

Identification and characterization of autochthonous lactic acid bacteria from Pico cheese with potential as starter cultures

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ABSTRACT

Pico Cheese is a traditional, artisanal product of the Azores that risks extinction. Its natural microflora holds promise as a source of lactic acid bacteria (LAB) for starter culture development. The aim of the present work was to identify and characterize previously isolated LAB from Pico Cheese, having in mind their potential utilization as starter cultures.

Three LAB isolates (L1C1R5, LIC1E3, and L1C1E6) were identified by sequencing the 16S rRNA gene and their relevant physiological properties were investigated. Safety-related phenotypic properties (hemolysin, gelatinase, thermonuclease, histidine decarboxylase, and resistance/sensitivity to 22 antibiotics distributed among the sulfamide, quinolone, β -lactam, aminoglycoside, lincosamide, phenicol, glycopeptide, macrolide, ansamycin and tetracycline families) and technological characteristics (acidification rates, caseinolysis, lipolysis, diacetyl production and enzymatic activities) were studied.

The isolates were identified as *Lactobacillus paracasei* (L1B1E3), *Leuconostoc pseudomesenteroides* (L1C1E6), and *Enterococcus faecalis* (L1C1R5). None of the isolates displayed hemolysin, gelatinase, thermonuclease or histidine decarboxylase activity. Isolates L1C1E6 and L1B1E3 were resistant against a low number (6 – 8) of the tested antibiotics, but L1C1R5 (an enterococcus) showed phenotypic resistance to 15 of the tested antibiotics. L1B1E3 and L1C1R5 showed fast acidification (pH 3.8 after 72h). L1C1R5 and L1C1E6 showed caseinolytic activity and were citrate+. L1B1E3 and L1C1E6 showed α -chymotrypsin and peptidase activities. Esterase activities were present in isolates L1B1E3 and L1C1R5, although lipolytic activity could not be detected when tributyrin was used as a model lipid. The studied isolates displayed physiological properties that may contribute positively to cheese aroma and no isolate displayed any of the tested virulence factors. Antibiotic resistance should be further investigated, especially in the case of *E. faecalis* L1C1R5 and *Lc. pseudomesenteroides* L1C1E6, since these species do not have QPS status.

1. INTRODUCTION

Pico Cheese is an artisanal product of Pico Island (Azores, Portugal) that is made from raw cow milk. It has a very low cylindrical shape, a distinct, mild flavour and a very short maturation time (21 days). It obtained Protected Denomination of Origin Status in 1996. At that time, more than a dozen cheesemaking units were active throughout the island and their product was sold all over the Azores and in mainland Portugal. Presently, Pico Cheese risks extinction, with only three artisanal units still working. The bacterial consortium that contributes to Pico Cheese's unique characteristics is still little studied [1, 2]. Enterococci, lactobacilli and leuconostcs are part of the Pico Cheese lactic microbiota [2] and these autochthonous lactic acid bacteria (LAB) could be used to improve the cheese safety and quality if added as starter/adjunct cultures.

Hoier *et al.* [3] presented a list of criteria for the screening of LAB for cheesemaking. These criteria encompass isolate identity, safety-related characteristics as well as technologically-relevant properties. The aim of the present work was to identify and characterize previously isolated LAB from Pico Cheese, having in mind their potential utilization as starter cultures, by establishing their identity, studying the production of pathogenicity markers, antibiotic sensitivity/resistance patterns, acidification capacity, caseinolysis, lipolysis, diacetyl production and relevant enzymatic activities.

2. MATERIALS AND METHODS

2.1. Isolates

Three Gram-positive, catalase-negative, oxidase-negative isolates obtained from Pico cheese in previous works [1] L1B1E3, L1C1E6 and L1C1R5 were used in this study.

2.2. 16S rRNA Gene Sequencing for Bacterial Identification

DNA was extracted with UltraClean® Microbial DNA Isolation Kit (MoBio, Carlsbad, CA) and amplified with universal bacterial primers 46F and 1409R. Sequences were edited using the software Sequencher 4.8 (Gene Codes, Ann Arbor, Michigan). Alignment was accomplished with Greengenes [3] and manually corrected with the BioEdit editor (www.mbio.ncsu.edu/BioEdit/bioedit.html), guided by 16S rRNA secondary structure considerations. The BLAST program [4] was employed for the similarity search. Sequence Identity Matrix values were calculated with the BioEdit editor.

2.3. Safety-related properties

Hemolysin, gelatinase, thermonuclease and histidine decarboxylase production were assessed in the appropriate culture media [5–8]. Resistance/sensitivity to chloramphenicol, nalidixic acid, ofloxacin, amoxicillin/clavulanic acid (2:1), carbenicillin, penicillin G, piperacillin, kanamycin, streptomycin, gentamycin, netilmycin, tobramycin, cephalothin, cefotaxime, ceftriaxone, ceftazidime, clindamycin, erythromycin, rifampicin, sulfamethoxazole /trimethoprim, tetracycline and vancomycin was evaluated by the Kirby-Bauer test [9].

