Short Communication

Characterization of *Leuconostoc* strains isolated from fresh anchovy (*Engraulis anchoita*)

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The biomass value of anchovy (*Engraulis anchoita*), the most abundant fish species in the Southwest Atlantic Ocean, is considered to be around 4,300,000 tons, with a maximum sustainable yield of 1,140,000 tons (Cabrer et al., 2002), being at present under exploited at 27,800 tons/year (SAGPyA, 2008). Recently, as part of the FAO's project Towards a Sustainable Aquaculture: Selected Issues and Guidelines, which addresses the use of wild fish to feed cultured fish and its implications to food security and poverty alleviation, a study on the use of small pelagic fish for new product development has been carried out (Madureira et al., 2007). Although this resource is shared by Brazil, Uruguay and Argentina in the so-called "anchoita Bonaerense" stock, anchovy is processed for human consumption only in Argentina, where a diversified production (whole frozen, salt-cured, marinated and tinned anchovy fillets) targets mainly the external market. *E. anchoita* is described as a species with high-lipid content which undergoes significant variations according to the time of the year (Yeannes and Casales, 1995). Due to lipid oxidation, anchovy is highly perishable; however high-polyunsaturated fatty acids (omega-3) content (Massa et al., 2007) makes ancho-

vy a healthy raw material; its dietary intake is in fact inversely associated with cardiovascular diseases in population studies (Cicero et al., 2009).

Although it has often been suggested that lactic acid bacteria (LAB) found on fish might be regarded as land-borne contaminants, species of certain genera are not uncommon in aquatic environments (Stiles and Holzapfel, 1997). Several studies have demonstrated that LAB have been found in marine and freshwater fish (mainly from the gastrointestinal tract and gills) and their surrounding environment (Bucio et al., 2006; González et al., 2000; Ringø et al., 2000, 2002; Seppola et al., 2006). Moreover, LAB have recently become a major source of concern in aquaculture due to their pathological significance (Michel et al., 1997). On the other hand, LAB growth on fish may be stimulated by different processes (drying, salting, marinating and vacuum-packaging) constituting the major part of the final product microbiota (Gancel et al., 1997; Lyhs et al., 2002; Thapa et al., 2006; Tomé et al., 2007). Since Argentina has a long tradition processing fresh raw anchovy for human consumption, a preliminary study was conducted to identify LAB in view to be used as a functional starter culture for anchovy-based product diversification.

Fresh anchovies (*E. anchoita*), caught between 34 and 36° Atlantic South latitude were provided by a fish processing factory from Mar del Plata, Argentina. For microbial analysis, samples from the 2005–2006 fish

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catch season were aseptically collected and transported to the laboratory in ice boxes. For isolation, three independent 10 g anchovy samples were stomached (400, Seward, UK) with 90 ml of saline solution (0.85% NaCl) for 2 min. Dilutions were plated on PCA and MRS agar (Britania, Argentina) and incubated (30°C, 48 h) and (25°C, 5 days) for total viable and LAB enumeration, respectively. From each sample, random colonies (>100) from MRS were picked and streaked again on fresh agar plates. Even though some strains were not able to be recovered, purified Gram positive, catalase negative isolates were stored at -80°C in MRS + glycerol (20%) for further characterization.

The analyzed fresh anchovies exhibited total viable counts of $5.87 \pm 0.19 \log \text{CFU g}^{-1}$ while LAB counts were in the range of 3.58 ± 0.92 to $4.43 \pm 1.67 \text{CFU g}^{-1}$. Raw anchovy's pH value was in the range of 6.42 ± 0.07 . The low bacterial counts observed in this study for fresh anchovy are in agreement with those reported by Pons-Sánchez-Cascado et al. (2005) and Fuselli et al. (1998); the high fish flesh pH (> 6.0) found being correlated with the low carbohydrate content and the low lactic acid produced post-mortem (Gram and Huss, 1996).

For genotypic characterization of bacterial isolates, total DNA was extracted using the guanidium thiocyanate method of Pitcher et al. (1989) modified by Björkroth and Korkeala (1996). Restriction endonuclease analysis (REA) with *Hind*III (New England Biolabs, Beverly, MA, USA), Southern transfer, hybridization and ribotyping were performed as previously described (Björkroth and Korkeala, 1996). The OligoMix5 probe used was described by Regnault et al. (1997). Genomic blots were performed using a vacuum device (Vacugene, Pharmacia, Uppsala, Sweden).

