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Safety aspects, genetic diversity and technological characterisation of wild-type *Streptococcus thermophilus* strains isolated from north Italian traditional cheeses

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ABSTRACT

Antibiotic susceptibility, antimicrobial activity, genotypic and technological properties of 52 *Streptococcus thermophilus* isolates, collected from four north Italian traditional cheeses, was investigated. RAPD-PCR, was used to study genetic variability and distinguish closely related strains; the results showed a high degree of heterogeneity among isolates. With regard to technological properties, after 6 h of incubation in milk 25% of the streptococcal strains could be defined as fast acid producers, while after 24 h the majority of isolates (79%) displayed only weak acidification activity. Reduction activity was generally low; in fact, none of these *S. thermophilus* strains showed a Eh < -102 mV). All the studied *S. thermophilus* were susceptible to ciprofloxacin, levofloxacin, penicillin G, ampicillin, mupirocin, nitrofurantoin, quinupristin/dalfopristin and rifampicin. Nine isolates were classified as resistant to tetracycline, 6 to streptomycin, 5 to oxacillin, 3 to erytromycin, 3 to vancomycin and only one to chloramphenicol. PCR-based detection did not identify any of the common genetic determinants for vancomycin (*van*A, *van*B, *van*C1, *van*C2, *van*C3, *van*D, *van*E, *van*G) or erythromycin (*erm*B and *erm*C). The genetic basis of the tetracycline resistance phenotype in these strains was linked to *tet*S-*tet*L genes (8 isolates) or the *tet*M gene (1 isolate), and the integrase element *int* of the Tn*916*/Tn*1545* family of transposons was negative. Four strains were able to produce antimicrobial compounds against *Clostridium tyrobutyricum*. The study provides new evidence of the resistance of *S. thermophilus* to antimicrobial agents, confirming the importance of including an accurate safety assessment of phenotypic/biotechnological data when carrying out strain selection for dairy applications.

1. Introduction

Streptococcus thermophilus is a thermophilic lactic acid bacteria (LAB) of major importance for the dairy industry. This species is widely used as a starter to produce fermented milk products because of its capacity to produce lactic acid by catabolising lactose, thus contributing to milk acidification and organoleptic properties (Giraffa, Paris, Valcavi, Gatti, & Neviani, 2001). *S. thermophilus* has the status of GRAS (Generally Recognized as Safe) in the USA and a Qualified Presumption of Safety (QPS) status in the European Union, due to its long history of safe use in food production. At present *S. thermophilus* is considered the second most important species of industrial LAB after *Lactococcus lactis*, with a market value of around 40 billion US$; over 10^{21} live cells are ingested annually by humans (Iyer, Tomar, Uma Maheswari, & Rameshwar Singh, 2010). In milk fermentation, *S. thermophilus* plays a role not only in the production of lactic acid, but also contributes to several other important technological properties such as reduction and peptidase activities.

Oxidation-reduction potential (Eh) is a major factor in determining the type of microorganisms that will grow in cheese, and excludes obligate aerobe growth in the cheese interior; in addition, Eh contributes to the creation of conditions necessary for balanced flavor development (Brasca, Morandi, Lodi, & Tamburini, 2007). Indeed, a low Eh value in the cheese indicates anaerobic conditions necessary for reactions resulting in good quality flavor (Urbach, 1995). Considerable variation has been detected among the different LAB species tested for their ability to change the redox potential (Brasca et al., 2007). *S. thermophilus* is widely present in natural milk and whey cultures used in the production of Italian cheeses of protected designation origin (PDO), and artisanal ones such as Asiago, Fontina, Gorgonzola, Grana Padano, Montasio, Monte Veronese, Mozzarella, Parmigiano Reggiano, Pecorino Toscano e Provolone and Taleggio cheese (Andrighetto, Borney, Barmaz, Stefanon, and Lombardi (2002); Beresford, Fitzsimons, Brennan, & Cogan, 2001; Bizzarro, Torri Tarelli, Giraffa, & Neviani,

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2000; Coppola, Parente, Dumontet, & La Peccerella, 1988; Dolci et al., 2009; Giraffa et al., 2001; Lazzi et al., 2009; Marino, Maifreni, & Rondinini, 2003). *S. thermophilus* can also be used as a bio-preservative to control the growth of pathogenic and spoilage bacteria in dairy products; in fact, one special benefit of the presence of such bacteria in cheeses is the production of bacteriocins (thermophilins) inhibiting *Clostridia*, *Listeria monocytogenes*, enteropathogenic and Gram negative bacteria (Ivanova et al., 1998; Kabuki, Uenish, Watanabe, Seto & Nakajima, 2007). Some wild *S. thermophilus* strains isolated from raw milk and traditional Greek yogurt have been shown to exert an inhibitory effect on *Clostridium sporogenes*, *Cl. butyricum, Cl. tyrobutyricum*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Bacillus cereus* (Aktypis & Kalantzopoulos, 2003; Kabuki et al., 2007; Mathot, Beliard, & Thuault, 2003). In recent years, increasing attention has been paid to the possibility that lactic acid bacteria could serve as reservoirs for antibiotic resistance determinants, the risk being that there could be gene transfer to many food-borne commensal bacteria and other pathogenic bacteria. However, few data are available on the antibiotic susceptibility of *S. thermophilus*, despite the abundance of this bacterium in dairy products (Ammor, Flórez, & Mayo, 2007; Mathur & Singh, 2005).

