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1 Identification of lactic acid bacteria from spoiled, vacuum-packaged ‘gravad’ rainbow
2 trout using ribotyping

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Abstract

A total of 296 lactic acid bacteria (LAB) isolated from spoiled, vacuum-packaged 'gravad' rainbow trout stored at 3°C and 8°C were characterised and identified using a molecular approach. The isolates were initially grouped according to their *HindIII* restriction endonuclease profiles and further identified to species level using a rRNA gene restriction pattern (ribotype) identification database. *Lactobacillus sakei*, *Lactobacillus curvatus* and *Carnobacterium piscicola* were the three main species detected. Only one isolate was identified as *Carnobacterium divergens*. Most of the carnobacteria were found in the samples stored at 3°C. The relative proportion of *L. sakei* was higher in the samples stored at 8°C.

Key words: Fish; Spoilage; 'Gravad'; Vacuum-packaging; Lactic acid bacteria; Identification; Restriction endonuclease analysis; Ribotyping

1. Introduction

Among the traditionally manufactured fish products in the Nordic countries, sugar-salted ('gravad') fish is of considerable importance. This product is characterised by a salt content of 3 to 6% and a pH higher than 5. The fish, mostly fillets, is preserved by addition of salt and sugar, also dill and other spices are added. In commercial manufacture the fillets are sliced, usually vacuum-packaged and stored at chilled temperatures. Vacuum-packaged 'gravad' fish belongs to the ready-to-eat products and is eaten raw without further heating. For the product studied here the manufacturer had declared a shelf-life of 18 days at $\leq 3^{\circ}\text{C}$.

During the storage of vacuum-packaged 'gravad' fish products, a complex microflora of different species develops. The dominating bacterial groups have been Gram-negative, oxidase-positive bacteria (Lyhs et al., 2000) or lactic acid bacteria (LAB) (Knøchel, 1983; Leisner, 1992). The development of a variable microflora associated with spoilage has been observed also in vacuum-packaged cold-smoked fish products (Civera et al., 1995; Truelstrup Hansen, 1995; Leroi et al., 1998; Lyhs et al., 1998; Paludan-Müller et al., 1998).

Due to the increasing popularity of vacuum-packaged 'gravad' fish products a better understanding of the spoilage factors as well as the role of the spoilage bacteria is needed. The characterisation of LAB from vacuum-packaged 'gravad' fish products has mainly been based on the traditional biochemical and physiological tests (Leisner, 1992; Jeppesen and Huss, 1993; Leisner et al., 1994). Since phenotyping alone has been found insufficient in the identification of many psychrotrophic spoilage LAB (Björkroth et al., 1998, 2000; Lyhs et al., 1998), molecular identification has been recommended (Gancel et al., 1997; Björkroth and Korkeala, 1996b; Björkroth et al., 1998; Lyhs et al., 1998). Ribotyping has been applied with success for the

1 identification of the main spoilage LAB in different fish products, such as vacuum-
2 packaged, cold-smoked rainbow trout (Lyhs et al., 1999) and marinated herring (Lyhs
3 et al., 2001).

4 The aim of this work was to characterise and identify the spoilage LAB of
5 vacuum-packaged 'gravad' rainbow trout slices stored at 3°C and 8°C. During the
6 study the isolates were initially grouped according to their restriction endonuclease
7 (REA) profiles and strains representing each group were further identified to the
8 species level using a rRNA gene restriction pattern (ribotype) database.

