Characterization and identification of lactic acid bacteria from freshwater fishes

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This work reports on the characterization and identification of 249 strains of lactic acid bacteria (237 rods and 12 cocci) isolated from freshwater fish (mainly wild brown trout) and their surrounding environments. Approximately 90% (226 strains), not able to grow on Acetate (Rogosa) agar, exhibited a high degree of phenotypic homogeneity and were assigned to the genus Carnobacterium. Only a reduced number of strains (22) were allocated to the genera Lactobacillus, Enterococcus, Lactococcus or Vagococcus. One strain remained unidentified at genus level. Most Carnobacteria were identified as C. piscicola (156), whereas the species C. divergens was represented only by 10 strains. A total of 59 carnobacteria remained unidentified at species level. After a numerical taxonomy classification based on phenotypic features, at a similarity level of 86% ($\text{SSM}$), and with an average linkage algorithm (UPGMA) of clustering, 13 clusters were formed, seven of which contained three or more strains (regarded as significant clusters). Clusters I and II were labelled as Enterococcus durans and Lactobacillus spp, respectively. Clusters III to VI were assigned to C. piscicola, whereas cluster VII was ascribed to the species C. divergens. An accurate discrimination among Carnobacteria and Lactobacillus was achieved through the use of numerical taxonomy, although the separation between Carnobacterium species was not so clearly defined. Phenotypically, the distinction between both species relied only on a reduced number of tests (growth at 40°C and mannitol fermentation). The ability of Carnobacteria to degrade proteinaceous and lipidic compounds was scarce.

Introduction

The lactic acid bacteria (LAB) are a group of Gram-positive rod- and coccus-shaped organisms that have less than 55% mol G+C content in their DNA. They are non-sporeforming, non-motile and produce lactic acid as their major end product during the fermentation of carbohydrates. The taxonomy of LAB has changed considerably during the last few years and, at present, this group comprises the following genera: Aerococcus, Alloicoccus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tétragrenococcus, Vagococcus and Weissella (Stiles and Holzapfel 1997, Axellson 1998). Since many of these organisms are typically involved in a large number of food fermentations and play also an important role as spoilage microflora, a great deal of information is available on the characterization and identification of food isolates (Salminen and
von Wright 1998). By contrast, taxonomic studies on LAB populations obtained from poikilotherm animals are rare. Although LAB are not considered to belong to aquatic environments, species of certain genera (i.e. *Carnobacterium* or *Vagococcus*) are not uncommon in freshwater fish and river water (Stiles and Holzapfel 1997). Furthermore, some members of these genera are isolated from diseased fish (Hii et al. 1984, Austin and McIntosh 1991, Baya et al. 1991, Hammes et al. 1992).

This article reports a detailed study of the phenotypic characteristics of a LAB population obtained from freshwater fish and their habitats, also including their identification by classical schemes and numerical taxonomy.

**Materials and Methods**

**Bacterial strains**

A total of 249 Gram-positive, oxidase- and catalase-negative strains (237 rods and 12 cocci) were examined. All strains were selected from Tryptone Soya agar (TSA, Oxoid, Basingstoke, UK) plates incubated at 30°C (171 strains) and 7°C (78 strains). Of these 202 were obtained from the skin, gills and intestines of wild specimens of brown trout and their surrounding habitats, 30 from farmed specimens of rainbow trout (skin, gills and water) and 17 from wild specimens of pike (gills and water). Inocula of strains were grown in BM broth (Wilkinson and Jones, 1977), which is a modified MRS medium, with sodium acetate and ammonium citrate omitted, for 36 h at 28°C. Control plates of BM agar were used to ensure viability and purity of inocula.

The following reference strains were also included in this study: *Carnobacterium divergens* (DSM 20623), *Carnobacterium piscicola* (ATCC 35586), *Lactobacillus fermentum* (ATCC 9328), *Lactobacillus sakei* subsp. *sakei* (DSM 20017), *Lactobacillus plantarum* (ATCC 8014), *Lactobacillus curvatus* subsp. *curvatus* (DSM 20019), *Enterococcus faecium* (ATCC 14432) and *Lactococcus lactis* subsp. *lactis* (ATCC 11007).

