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Lactobacillus sakei/curvatus is the prevailing lactic acid bacterium group in spoiled maatjes herring

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Abstract

A total of 164 lactic acid bacteria (LAB) isolated from spoiled maatjes herring stored in air and under modified atmosphere at 4 or 10 °C were characterised and identified using an rRNA gene restriction pattern (ribotype) database. The isolates were initially grouped according to their *Hin*dIII restriction endonuclease profiles and further identified to species level using numerical analysis. *Lactobacillus sakei*, *Lactobacillus curvatus* and strains of the *L. curvatus* spp./*Lactobacillus fuchuensis* group were the main species detected. Of all the isolates, six were identified as *Lactococcus* spp.

Keywords: Maatjes herring; Modified-atmosphere packaging; Spoilage; Lactic acid bacteria; Ribotyping

1. Introduction

Maatjes herring is a lightly salted and fermented ready-to-eat fish product, which is very popular in the Netherlands. The term ''maatjes'' refers to herring caught just before its first spawning between May and July, and is characterised by a distinct level of subcutaneous fat of 16–20%. After being caught, the herring is gibbed and lightly cured. The remaining appendices pyloricae produce tryptic enzymes (Priebe, 1980; Luten, 1997), which stimulate a fermentation process resulting in typical maatjes product characteristics. After brining, the fish undergoes a ripening period of up to 1 day before being vacuum packaged and stored frozen until further use. For retail, the product is thawed, filleted and sold loose or packaged under modified atmosphere and stored at chilled temperatures.

The occurrence of lactic acid bacteria (LAB) in high numbers in modified-atmosphere packaged (MAP) fish products after a few weeks' storage at chilled temperatures has also been reported previously (Stenström, 1985; Nieper and Stockemer, 1995; Hong et al., 1996; Emborg et al., 2000; Franzetti et al., 2001). When studying packed herring (Heringsfilet nach Matjesart) at retail level, Nieper and Stockemer (1995) determined 80% of the total bacteria count as LAB at the end of the shelf-life. Lyhs et al. (2007) detected 10^2–10^4 and 10^6 cfu/g LAB in sensorial-spoiled MAP maatjes herring at 4 and 10 °C, respectively. It is known that within the LAB there are specific strains causing spoilage. Lactic acid bacterium strains have been found to be able to produce some characteristic off-odours associated with spoiled vacuum-packaged cold-smoked salmon (Truelstrup Hansen, 1995; Joffraud et al., 2001). On the other hand, some LAB did not produce any spoilage off-odour in the same fish product (Leroi et al., 1996, 1998; Paludan-Müller et al., 1998). In semi-preserved marinated fish products, they are known to cause a spoilage process called 'protein swell' (Meyer, 1956, 1962; Lyhs et al., 2001). However, the fact that the spoilage potential is not the same in all LAB emphasises the need for species identification. Furthermore, the spoilage process of maatjes herring is still not clear. The great popularity of these fish products calls for a better understanding of the spoilage factors as well as of the role of the spoilage bacteria including LAB.

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Using ribotyping, it has been possible to successfully identify the main spoilage LAB in different fish products, such as vacuum-packaged, cold-smoked and 'gravad' rainbow trout and marinated herring (Lyhs et al., 1999, 2001, 2002, 2004). To the authors' knowledge, no data exist on the identification of LAB isolated from spoiled, packaged and unpacked maatjes herring.

The aim of this study was to identify the spoilage LAB of maatjes herring stored in air and under modified atmosphere at 4 and 10 °C using an rRNA gene restriction pattern (ribotype) database. The present paper can be considered as related to the one by Lyhs and Schelvis-Smit (2005) focusing on the development of a Quality Index Method (QIM) scheme for maatjes herring, and to the one by Lyhs et al. (2007) describing the microbiological and sensory changes of maatjes herring produced in the Netherlands during storage in air and under modified atmosphere at 4 and 10 °C.