10 **2. Material and methods**

11 *2.1. Bacterial strains*

12 A total of 296 bacterial strains originating from spoiled, vacuum-packaged
13 'gravad' rainbow trout slices stored at 3°C or 8°C (Lyhs et al., 2000) were
14 characterised. Determination of the spoilage had been based on both sensorial and
15 microbiological analyses. At the time of spoilage the levels of LAB had been from
16 10^4 - 10^6 cfu/g and 10^5 - 10^7 cfu/g in the products stored 3°C or 8°C, respectively. The
17 strains were considered as LAB since they all grew on MRS agar (Oxoid,
18 Basingstoke, United Kingdom) and were Gram-positive and catalase-negative. Totals
19 of 128 and 168 isolates originating from samples stored at 3°C and 8°C, respectively,
20 were studied. All strains were stored at -70°C in MRS broth (Difco, Detroit,
21 Michigan, USA). Before use, they were subcultured overnight in 10ml MRS broth
22 (Difco) at 25°C and then plated on MRS agar (Oxoid). The plates were incubated
23 anaerobically at 25°C for 5 days in an anaerobic jar with a H₂+CO₂ generating kit
24 (Oxoid).

1 2.2. *Isolation of DNA, restriction endonuclease analysis (REA) and determination of*
2 *rRNA gene restriction patterns (ribotyping)*

3 DNA was isolated according to the guanidium thiocyanate method of Pitcher
4 et al. (1989) modified by Björkroth and Korkeala (1996a) with a combined
5 mutanolysin (Sigma Chemical Company, St. Louis, MO, USA) and lysozyme
6 (Sigma) treatment. Restriction endonuclease digestion of 6µg of DNA was done
7 according to the manufacturer's instructions with *HindIII* (New England Biolabs,
8 Beverly, MA, USA). REA, southern transfer, hybridisation and the cDNA probe for
9 rRNA gene restriction patterns (ribotypes) were prepared as described by Björkroth
10 and Korkeala (1996a).

11 The similarity between all isolates was initially checked visually using *HindIII*
12 REA. One strain from the groups formed by REA was further subjected to ribotyping.
13 *HindIII* was chosen because it has been found to provide species-specific patterns for
14 various spoilage LAB (Björkroth and Korkeala, 1996a, 1997; Björkroth et al., 1998,
15 2000).

16
17 2.3. *Ribotyping data management*

18 The membranes were scanned with a Hewlett-Packard ScanJet 4c/T tabletop
19 scanner (Boise, Idaho, USA). Numerical analysis of the ribopatterns was performed
20 using the Gelcompar II 1.0 software package (Applied Maths, Kortrijk, Belgium), as
21 recommended by the manufacturer. Based on internal controls, 1.8% position
22 tolerance was allowed for the bands. The similarity between all pairs was expressed
23 by Dice coefficient correlation, and the unweighted pair-group method with arithmetic
24 averages (UPGMA) was used for the construction of the dendrogram. The
25 ribopatterns were compared with the corresponding patterns in the LAB database at

the Department of Food and Environmental Hygiene, University of Helsinki, Finland. It comprises patterns of all relevant spoilage LAB in the genera of *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Weissella* (Björkroth and Korkeala, 1996b, 1997; Björkroth et al., 1998, 2000; Lyhs et al., 1999). Identification of the fish isolates was made on the basis of locations of the type and reference strains in the clusters formed.

3. Results

*Hind*III REA of the 296 LAB isolates resulted in formation of 37 groups possessing group-specific REA patterns (Table 1). Eleven different ribopatterns (H1-H11) were obtained when one strain from each group was further ribotyped. Fig. 1 shows the different ribotypes obtained and the UPGMA clustering based on the similarity of the patterns.

Table 2 shows the distribution of the LAB isolates originating from 3°C and 8°C. Most of the *L. curvatus* subsp. *curvatus* strains were found in the samples stored at 8°C. *L. sakei* subsp. *sakei/carnosum* strains occurred mainly in the samples stored at 8°C whereas most of the carnobacterial strains were found in the samples stored at 3°C.

