

ORIGINAL ARTICLE

Selection of raw cow's milk thermophilic lactic acid bacteria obtained from southwest Parana, Brazil, with potential use as autochthonous starter

Seleção de bactérias ácido-láticas termófilas de leite cru bovino obtido no sudoeste do Paraná, Brasil, com uso potencial como fermento autóctone

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Abstract

This work aimed to isolate and identify *Streptococcus* and *Lactobacillus* species from raw cow's milk obtained from Southwest Paraná - Brazil. We used randomly amplified polymorphic DNA (RAPD)-PCR to identify and type 58 *Streptococcus* and 48 *Lactobacillus* isolates, of which 04 *Streptococcus thermophilus* and 02 *Streptococcus macedonicus* were confirmed by species-specific PCR and by sequencing of the 16S ribosomal RNA of 02 *Streptococcus lutetiensis/infantarius*, 10 *Lactobacillus fermentum*, 03 *Lactobacillus delbrueckii* subspecies *bulgaricus*, 01 *Lactobacillus rhamnosus/casei* and 02 *Lactobacillus helveticus*. The results indicated predominance of *Streptococcus thermophilus* and *Lactobacillus fermentum*. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subspecies *bulgaricus* strains were tested on the basis of their acidification kinetics. Considerable variation between the *Streptococcus thermophilus* was observed for the maximum rate of acidification (V_m), with a maximum of -4.5 and minimum of -4.2 pH milliunits min^{-1} . The *Lactobacillus delbrueckii* subspecies *bulgaricus* showed values between -8.4 and -7.1 pH milliunits min^{-1} . These results suggest that strains characterized as having a high acidifying capacity, could be used as starters in cheesemaking. The ferments presented an excellent performance in the acidification process, generating adequate curves, characteristics of a starter culture.

Keywords: Indigenous bacteria; *Streptococcus*; *Lactobacillus*.

Resumo

A proposta deste trabalho foi isolar e identificar espécies de *Streptococcus* e *Lactobacillus* a partir de leite cru de vaca coletado no sudoeste do Paraná, Brasil. A reação de amplificação aleatória de DNA polimórfico (RAPD)-PCR foi aplicada para identificação e tipificação de 58 *Streptococcus* e 48 *Lactobacillus* isolados, dos quais quatro



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Streptococcus thermophilus e dois *Streptococcus macedonicus* foram confirmados por PCR espécie-específica e, por sequenciamento do RNA ribossômico 16S, dois *Streptococcus lutetiensis/infantarius*, dez *Lactobacillus fermentum*, três *Lactobacillus delbrueckii* subespécie *bulgaricus*, um *Lactobacillus rhamnosus/casei* e dois *Lactobacillus helveticus*. Os resultados indicaram que os *Streptococcus thermophilus* e *Lactobacillus fermentum* foram predominantes. As estirpes *Streptococcus thermophilus* e *Lactobacillus delbrueckii* subespécies *bulgaricus* foram avaliadas de acordo com a cinética de acidificação. Observou-se variação considerável entre o *Streptococcus thermophilus* para a taxa máxima de acidificação (V_m), com um máximo de -4,5 e mínimo de -4,2 pH miliunidades min^{-1} . Os *Lactobacillus delbrueckii* subespécie *bulgaricus* apresentaram valores entre -8,4 e -7,1 pH miliunidades min^{-1} . Estes resultados sugerem que as cepas caracterizadas com elevada capacidade de acidificação poderiam ser utilizadas como bactérias iniciadoras (*starters*) na fabricação de queijos. Os fermentos apresentaram excelente desempenho no processo de acidificação, gerando curvas adequadas, características de uma cultura iniciadora.

Palavras-chave: Bactérias nativas; *Streptococcus*; *Lactobacillus*.