2. Material and methods

2.1. Bacterial strains

A total of 164 LAB strains originating from spoiled maatjes herring stored in air and under modified atmosphere stored at 4 or 10 °C were characterised. Determination of the spoilage had been based on both the sensorial and microbiological analyses (Lyhs et al., 2007). A total of 53 isolates originating from samples stored under air (31 and 22 isolates from samples stored at 4 and 10 °C, respectively) and 111 isolates from the MAP samples (56 and 55 isolates from the samples stored at 4 and 10 °C, respectively) were studied.

The strains were considered as LAB since they all grew on MRS agar (Tritium Mikrobiologie, Veldhoven, The Netherlands) and were Gram positive and catalase negative. All the strains were stored at −70 °C in MRS broth (Tritium Mikrobiologie). Before use, they were subcultured overnight in 10 ml MRS broth (Tritium Mikrobiologie) at 25 °C and then plated on MRS agar (Tritium Mikrobiologie). The plates were incubated anaerobically at 25 °C for 5 days in an anaerobic jar with an $H_2 + CO_2$ generating kit (Merck, Germany).

2.2. Isolation of DNA, restriction endonuclease analysis (REA) and RFLP of 16 and 23S rRNA encoding gene for LAB species identification

Cells harvested from 1 to 2 ml of MRS broth culture were used for DNA analyses. DNA was isolated by the guanidium thiocyanate method of Pitcher et al. (1989) as modified by Björkroth and Korkeala (1996a) by a combined lysozyme and mutanolysin treatment (Sigma, St. Louis, Missouri). Restriction endonuclease treatment of 8 μg of DNA was done using *Hin*dIII restriction enzyme (New England Biolabs, Beverly, MA, USA) as recommended by the manufacturer. DNA fragments were separated by agarose gel electrophoresis and the resulting fingerprint patterns transferred onto a nylon membrane via Southern blotting using a vacuum-blotting device (Vacugene, Pharmacia, Uppsala, Sweden). Ribotyping was performed using a cDNA probe reverse transcribed (AMV-RT, Promega, Madison, Wisconsin) from 16 and 23S rRNA and digoxigenin labelled with Dig DNA Labelling Kit (Roche Molecular Biochemicals, Mannheim, Germany) as described by Blumberg et al. (1991). Membranes were hybridised at 58 °C overnight and detection of the digoxigenin-labelled fragments (ribopatterns) was performed as recommended by Roche Molecular Biochemicals.

2.3. LAB database and numerical pattern analysis

The *Hin*dIII ribopatterns were compared to the corresponding patterns in the previously established LAB database of the Department of Food and Environmental Hygiene, University of Helsinki, Finland. This database comprises patterns of all the relevant food-associated LAB in the genera of *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Weissella* (Björkroth and Korkeala, 1996b, 1997; Björkroth et al., 1998, 2000, 2005; Susiluoto et al., 2003; Koort et al., 2004a, 2005; Lyhs et al., 2004). It utilises 16 and 23S rRNA gene *Hin*dIII RFLP patterns of over 300 type and reference strains as operational taxonomic units in numerical analyses. The isolates are identified based on the locations of type and reference strains within the clusters. Reliability of the clusters to distinguish between different species has been evaluated in several polyphasic taxonomy studies of LAB (Björkroth et al., 1998, 2000; Koort et al., 2004a, b, 2005; Koort, 2006).

For the numerical analysis, the ribopatterns were scanned using a Hewlet Packard (Boise, Idaho) ScanJet 4c/T scanner. The patterns were normalised based on the mobility of standards and a similarity matrix was created using the BioNumerics 4.1 software package (Applied Maths, Sint-Martens-Latem, 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 construction of the dendrogram. Based on the use of internal controls in the database, pattern optimisation and band position tolerance of 0.5 and 1.5, respectively, were allowed.