The ribopatterns were compared with the corresponding patterns in the LAB database at the Department of Food and Environmental Hygiene, University of Helsinki, Finland. The thirteen selected strains were analyzed by ribotyping and the resulting hybridization *Hind*III banding patterns were used to generate a dendrogram for cluster division and LAB species delineation (Fig. 1). The LAB analyzed were assembled in two clusters at a similarity level of 50% with ribotypes corresponding to *Leuc. mesenteroides* and *Leuc. carnosum* species. Cluster I contained nine isolates (SACB53/02a/07/01S/04a/09/21/02/03) with the ribotype of *Leuc. mesenteroides* type strains [*Leuc. mesenteroi-*

Fig. 1. Schematic of *Hind*III ribopatterns and their numerical analysis presented as a dendrogram.

Scale from 60 to 100 shows the percentile similarity. (*) SACB04a is duplicated. *Leuc. mesenteroides* subsp. *mesenteroides* LMG7939, DSM20343^T, *Leuc. mesenteroides* subsp. *dextranicum* LMG17954, LMG11318, and DSM20484^T, *Leuc. mesenteroides* subsp. *cremoris* CCUG21965^T and *Leuc. carnosum* NCFB2776^T were used as reference strains.

des subsp. *mesenteroides* (LMG7939, DSM20343^T), *Leuc. mesenteroides* subsp. *dextranicum* (LMG17954, LMG1318, and DSM20484^T) and *Leuc. mesenteroides* subsp. *cremoris* (CCUG21965^T); three of these isolates (SACB07/01S/04a) clustered with *Leuc. mesenteroides* type strains at a similarity of 100%, while six isolates (SACB53/09/21/02a/02/03) merged at a similarity level of 90–95%. Cluster II contained four isolates (SACB01/04/05/01a) with a similarity of 100% with the ribotype of *Leuc. carnosum* type strain (NCFB2776^T). Strain differentiation based on biochemical and physiological traits (Björkroth and Holzapfel, 2006; Elliot and Facklam, 1993) confirmed the genotypic characterization. Although dextran production was a variable phenotype, strains SACB01S/02/03/07/21 and 53 may be classified as *Leuc. mesenteroides* subsp. *mesenteroides* (Table 1). On the other hand, SACB02a/04a and 09 strains exhibited a pattern corresponding to *Leuc. mesenteroides* subsp. *dextranicum* while strains SACB01/01a/04 and 05, with variable sugar fermentation patterns, were classified as *Leuc. carnosum*.

A considerable polymorphism among *Leuconostoc* strains was observed when RAPD-PCR analysis was conducted using a set of 4 primers [M13 (Huey and Hall, 1989); RAPD1, RAPD2 (Cocconcelli et al., 1995) and XD9 (Moschetti et al., 1998)]. Reproducible M13- and RAPD1-fingerprinting results were obtained in duplicated PCR reactions and using DNA prepared from separated cultures of the same strain; the fingerprints showed differences in the number of bands, fragment size and intensity. In contrast, low amplification effectiveness was observed with primers RAPD2 and XD9 for *Leuc. mesenteroides* strains and absolutely no amplification was obtained with primer RAPD2 for *Leuc. carnosum* strains (data not shown). Figure 2 shows M13 and RAPD1 profiles. Eleven profiles were determined with primer RAPD1 (7 of them for *Leuc. mesenteroides*, including the type strains for the subspecies *mesenteroides* and *dextranicum*, and 4 for *Leuc. carnosum*), while 7 different banding patterns were observed with primer M13 (5 for *Leuc. mesenteroides* and 2 for *Leuc. carnosum*). The M13- and RAPD1-

Table 1. Biochemical, physiological, M13- and RAPD1-RAPD profiles of isolated strains.

	^ψ Acid from			Production of		Growth with	RAPD profiles	
	Arabinose	Fructose	Trehalose	^φ Dextran	^ω CO ₂	6.5% NaCl	M13	RAPD1
<i>Leuc. mesenteroides</i> [*]								
subsp. <i>mesenteroides</i> ^a	+	+	+	+	±	+	G	k
subsp. <i>dextranicum</i> ^b	–	+	+	+	±	–	H	l
subsp. <i>cremoris</i> ^c	–	–	–	–	–	–	I	j
SACB01S	+	+	+	+	+	+	E	e
SACB02	+	+	+	–	+	+	B	a
SACB03	+	+	+	–	+	+	B	a
SACB07	+	+	+	–	+	+	B	b
SACB21	+	+	+	+	+	+	C	e
SACB53	+	+	+	+	+	+	B	c
SACB02a	–	+	+	+	+	+	E	d
SACB04a	–	+	+	+	–	+	C	d
SACB09	–	+	+	+	+	+	C	d
<i>Leuc. carnosum</i> [*]	–	+	+	+	+	ND	ND	ND
SACB01	+	+	+	+	+	+	A	f
SACB01a	+	+	+	+	+	+	D	g
SACB04	–	+	–	+	+	+	D	h
SACB05	–	+	+	+	+	+	D	i