1 Introduction

The lactic acid bacteria (LAB) are easily found as autochthonous microflora in raw milk (Rodríguez et al., 2000). In the current industrial food production, LAB are mostly focused on six genus and/or species: *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Oenococcus oeni* and *Streptococcus salivarius* subspécie *thermophiles* (Klaenhammer et al., 2002; Ray & Bhunia, 2014). *Streptococcus thermophilus* (*S. thermophilus*) adapts to the milky environment and can be isolated only from the milk. The amount of this microorganism in raw milk is small. Incubation at temperatures $> 40^\circ\text{C}$ presents as an exclusive and selective means to enrich *S. thermophilus* from a mixed population (Harnett et al., 2011). *Lactobacillus* bacteria are not present after the milk has been milked aseptically, but the contamination may occur from contact with soil, manure, grass, silage or other foods adhered to the udder, as well as milking and milk storage equipment (De Angelis & Gobetti, 2011). There is a great demand for recovering microorganisms' starters from wild LABs of milk or raw dairy products (Wouters et al., 2002). Thermophilic LAB such as *S. thermophilus*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subspécies *bulgaricus* or subspécie *lactis* (*L. delb.* subsp. *bulgaricus* or *lactis*) have long been used as starter cultures in the production of fermented milks and various Swiss and Italian cheeses (Iyer et al., 2010; Rizzello & De Angelis, 2011). Since the intrinsic bacteria promote slow acidification, the function of the starter cultures is to accelerate the process and their addition maintains the uniformity of the products; however, the biota of the raw milk can be lost (Wouters et al., 2002). Rather than acidifying milk, LABs have the function of altering the flavor and texture properties of cheeses and inhibiting the development of pathogens and deteriorants (Leroy & De Vuyst, 2004; Lahtinen et al., 2012). The acidification rate during the fermentation period can be described from the pH curves and the following kinetic parameters are calculated by: the maximum acidification rate ($V_m = \text{dpH}/\text{dt}$), expressed as pH milliunits min^{-1} , which is the maximum slope of the acidification curve; and the time (T_m), expressed as minutes, at which V_m occurred (Zanatta & Basso, 1992). These parameters have been widely studied (Kristo et al., 2013; Lucas et al., 2004; Chammas et al., 2006; Almeida et al., 2008; Damin et al., 2008). The *S. thermophilus* and *Lactobacillus* species have been used for centuries in the elaboration of fermented food and are considered Generally Recognized as Safe (GRAS) (Iyer et al., 2010; Gharaei-Fathabad & Eslamifar, 2011). The use of the selected strains are important biological and genetic resources, and could assure cheese quality and typicality, and help protect the traditional process of fermented products. The southwest Parana region, Brazil, has been increasing its milk production in the last years, and, considering the main dairy regions of the state, was the region that showed the greatest growth in herd size and milk production between 2008 and 2013. During this period, production doubled, making it the leading dairy region of the state. Thus, actions that can contribute to the development and enhancement of the dairy chain are important and should be considered.

This work aimed to isolate, identify, characterize and select the thermophilic lactic microflora of raw cow's milk obtained from Southwest Parana-Brazil, in order to clarify the potential technological role of *S. thermophilus* and *L. delb. subsp. bulgaricus* as autochthonous starter cultures.

2 Material and methods

2.1 Strains and growth conditions

Twenty milk samples were aseptically collected from bulk tanks in dairy farms and dairies in southwest Paraná - Brazil and transported to the laboratory under refrigerated conditions. These samples were subjected to heat treatment at 63 °C for 12 min and then incubated at 44 °C for approximately 12 h (acidity between 0.225 to 0.270 lactic acid 100 mL⁻¹). The *Streptococcus* strains were grown in M17 Agar Base (HIMEDIA): 44 °C for 48 h and the *Lactobacillus* in Man, Rogosa & Sharpe (MRS) medium (HIMEDIA): pH 5.45, 44 °C for 72 h (International Organization for Standardization, 2003). The morphology, Gram stain and catalase activity were also checked (Silva et al., 2010). The Enterococci were counted on Kanamycin Aesculin Azide Agar (KAA, Oxoid) after 24 h at 37 °C under aerobic conditions (Baylis, 2007). For each culture, 10-12 colonies identified as *S. thermophilus* and *Lactobacillus* strain were purified by streaking onto M17 and MRS agars, respectively. The cultures were maintained at -40 °C in 0.25 mL of M17 or MRS broths, supplemented with freezing mix (20 mM MgSO₄ and 80% glycerol) and 0.5 mL of reconstituted powdered milk, denominated the Stock Culture 1 (SC1), and numbered from S01 to S150 for *Streptococcus*, and from L01 to L141 for *Lactobacillus* species. Subsequently the colonies were slightly thawed and, with the help of disposable loops (1 µL), were inserted into microtubes containing their respective solidified culture media (M17 and, MRS acidified at pH 5.45). The tubes were then incubated at 44 °C in aerobiosis (*Streptococcus*) and anaerobiosis (*Lactobacillus*) until growth. The strains were conditioned in boxes with support for microtubes and sent to the BIOAGRO INSTITUTE (Thiene, Italy) for DNA extraction.