**Characterization tests for all isolates**

Except where otherwise stated, strains were cultured at 28°C for 48 h in BM broth and/or BM agar as basal media. The following tests were performed as previously described (Prieto et al. 1992, García-Armesto et al. 1993, Hii et al. 1984): Gram reaction, motility in GI medium (Difco, Detroit, Michigan, USA), morphological and cultural characteristics (BM agar and BM broth), growth on Acetate agar (Rogosa agar, Oxoid), growth at 4, 8 and 45°C (10 d), growth at 37, 40 and 42°C (48 h), salt tolerance (4, 7 and 10% NaCl), O/F test, acid production from carbohydrates (1% w/v, L-arabinose, amygdalin, cellobiose, D-fructose, D-galactose, gluconate, inulin, lactose, maltose, mannitol, D-mannose, melezitose, melibiose, raffinose, rhamnose, D-ribose, salicin, sucrose, sorbitol, L-sorbos, trehalose, D-xylose and xylitol) in BM broth (glucose and meat extract free) with chlorophenol red (Sigma, St. Louis, Missouri, USA) as indicator, aesculin hydrolysis in aesculin broth, Voges-Proskauer test in MRVP medium (Oxoid), production of dextran from glucose, arginine hydrolysis, H₂S production from sodium thiosulphate (in TSI-Oxoid-slants) and ß-haemolysis on sheep red blood cells (González-Serrano 1996). The hydrolysis of Tweens 20 and 80, egg yolk, tributyrin, casein, gelatin and starch was investigated on TSA (Oxoid) plates according to Prieto et al. (1992).

**Further discriminant tests and classical identification**

Identification of the 237 rods at the species level was done according to several authors (Collins et al. 1987, Schillinger and Lücke 1987, Hii et al. 1984, Hammes et al. 1992). To achieve a better discrimination of these bacteria from related genera (i.e. *Erysipelothrix, Listeria*), two additional tests were performed: H₂S production from sodium thiosulphate (in TSI-Oxoid-slants) and ß-haemolysis on sheep red blood cells (González-Serrano 1996).

Identification of the 12 cocci at the species level was performed by using the schemes of Schleifer and Kilpper-Bälz (1987), Collins
et al. (1989), Wallbanks et al. (1990), Williams et al. (1990), Hammes et al. (1992) and Axelsson (1998). Additional tests assayed were: ability to grow at 10°C, salt tolerance (6-5%), growth at pH 9-6, CO₂ from glucose, acid production from tagatose (Prieto et al., 1992), haemolytic activity on sheep red blood cells (González-Serrano, 1996), tolerance to 40% bile salts (Jayne-Williams, 1976), and starch and hippurate hydrolysis (Mölín and Ternström, 1982).

Numerical analysis

All tests were repeated on 10% of the strains. The average probability of error (P) was calculated according to Sneath and Johnson (1972). Four characteristics [acid production from cellobiose (all positive), gas from glucose in BM agar, and acid production from L-sorbose and xylitol (all negative)] were identical for all strains and therefore were omitted from the numerical analysis. The remaining 45 characters (those cited in Table 1 as well as cell morphology, O/F test, and acid production from trehalose) were scored as negative (0), positive (1) or doubtful (2). The simple matching coefficient (S_{SM}) of Sokal and Michener (1958) was used and clustering was achieved by unweighted pair group average linkage (Sneath and Sokal, 1973). The software employed was SPSS.PC+V3.1 (SPSS Inc., Chicago, Illinois, USA) for clustering and BASIC computer programs developed by Prieto (1990) for the calculation of the similarity coefficients among strains.

The cophenetic correlation coefficient r_{cs} (Sneath and Sokal, 1973), a measure of agreement between the similarity values derived from a phenogram and those of the original similarity matrix, was calculated with the NTSYS-PC V1.80 program (Exeter Software, New York, USA). Character frequency tables generated by using the LOTUS 1, 2, 3 V2.0 program (Lotus Development Corporation, Texas, USA), were used to determine the most discriminatory characters.

