Cultivability of *Streptococcus thermophilus* in Grana Padano cheese whey starters

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Received 2 August 2005; revised 9 January 2006; accepted 16 January 2006.
First published online 15 February 2006.

doi:10.1111/j.1574-6968.2006.00155.x

Editor: Bruce Ward

Keywords
Streptococcus thermophilus; whey starters; Grana Padano cheese; microbial cultivability; length heterogeneity-PCR.

Abstract

The application of a culture-independent approach, that of reverse transcriptase-length heterogeneity-PCR coupled with epifluorescence microscopy, allowed us to observe that *Streptococcus thermophilus* is metabolically active, but only partially cultivable in Grana Padano cheese whey starters. A short preincubation of the starters in sterile skimmed whey was followed by cultivation in sterile skimmed whey-enriched M17. This procedure restored the cultivability of *S. thermophilus* and enabled us to detect *S. thermophilus* at ranges (10^7–10^8 CFU mL^{-1}) which have rarely been reported in these cultures. The use of cheese whey as a cultivation-revitalization substrate can be useful to obtain an unbiased picture of the microbial composition of whey starters for Grana Padano cheese, thus avoiding an underestimation of *S. thermophilus* in these cultures.

Introduction

In the microbiology of Grana Padano cheese, one of the most widespread Italian raw milk, hard-cheese varieties, the whey starter cultures play an acknowledged role in the achievement of the sensory characteristics of the cheese. Such cultures are composed of complex associations of thermophilic lactic acid bacteria (LAB) belonging to *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *lactis* and ssp. *bulgaricus*, *Lactobacillus fermentum*, and *Streptococcus thermophilus*, which become dominant within the early 24–48 h of manufacturing. By lactic fermentation, whey cultures essentially allow the curd acidification, which favors the cheese whey drainage (Giraffa *et al.*, 1997, 1998; Beresford *et al.*, 2001).

The microbial ecology of natural whey starters appears complex and is still not completely understood. Grana cheese is made of raw milk, but, in light of the present knowledge, the microbial composition of the whey starters only in part corresponds to the raw milk composition. *Streptococcus thermophilus*, lactococci, and mesophilic lactobacilli are rarely isolated from these starters (Andrighetto *et al.*, 2004; Lazzi *et al.*, 2004). This could depend mainly upon the Grana cheese technology, the method of the whey starter preparation, nutritional requirements, bacteriophage attack, and ecological phenomena (inhibitions, stimulations). With regard to this last point, inhibition of LAB isolated from whey starters has been shown to be an important strain-selecting factor taking place during preparation of whey cultures (Giraffa *et al.*, 1996). The variable presence of thermophilic LAB species and strains in Grana Padano cheese whey starters can affect both the cheese-making process and the sensory characteristics of the ripened cheese. Whereas a number of studies deal with biodiversity and phenotypic characteristics of thermophilic lactobacilli (Giraffa *et al.*, 2000; Gatti *et al.*, 2004), little is known about the presence and role of *S. thermophilus* in these cultures.

It is widely accepted that plate culturing techniques reveal a small portion (i.e. viable and cultivable) of the true microbial population in natural ecosystems. This can essentially be explained by two ecological phenomena: (i) an inability to detect novel microorganisms, which might not be cultivable with known media, and (ii) an inability to recover known microorganisms that are either stressed or actively growing but which enter a noncultivable state (Fleet, 1999). Therefore, culture-independent strategies to study the microbial diversity in complex foods have been developed (ben Omar & Ampe, 2001; Giraffa & Neviani, 2001; van Beck & Priest, 2002; Randazzo *et al.*, 2002; Duthoit *et al.*,

2003; Andrighetto *et al.*, 2004; Henri-Dubernet *et al.*, 2004). In this study, we show the limitations related to the enumeration of streptococci using traditional plating media. A simple revitalization step coupled with a modified M17 medium gave better recovery of *S. thermophilus* from these cultures.

