Characterization of dominant lactic acid bacteria isolated from São Jorge cheese, using biochemical and ribotyping methods

J.M. Kongo[1,2], A.J. Ho[3], F.X. Malcata[1] and M. Wiedmann[3]

1 Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, Porto, Portugal
2 Centro de Investigação em Recursos Naturais, Departamento de Biologia, Universidade dos Açores, Ponta Delgada, Portugal
3 Department of Food Science, Cornell University, Ithaca, NY, USA

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Correspondence
F. Xavier Malcata, Escola Superior de Biotecnologia, Rua Dr. António Bernardino de Almeida, P-4200-072 Porto, Portugal.
E-mail: fxmalcata@esb.ucp.pt

Abstract

Aims: To identify, using phenotypic and genotypic methods, the dominant lactic acid bacteria (LAB) present in São Jorge cheese – one of the 11 Portuguese cheeses currently bearing an *Appéllation d'Origine Protegée* status.

Methods and Results: A total of 225 isolates from milk, curd and cheeses throughout ripening were identified to the genus level, 108 to the species level and ten to the strain level. Phenotypic methods indicated that lactobacilli, followed by enterococci, were the dominant bacteria. The most frequently isolated species were *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Enterococcus faecalis* and *Enterococcus faecium*. Ribotyping differentiated three *L. paracasei*, two *E. faecalis* and one *Lactobacillus plantarum* types. *Enterococcus* spp. exhibited the highest esterase and β-galactosidase activities among all isolates.

Conclusions: The dominant LAB in São Jorge cheese are *L. paracasei*, *L. rhamnosus*, *E. faecalis* and *E. faecium*. *Enterococcus* likely plays a leading role upon acidification and aroma development in said cheese.

Significance and Impact of the Study: Our results support that a combination of conventional biochemical methods with genotypic methods allows for a thorough characterization and identification of isolates. Despite the limited number of isolates subject to molecular subtyping, a few specific *Enterococcus* and *Lactobacillus* strains were found that are promising ones for development of a starter culture. Hence, *L. paracasei* and *E. faecalis* are good candidates for a tentative starter culture, designed for manufacturing of São Jorge cheese at large – which takes advantage of actual isolates, in attempts to eventually standardize the quality of said cheese variety.

Introduction

The unique flavour of a given cheese variety is the consequence of a complex combination of proteolytic, glycolytic and lipolytic activities – which take place mainly during cheese ripening, and which are often brought about by lactic acid bacteria, LAB (Fox and McSweeney 1996; Steele *et al.* 1997; Sousa *et al.* 2001; Hayaloglu *et al.* 2004). Characterization of adventitious LAB present in artisanal raw milk cheeses is therefore both a challenge and a relevant issue – as it may help one understand the role of such micro-organisms upon cheese ripening, and eventually lead to more rational cheese-making practices and educated improvement of specific cheese characteristics (Centeno *et al.* 1996; Medina *et al.* 2001). This realization justifies the increased interest in genotypic and phenotypic studies focussed on wild LAB, isolated from traditionally manufactured dairy products (Cogan *et al.* 1997; Baruzzi *et al.* 2000; Coppola *et al.* 2003).

São Jorge is a semi-hard, traditional Portuguese cheese, manufactured from raw bovine milk on the Azores island of the same name. This cheese was granted an *Appéllation d'Origine Protegée* (AOP) status in 1986, and is still produced in small dairies – where whole fresh milk is mixed with whey from the previous batch (which consequently acts as a natural starter). Acidification at 30°C is followed by curd cooking at 37°C, whey draining off, curd pressing and cheese salting – and finally cheese ripening, for 6–12 months. The ripening process thus receives the combined contributions of the indigenous microbiota in raw milk and whey. However, and as happens with manufacture of similar products (Holzapfel 2002; Mannu and Paba 2002; Poznanski *et al.* 2004), São Jorge cheeses lack consistency in their final product quality, partly because of somehow unpredictable microbial population. One way to alleviate this constraint is via development of specific starters or adjuncts – which may aid toward production of less variable final products, yet without significant loss of their typical flavour.

Identification and characterization of LAB in foods is often achieved via studying their carbohydrate fermentation patterns – complemented with such technological features as acidification activity and aroma development. On the contrary, recent developments in molecular biology and increased knowledge of genomic structures of many bacterial groups have turned molecular methods into common practice – especially when seeking reliable identification results (Beresford *et al.* 2001; Gatti *et al.* 2003; Andrighetto *et al.* 2004; Dutoit *et al.* 2005; Sanchez *et al.* 2006). Molecular typing techniques include e.g. pulsed-field gel electrophoresis, PCR-based methods, DNA sequencing and ribotyping. The latter is based on restriction digestion of bacterial chromosomal DNA, followed by Southern hybridization with a ribosomal operon probe; this can be performed manually – as in classical multi-step Southern blotting, or mechanically – as in automated ribotyping (Wiedmann 2002).