2.4. Technological characteristics

Acidification in MRS medium (in triplicate), caseinolysis in Skim Milk Agar [10], lipolysis in Tributyrin Agar [11], diacetyl production in milk by the Voges-Proskauer test [10], and the API Zym enzymatic activity profile (alkaline phosphatase, C4 esterase, C8 esterase-lipase, C14 lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naftol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, and α -fucosidase) were determined for the three isolates under study.

3. RESULTS AND DISCUSSION

3.1 Identity of the isolates

The phylogenetically closest neighbours identified by BLAST of the isolates L1B1E3, L1C1E6 and L1C1R5 belong to the phylum of Firmicutes, *Lactobacillus paracasei*, *Leuconostoc pseudomesenteroides* and *Enterococcus faecalis*, respectively. The sequence similarity values were higher than 98% for all the sequences.

3.2 Safety-related properties

None of the 3 isolates displayed any of the pathogenicity markers under study. None of the studied isolates was resistant to chloramphenicol, amoxicillin/clavulanic acid, penicillin G, piperacillin, carbenicillin, erythromycin, cephalothin, rifampicin, and tetracycline (data not shown). Resistance patterns to other antibiotics are shown in Table 1. A high proportion of resistance to the 22 antibiotics tested was observed among the isolates studied. However, the used methodology does not allow to distinguish between innate and acquired resistance. Further studies are, thus, needed in order to assess the potential for horizontal transfer of the antibiotic resistance genes in these isolates before contemplating their use for cheesemaking.

Table 1. Antibiotic resistance patterns in three LAB isolates obtained from Pico cheese.
S=sensitive; R=resistant; I=intermediate

Families	Antibiotics	<i>Lb. paracasei</i> (L1B1E3)	<i>Lc. pseudomesenteroides</i> (L1C1E6)	<i>E. faecalis</i> (L1C1R5)
Quinolones	Nalidixic acid	R	R	R
	Ofloxacin	I	R	R
Aminoglycosides	Kanamycin	R	I	R
	Netilmycin	R	S	R
	Gentamycin	R	S	R
	Streptomycin	R	S	R
	Tobramycin	R	S	R
Cephalosporins	Cefotaxime	S	S	R
	Ceftazidime	R	R	R
	Ceftriaxone	S	R	R
Lincosamides	Clindamycin	R	S	R
Glycopeptides	Vancomycin	S	R	S
Sulfamides	Sulfamethoxazole/trimethoprim	S	R	S

3.3 Technological properties

The results are summarized in Table 2. None of the studied isolates was able to degrade tributyrin. *Lb. paracasei* was not caseinolytic and did not produce diacetyl. However, it

presented enzymatic activities that can be of interest in cheesemaking (esterases, proteases and peptidases) and acidified MRS medium to pH 3,8 within 72h. *Lc. pseudomesenteroides* L1C1E6 and *E. faecalis* L1C1R5 were both able to degrade casein and to produce diacetyl. The former acidified MRS medium to pH 3,8 within 72h, while the latter reached a pH of only 4,4 within the studied period of time. Peptidase and protease activities were present in *Lc. pseudomesenteroides* L1C1E6, while *E. faecalis* had esterase and phosphatase activity.

Table 2. Technological properties of three LAB isolates obtained from Pico cheese.

Properties	<i>Lb. paracasei</i> (L1B1E3)	<i>Lc. pseudomesenteroides</i> (L1C1E6)	<i>E. faecalis</i> (L1C1R5)	
Enzyme activity (nmole utilized substrate)	C4 Esterase	10	0	20
	C8 Esterase-lipase	20	0	20
	Leucine arylamidase	≥40	0	0
	Valine arylamidase	≥40	5	0
	Cystine arylamidase	5	5	0
	Trypsin	0	5	0
	α-Chymotrypsin	5	0	0
	Acid phosphatase	0	0	5
	Naftol-AS-BI-fosfohydrolase	0	0	10
	α-Glucosidase	30	0	0
	n-Acetyl-β-glucosaminidase	10	5	0
pH after 72 h	3,8	3,8	4,4	
Caseinolysis	-	+	+	
Lipolysis (tributyryn)	-	-	-	
Diacetyl production	-	+	+	

4. CONCLUSIONS

All studied isolates displayed interesting technological properties and lacked the studied pathogenicity markers. However, antibiotic resistance by these strains needs to be further studied and their potential for cheesemaking needs to be assessed in model cheese systems.

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