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Characterisation of lactic acid bacteria from spoiled, vacuum-packaged, cold-smoked rainbow trout using ribotyping

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Abstract

A total of 405 lactic acid bacteria (LAB) isolated from spoiled, vacuum-packaged, salted, sodium nitrite- or potassium nitrate-treated, cold-smoked rainbow trout stored at 4°C or 8°C were characterised and identified using a molecular method. The isolates were initially classified according to their restriction endonuclease profiles using *Hind*III and *Eco*RI restriction endonucleases and further characterised by rRNA gene restriction patterns (ribotypes). Numerical analysis of these ribopatterns was performed together with 19 reference LAB strain patterns in order to identify the isolates to species level. The strains were divided with *Hind*III and *Eco*RI ribopatterns into ten and nine clusters at the similarity level of 65% and 50%, respectively. The *Leuconostoc*-clusters and the *Lb. sakei*/*Lb. curvatus*-clusters formed the two main groups. Only one isolate was identified as *Lactobacillus plantarum* and no *Carnobacterium* strains were discovered. For both enzymes, the 35 isolates possessing six individual ribotypes and forming five clusters could not be identified further with the reference strains used. The relative proportion of *Leuconostoc mesenteroides* subsp. *mesenteroides* was higher in all samples stored at 4°C. Most of the *Leuconostoc citreum* were found in the samples stored at 8°C, and particularly in the nitrite-treated samples.

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

1. Introduction

Lactic acid bacteria (LAB) comprise the major component of the spoilage flora, which develops on vacuum-packaged, lightly preserved fish products after a few weeks' storage at chilled temperatures. Magnússon and Traustadóttir (1982) found LAB dominating in vacuum-packaged, cold-smoked herring, Shimasaki et al. (1994), Civera et al. (1995), Truelstrup Hansen (1995), Leroi et al. (1998) Lyhs et al. (1998) and Paludan-Müller et al. (1998) in vacuum-packaged, cold-smoked salmon and rainbow trout, as well as Jeppesen and Huss (1993) and Leisner et al. (1994) in sugar-salted ('gravad') fish. In contrast to the spoilage role of these bacteria, the existence of LAB in a product is sometimes beneficial. Production of natural antagonistic substances by LAB may prolong the shelf life and inhibit the growth of pathogens contaminating the product (Bacus and Brown, 1981; Lindgren and Dobrogosz, 1990). This concept is known as biopreservation and recently there has been a great deal of interest in its practical application.

The use of biopreservation and other techniques increasing shelf-life of fish products demand a better understanding of the factors associated with spoilage. A primary area requiring further investigation is the variety of different spoilage LAB in fish products. The identification of LAB has mainly been based on traditional biochemical and physiological tests, which are not only time-consuming, but also often produce very controversial results. Moreover, there are certain species that cannot readily be distinguished by phenotypic characters (Schleifer et al., 1995). When fish products have been studied, phenotyping of LAB has resulted in unreliable species identification. LAB originating from vacuum-packaged, salted, smoked herring gave atypical profiles (Gancel et al., 1997), and in a study of the spoilage flora of vacuum-packaged, salted, cold-smoked rainbow trout fillets (Lyhs et al., 1998), we also concluded that phenotyping is not always reliable for

1 identification of the spoilage LAB. In both cases, molecular identification of the LAB to
2 species level was recommended.

3 Several molecular methods have been used for strain typing or characterisation
4 of LAB (Hill and Hill, 1986; Tannock et al., 1990; Welsh and McClelland, 1990; Williams et
5 al., 1990; Duffner et al., 1993; Dykes et al., 1993; Jaho et al., 1993; Roussel et al., 1993;
6 Björkroth et al., 1996a, 1996b), but only some of these applications have been used for
7 establishing species identification libraries. Paludan-Müller et al. (1998), studying the role of
8 *Carnobacterium* species in the spoilage of vacuum-packaged, cold-smoked salmon, used
9 whole-cell-protein patterns for identification of the carnobacteria. Simple restriction of DNA
10 (Manchini and Parini, 1983; Ståhl et al., 1990) has been used as a tool for separating different
11 species of lactobacilli, and ribotyping (Grimont and Grimont, 1986), which is based on
12 patterns generated from the ribosomal RNA genes only, has also been applied with success
13 for LAB (Rodtong and Tannock, 1993; Björkroth and Korkeala, 1996a, 1996b; Björkroth et
14 al., 1998).