2.2 DNA extraction, RAPD-PCR, species-specific pcr and analysis sequencing of the 16S ribosomal RNA

In all, 58 *Streptococcus* and 48 *Lactobacillus* strains were revitalized by streaking (M17: 44 °C for 48 h and MRS: 37 °C for 72 h). Each strain was inoculated into 10 mL of the respective broth and incubated overnight. The cells were pelleted by centrifugation at 13.200 rpm for 5 min, the supernatant discarded, and the cell mass prepared for freezing according to the same procedure described for SC1, and stored at -80 °C, Stock Culture 2 (SC2). The isolates (SC2) were grown overnight at 37 °C or 44 °C in 10 mL of M17 or MRS broths, and the DNA extracted using the MicroLYSIS kit (Labogen, Italy) following the manufacturer's instructions. RAPD-PCR reactions were carried out with the primer D11344 (5' AGT GAA TTC GCG GTC AGA TGC CA3'). The following amplification conditions were used: an initial step of 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 42 °C for 1 min, 72 °C for 1.5 min, and a final step at 72 °C for 10 min (Akopyanz et al., 1992). The amplification products were separated by electrophoresis on 1.5% (w/v) agarose gels in 0.5% TBE buffer (54 g of Tris, 27.5 g of boric acid and 20 mL of 0.5 M EDTA at pH 8.0). Grouping of the RAPD-PCR profiles was obtained using the Gel Compar 4.1 software package (Applied Maths, Kortrijk-Belgium) with the Pearson product moment correlation coefficient, and the Unweighted Pair Group Method with the Arithmetic Average (UPGMA) clustering algorithm (Vauterin & Vauterin, 1992). Assignments to the species *S. thermophilus* and *Streptococcus macedonicus* (*S. macedonicus*) were confirmed for some strains by a species-specific PCR based on the amplification. The reactions were carried out with primers for *S. Thermophilus* (ST1: CAC TAT GCT CAG AAT ACA; ST2: CGA ACA GCA TTG ATG TTA) and 30-cycle amplification conditions as follows: 90 °C for 30 s, 54 °C for 70 s and 70 °C for 30 s according to Lick et al. (1996), and for *S. macedonicus* (MAC1: ACT GCG CTG TGG GAA GTC;

MAC2: CCT TCT CCC GAA GTT ACG) with the following amplification conditions: 94 °C for 3 min and 30 cycles: 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min (Lombardi et al., 2004). The DNA sequences were thus obtained (Klijn et al., 1991). The following strains were used as references in the genetic assays: *S. macedonicus* 893^T and *S. thermophilus* 85^T, for *L. delb.* subsp. *bulgaricus* 92^T, *L. delb.* subsp. *lactis* 137^T and *L. helveticus* 303^T.

2.3 Acidification rate

The acidification kinetics was evaluated in the Micros automatic system for pH measurement and data acquisition (Conegliano, Italy). One hundred microliters of an overnight culture in M17 or MRS broths were inoculated into 10 mL of reconstituted skim milk and incubated at 37 °C until coagulation. Two milliliters were then inoculated into 250 mL glass bottles containing 200 mL of reconstituted skim milk and incubated at 37 °C (*S. thermophilus*) or 44 °C (*L. delb.* subsp. *bulgaricus*) for 16 h. The pH was measured continuously by introducing the electrodes, disinfected with ethanol, through holes in the screw cap, together with the Micros integrated data acquisition system (Conegliano, Italy), (Zanatta & Basso, 1992). A control culture (S439C) was also used to determine the acidification rate for *S. thermophilus*.