Results

Since 226 rods were acetate sensitive and did not grow in presence of 8% NaCl nor at 45°C, they were assigned to the genus *Carnobacterium*. Using diagnostic tables (Collins et al. 1987, Schilling and Lücke, 1987, Hiu et al. 1984, Hammes et al. 1992), 156 of them were identified as *C. piscicola*, 10 as *C. divergens* and one as *C. mobile*. The remaining 59 carnobacteria, which were Voges-Proskauer-negative, could not be identified to species level. Another 11 rods matched the description of the genus *Lactobacillus*, the species found being: *L. plantarum* (two isolates), *L. sakei* subsp. *sakei*, *L. sharpeae*, *L. mali*, *L. curvatus* subsp. *curvatus* (one isolate from each species) and unidentified (five isolates). Among cocci, eight strains belonged to the genus *Enterococcus* (*E. durans*, six isolates, and *Enterococcus* spp., two isolates), two belonged to the genus *Vagococcus* (*V. fluvialis*), one belonged to the genus *Lactococcus* and one remained unidentified.

In the numerical analysis, the cophenetic correlation coefficient was 0.879 and the average probability of an erroneous result (P) was 2.6%, which would not produce a significant distortion of the taxonomic structure. Defined at a similarity level (S_{SM}) of 86%, 13 clusters were formed (Fig. 1). Seven of them (I–VII) were regarded as significant clusters (three or more strains) and grouped 236 strains (231 environmental and five reference strains), and six were labelled as small clusters (each one containing two strains). From the nine unclustered strains, two were reference strains and seven were from fish samples. In Table 1, the characters useful for the distinction among significant clusters are shown.

Cluster I represented *E. durans*. It was composed of six Gram-positive, non-saccharolytic, cocci, that produced arginine dihydrolase and grow at 45°C, but not at 4°C.

Cluster II contained three *Lactobacillus* reference strains: *L. fermentum*, *L. sakei* subsp. *sakei* and *L. plantarum*. All three could grow in Rogosa agar, and produced acid from lactose. None were positive for the Voges–Proskauer test.

Cluster III grouped eleven strains, all ascribed to genus *Carnobacterium*. Ten were assigned to the species *C. piscicola*, whereas one strain remained unidentified at this level. All were not able to grow at 4°C and 40°C. They were positive for the Voges–Proskauer test,
produced acid from gluconate, though not from lactose and melezitose and were negative for the hydrolysis of arginine.

The 35 strains included in cluster IV were also carnobacteria. All yielded colonies larger than 2 mm in diameter and were able to grow at 4°C, but not at 40°C. Twenty-four strains fitted the description of C. piscicola, but 11 were Voges–Proskauer-negative. This cluster was also assigned to C. piscicola.

Cluster V contained six salt-tolerant strains, all assigned to C. piscicola. They were also positive for the Voges–Proskauer test, able to hydrolyse arginine, acid producers from melezitose and, unlike strains included in cluster IV, showed a narrow range of growth temperature.

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**Table 1.** Distinguishing features among significant clusters containing lactic acid bacteria

<table>
<thead>
<tr>
<th>Cluster</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. durans</td>
<td>6</td>
<td>3</td>
<td>11</td>
<td>35</td>
<td>6</td>
<td>165</td>
<td>10</td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>50*</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C. piscicola</td>
<td>33</td>
<td>64</td>
<td>64</td>
<td>50</td>
<td>50</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>C. piscicola</td>
<td>33</td>
<td>64</td>
<td>64</td>
<td>50</td>
<td>50</td>
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<td>33</td>
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<tr>
<td>C. piscicola</td>
<td>33</td>
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<td>64</td>
<td>50</td>
<td>50</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>C. diversgens</td>
<td>33</td>
<td>64</td>
<td>64</td>
<td>50</td>
<td>50</td>
<td>33</td>
<td>33</td>
</tr>
</tbody>
</table>

| Number of strains | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Colony morphology: | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| creamy consistency | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| smooth edges | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| flat colonies | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| convex colonies | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Ø < 1 mm | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Ø 1 mm–2 mm | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Salt tolerance: | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| 7% NaCl | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| 10% NaCl | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Temperature of growth: | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| 4°C | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| 40°C | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| 45°C | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Voges–Proskauer test | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Arginine hydrolysis | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Growth in acetate agar | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Acid production from: | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Amygdalin | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| L-arabinose | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Galactose | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Gluconate | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Mannitol | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Melitose | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Sucrose | 50* | 1 | 1 | 1 | 1 | 1 | 1 |
| Sorbitol | 50* | 1 | 1 | 1 | 1 | 1 | 1 |