15 The aim of this study was to characterise and identify the main spoilage LAB of
16 vacuum-packaged, salted, sodium nitrite-or potassium nitrate-treated, cold-smoked rainbow
17 trout stored at 4°C or 8°C. The isolates were initially classified according to their restriction
18 endonuclease (REA) profiles and further identified to species level using a rRNA gene
19 restriction pattern (ribotypes) identification database.

20 21 **2. Material and methods**

22 23 **2.1. Bacterial strains**

24 A total of 405 previously phenotyped LAB (Lyhs et al., 1998) were included in
25 this study. The isolates originated from spoiled vacuum-packaged, salted, sodium nitrite-or

potassium nitrate-treated, cold-smoked rainbow trout, stored at 4°C or 8°C. All strains were stored at -70°C in MRS broth (Difco, Detroit, Michigan, USA). Before use, they were subcultured overnight in 10ml MRS broth at 30°C, plated on MRS agar (Oxoid, Basingstoke, UK) and incubated anaerobically at 25°C for 5 days using a model BR 38 gas-generating kit (Oxoid) in an anaerobic jar. Table 1 shows the reference strains ribotyped for the identification database with *EcoRI* and *HindIII*. These strains were selected to cover the range of phenotypic groups obtained in the previous study (Lyhs et al., 1998) of the fish isolates.

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

DNA was isolated according to the guanidium thiocyanate method of Pitcher et al. (1989) modified by Björkroth and Korkeala (1996) with combined mutanolysin (Sigma Chemical Company, St. Louis, MO, USA) and lysozyme (Sigma) treatment. Restriction endonuclease digestion of 6µg of DNA was done according to the manufacturer's instructions with *EcoRI* and *HindIII* (New England Biolabs, Beverly, MA, USA). REA, southern transfer, hybridisation and the cDNA probe for rRNA gene restriction patterns (ribotypes) were prepared as described by Björkroth and Korkeala (1996). The similarity between all isolates was initially checked using *EcoRI* and *HindIII* REA. One strain from each different pattern group was further ribotyped.

2.3. Ribotyping data management

The membranes were scanned with a Hewlett-Packard ScanJet 4c/T tabletop scanner (Boise, Idaho, USA). Numerical analysis of the ribopatterns was performed with a GelCompar system (version 4.0; Applied Maths, Kortijk, Belgium), as recommended by the manufacturer. The similarity between all pairs was expressed by Dice coefficient correlation,

and the unweighted pair-group method with arithmetic averages (UPGMA) was used for the construction of the dendrogram. Identification of the fish isolates was made on the basis of the reference strains locations in the clusters formed.

3. Results

REA of the 405 LAB isolates resulted in formation of 25 groups. When one isolate from each group was further ribotyped, 15 and 16 patterns were obtained with *Hind*III and *Eco*RI, respectively (Table 2).

The similarity between the isolates clusters and with some of the reference strains used is shown in Fig. 1 and 2, for *Hind*III and *Eco*RI, respectively. Using *Hind*III, ten clusters were defined at a similarity level of 65%. In cluster I, one isolate and the type strain of *Lactobacillus plantarum* (ATCC 14971) clustered at a similarity level of 100%. In cluster III, two different ribotypes representing 19 isolates, merged at a similarity level of 70%. Cluster IV contained a ribotype possessed by two isolates and the ribotype of *Lactobacillus curvatus* subsp. *curvatus* type strain (ATCC 25061), merging at a similarity level of 100%. Eleven isolates possessed one ribotype forming cluster V. Cluster VI was formed by one identical ribotype (similarity level 100%) from 94 isolates and the type strain of *Leuconostoc citreum* (LMG 9824^T). Cluster VIII contained the different types gained from 136 isolates, together with the type strains of *Lactobacillus sakei* subsp. *carosum* (CCUG 31331), *Lactobacillus sakei* subsp. *sakei* (ATCC 15521) and *Lactobacillus curvatus* subsp. *melibiosus* (CCUG 34545). At a similarity level of 78%, two subclusters, VIIIa and VIIIb, were formed. Subcluster VIIIa has three different ribopatterns of 47 isolates and the ribotype of the *Lactobacillus sakei* subsp. *carosum* (CCUG 31331) type strain, merging at a similarity level of 87%. Subcluster VIIIb is associated with 89 isolates possessing two different ribotypes and the type strains of *Lactobacillus sakei* subsp. *sakei* (ATCC 15521) and *Lactobacillus curvatus*