The aim of this research was to identify the dominant LAB in São Jorge cheese, at various stages of ripening – using state-of-the-art biochemical and (automated) ribotyping tools.

Materials and methods

Sampling and plating

Samples (100 ml or 100 g, as appropriate) of raw milk, whey, curd and cheese were aseptically collected on a monthly basis from seven dairies located in Azores and certified for manufacture of São Jorge cheese. Samples were transferred into sterile individual bags or flasks, transported to our laboratory in insulated coolers containing cold packs, and analysed within 10 h of collection.

From each sample, 25 ml or 25 g (as appropriate) portions were aseptically transferred into flasks containing 100 ml of 2% (w/v) sodium citrate at 40 ± 2°C and homogenized for 10 min, and then decimally diluted in 0·1% (w/v) peptone saline water. Finally, a 1-ml aliquot of each dilution was plated in duplicate on appropriate media (all from Oxoid, Basingstoke, UK) and incubated as follows: MSE (Mayeux Sandine and Elliker) agar, for leuconostoc, at 22°C for 4 days; M17 agar, for lactococcus, at 30°C for 24 h; Rogosa agar, for lactobacillus, at 30°C for 5 days; and KAA (Kanamycin Aesculin Azide) agar, for enterococcus, at 37°C for 24 h. After incubation, five colonies at random were isolated from each medium, purified, and stored at −30°C in vials containing MRS broth with a final concentration of 20% (w/v) glycerol. For identification and characterization purposes, isolates were thawed and plated on MRS agar, and incubated accordingly.

Phenotypic characterization

A total of 225 isolates were subject to identification. All isolates were presumptively identified by morphology using microscopy, and their genera were confirmed using the following tests: Gram-staining, catalase reaction, growth in MRS broth at 15°C for 7 days and at 45°C for 2 days, and ability to produce CO_2 from glucose in MRS broth tubes containing Durham bells.

From the isolates identified to the genus level, a total of 108 were further identified to the species level using the following API tests: API CHL 50 and API 20 Strep kits (BioMérieux, Marcy-l'Etoile, France), following manufacturer's instructions, and incubated at 30°C for 24 and 48 h respectively.

Automated ribotyping

Selected isolates identified to the species level were further characterized using automated ribotyping, with *Eco*RI as enzyme, and the Riboprinter Microbial Characterization System (DuPont-Qualicon, Wilmington, DE, USA). This process involved *Eco*RI-mediated digestion of chromosomal DNA, followed by Southern hybridization and probing with an *Escherichia coli rrn*B rRNA operon probe (Wiedmann 2002). Images were acquired with a charge-coupled device camera and processed with a suitable software – that normalizes fragment pattern data of band intensity and relative band position (as compared with the corresponding molecular weight marker), hence providing a high degree of reproducibility (Wiedmann 2002).

Genus	Source			
	Curd	5-week cheese	12-week cheese	22-week cheese
Lactobacillus	11 (19·0%)	22 (40·7%)	28 (53·9)	32 (52·5%)
Pediococcus	–	2 (3·7%)	3 (5·8%)	6 (9·8%)
Lactococcus	4 (6·9%)	2 (3·7%)	2 (3·9%)	–
Enterococcus	36 (62·1%)	20 (37·0%)	16 (30·8%)	19 (31·2%)
Leuconostoc	4 (6·9%)	5 (9·3%)	2 (3·9%)	4 (6·6%)
ND	3 (3·4%)	3 (5·6%)	1 (1·9%)	–
Total	58 (100%)	54 (100%)	52 (100%)	61 (100%)

ND, not determined.

Table 1 Identification of dominant LAB genera found – number (and percent) of isolates at various ripening stages in São Jorge cheese using biochemical methods (n = 225)

Species	Degree of confidence				
	Excellent	Good	Acceptable	Doubtful	Unacceptable
Lactobacillus paracasei	5	12	9	2	1
Lactobacillus rhamnosus	–	3	4	5	3
Lactobacillus coryniformis	–	–	2	2	2
Lactobacillus plantarum	1	2	2	–	–
Enterococcus faecalis	7	5	9	8	2
Enterococcus faecium	–	5	7	4	3
Lactococcus lactis	1	2	–	–	–
Total	14	29	33	21	11

Table 2 Identification of dominant LAB species found – number of isolates in São Jorge cheese using API methods (n = 108)

Enzyme profiling

A total of 14 isolates of the dominant genera and species were further characterized, in terms of their enzymatic profile, using the API-ZYM kit (BioMérieux).