Materials and methods

Whey starters collection

The whey cultures considered in this study were sampled from eight dairy plants situated in different areas of Lombardia and Emilia Romagna regions. Culture samples were collected just before being added to the vat milk, cooled at 4–6 °C at the dairy plant, and shipped under refrigerated transport to the laboratory, where they were immediately analyzed.

Preparation of sterile skimmed whey

Sterile skimmed whey (SSW) was used both as a medium and medium ingredient. SSW was prepared by dissolving 50 g of commercial, cheese whey powder (type 04-SILP-M-1, Borghi-Porotto, Ferrara, Italy) in 1 L distilled water. After centrifugation (7000 ***g***, 10 min, 4 °C), reconstituted whey was prefiltered through 1.0 μm unit filtrations (mod. Polycap 75 SPF; Whatman Inc., Clifton, NY), and then filtered through 0.22 μm unit filtrations (mod. Stericup; Millipore Italia, Milano, Italy).

Microbial counts of thermophilic LAB

Thermophilic lactobacilli were counted in whey agar (WA) medium, according to Gatti *et al.* (2003). WA was prepared by mixing (vol : vol) sterile agar solution (30 g L^{-1}) with SSW. Plates were incubated at 42 °C for 48 h under anaerobic conditions (Anaerogen™, Oxoid, Basingstoke, UK). Thermophilic lactobacilli were also counted on MRS agar (Biokar, Beauvais, France), acidified to pH 5.4 with acetic acid, using the same incubation conditions applied to WA. The counts of thermophilic streptococci were performed in M17 agar (Scharlau Chemie, Barcelona, Spain) at 37 °C for 48 h under aerobic conditions. Thermophilic streptococci counts were also performed after a procedure aimed at better recovering these bacteria from the whey. In brief, 1 mL whey starter was preliminary incubated at 30 °C for 30 min in 9 mL of SSW. Thermophilic streptococci were then counted in M17 agar containing 7% (vol : vol) of SSW (M17-SSW). All microbial counts were carried out in duplicate and the standard deviation of mean values was calculated.

Isolation and identification of thermophilic streptococci from whey starters

Ten colonies from M17-SSW agar plates after the recovery treatment were selected by morphology, picked, and grown in appropriate media. After total DNA extraction, strains were typed by PCR fingerprinting as described previously (Gatti *et al.*, 2003). Then, a reduced number of strains were identified by 16S rRNA gene sequencing. The identification was performed through BlastN (www.ncbi.nlm.nih.gov/BLAST) alignment of the obtained sequences with the 16S rRNA gene sequences of LAB available from the EMBL database. DNA extraction and sequencing were performed as described previously (Giraffa *et al.*, 2003).

Extraction of total RNA

Total RNA from Grana cheese whey starters was extracted according to the following protocol. One milliliter of whey samples was centrifuged at 7000 ***g*** for 10 min and the cell pellet was washed twice in TE buffer (10 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} EDTA, pH 7.5). Pellets were resuspended in 100 μL of TES buffer (0.1 g L^{-1} sucrose in TE buffer) containing lysozyme (20 mg mL^{-1}) and incubated at 37 °C for 30 min. Extraction of RNA was performed using Trizol Reagent (Invitrogen SrL, Milano, Italy) following the instructions given by the supplier. RNA was precipitated with 500 μL of isopropanol by incubation for 10 min at room temperature. The pellets were washed twice with 1 mL ethanol (75% vol : vol), air dried, dissolved in 100 μL of diethyl pyrocarbonate-treated water, and treated with DNAse according to the protocol included in the Amplification Grade DNAase I kit (Sigma-Aldrich, Milan, Italy). Pure RNA extracts were stored at − 80 °C until use.