3 Results and discussion

3.1 Identification of the isolates

The bacterial isolates were subjected to Gram staining and to the catalase test, and identified as Gram-positive and catalase-negative *bacilli* or *cocci*. Enterococci are the microorganisms that inhabit the ecosystem of raw milk, but were not found in the samples under examination. Their presence could cause confusion in the identification of the LAB bacteria of interest. To selectively enumerate enterococci as unique components, the culture medium used is Kanamycin-aesculin-azide (KAA). Enterococci are commonly observed in association with a diverse microflora, so any other members of the LAB make it difficult to examine a mixed microflora (Domig et al., 2003), since they may survive low pasteurization temperatures (Giraffa, 2003).

3.2 Identification of *Streptococcus* and *Lactobacillus* species

The numerical elaboration of the combined RAPD-PCR profiles of the 58 natural isolates and the two type/reference strains (85^T for *S. thermophilus* and 893^T for *S. macedonicus*) clustered by the UPGMA analysis, resulted in the dendrogram shown in Figure 1.

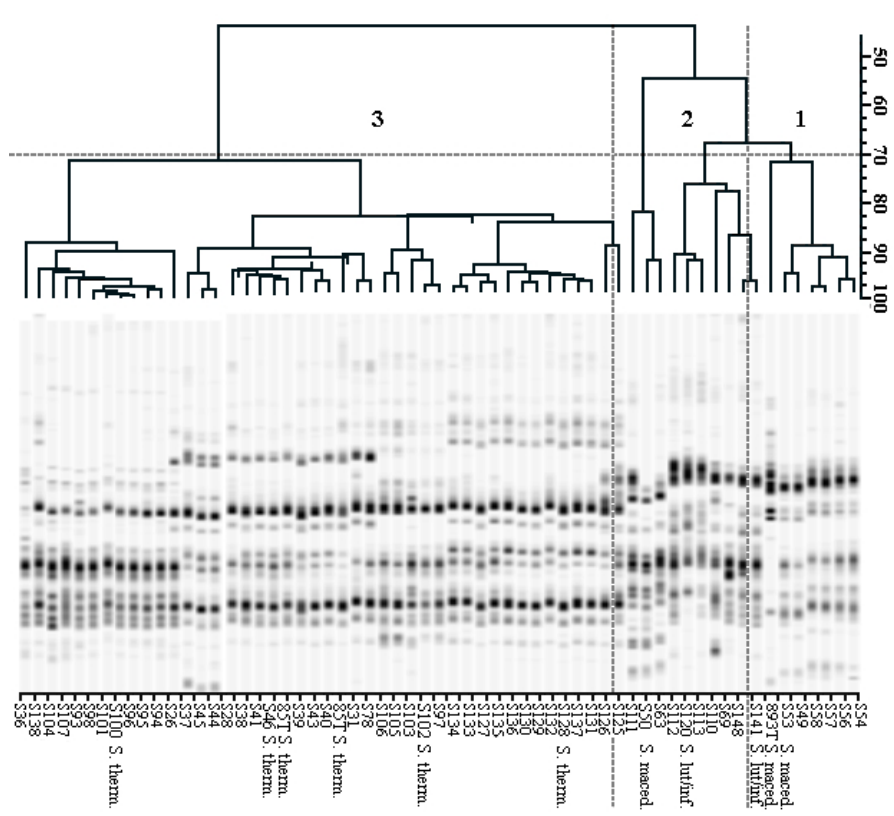


Figure 1. Cluster analysis of the RAPD-PCR profiles of *Streptococcus* type/reference strain isolates originating from raw milk.