Results

Phenotypic characterization

The biochemical methods selected for this study allowed identification of a total of 225 isolates to the genus level (Table 1), and of a total of 108 isolates to the species level (Table 2). Inconclusive biochemical profiles did not permit identification of 3% of the isolates (Table 1).

Among all isolates, *Lactobacillus* represented 41%, *Enterococcus* 40%, *Leuconostoc* 7%, *Pediococcus* 5% and *Lactococcus* 4%. However, 62% of the isolates in curd were identified as *Enterococcus*, whereas *Lactobacillus* accounted for 19%, and *Lactococcus* and *Leuconostoc* for 7% each. The latter two genera were detected at earlier maturation stages, and only in some curds. From week 5 of maturation on, the proportion of *Lactobacillus* isolates increased – and the said genus eventually became dominant (53%) by week 22, while the frequency of *Enterococcus* decreased and eventually levelled off at 30%; the proportion of other groups remained essentially constant.

The results of API-based identification of the 108 isolates, i.e. 55 *Lactobacillus*, 3 *Lactococcus* and 50 *Enterococcus*, are tabulated in Table 2. Among the *Lactobacillus* genus, 29 (i.e. 53% of all isolates) were identified as *Lactobacillus paracasei*, 15 (27%) as *Lactobacillus rhamnosus*, 6 (11%) as *Lactobacillus coryniformis* and 5 (9%) as *Lactobacillus plantarum*. Among the *Enterococcus* genus, 31 (i.e. 62% of all isolates) were identified as *Enterococcus faecalis* and 19 (38%) as *Enterococcus faecium*. Finally, three isolates were identified as *Lactococcus lactis*. The API system employed was able to classify 13% of the isolates at an excellent ID level, 27% at a good ID and 31% at an acceptable ID. More than 67% of *Enterococcus* spp. and more than 70% of *Lactobacillus* spp. were thus classified from excellent to acceptable via such an API approach.

Automated ribotyping

Among the 14 isolates classified at an excellent ID level by the API system, ten were further subject to automatic *Eco*RI ribotyping; the resulting patterns are shown in Fig. 1.

All isolates were identified to species and subspecies levels, with a similarity index not below 0·90 – except for Q2B and Q1C, which were identified with 0·89, and Q1B, which was identified with 0·87. The similarity required for identification is 0·85.

Label	DuPont ID Label	RiboGroup	RiboPrint™ Pattern
			1 kbp 5 10 15 50
Q3B	Lactobacillus paracasei ss. paracasei	EcoRI 116-1036-S-1	
Q2B	Lactobacillus paracasei ss. paracasei	EcoRI 116-1036-S-3	
Q1C	Lactobacillus paracasei ss. paracasei	EcoRI 116-1036-S-5	
Q3A-LB	Lactobacillus paracasei ss. paracasei	EcoRI 116-1036-S-3	
Q1A	Lactobacillus paracasei ss. paracasei	EcoRI 116-1036-S-1	
Q2A-LB	Lactobacillus plantarum	EcoRI 116-178-S-6	
M1B	Enterococcus faecalis	EcoRI 116-366-S-5	
Q3A-EC	Enterococcus faecalis	EcoRI 116-366-S-5	
Q2A-EC	Enterococcus faecalis	EcoRI 116-366-S-1	
Q1B	Enterococcus faecalis	EcoRI 116-1036-S-4	

Figure 1 Identification and characterization of selected LAB strains found in São Jorge cheese using automated ribotyping.

Table 3 Enzyme profiling of 14 representative LAB isolates found in São Jorge cheese using the API-ZYM method

	Genus					
	Lactobacillus			*Enterococcus*		
	Per cent of strains giving indicated reaction*			Per cent of strains giving indicated reaction*		
Enzyme	–	+	++	–	+	++
Alkaline phosphatase	100			100		
Esterase	100			100		
Lipase	100				25	75
Leucine aminopeptidase	12	88				100
Cystine aminopeptidase	100				100	
α-Chymotrypsin	100				100	
Acid-phosphatase	88	24		15	75	
Phosphoamidase		100			100	
β-Galactosidase		100			50	50
β-Glucuronidase		100			25	75
α-Glucosidase	100			100		
β-Glucosaminidase	100			100		

*API-ZYM color: grade 0, negative (–); grades 1 through 3, positive (+); grades 4 and 5, plus positive (++).

Lactobacillus spp. isolates Q3B and Q1A matched to the same ribogroup and thus represented the same ribotype, as well as isolates Q2B and Q3A-LB, respectively (all isolated from cheeses of two different dairies); therefore, a total of three *L. paracasei* types could be identified. The four *E. faecalis* isolates were classified into three ribogroups; however, visual inspection indicated that ribogroups 116-366-S-5 and 116-366-S-1 were very similar – so the three isolates (M1B, Q3A-EC and Q2A-EC) were considered to represent the same ribotype.