Length heterogeneity-RT-PCR

Length heterogeneity (LH)-RT-PCR analysis was carried out on whey starters. Domain A of the variable region of the 16S rRNA gene was reverse transcribed and subsequently amplified using LH-PCR primers described previously (Lazzi *et al.*, 2004). One-step RT-PCR was performed using the Gene Amp EZ rTth RNA PCR Kit (Applera Italia, Monza, Italy) according to the instructions given by the manufacturer. Reactions were carried out in a final volume of 25 μL containing 5 μL of 5 × EZ buffer, 3 μL of deoxynucleoside triphosphate (dNTP) mix (containing 10 mmol L^{-1} each dNTP), 2.5 μL of 25 mmol L^{-1} Mn $(OAc)_2$ solution, 1.25 μL of each primer solution (10 mmol L^{-1}), 1 μL of rTth DNA polymerase (2.5 U mL^{-1}), and 2.5 μL of total RNA (diluted up to 100 ng μL^{-1}). Segments of 16S rRNA gene were reverse transcribed by an initial incubation at 60 °C for 30 min. The resulting cDNA was amplified by PCR using the following conditions: after one cycle of 94 °C for 1 min, 20 cycles of

94 °C for 15 s (denaturing), 49 °C for 30 s (annealing), and 72 °C for 30 s (extension) were performed. Final extension was carried out at 72 °C for 7 min. Reactions were carried out in a 9700 PCR system (Applied Biosystems, Foster City, CA). PCR without a reverse transcription step was performed to verify the absence of contaminant DNA.

Dominant community composition of the different starter samples was evaluated after separation of RT-PCR products by capillary electrophoresis on an ABI Prism 310 Genetic Analyser (Applied Biosystems) using conditions described previously (Lazzi *et al.*, 2004). Runs were performed under denaturing conditions and resulting electropherograms were analyzed using Genescan software (version 3.1; Applied Biosystems). The size, in basepairs, of the amplified fragments was estimated by reference to the internal size standard using the Local Southern method. The taxonomic assignment of the amplified fragments was obtained after comparison to the LH-PCR reference database for LAB (Lazzi *et al.*, 2004). Only peak heights scaled above 150 relative fluorescence units (RFU) were considered for the analyses.

Estimation of bacterial viability

Viability of bacterial populations in whey starters was evaluated using the LIVE/DEAD *Bac*Light bacterial viability kit (Molecular Probes Inc., Cambridge Bioscience, Cambridge, UK) according to the indications of the manufacturer. The kit is based on mixtures of the green fluorescence nucleic acid stain, SYTO9, that labels all cells and the red fluorescence nucleic acid stain, propidium iodide, that penetrates only bacteria with damaged membranes and quenches the green stain SYTO9. Thus, bacteria with intact cell membranes stain green, whereas bacteria with damaged membranes stain red. Aliquots of 10 mL of whey were centrifuged (7000 ***g***, 10 min, 4 °C) to eliminate the interference of the lipid phase. The pellet was resuspended in 10 mL sterile water, and this cleaning step was repeated twice. The resuspended pellets were 2.5-fold diluted and 1 mL aliquots were stained with the two dyes (700 μL SYTO9 + 1 mL propidium iodide) and incubated in the dark for 15 min at room temperature. Then, bacteria were captured by filtration through 0.2 μm pore size black polycarbonate, disc-supported, membrane filters (13 mm in diameter, Millipore, cod. GTBPO1300, Millipore Italia). The filters were dried on filter paper and mounted on clean glass slides with a fine smear of immersion oil. A drop of the oil was placed on top of the filter followed by a cover slip that was pressed down. Bacterial cells were examined under an Axioskop 40 FL microscope (Carl Zeiss SpA, Milano, Italy) equipped with an HBO 50 W mercury lamp. Carl Zeiss filter set 10 was used for SYTO9 (excitation wavelength, 450–490 nm; emission wavelength, 515–565 nm); Carl Zeiss filter set 15 was used for propidium iodide (excitation wavelength, 546 nm; emission wavelength, 590 nm). Viable and membrane-damaged cells of both coccus-shaped and rod-shaped bacteria were counted using the Axiovision software (version 4.1, Carl Zeiss) according to the manufacturer's indications. The number of cells calculated by the software were multiplied by 1.17×10^4, a coefficient which took into account sample dilution and the counting field (0.7 cm^2). A detection limit of 10^6 cells mL^{-1} was experimentally verified.