We observed genotypic heterogeneity among the isolated strains, confirmed by the low coefficient of similarity (64%), and three clusters (labelled 1 to 3). Strains $\geq 90\%$ of similarity coefficient can be classified as being extremely close genotypically, and perhaps even identical (Morandi & Brasca, 2012). Cluster 1 comprised the *S. macedonicus* strain, with six isolates, while cluster 2 contained the *Streptococcus lutetiensis/infantarius* type strain. Finally, most of the isolates (41 of 58) were grouped in cluster 3, with the strain type of *S. thermophilus*. A species-specific PCR analysis of a number of isolates (Figure 1) confirmed the assignment of the isolates included in cluster 1 as the species *S. macedonicus* (S53); also the assignment of strain S50 (cluster 2) was confirmed as this strain, and cluster 3 as *S. thermophilus* (S46, S100, S102 and S128). The strains S36, S63 and S111 did not belong to any of the types/references considered in this work. The strains, S120 and S141, in cluster 2 were confirmed by genetic sequencing as *S. lutetiensis/infantarius*, and 70.7% of the isolates obtained from the raw milk were identified as *S. thermophilus*. This microorganism is largely found 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, Parmegiano Reggiano, Pecorino Toscano, Provolone and Taleggio cheeses (Coppola et al., 1998; Bizzarro et al., 2000; Beresford et al., 2001; Giraffa et al., 2001; Andrighetto et al., 2002; Marino et al., 2003; Dolci et al., 2009; Lazzi et al., 2009). *S. thermophilus* isolates represented the majority in both the pasteurized milk (63 °C for 30 min) samples and the pasteurized milk samples incubated at 42 °C for 24 h, followed by *L. delb. subsp. bulgaricus* and *S. macedonicus* (Almeida et al., 2009). *S. macedonicus*, which was first isolated from Greek Kasser cheese, has been described in Italian cheeses. Given that most of the isolates studied originated from raw milk cheeses, the authors of this paper speculated that this organism could arise from the raw milk (Tsakalidou et al., 1998; Andrighetto et al., 2002; Gatto et al., 2002). The use of *S. macedonicus* is more efficient as primary culture rather than adjunct starter (Lombardi et al., 2004; Anastasiou et al., 2007) is of interest, and also that it has multifunctional properties

(13.3%) (Abdullah & Osman, 2010). The diversity of the *Lactobacilli* present in milk indicates a variation of the species according to area, caused mainly by differences in climate, vegetation and feed supplied to the animals.

Our results indicated that *Streptococcus thermophilus* and *Lactobacillus fermentum* were predominant. In recent research about microbial diversity in Brazilian artisanal cheeses (made with raw milk), the predominate genera were of *Streptococcus*, *Leuconostoc* and *Lactococcus* (Kamimura et al., 2019). The occurrence of one or other BALs type may be related to the origin of the raw material, if were isolated from raw milk, pasteurized and/or from the cheese and in this case the maturation time may influence in the microbiota.

3.3 Acidifying activity of lactic bacteria

Cultures Starters can be defined as preparations containing microorganisms that are developed by the fermentation of a certain substrate, such as lactose, present in the medium and its transformation into lactic acid, in the initial stage of the production of fermented milks and cheeses. A useful rule is to decrease the value to < 5.3 in the milk in approximately 6 h at 30 °C to 37 °C, depending on the dairy product. The low pH inhibits undesirable organisms, and, in fermented milks, promotes the formation of the gel. Starters may also impart other benefits for health (Beresford et al., 2001; De Angelis & Gobbetti, 2011; Harnett et al., 2011). Thus, for the production of starters, it is important that the strains exhibit high fermentation rates in order to ensure that the pH values of the cheeses reach a value close to 5.2 in less than 6 hours. Strains of *S. thermophilus* and *L. delb. subsp. bulgaricus* were selected to verify the acidification rate and the results of the analysis can be seen in Table 1.

Table 1. Kinetic parameters of the strains of *S. thermophilus* and *L. delbrueckii* subsp. *Bulgaricus*.

Strain	Isolate code	V_m dpH dt ^{-1a}	T_{max} (min) ^b	$T_{pH5.2}$ ^c
<i>S. thermophilus</i>	S439C ^d	-14.5	185	222
	S128	-12.8	206	277
	S28	-14.5	152	226
	S104	-13.1	209	255
	S100	-14.2	212	261
	S103	-13.0	224	272
	S93	-13.8	211	267
	S102	-12.1	196	284
	S125	-7.2	396	526
	S63	-5.0	391
	S111	-5.3	384
	S141	-5.1	418
	S46	-6.5	382
	S43	-6.2	159
	S50	-5.4	649
S53	-4.2	532	
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	LB120	-13.7	563	579
	LB140	-8.4	242	329
	LB34	-8.4	263	301
	LB134	-8.0	302	356
	LB33	-7.7	256	330
	LB28	-7.1	242	326

^a V_m , corresponds to the maximum slope of the acidification curve and represents the maximum acidification rate (pH milliunits min⁻¹). ^bTime at which the maximum acidification rate (V_m) occurred. ^cTime at which the pH reached 5.2. ^dControl culture.