^{*}Phenotype of type strains according to Björkroth and Holzapfel (2006) and Elliott and Facklam (1993); type strains from CERE-LA culture collection ^aCRL742, ^bCRL982 and ^cCRL983; ND: not determined. ^ψMRS-phenol red (0.0018%) broth free of glucose supplemented with each sugar (2%), ^φMcCleskey et al. (1947); ^ωGibson and Abd-El-Malek (1945).

Fig. 2. RAPD profiles of *Leuconostoc* strains isolated from anchovy obtained with (A) M13 primer: Lane 1: 100-bp ladder; lanes 3–9, 13 and 14: *Leuc. mesenteroides*; lanes 2 and 10–12: *Leuc. carnosum*; lanes 15: *Leuc. gelidum* CRL542; lane 16: *Leuc. mesenteroides* subsp. *mesenteroides* CRL742; lane 17: *Leuc. mesenteroides* subsp. *dextranicum* CRL982 and lane 18: *Leuc. mesenteroides* subsp. *cremoris* CRL983 and (B) RAPD1 primer: Lane 1: 100-bp ladder; lanes 2–10: *Leuc. mesenteroides*; lanes 11–14: *Leuc. carnosum*.

Letters at the bottom indicate the different profiles obtained.

banding patterns of the isolates showed no similarities to reference strain band profiles (Fig. 2). Moreover, identical phenotypes and RAPD profiles (M13-B and RAPD1-a) were observed for SACB02 and SACB03 strains suggesting the same strain could have been isolated twice.

Among the *Leuconostoc* commonly associated with fish and fish products, *Leuc. mesenteroides* was early

identified by traditional methods from seafood (Mauguin and Novel, 1994) and specifically, *Leuc. mesenteroides* subsp. *mesenteroides* from the intestinal content of salmonids using PCR amplification of 16S rRNA (Balcázar et al., 2007). Here, a significant genetic heterogeneity within the *Leuc. mesenteroides* and *Leuc. carnosum* isolated strains was observed using primers RAPD1 and M13 (Fig. 2; Table 1). Indeed, three different RAPD1 profiles (g, h and i) for *Leuc. carnosum* isolates with the M13-D profile were detected. In addition, M13 profiles C and E were common to both *Leuc. mesenteroides* subspecies; in contrast, RAPD1-d profile was specific for *Leuc. mesenteroides* subsp. *dextranicum*. A lack of fit to reference type strains for *Leuc. mesenteroides* biotypes discriminated by phenotypic characterized and RAPD fingerprinting was observed; this may be explained by their origin (CRL742 from olives; CRL982 and CRL983 from dairy products). Intraspecific diversity for *Leuc. mesenteroides* isolated from vacuum-packaged meat products and traditional cheeses has also been observed using different molecular typing approaches (Aznar and Chenoll, 2006; Cibik et al., 2000; Pérez et al., 2002; Villani et al., 1997), RAPD-PCR allowing reproducible and rapid differentiation within *Leuconostoc* species complementing preliminary identification by other molecular methods. The technological and safety characteristics of isolated strains showed that 6 (SACB01S/07/21/53/04a/09) out of 9 *Leuc. mesenteroides* strains were able to grow in the presence of 10% NaCl. In addition, positive amplification of a ~907 bp fragment of the *gdh* gene was shown for *Leuc. mesenteroides* subsp. *dextranicum* (all strains), *Leuc. carnosum* (SACB01/01a) and *Leuc. mesenteroides* subsp. *mesenteroides* (SACB03) (data not shown), suggesting that the generated amino acid from fish protein would be transformed into aroma compounds by these strains, as reported by Fernández de Palencia et al. (2006). Regarding safety features, an anti-*Listeria* compound was detected for SACB21 strain while 11 out of 13 *Leuconostoc* strains were unable to decarboxylate tyrosine and histidine (data not shown). The different *Leuconostoc* genotypes found in this study provide diversity of metabolic activities which are of great interest to enhance the quality of the final product, offering safety, health, marketing and technological advantages. Although the presence of *Leuconostoc* in marine and freshwater fish has been reported, as far as we know this study provides the first

evidence of its presence in fresh anchovy, some of these strains exhibiting traits that may play a role in the development of new anchovy-based products.

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