These considerations raise several questions that the present work addresses. First, what, actually, is the antibiotic susceptibility, and the distribution, of resistance genes of wild *S. thermophilus* in raw milk cheeses? The second question concerns the contribution of pathogenic and undesirable bacteria of wild *S. thermophilus* to inhibition, while a third question concerns the relationships among strains that display different acidifying and reducing activities.

In order to find answers to these questions the present study considered 52 *S. thermophilus* strains collected from four north Italian traditional cheeses (Bitto, Formagèla Valseriana, Semuda and Valtellina Casera) made using raw milk and different technologies; the strains were characterized in order to study their genotypic biodiversity, technological properties (acidifying and reducing activity, salt tolerance), and antibiotic susceptibility, as well as the distribution of tetracycline, erytromycin, vancomycin resistance genes, and the ability to produce antibacterial substances.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A group of 52 *S. thermophilus* was selected from the strain collection of the Institute of Sciences of Food Production of the National Research Council of Italy (CNR-ISPA), according to the cheese of origin and the production area (Table 1). Samples of milk cultures, whey cultures, curd and cheeses were obtained from different dairy environments in the Lombardy region (Northern Italy). Thirty-six strains were collected during the manufacture of two Italian artisanal cheeses of protected denomination origin (PDO), Bitto and Valtellina Casera, both produced in the Valtellina valley but using different cheesemaking technologies. Bitto is a cooked cheese, made *in situ* only during the summer season in pastures at an altitude of at least 1500 m, while Valtellina Casera is a semi-cooked cheese produced all year round in the valley floor. From another cheese, Formagèla Valseriana, a traditional cheese curdled by acidification with natural milk cultures, 11 isolates were recovered from the production chain, as were 5 isolates from Semuda, a cheese from a restricted Italian alpine area (north of Lake Como), produced with milk allowed to rest for at least 12 h and partially skimmed after the natural surfacing of the cream. Before each experiment the cultures were incubated overnight at 37 °C, in M17 broth (Scharlau Microbiology, Barcelona, Spain).

Table 1
Origin of the 52 *S. thermophilus* strains investigated in this study.

Cheese	Area	Total strains	Source	n°
Bitto	Valtellina	18	Whey culture	3
			Curd	12
			Cheese	3
Formagèla Valseriana	Valle Seriana	11	Milk culture	8
			Curd	2
			Cheese	1
Semuda	Alto Lario	5	Cheese	5
Valtellina Casera	Valtellina	18	Curd	14
			Cheese	4

2.2. DNA extraction

The isolates were grown overnight at 37 °C in 10 mL of M17, and DNA was extracted using the Microlysis kit (Labogen, Rho, Italy) following the manufacturer's instructions.

2.3. Randomly amplified polymorphic DNA (RAPD) analysis

RAPD-PCR was used to explore the genetic diversity of the *S. thermophilus* strains. RAPD-PCR reactions were performed with primers M13 (5′-GAGGGTGGCGGTTCT-3′), D11344 (5′-AGTGAATTCGCGGTCAGATGCCA-3′) and D8635 (5′-GAGCGGCCAAAGGGAGCAGAC-3′), while amplification conditions, as well as electrophoresis and the analysis of the amplification products, were as previously described (Andrighetto et al., 2002; Morandi, Brasca, Andrighetto, Lombardi, & Lodi, 2006). Grouping of the RAPD-PCR profiles was obtained with the BioNumeric 5.1 software package (Applied Maths, Kortrjik, Belgium) using the UPGMA (unweighted pair group method using arithmetic averages) cluster analysis. The reproducibility value of the RAPD-PCR assay, calculated from two repetitions of independent amplification of LAB type strains, was higher than 90%.

2.4. Growth- temperature and salt tolerance

The strains were tested for their ability to grow at 15 and 45 °C in M17 broth, salt tolerance (growth with 2%, 4% and 6.5% of NaCl in M17 broth), and their activity in litmus milk. All these tests were performed twice. The strains were stored at −18 °C in litmus milk.

2.5. Acidification ability

A multi-channel pH-meter (Cinac version 3 Ysebaert, Frepillon, France) was used to follow the pH values in the milk during 24 h of incubation at 37 °C. Strains were inoculated at a level of 1% in reconstituted sterile non-fat dry milk (10% w/v). Combined pH electrodes (InLab 51343050, Metler Toledo, Greifensee, Switzerland) were standardised using two buffers (pH 4.0 and pH 7.0) and cleaned after each run using a pepsin/HCl solution. The pH measure was estimated as the mean value of two replicates for each bacterial strain.

The acidification rate was calculated as ΔpH ($\Delta pH = pH_{\text{zero time}} - pH_{\text{at time}}$). Values of ΔpH after 6 h (ΔpH_6) and 24 h (ΔpH_{24}) were used to compare the acidifying activity of the strains.

2.6. Redox activity (Eh)

An Eh-meter (pH302 Hanna Instruments, Villafranca Padovana, Italy) was used to follow the redox values (Eh) in the milk during the 24 h incubation period at 37 °C. The strains were inoculated at