The maximum acidification rates (V_m) of the *S. thermophilus* and *L. delb. subsp. bulgaricus* isolates, the times (T_m) required for the different strains to reach the V_m values, and the time taken to reach pH 5.2 can

be seen in Table 1. *S. thermophilus* strains are defined as “fast acidifiers” and show a mean V_m value of -10.73 pH milliunits min^{-1} (Zanatta & Basso, 1992), but 46.67% of the isolates examined in this study showed V_m values below this reference. The V_m values ranged between -8.4 and -7.1 pH milliunits min^{-1} in the six samples of *L. delb. subsp. bulgaricus* studied. A study of these pure cultures isolated from fermented milks and known as “laban”, showed maximum acidification rates of -10.5 pH milliunits min^{-1} (Chammas et al., 2006). The symbiotic relationship between *S. thermophilus* and *L. delb. subsp. bulgaricus* has long been used in the manufacture of fermented milks and various cheeses. Co-cultures achieve higher V_m values, typical of fast strains, with reported V_m values of -14.5 and -13.75 pH milliunits min^{-1} (Oliveira & Damin, 2003; Almeida et al., 2009), respectively. The *S. thermophilus* strains S28, S104 and S128 showed acidifying activity with a reduction in pH to about 5.2 in similar period time to that of the standard (222 min.), and the *L. delb. subsp. bulgaricus* strains all showed similar acidification times with a minimum value of 301 min for L34 and maximum value of 356 min for L134.

Two yeasts, A and B, were developed with best strains. The yeast A composed of strains S28 and S128 (*S. thermophilus*) and strain LB134 (*L. delbrueckii* sp. *bulgaricus*), and B composed of strains S98 and S104 (*S. thermophilus*) and a strain LB134 (*L. delbrueckii* sp. *bulgaricus*). The acidity of fermentation was evaluated during the preparation of the Santo Giorno cheese typical of Southwestern region of Parana - Brazil, according to the methodology described by Pereira et al. (2017), the results of which are showed in Figure 3.

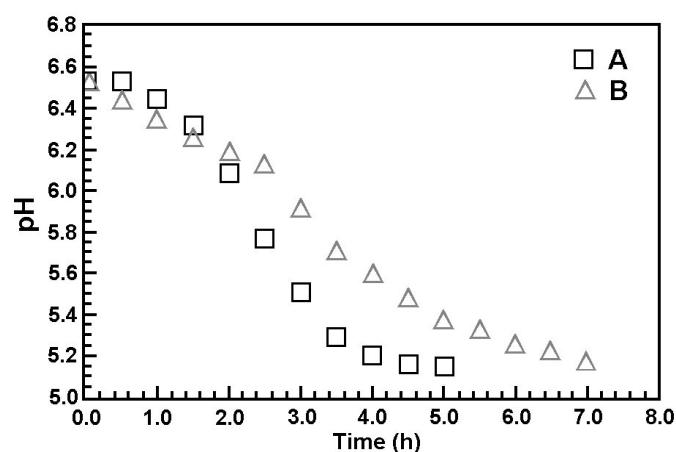


Figure 3. Average acidification curve of ferments A and B.

We observed that the ferments presented an excellent performance in the acidification process, generating adequate curves, characteristics of a starter culture. The average pH of the dough was reduced to a value of about 5.20 in a maximum time of 4 hours for yeast A and 7 hours for yeast B.

4 Conclusions

The results indicate that cow's milk obtained from Southwest Paraná, Brazil, exhibits a wide diversity of thermophilic LAB (*S. thermophilus*, *S. macedonicus*, *S. lutetiensis/infantarius*, *L. helveticus*, *L. fermentum*, *L. delb. subs. bulgaricus*, *L. rham/casei*), where their separation into clusters can define distinct groups of strains. Such information is essential and fosters evidence that natural environments are rich in biodiversity and carrying sources of strains and species, as example LAB group. *S. thermophilus* and *L. fermentum* are predominant in the milk analyzed and may play an important role in their quality. The acidification rates suggest that the strains S128, S28, S104, S100, S103, S93, S102, L140, L34, L134, L33 and L28 have

potential as autochthonous starter cultures in cheese manufacture. A well characterized bacterial culture can serve as the source for the construction of a starter with well-defined functional properties.

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