4. Discussion

Using ribotyping, a good species-specific clustering was obtained. The largest group, 54% of all isolates, should be considered as *L. sakei* since there is strong evidence that *L. curvatus* subsp. *melibiosus* type strain should be classified as *L. sakei*. E. Falsen, the curator of the CCUG culture collection, has noticed that *L. curvatus* subsp. *melibiosus* type strain and *L. sakei* subsp. *carnosum* are adjacent

1 phenotypically and cluster together also in the dendrograms based on whole-cell
2 protein patterns (Falsen, 1999). This controversial situation has also been noticed in
3 other studies about meat (Mäkelä et al. 1992; Björkroth and Korkeala, 1996b) and fish
4 products (Lyhs et al., 1999). By the means of phenotypic identification, *L. sakei* has
5 been observed also before to be present either as a main organism or with other LAB
6 dominating in vacuum-packaged 'gravad' fish products. Jeppesen and Huss (1993)
7 identified 25 out of a total of 37 LAB isolates originating from vacuum-packaged
8 'gravad' salmon, mackerel and Greenland halibut stored at 5°C and 10°C for 2 to 4
9 weeks as *L. sakei* whereas Leisner et al. (1994) found only four out of a total of 18
10 LAB from vacuum-packaged 'gravad' fish products to be *L. sakei*.

11 Considering *L. curvatus* subsp. *melibiosus* as *L. sakei*, *L. curvatus* subsp.
12 *curvatus* strains formed the second largest group, 27% of all isolates studied. The
13 occurrence of *L. curvatus* in such high numbers has not previously been reported. In
14 the study of Leisner et al. (1994) only one from the total of 18 LAB strains from
15 vacuum-packaged 'gravad' fish products was identified as *L. curvatus*. Because
16 phenotypic tests have been found insufficient to distinguish between *L. curvatus* and
17 *L. sakei* (Dykes and von Holy, 1994), the proportions of these two species reported in
18 the earlier studies may not have been totally correct. Known already as a typical
19 spoilage organism in different meat products (Björkroth and Korkeala, 1996a, 1996b;
20 Holzapfel and Gerber, 1986; Mäkelä and Korkeala, 1987; Mäkelä et al., 1992; Stiles
21 and Holzapfel, 1997), *L. curvatus* may, however, play an important role also in the
22 spoilage of vacuum-packaged 'gravad' fish products. Due to the limited number of the
23 bands in *HindIII* ribopatterns, *L. curvatus* subsp. *curvatus* and *C. divergens* type
24 strains showed a similarity level of 72%. Lower similarity values for these species can
25 be obtained using *ClaI* or *EcoRI* digestion enzymes (Björkroth and Korkeala, 1996b).

1 The third major LAB group consisted of carnobacteria. Fifty-five isolates and
2 one isolate were identified as *C. piscicola* and *C. divergens*, respectively. In contrast
3 to the present findings, the majority of the predominating LAB in vacuum-packaged
4 'gravad' fish products stored at chilled temperatures has occasionally been assigned as
5 carnobacteria (Leisner, 1992, Leisner et al., 1994). *C. piscicola* has been associated
6 with fresh fish and with packaged, chill-stored fresh fish (Baya et al., 1991; Stoffels et
7 al., 1992; Ringo et al., 2000) and strains have been detected from different fish
8 species, e.g. from rainbow trout (Hiu et al., 1984; Starliper et al., 1992). Variable
9 proportions of carnobacteria have also been associated with the spoilage flora of
10 vacuum-packaged cold-smoked fish products stored at chilled temperatures. The
11 identification of carnobacteria in these studies had been done using either
12 phenotypical (Gancel et al., 1997; Leroi et al., 1998, 2000) or genotypical tests
13 (Paladuan-Müller et al., 1998; Lyhs et al., 1999). Reasons for the variability in the
14 determination of carnobacteria may be due to the different growth conditions, species
15 identification methods used and/or the spoilage degree of the product studied.