1 All strains were negative for that characteristic.
2 All strains were positive for that phenotypic test.
* Percentage of strains positive for each phenotypic property. All strains included in taxons I to VII gave non-pigmented colonies, were tolerant to NaCl concentrations of 4% and able to grow at 8°C and 37°C, hydrolysed aesculin, produced acid in air from D-fructose, D-mannose and D-ribose, but not from D-xyllose, and were unable to produce H2S from cysteine, dextrane from glucose, and gas from glucose in BM agar containing arginine.
Cluster VI, the largest, comprised 164 field strains and the *C. piscicola* type strain. Most field isolates included in this group had been assigned to the species *C. piscicola* (114), two were identified as *C. divergens* and one as *C. mobile*. The remaining 47 isolates were negative for the Voges–Proskauer test. At 94% similarity (SSM), this cluster could be divided...
into more than 10 subclusters. Most strains grouped in this cluster shared some cultural characteristics on solid media (such as the convexity of colonies and the entire smooth edges). Other typical features of these strains were the production of acid from gluconate and galactose. Despite the heterogeneity of this cluster, it was finally assigned to *C. piscicola*.

Seven out of 10 strains grouped in cluster VII were identified as *C. divergens*. This group also included the reference strain of *C. divergens* included in our study. Therefore, the cluster was labelled as *C. divergens*. It should be mentioned that two field strains, also grouped in this taxon, were capable of producing acid from mannitol (thus identified as *C. piscicola*). The most distinctive features of the strains contained in cluster VII were the ability to hydrolyse arginine but not to produce acid from galactose, inulin and lactose. In addition, 70% of strains, all assigned to species *C. divergens*, were able to grow at 40°C.

Among non-significant clusters, it is interesting to note the one formed by two strains of *Vagococcus fluvialis*.

Frequency rates of hydrolytic activity of carnobacteria over several substrates depended on the substrate assayed. Thus, whereas 74% showed lipolytic activity over tributyrin, only 4% and 8%, respectively, were able to degrade casein and Tween 20 and 80, and lecithin. None of the strains tested hydrolysed starch neither gelatine.

**Discussion**

Most lactic acid bacteria isolated in our study (c. 91%) were assigned to *Carnobacterium*. Strains belonging to this genus, or to the former species *L. divergens* and *L. carnis* (now renamed as *Carnobacterium*), have been isolated from a wide range of foods such as meat (Holzapfel and Gerber 1983, Shaw and Harding 1985), poultry (Thornely and Sharpe 1959), mould-ripened cheese (Milliere et al. 1994) and fish and seafood (Hiu et al. 1984, Mauguin and Novel 1994).

As in our study, several authors (Feresu and Jones 1988, Holzapfel and Gerber 1983, Milliere et al. 1994) have highlighted the difficulties or even the inability of carnobacteria to develop on MRS agar, whereas they displayed growth on non-selective media (Milliere et al. 1994). Thus, we utilized BM agar (Wilkinson and Jones 1977) for most tests in order to minimise the likely inhibitory effects of sodium acetate and diammonium citrate on this bacterial group. As stated by several investigators (Baya et al. 1991, Montel et al. 1991), the inability of isolates to grow in acetate agar supported their assignation to the genus *Carnobacterium*. Moreover, the inhibition of their growth at temperatures above 40°C was also taken as criterion for discrimination of this genus from other related genera. Some tests, such as the production of gas in arginine-MRS medium and the hydrolysis of arginine, both considered as useful for the phenotypic differentiation of carnobacteria from other related genera, were not discriminatory in our study (Table 1). Indeed, the type strains of *Carnobacterium* included in our research were negative for both tests. The ability of carnobacteria to produce gas from glucose is variable, frequently negative (Collins et al. 1987), although sometimes is difficult to detect (Shaw and Harding 1985). The hydrolysis of arginine could have been influenced by the concentration of glucose (2%) of the medium. In this sense, Mauguin and Novel (1994) and Pilet et al. (1995) have observed that carnobacteria included in their studies were not able to hydrolyse arginine in the presence of 2% glucose, but did in the presence of 0·05% or 0·005% glucose.