1 subsp. *melibiosus* (CCUG 34545). Cluster X consisted of three different patterns associated
2 with 136 isolates and the pattern of the type strain of *Leuconostoc mesenteroides* subsp.
3 *mesenteroides* (DSM 20343), clustering at a similarity level of 66%.

4 Using *EcoRI*, nine clusters were defined at a similarity level of 50%. Two
5 distinct subclusters, subcluster IIIa and IIIb, were identified at a similarity level of 60%.
6 Subcluster IIIa contained nine types and the type strains of *Lactobacillus sakei* subsp.
7 *carnosum*, *Lactobacillus curvatus* subsp. *melibiosus* and *Lactobacillus sakei* subsp. *sakei*,
8 merging at a similarity level of 70%. The strains forming subcluster IIIb and the type strain of
9 *Lactobacillus curvatus* subsp. *curvatus* merged at a similarity level of 88%. Cluster V
10 included one of the fish isolates and the type strain of *Lactobacillus plantarum* (ATCC
11 14971), clustering at a similarity level of 100%. In cluster VI, two different ribotypes,
12 possessed by 21 isolates, merged at a similarity level of 50%. Cluster VII consisted of the
13 100% identical ribotypes associated with 94 isolates and the type strain of *Leuconostoc*
14 *citreum* (LMG 9824T). The ribotype appearing in 136 isolates and the ribotype of the type
15 strain of *Leuconostoc mesenteroides* subsp. *mesenteroides* (DSM 20343) merged at a
16 similarity level of 55%, forming cluster VIII. In cluster IX, one ribotype is possessed by
17 eleven isolates.

18 High numbers of *Lactobacillus sakei* subsp. *sakei* and *Lactobacillus sakei*
19 subsp. *carnosum* occurred in the nitrate-treated samples stored at both storage temperatures
20 (Table 3). The relative proportion of *Leuconostoc mesenteroides* subsp. *mesenteroides* was
21 higher in all kind of samples stored at 4°C than in samples stored at 8°C. Most of the
22 *Leuconostoc citreum* were found in the samples stored at 8°C, and especially in the nitrite-
23 treated samples.

4. Discussion

Ribotyping allowed the differentiation of two clusters of leuconostocs; one containing the *Leuc. mesenteroides* subsp. *mesenteroides* (cluster X for *Hind*III and IX for *Eco*RI), and the other the *Leuc. citreum* (cluster VI for *Hind*III and VII for *Eco*RI) type strain. In the present study, the largest LAB group, 57% of all isolates, was considered as leuconostocs; 33% as *Leuc. mesenteroides* and 23% as *Leuc. citreum*. Jeppesen and Huss (1993) identified all LAB from vacuum-packaged, minced herring as *Leuconostoc* spp. However, Mauguin and Novel (1994) found that only eight of 86 LAB isolated from various samples of seafood belonged to the genus *Leuconostoc*. Moreover, Truelstrup Hansen (1995), in characterising 168 LAB from spoiled, cold-smoked, vacuum-packaged salmon, identified only 4.5% as *Leuc. mesenteroides*. In our study, the majority of *Leuc. mesenteroides* subsp. *mesenteroides* were found in the samples stored at 4°C (Table 3). *Leuc. citreum* (*amelibiosum*) has been isolated from various meat products (Mäkelä et al., 1992) and from raw, minced, freshwater fish, used for the production of a Thai low-salt, fermented fish product called *som-fak* (Paludan-Müller et al., 1999). However, to the author's knowledge, it has not previously been reported to be associated with vacuum-packaged, cold-smoked fish products. High numbers of *Leuc. citreum* occurred in the nitrite-treated samples stored at 8°C. One reason for the low numbers of leuconostocs detected with phenotypical patterns during the first study (Lyhs et al., 1998) may be the bias caused by phenotypical testing. Most of the *Leuconostoc* isolates studied here grew on Rogosa selective *Lactobacillus* (SL) agar, resembling heterofermentative lactobacilli.