16 Comparing the spoilage LAB in vacuum-packaged 'gravad' and cold-smoked
17 rainbow trout, a difference in the composition of the spoilage LAB can be seen. Thus,
18 in 'gravad' fish no leuconostocs were found. In a study of vacuum-packaged, salted,
19 cold-smoked rainbow trout fillets, with or without the addition of nitrate or nitrite,
20 stored at 4°C and 8°C, Lyhs et al. (1999) detected leuconostocs forming the largest
21 group in addition to *L. sakei* and only few *L. curvatus* strains were detected.
22 Carnobacteria were not observed at all. In a study of vacuum-packaged cold-smoked
23 salmon stored for 5 weeks at 5°C Leroi et al. (2000) reported the sensitivities of
24 carnobacteria and lactobacilli to salt and smoke. When salt or smoke treatments were
25 studied separately, the sensitivities of the two genera of LAB were identical.

Carnobacteria, however, were more sensitive to the combination of low salt concentrations and smoke treatment. It seems that the combined use of salt and smoke creates a growth hurdle affecting carnobacteria more than lactobacilli. Lücke (1996) has suggested that heavy salting tends to suppress growth leuconostocs and carnobacteria in manufacture of raw meat products.

In 'gravad' fish salt in a relatively high concentration is the only preservative besides sugar. Another major factor behind the reported differences in fish spoilage populations is apparently related to the handling of the fish. Krüger (1973) has suggested that LAB contaminate fish products during the processing as a secondary contamination. Depending on the species occurring in the initial microflora and in the in-house flora, psychrotrophic *L. sakei*, *L. curvatus* or carnobacteria may occur in high initial levels. Lactobacilli may recover faster from the processing stress and overgrow carnobacteria in the later storage as Schillinger and Lücke (1986) have indicated in respect to meat processing and Leroi et al. (2000) to vacuum-packaged cold-smoked salmon manufacture. This together with the additional suppression of the carnobacteria due to the salt used, may be the reason for *L. sakei*/*L. curvatus* predominance in this 'gravad' fish product.

5. Conclusion

Using ribotyping, it could be concluded that *L. sakei*, *L. curvatus* and *C. piscicola* were the major lactic acid bacterium species associated with this spoiled fish product.

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1 Table 1

2 Number of isolates, types obtained by *Hind*III restriction endonuclease

3 analysis and ribotypes of LAB from spoiled, vacuum-packaged ‘gravad’

4 rainbow trout slices stored at 3°C and 8°C

REA type	Ribotypes	No. of isolates
1-7	H1	121
8-24	H2	72
25-29	H3	20
30	H4	18
31	H5	1
32	H6	8
33	H7	1
34	H8	50
35	H9	3
36	H10	1
37	H11	1

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1 Table 2

2 Distribution of the lactic acid bacteria isolated from spoiled, vacuum-packaged 'gravad' rainbow trout slices stored at 3°C and 8°C

Storage temperature (°C)	No. of isolates	<i>Lactobacillus</i> <i>curvatus</i> subsp. <i>curvatus</i>	<i>Lactobacillus</i> <i>curvatus</i> subsp. <i>melibiosus</i> ^a	<i>Lactobacillus</i> <i>sakei</i> subsp. <i>sakei</i>	<i>Lactobacillus</i> <i>sakei</i> subsp. <i>carosum</i>	<i>Carnobacterium</i> <i>piscicola</i>	<i>Carnobacterium</i> <i>divergens</i>
3	128	24 (19%) ^b	59 (46%)	4 (3%)	3 (2%)	37 (29%)	1 (1%)
8	168	57 (34%)	62 (37%)	14 (10%)	17 (10%)	18 (11%)	0

3 ^a Classification as *L. curvatus* doubtful. This species shows strong genotypic and phenotypic association with both *L. sakei* subspp. (Mäkelä et al., 1992;
4 Björkroth and Korkeala, 1996a; Falsen, 1999; Lyhs et al., 1999).

5 ^b Percentage of all strains included in the study at each storage temperature.

1 Legend to the figure:

2

3 Fig. 1. Dendrogram and schematic banding patterns based on *Hind*III ribopatterns. H1 to H11 represent restriction endonuclease analysis based

4 groups of lactic acid bacterium isolates from spoiled, vacuum-packaged 'gravad' rainbow trout slices stored at 3°C or 8°C.

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