IDENTIFICATION OF LACTIC ACID BACTERIA IN "MORCILLA DE BURGOS" BY RIBOTYPING

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Background

Lactic acid bacteria (LAB) comprise the major component of the spoilage flora in vacuum and modified atmosphere packaged blood sausage "Morcilla de Burgos" (Santos, 2001). In a previously work the LAB flora from this product was identified by means of phenotypic and biochemical characteristics (Santos et al., 2001). According to that study *W. viridescens*, *Leuconostoc mesenteroides*, *Leuc. carnosum* and *W. fructosus* were the predominant species in this kind of product. Although classical approach to bacterial identification based on morphological, physiological and biochemical features provided reasonable results these phenotypic properties did not provide sufficient basis for the reliable identification of some LAB. Genotypic methods have a higher discriminatory power and in this sense the efforts of the current bacterial taxonomy are oriented to a polyphasic approach which involves phenotypic and genotypic characterisation (Vandamme et al., 1996). Ribotyping technique which combines Southern hybridisation of chromosomal DNA fingerprints with the uses of *Escherichia coli* rRNA probes is a powerful tool in the classification of LAB (Björkroth and Korkeala 1997, Björkroth et al., 1998; Lyhs et al., 2000; Björkroth et al., 2000).

Objetives

The aim of this work was to identify by ribotyping the LAB strains isolated from "morcilla" produced in Burgos region previously phenotypically characterized. Additionally, results of both phenotypic and genotypic identification methods were analysed.

Methods

Origin of the strains: A total of 174 LAB, previously characterised by phenotypic methods (Santos et al., 2001), were included in this study. The isolates originated from unspoiled and spoiled "morcilla" aerobically, vacuum and modified atmosphere packaged.

Ribotyping: *Hin*dIII restriction enzyme (New England Biolabs, Beverly, Mass.) was used for ribotyping. DNA was isolated by the guanidium thiocyanate method of Pitcher et al. (1989) as modified by Björkroth and Korkeala (1996a) by the combined lysozyme and mutanolysin (Sigma; St. Louis, Missouri, USA) treatment. Restriction endonuclease treatment of 3 µg of DNA was done as specified by the manufacturer (New England Biolabs, USA) and REA as described before (Björkroth and Korkeala, 1996a). Before southern blotting, REA patterns were inspected visually in order to obtain preliminary information of the clonal variation. Genomic blots were made using a vacuum device (Vacugene, Pharmacia, Uppsala, Sweeden) and rDNA probe for ribotyping was labelled by reverse transcription (AMV-RT, Promega, Madison, Wisconsin and Dig DNA Labelling Kit, Roche Molecular Biochemicals, Mannheim, Germany) as previously described by Blumberg et al. (1991). Membranes were hybridised at 68ºC as described by Björkroth and Korkeala (1996a).

Pattern analysis: The *Hin*dIII ribopatterns were compared with the corresponding patterns in the previously established LAB database of the Department of Food and Enviromental Hygiene, University of Helsinki, Finland. These comprise patterns of all relevant spoilage LAB in the genera of *Carnobacteria*, *Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Weissella* (Björkroth and Korkeala, 1996b, Björkroth and Korkeala 1997, Björkroth et al., 1998, Lyhs et al., 2000, Björkroth et al., 2000). For numerical analysis, ribopatterns were scanned using a Hewlett Packard (Boise, Idaho, USA) ScanJet 4c/T scanner and analysed using the GelCompar II software package (Applied Maths, Kortrijk, Belgium). The similarity between all pairs was expressed by Dice coefficient correlation and UPGMA (unweighed pair group method using arithmetic averages) clustering was used for the construction of the dendrogram.