3. Results and discussion

Using ribotyping, it was possible to identify the main spoilage LAB of sensorial-spoiled maatjes herring stored in air and under modified atmosphere at 4 and 10 °C to species level. Fig. 1 shows the different ribotypes obtained and the UPGMA clustering based on the similarity of the patterns. Two main clusters were defined: one containing *Lactobacillus* spp. (L.) (93%) and the other containing

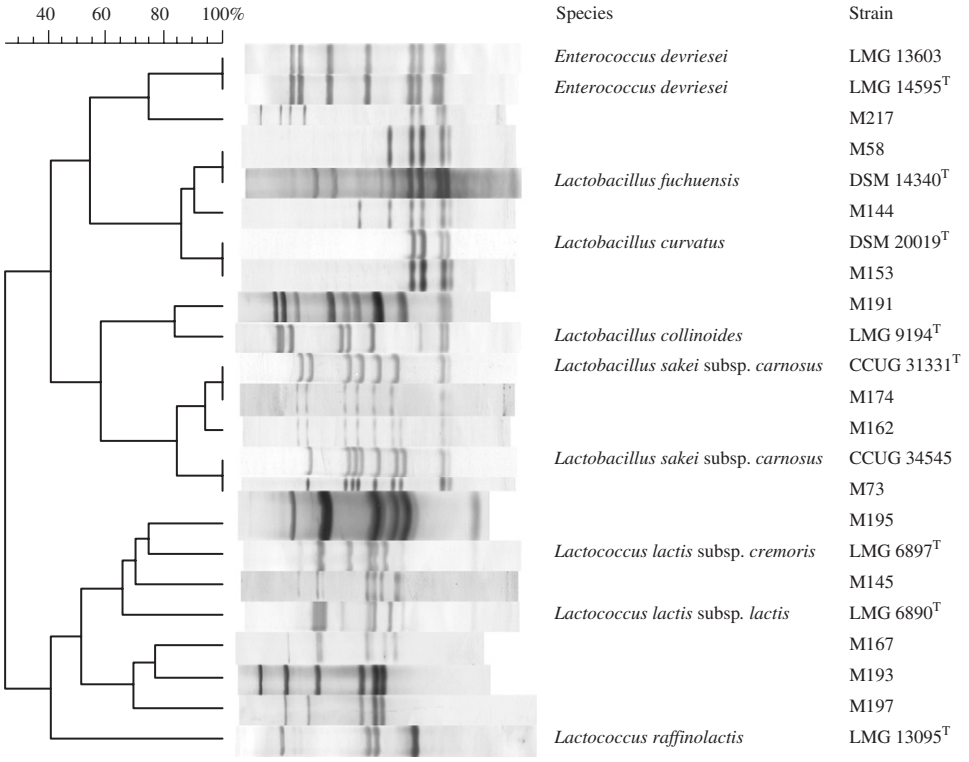

Fig. 1. *Hin*dIII 16 and 23S RFLP patterns obtained from the lactic acid bacterium strains detected in spoiled maatjes herring stored in air and under modified atmosphere at 4 and 10 °C and numerical analysis of the patterns presented as a dendrogram.

Lactococcus spp. (Lc.) (4%). During the processing of maatjes herring, the fish is gibbed, meaning that the appendices pyloricae remain in the fish. LAB (*Streptococcus, Leuconostoc*, *Lactobacillus* and *Carnobacterium*) are known to belong to the normal microflora of the intestines in healthy fish (Ringø et al., 1998, 2000). Kraus (1961) isolated lactobacilli from the whole intestine tract of herring (*Clupea harengus*). It might be possible that, during the ripening period of maatjes herring, *Lactobacillus* spp. migrate from the remaining intestines into the muscle and are selected later due to packaging and storage characteristics. It could also be possible that LAB from the processing environment contaminate the maatjes herring after thawing and during further processing as suggested earlier for other fish products (Krüger, 1973; Lyhs et al., 2001; Dalgaard et al., 2003).

Lactobacillus sakei subsp. *carnosum* and *Lactobacillus curvatus* formed with 42% and 30% of all isolates, respectively, the two major groups at both atmospheres and temperatures (Table 1). The occurrence of *L. sakei* and *L. curvatus* in different numbers in vacuum-packaged 'gravad' or cold-smoked spoiled fish products has also been reported previously (Jeppesen and Huss, 1993; Truelstrup Hansen, 1995; Gancel et al., 1997; Leroi et al., 1998; Paludan-Müller et al., 1998; Lyhs et al., 1999, 2001). In contrast, a study of packed herring (Heringsfilet nach Matjesart), Nieper and Stockemer (1995) found mostly *Lactobacillus brevis*, but also *Lactobacillus buchneri*, *Lactobacillus delbrückii* and *Weissella viridescens* at the end of the shelf-life using an API system for identification. It is known that the phenotyping of spoilage LAB in fish products may result in unreliable species identification (Gancel et al., 1997; Lyhs et al., 1998). This may explain the different findings compared to the present study.