Enzyme profiling

The enzymatic profiles of ten *Lactobacillus* and four *Enterococcus* spp., selected at random from the aforementioned 14 isolates, are depicted in Table 3. Lipase activity was detected only in the *Enterococcus* isolates – which also showed distinctly higher leucine-aminopeptidase, β-galactosidase and β-glucuronidase activities than their *Lactobacillus* counterparts.

Discussion

Our experimental results indicate clearly that lactobacilli and enterococci are the dominant bacteria in the production chain of São Jorge cheese characterized here. Among lactobacilli, two species appeared as dominant: *L. paracasei* and *L. rhamnosus*; on the contrary, *E. faecalis* and *E. faecium* predominated among enterococci. This observation is in agreement with previous studies, which reported high

viable counts of *Enterococcus* and *Lactobacillus* spp. in artisanal AOP cheeses manufactured from raw milk (Centeno *et al.* 1996; Macedo *et al.* 2000; Garcia *et al.* 2002; Marino *et al.* 2003; Jurkovic *et al.* 2006). Furthermore, our results pertaining to the evolution patterns of the dominant microbial population (i.e. lowering of *Enterococcus* viable counts with ripening time and eventual dominance of *Lactobacillus* by the end of maturation) agree with data reported elsewhere (Zárate *et al.* 1997; Poznanski *et al.* 2004; Aquilanti *et al.* 2006). Mannu and Paba (2002) also claimed that, in Pecorino Sardo raw ewe's milk cheese, the strains that dominate in the first stage of ripening do not necessarily dominate later in ripening. We found that among lactobacilli, *L. rhamnosus* was more commonly detected in the early to mid phases, while *L. paracasei* was present throughout the whole ripening period of São Jorge cheese.

In general, each factory has its own in-house contaminants (de Angelis *et al.* 2001); however, no major difference was found among the distribution patterns of the various microbial groups in the (representative) dairy farms considered (results not shown). This may be explained (at least partially) by the habit of sharing whey starters among dairy farmers, or because few isolates were ribotyped so a different situation could have been observed if more isolates had been considered.

Milk acidification, a consequence of lactose metabolism brought about by LAB, is a critical step in cheese manufacturing; this metabolic event requires presence of β-galactosidase to initially break down the major milk disaccharide (viz. lactose). Considerable β-galactosidase activity would accordingly be expected in both *Lactobacillus* and *Enterococcus* spp.; however, this was not the case, as very poor activity thereof was detected, especially in *Lactobacillus* isolates. Consequently, initial acidification in São Jorge cheese curd is likely the result of contributions by other micro-organisms which could not be isolated via our culturing methods (Suzzi *et al.* 2000). On the contrary, *E. faecalis* has been claimed to exhibit substantial esterase activity – which is relevant towards lipolysed aroma development (Oterholm *et al.* 1968; Dupuis *et al.* 1993; Tsakalidou *et al.* 1993; Sarantinopoulos *et al.* 2001; Moreno *et al.* 2006). The high activity of leucine-aminopeptidase, an enzyme involved in metabolism of that amino acid exhibited by *Enterococcus* spp., may also be important in strengthening of cheese taste. The aforementioned enzymatic activities, which convey to the *Enterococcus* genus a probable role in cheese flavour development, have as well been reported by other authors (Gardiner *et al.* 1999; Gelsomino *et al.* 2001) as contributing to the specific flavour profiles of several varieties of regional cheeses.

The isolates, which were previously identified via API 50 CHL as good to excellent, were well discriminated to the strain level by ribotyping. However, identification results via API of most *L. rhamnosus* were not reliable. This apparent difficulty in resolving closely related species, e.g. species such as *L. paracasei* and *L. rhamnosus* by biochemical methods, has also been reported elsewhere (Tynkkynen *et al.* 1999; Aquilanti *et al.* 2006). In these cases, the only reliable identification approach is to resort to genotypic methods. Technical constrains did not allow ribotyping of the *L. rhamnosus* isolated from São Jorge cheese. Mannu *et al.* (2000) considered that two molecular techniques may be necessary for a thorough and accurate typing in order to better distinguish between closely related isolates (as is the case of *L. paracasei* and *L. rhamnosus*) or isolates of the same clone lineage. The said approach will be applied in future work to type *L. rhamnosus* isolates. Confirmation and full characterization of these isolates may reveal strains exhibiting probiotic features (Alander *et al.* 1997; Hansen 1997; Abou-Dawood 2003), and this would be an interesting finding, in health terms, for São Jorge cheese. Finally, two ribotypes of *E. faecalis* were differentiated among the four *E. faecalis* isolates characterized.

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