Results and discussion

Using *Hin*dIII fifteen clusters were defined at a similarity level of 70%. Reference strains were found in all clusters except in Cluster VII where not reference strain pattern was found. Cluster VI was the biggest one with 75 isolates (42.6%) and the type strain *Weissella viridescens* ATCC 12706^T. Table 1 shows the identification results of the strains by phenotypic characteristics and ribotyping. According to the results most of the strains included in the species *W. viridescens*, *Leuc. mesenteroides* and *Leuc. carnosum*, all the strains of *L. sakei* and *L. curvatus*, and half of the strains *W. confusa* were correctly classified by both methods (around 70% of the total isolates). However ribotyping identification do not consider the presence of *L. fructosus*, *L. sanfrancisco* and *Carnobacterium piscicola* and establish the presence of other species like *W. cibaria*, species from *Lactococcus* genus and also a group of bacteria which has not been identified. Many authors have reported the difficulty of identification of leuconostocs by phenotypic means due to the great heterogeneity in biochemical and physiological characteristics (Milliere et al., 1989; Shaw and Harding, 1989; Björkroth et al., 1998). However in this case most leuconostocs were correctly classified by phenotypic characteristics. Ribotyping revealed also the presence of species like *Leuc. pseudomesenteroides*, *Leuc. lactis*, *Leuc. carnosum*, *Leuc. citreum* and *Leuc. gasicomitatum*. The latest species has been recently described by Björkroth et al. (2000) in spoiled tomato-marinated raw broiler meat strips.

Although isolates from species *Leuc. mesenteroides* were not assigned to any subspecies, these bacteria resembled more *Leuc. mesenteroides* subsp. *mesenteroides* and *Leuc. mesenteroides* subsp. *dextranicum* than *Leuc. mesenteroides* subsp. *cremoris* since our isolates were dextran positive and fermented other sugars than galactose. These facts were confirmed by ribotyping analyses. The group phenotypically classified as *W. confusa* really comprised two species (*W. confusa* and *W. cibaria*) according to ribotyping. These *W. cibaria* isolates unlike strains of *W. confusa* presented a weak fermentation of xylose and did not ferment ribose. Species belonging *Lactococcus* clusters according to ribotyping results had been phenotypically misidentified as *Carnobacterium piscicola* due to the fact that they presented rod shape at the microscopy. Different works have reported that electronic microscopy offer better results in the determination of the shape of this genus than phase contrast microscopy (Mauguin and Novel, 1994). *L. fructosus* and *L. sanfrancisco* species are quite similar to *W. viridescens* according to Schillinger and Lücke scheme (1987) and are rarely found in meat and meat products which is confirmed by ribotyping (Table 1). Cluster VII comprised isolates with irregular shape belonging to *L. fructosus*, *L. sanfrancisco* and *W. viridescens* species according to phenotypic characteristics. This group of LAB could be a new variant of *W. viridescens* species or a different species but more information like DNA homology studies and whole-cell protein analysis is necessary in order to confirm the identity of these strains.

Conclusion

Ribotyping has been probed an excellent tool for the identification of LAB isolated from "Morcilla de Burgos", especially for those LAB previously assigned to different species according to the phenotypic characteristics.

Only the identity of the seventeen strains of Cluster VII remains uncertain, due to they do not fit well with any of the reference strains tested and more studies are necessary to get the complete identification of this group.

Pertinent literature

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Dates in the form of tables:

Table 1. Phenotypic and genotypic identification of LAB isolates from "morcilla de Burgos".

No. of strains	Phenotypic identification	No. of strains	Ribotyping	Code[a]
74	W. viridescens	68	W. viridescens	C
		4	Cluster VII	I
		1	*Leuc.*	I
		1	NT[b]	
20	*Leuc. Mesenteroides*	19	*Leuc.*	C
		1	*Leuc.*	U
17	*Leuc. Carnosum*	15	*Leuc.*	C
		2	NT	
5	*Leuconostoc sp.*	2	*Leuc. lactis*	U
		1	*Leuc.*	U
		1	*Leuc. citreum*	U
		1	NT	
20	*W. confusa*	11	*W. confusa*	C
		9	*W. cibaria*	U
10	*L. fructosus*	10	Cluster VII	I
7	*L. sanfrancisco*	5	*W. viridescens*	I
		2	Cluster VII	I
1	*Pediococcus*	1	*Pediococcus*	U
5	*L. sakei*	5	*L. sakei*	C
1	*L. curvatus*	1	*L. curvatus*	C
5	*Carnob.piscicola*	2	*Lactococcus*	I
		1	*Lactococcus*	I
		2	*Pediococcus*	I
11	*Lactobacillus sp.*	5	*W.confusa*	I
		2	*W. viridescens*	I
		1	*W. cibaria*	I
		1	*Pediococcus*	I
		1	*Leuc.*	I
		1	Cluster VII	I

[a]: C: species correctly identified by phenotypic methods; U: genus correctly determined by phenotypic methods; I: species incorrectly identified by phenotypic methods.

[b]: NT: not tested by ribotyping.