Results

Community fingerprinting

Figure 1 shows an example of the typical microbial composition of a whey starter obtained through LH-RT-PCR. *Lactobacillus helveticus* (peak size 334 ± 1 bp), *Lactobacillus delbrueckii* ssp. *lactis*/*bulgaricus* (331 ± 1 bp), and *Streptococcus thermophilus* (peak size 319 ± 1 bp) were present in all the cultures analyzed. Five out of the eight cultures also contained *Lactobacillus fermentum* (343 ± 1 and 346 ± 1 bp). *Streptococcus thermophilus* was present within the major species in three (i.e. starters 2, 5, and 6) out of the eight cultures studied (Fig. 1, starter 6 shown as example). The peaks sized 289 ± 1 and 307 ± 1 bp were secondary peaks of *L. helveticus* resulting from sequence polymorphism of the 16S rRNA gene (data not shown).

Analysis of the whey starters

Fluorescence microscopy of the eight whey starters revealed bright green cells of both streptococci and lactobacilli (Fig. 2; starter 8 shown as example) and enabled us to estimate a total viable population ranging between 10^7 and 10^8 CFU mL^{-1}, and between 10^8 and 10^9 CFU mL^{-1},

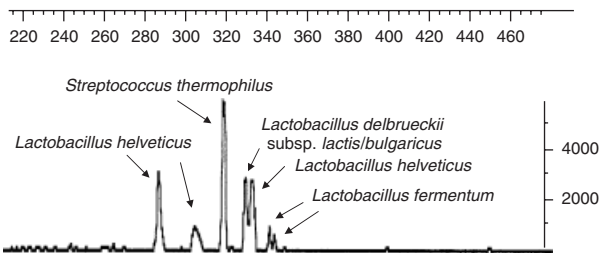

Fig. 1. Typical pattern of a Grana Padano cheese whey starter obtained by length heterogeneity (LH)-RT-PCR. Electropherogram represents RT-PCR products of Domain A of the variable region of the 16S rRNA gene, which were reverse transcribed and subsequently amplified from total RNA extracted from the whey starter. The x axis represents the size, shown at the top, of the amplified products in base pairs and estimated by comparison with the tetra-methylcarboxyrhodamine-labelled GS500 internal size standard (Applera Italia). The y axis represents relative fluorescence units. The bacterial species indicated are assigned according to the reference LH-PCR database (Lazzi *et al.*, 2004).

respectively (Table 1). The counts of thermophilic lactobacilli cultivable on MRS resulted generally lower (of about 1-log) than those obtained by both WA and epifluorescence microscopy. The counts of thermophilic streptococci cultivable on M17 resulted significantly lower than fluorescence microscopy counts in seven out of the eight cultures analyzed; a large proportion (68.4–99.9%) of the streptococcal population in these seven cultures was deemed to be viable but uncultivable in M17 (Table 1). According to RT-LH-PCR data of the cultures, this population was supposed to be metabolically active also.

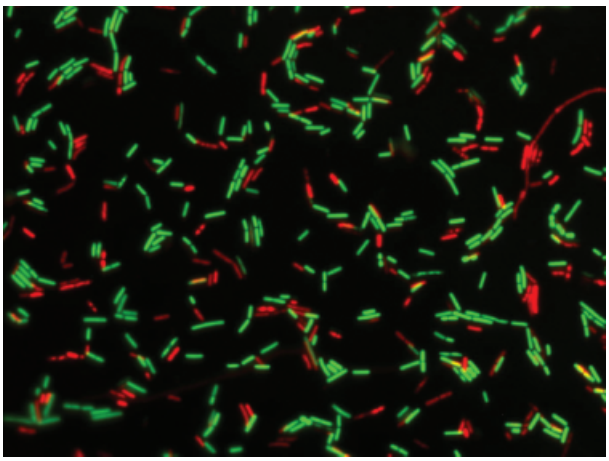

Fig. 2. Fluorescence microscope images of a whey starter culture treated with *Bac*Light. Live cells stain green; stressed/dead cells stain red. In this sample (i.e. starter 8), lactobacilli and streptococci accounted *c.* 9 and 7 log cells mL^{-1}, respectively (see Table 1). On the right-hand side of the image, viable, green-stained streptococci are clearly visible.

