding[1]	80.2		19.8		...	
6 h	78.5	5.5	18.4	4.2	3.2	0.3
48 h	41.8	4.0	36.3	4.2	22.0	2.8
Cheese outside (mean of the zones A, B, and C)						
6 h	80.0	5.6	18.0	3.5	2.0	0.1
48 h	53.3	5.2	19.5	3.3	27.2	3.5
Cheese interior, (mean of the zones F, D, and E)						
6 h	77.0	5.5	18.7	4.8	4.3	0.5
48 h	30.3	2.7	53.0	5.0	16.7	2.0

[1]At molding, lactobacilli were isolated from the whole form (see Materials and Methods).

confirming the high sensitivity of the method. The high level of species specificity of the DNA probes has previously been demonstrated using a target DNA that derived from strains that were closely related phylogenetically (4, 5). In this manner, the dot-blotting technique allowed the exclusion of *L. acidophilus*, a species that is closely related phylogenetically to *L. helveticus* (18). The DNA fragment that was used as a probe for *L. delbrueckii* was not able to discriminate among its three subspecies, as has been reported also by Delley et al. (4). However, because *L. delbrueckii* ssp. *bulgaricus* and *L. delbrueckii* ssp. *lactis* are predominantly found in dairy products and *L. delbrueckii* ssp. *delbrueckii* is predominantly found in fermented vegetables (4, 18), the *L. delbrueckii* that was found in the isolates most probably belonged to the *bulgaricus* and *lactis* subspecies.

Lactobacillus helveticus was confirmed to be the prevalent species in the natural whey starter and in the cheese 6 h after molding, which was consistent with the previous findings in Grana cheese (10, 18). *Lactobacillus delbrueckii* was the second most represented species in the whey starter and in the cheese, but heterofermentative lactobacilli, primarily *L. fermentum*, were also isolated from the curd within 6 and 48 h. This result confirms other findings on starter composition (3, 10), indicating that thermophilic lactobacilli belonging to these three species constitute the majority of the lactic acid microflora that were recovered from cheese during the initial stages of ripening. The homofermentative species are known to play a key role in curd acidification, and the heterofermentative lactobacilli are responsible for the gas formation and the typical minute holes formed during Grana ripening (3).

The variation in heating intensity across cheese regions seriously affected both the level and the species of LAB in cheese. In fact, we observed a different distribution of the species according to the cheese zones. *Lactobacillus helveticus* was predominant in the external region of the cheese, regardless of the moment of isolation, and *L. delbrueckii* predominated in the cheese core at 48 h after molding. These phenomena most probably reflect the different resistance of these two lactobacilli to the high temperatures conditions during the curd cooking (53°C for about 50 min in the case of Grana Padano cheese) and the subsequent holding at this temperature for a further 90 min before the curd is extracted (9). Neviani et al. (11) previously reported that *L. delbrueckii* ssp. *bulgaricus* seemed to adapt better to the temperature gradient conditions of Grana cheese than did *L. helveticus*.

The different distribution of the species throughout the cheese form did not seem to affect the species composition in the natural whey starter, which was traditionally obtained from the cheese making of the previous day (10). This result has an intrinsic technological relevance because variable starter composi-

tion might cause a different enzyme to be released, depending on the bacterial species and strains that exist, which might change the quality of Grana cheese accordingly (3, 10).

CONCLUSIONS

The initial phases of cheese making are considered to be essential for the successful production of Grana Padano cheese. Factors such as bacterial growth and acidification of milk and curd, which usually occur at different rates during the 48-h molding stage in the cheese form, are strictly correlated with the temperature gradient formed between the external and the internal regions of the cheese wheel. The overall growth of thermophilic lactobacilli is strongly affected, as is also the species distribution within the cheese form. This study represents the first contribution toward understanding the behavior of the complex microbial association of starter bacteria during Grana cheese making and demonstrates that the behavior of the thermophilic lactobacilli during the technological process is quite different from pilot plant experiments or laboratory simulations.

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