In the present study, 20 strains of the *L. curvatus* spp./*Lactobacillus fuchuensis* group occurred mainly in the MAP samples stored at 4 °C. *L. fuchuensis*, isolated from spoiled vacuum-packaged chilled beef, is phylogenetically closely related to *L. sakei* and *L. curvatus*, but nevertheless a quite separate species (Sakala et al., 2002). To the authors' knowledge it has never been isolated from any fish products.

Five strains belonging to *Lactococcus* spp. have been isolated from the maatjes herring stored in air at 4 °C. Lactococci in low numbers have been isolated from different kinds of either packaged or unpackaged lightly preserved fish products (Maugin and Novel, 1994; Paludan-Müller et al., 1998, 1999, 2002). Dąbrowski et al. (2002) studied low-salt (5–7% salt in fish tissue) herring without preservatives and stored at 8 °C and identified 30% of all isolates as *Lactococcus* spp. Hagi et al. (2004) reported that in carps caught in the summer from a freshwater lake, *Lc. lactis* has been predominating the intestinal LAB flora in the fish. Vihavainen et al. (2007) reported that lactococci among other specific meat-spoilage LAB were recovered from the air in a broiler processing plant. Thus the lactococci found in the present study might

Table 1
LAB identified from spoiled maatjes herring stored in air and under modified atmosphere at 4 and 10 °C using 16 + 23 S rRNA gene restriction patterns (ribotyping)-based database

Product	Storage temperature (°C)	Number of isolates	*L. sakei* subsp. *carnosum*	*L. curvatus*	*L. curvatus* spp./*L. fuchuensis* group	*L. collinoides*	*Lactococcus* spp.	*Enterococcus* spp.	Unidentified
Stored in air	4	31	17	14	0	4	5	1	0
Stored in air	10	22	5	6	1	0	0	0	0
Stored under MAP[a]	4	56	15	20	20	0	0	0	1
Stored under MAP[b]	10	55	32	10	9	0	1	2	1
Total		164	69	50	30	4	6	3	2

[a]Low-oxygen MAP was used. At the time of isolating of the spoilage LAB the MAP contained CO_2 and O_2 concentrations of 40–50% and 0.1–0.6%, respectively.
[b]Low-oxygen MAP was used. At the time of isolating of the spoilage LAB the MAP contained CO_2 and O_2 concentrations of 50–60% and 0.2–0.5%, respectively.

either originate from the intestines of the herring or from the environment of the fish-processing plant.

No carnobacteria or leuconostocs were detected in this study. Besides lactobacilli both these species have been found in spoiled vacuum-packaged cold-smoked and "gravad" rainbow trout, respectively (Lyhs et al., 1999, 2001). It is known that of the psychrotrophic LAB, carnobacteria or leuconostocs tolerate less salt (Lücke, 1996; Lyhs et al., 2001). The salt concentration in maatjes herring usually varies between 0.5% and 4% and might be the reason for their absence in the present study (Luten, 1997; Lyhs et al., 2007). Leroi et al. (1998, 2000) observed in vacuum-packaged cold-smoked salmon that carnobacteria dominated the lactic acid bacterial flora during the first 2–3 weeks of storage and lactobacilli at the end of storage. Schillinger and Lücke (1986) suggested that in meat processing, lactobacilli might recover faster from the processing stress. It is possible that lactobacilli also overgrew carnobacteria in the later storage phase in the present fish product.

In conclusion, *L. sakei*, *L. curvatus*, *L. curvatus* spp./*L. fuchuensis* and *Lactococcus* spp. were the major LAB species associated with spoiled maatjes herring when stored in air and under modified atmosphere.

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