One milliliter aliquots of the whey cultures were preliminary incubated at 30 °C for 30 min in the presence of 9 mL SSW and counted in M17-SSW. After this treatment, the number of thermophilic streptococci increased, on average 10- to 1000-fold with respect to M17 counts (Table 1), giving statistically similar counts to viable cell counts (Table 1). Therefore, this simple procedure allowed a better cultivability of the thermophilic streptococci. Eighty strains were isolated by M17-SSW plates and, after PCR fingerprinting, 39 representative strains were further identified by 16S rRNA gene sequencing, coupled with BlastN analysis (data not shown). As expected through RT-LH-PCR, 35 out of 39 strains were determined to be *S. thermophilus*, whereas the remaining four could not be identified. This confirmed that (i) the uncultivable fraction of thermophilic streptococci in the whey cultures belonged to *S. thermophilus* and (ii) the recovery treatment was effective to fully restore the cultivability of this organism in Grana Padano whey cultures.

Discussion

A partial picture of the dominant microflora was achieved by studying the cultivable LAB fraction associated with the whey starters used for Grana Padano cheese. The application of a culture-independent approach was necessary to obtain a more complete view of the microbial diversity of these cultures. In particular, fluorescence microscopy using *Bac*Light staining was confirmed to be a valid method to rapidly evaluate and quantify viable bacterial cells directly from the whey starter cultures (Corich *et al.*, 2004). When coupled with culture-based analysis, fluorescence microscopy

Table 1. Microbial analysis of Grana Padano cheese whey starters (named as starter 1–8)

	Thermophilic lactobacilli			Thermophilic streptococci		
Starters	Viable cell count (log cells mL^{-1})*	MRS agar count (log CFU mL^{-1})	WA count (log CFU mL^{-1})	Viable cell count* (log cells mL^{-1})	M17 agar count (log CFU mL^{-1})	M17-SSW† agar count (log CFU mL^{-1})
Starter 1	9.00 ± 0.03	8.17 ± 0.13	8.92 ± 0.09	7.33 ± 0.18	< 4.00 (> 99.9)‡	7.46 ± 0.01
Starter 2	9.32 ± 0.29	8.37 ± 0.07	9.04 ± 0.18	7.91 ± 0.13	6.66 ± 0.03 (94.4)	7.96 ± 0.04
Starter 3	8.15 ± 0.04	6.96 ± 0.18	8.39 ± 0.03	< 6.00§	3.23 ± 0.01 (n.d.)	5.27 ± 0.04
Starter 4	8.99 ± 0.02	8.51 ± 0.03	9.17 ± 0.02	7.60 ± 0.20	6.30 ± 0.15 (94.9)	7.41 ± 0.03
Starter 5	9.03 ± 0.04	7.87 ± 0.13	8.80 ± 0.10	8.05 ± 0.06	7.55 ± 0.03 (68.4)	7.91 ± 0.09
Starter 6	9.35 ± 0.43	8.42 ± 0.02	9.44 ± 0.08	8.36 ± 0.12	7.35 ± 0.01 (90.2)	8.46 ± 0.06
Starter 7	9.16 ± 0.44	8.42 ± 0.12	9.80 ± 0.18	7.67 ± 0.04	5.27 ± 0.09 (99.6)	7.42 ± 0.01
Starter 8	8.97 ± 0.02	8.18 ± 0.14	9.04 ± 0.04	7.44 ± 0.14	< 4.00 (> 99.9)	6.97 ± 0.03

*Counts were performed by epifluorescence microscopy and LIVE/DEAD *Bac*Light bacterial viability kit (Molecular Probes Inc., Cambridge Bioscience). Green-stained cells were considered as 'viable'.

†SSW, sterile skimmed whey. Plating was carried out after a preliminary incubation (30 °C for 30 min) of 1 mL whey starter in 9 mL of SSW.

‡Percent values of the total streptococcal population calculated as viable but uncultivable in M17. These values are obtained after comparison between the viable cell counts and counts in M17 agar; n.d., not determinable.