Numerical taxonomy studies including ‘atypical’ lactic acid bacteria have been published previously (Feresu and Jones 1988, Shaw and Harding 1984). In our investigation, the use of numerical taxonomy have been of great value for the discrimination between *Carnobacterium* and other related lactic acid bacteria (Fig. 1). Thus, all clusters containing carnobacteria grouped at an 80% (SSM) similarity. Only the reference strain of *E. faecium* clustered, within this level, with carnobacteria. Below the 72% (SSM) level of similarity, strains included in clusters III–VII grouped together with the rest of lactic acid bacteria. Similar results were obtained by Feresu and Jones (1988) when they compared the ‘atypical’ lactic acid bacteria, comprised in the groups 2 and 3 of Thornley and Sharpe, with other lactic acid bacteria.
and related genera (Erysipelothrix, Brochothrix and Listeria). This method of bacterial classification has also been satisfactory for the distinction between C. divergens and C. piscicola (Fig. 1), although most carnobacteria not identified at species level are clustered with C. piscicola strains. This observation remarks the phenotypic closeness existing among Carnobacterium species. However, Montel et al. (1991) suggest that numerical taxonomy do not provide a clear method to achieve a proper separation within this genus.

Among genus Carnobacterium, the major species found in our study were C. piscicola (mainly grouped in clusters III to VI) and C. divergens (especially in cluster VII), which is in accordance with data reported by other investigators in fish (Pilet et al. 1995). The most distinguishing feature between C. piscicola and C. divergens was the ability of the latter to grow at 40°C, although the production of acid from mannitol was also of great value to achieve a proper separation of both carnobacteria as observed previously by Montel et al. (1991). Carnobacterium mobile was also detected, as well as other carnobacteria (most grouped in clusters III, IV and VI) not identified at species level (Carnobacterium spp). In general, phenotypic characteristics of C. piscicola strains isolated in our study mostly resembled that observed by Hiu et al. (1984) for L. piscicola and by Collins et al. (1987) for C. piscicola. Carnobacterium divergens strains also matched the phenotypic properties exhibited by the type strain of C. divergens studied by Collins et al. (1987) and by the strain V41 included in the investigation of Pilet et al. (1995). The Voges–Proskauer test, also considered as positive for C. piscicola and C. divergens, was negative for strains contained in clusters III to VI and labelled as Carnobacterium spp. These strains might constitute a sub-species within C. piscicola. Manchester et al. (1995) through the use of pyrolysis mass spectrometry, suggested the likely presence of sub-species of C. piscicola.

The occurrence of ‘typical’ lactobacilli in our samples was scarce (c. 0.44%) and their identification at species level was difficult to achieve. The presence of this bacteria in fish has been previously reported by others (Fricourt et al. 1994, Mauguin and Novel 1994, Valdimarsson and Gudbjörnsdottir 1984) and their assignation to previously known species has also been troublesome. Lactic acid cocci were also difficult to allocate within the genera and/or species known to be associated to foods. In general, identification schemes based on the study of phenotypic characteristics and developed for lactic acid cocci isolated from foods are not of great value for strains isolated from fish.

The role of carnobacteria in the spoilage of fish does not seem to be important, since the mere presence of high levels of this bacterial group in brown trout immediately after harversting (González-Serrano 1996) is not correlated with a shortening in their shelf-life if compared with other freshwater fish species kept under refrigeration conditions. Besides, the ability of carnobacteria to degrade proteinaceous and lipid compounds appears to be very low and limited to substrates such as tributyrin. These results are in accordance with those found by Feresu and Jones (1998), for the strains grouped in Thornley and Sharpe’s groups 2 and 3, and by Baya et al. (1991). Moreover, in the studies of Milliere et al. (1994) any hydrolytic activity could be seen, though visible growth was evident.

In summary, we consider that further studies are needed in order to elucidate the taxonomy of lactic acid bacteria from sources other than fermented foods and foods derived from terrestrial mammals.

Acknowledgements

We wish to thank Mr Carlos Bayón for kindly providing us with the brown trout specimens, and the Department of Animal Biology (University of León, Spain) for providing us with pikes. This work was supported by a grant from the Spanish Comisión Interministerial de Ciencia y Tecnología (Project Nº ALI94-0079). Dr C. González was beneficiary of a fellowship from the Spanish Ministerio de Educación y Ciencia.

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