§10^6 cells mL^{-1} was the lowest limit count of the method.

WA, whey agar (see methods).

Mean values ($\pm$ standard deviation) are indicated.

indicated that *Streptococcus thermophilus* is viable in almost all the starter cultures. In many cases, however, this organism was not fully quantifiable using the classical M17 count.

Length heterogeneity-PCR of domain A of the variable regions of the 16S rRNA gene is a useful tool to monitor microbial composition and population dynamics in artisan starter cultures (Lazzi *et al*., 2004). In the present work, the LH-PCR profiles were obtained from total RNA extracted from whey starters and amplified by RT-PCR. The use of RNA instead of DNA emphasized the sequence heterogeneity of the 16S rRNA gene, giving additional LH-PCR peaks, which had never been observed through direct RT-PCR analysis. As active bacteria have a higher number of ribosomes than dead cells, this approach gave an estimate of the relative activities of the major LAB populations present in the cultures. The sensitivity of the LH-PCR technique applied to the whey starters is *c*. 10^5 CFU mL^{-1} (Lazzi *et al*., 2004). Therefore, the presence of highly detectable (well above the baseline level, see Fig. 1) RT-LH-PCR peak signals for *S. thermophilus* underlines that a high proportion of this organism was metabolically active.

Our data demonstrated that *S. thermophilus* could not be fully cultivable in the M17 medium (which is a buffered medium), although this organism was often present within the major populations and viable in most of the starter cultures. Moreover, the lactobacilli counts in WA were higher than those in MRS. Taken together, these findings suggest that nutritional factors, either present in whey or produced by other microorganisms during growth in this mixed population, could most probably have stimulated the microbial growth. This is not surprising as the LAB community found in the whey starters is generally thermophilic and well adapted to the peculiar physical and chemical conditions (e.g. low pH and redox) of the whey substrate. To a great extent, the dependence on the substrate is so strict that bacteria multiply with difficulty or behave differently outside the cheese whey environment (Giraffa *et al*., 1996). Such observations were corroborated by our data. WA allowed a more effective evaluation of lactobacilli population than use of MRS medium. A treatment aimed at recovering *S. thermophilus* from whey starters, consisting of a short incubation in SSW and plating in M17 added with SSW, enabled us to obtain complete cultivability of this organism. Previous studies showed that bromocresol green WA performs better than MRS agar and M17 agar when *Lactobacillus delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, respectively, were enumerated in fermented milks (Yamany & Ibrahim, 1996).

Little is known about the effective role played by *S. thermophilus* in Grana Padano cheese manufacture, although it is widely assumed that an important growth of this organism during the early 20 h after cheesemaking is essential to obtain high quality cheeses at the end of ripening (Bottazzi, 1998). Although often indicated within the subdominant bacterial populations recoverable during lactic fermentation of whey cultures for Grana Padano cheese, *S. thermophilis* numbers presented here (10^7–10^8 CFU mL^{-1}) have rarely been reported (Giraffa *et al*., 1997; Beresford *et al*., 2001). Using randomly amplified polymorphic DNA-PCR and temperature gradient gel electrophoresis for the evaluation of the biodiversity of seven whey cultures for Grana Padano cheese, Andrighetto *et al*. (2004) did not find *S. thermophilus* at detectable levels. In a previous study, we found *S. thermophilus* (at estimated levels of 10^5–10^6 CFU mL^{-1}) in three out of eight Grana Padano whey starters (Lazzi *et al*., 2004). Therefore, data presented here indicated that this species can be considered as being within the prevalent and metabolically most important bacterial species recoverable from these cultures.

Acknowledgements

We are grateful to Mr Angelo Stroppa who collected the whey starter cultures analyzed in this work. We also thank Dr Lucia Fabiani, University of Parma (Italy), for technical support. This research was realized in part through the financial support of the Consorzio per la Tutela del Formaggio Grana Padano, Desenzano del Garda (Italy), which is administered by the Agricultural Research Service of the Region Lombardy.

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