The second largest group, 39% of the isolates, associated with *Lb. sakei/curvatus* type strains. The occurrence of *Lb. sakei/curvatus* in vacuum-packaged fish products has also been reported previously. In a study of LAB from different, lightly

1 preserved, chilled fish products, Jeppesen and Huss (1993) identified most of the 37 isolates
2 from vacuum-packaged, chilled fish products as *Lb. sakei*. Studying cold-smoked, vacuum-
3 packaged salmon, Truelstrup Hansen (1995) identified from 168 LAB 10%, as *Lb. sakei* and
4 50% as *Lb. curvatus*. Leroi et al. (1998), phenotyping LAB isolated from the same type of
5 fish product, assigned 10 of the 155 isolates as *Lb. sakei*. However, there has also been a
6 report of a seafood study (Mauguin and Novel, 1994) where no *Lb. sakei* was detected. In the
7 present study, high numbers of *Lactobacillus sakei* subsp. *sakei/carnosum* occurred in the
8 nitrate- treated samples stored at both storage temperatures.

9 Current taxonomy of *Lb. sakei* and *Lb. curvatus* divides these two species into 4
10 subspecies (Klein et al., 1996; Torriani et al., 1996). The *Lb. curvatus* subsp. *curvatus* cluster
11 is clearly distinguished (Figs. 1 and 2) by both enzymes from the clusters IIIa, VIIIa and
12 VIIIb, containing the type strains of the three other subspecies. When meat-associated *Lb.*
13 *sakei* strains were characterised by *EcoRI* and *HindIII* ribotyping (Björkroth and Korkeala,
14 1996), strain A210, possessing 84% homology in DNA-DNA reassociation with *Lb. sakei*
15 subsp. *sakei* type strain (Mäkelä et al. 1992), gave exactly the same *EcoRI* and *HindIII*
16 ribotypes as the “new” *Lb. curvatus* subsp. *melibiosus* type strain. In our study, as well, the
17 *Lb. curvatus* subsp. *melibiosus* type strain clusters together with the two *Lb. sakei* subspecies.
18 On the basis of our reassociation data together with these RFLP clusters, it is debatable
19 whether *Lb. curvatus* subsp. *melibiosus* type strain is really a *Lb. curvatus* species. However,
20 the studies performed by Klein et al. (1996) and Torriani et al. (1996) showed different
21 reassociation values and warrant this. More studies must be performed by means of DNA-
22 DNA reassociation with a larger number of strains from this cluster in order to clarify the
23 status of our strains.

24 Only one isolate could be identified as *Lb. plantarum*, which is often found in
25 seafood in small amounts. Isolating LAB from different vacuum-packaged, lightly preserved,

1 chilled fish products, Jeppesen and Huss (1993), Mauguin and Novel (1994) and Truelstrup
2 Hansen (1995) identified 3, 4 and 14 strains as *Lb. plantarum* from a total of 7, 77 and 168
3 LAB isolates, respectively. Gancel et al. (1997) found 11 of the 78 lactic acid bacteria from
4 vacuum-packaged, smoked and salted herring to belong to the *Lb. plantarum* – *pentosus*-
5 group.

6 No *Carnobacterium* strains were detected in this study. Carnobacteria have
7 previously been found in vacuum-packaged ‘gravad’ fish (Leisner et al., 1994). Gancel et al.
8 (1997) did not find any carnobacteria in fillets of vacuum-packaged, smoked and salted
9 herring and proposed that this may be due to the fish having been smoked, a hypothesis might
10 also apply to our fish product.

11 In this study, ribotyping was quite useful for the identification of the main
12 spoilage LAB to species level. Only a few random isolates could not be identified with the aid
13 of the reference strains used. Using this technique, we were able to conclude that *Leuc.*
14 *mesenteroides* subsp. *mesenteroides*, *Leuc. citreum*, *Lb. sakei* and *Lb. curvatus* were the
15 major species associated with spoilage of this fish product.

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Table 1. Lactic acid bacteria reference strains

Species	Strain designation and source ¹
<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>	DSM 20343
<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i>	CCUG 21965 ^T
<i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i>	DSM 20484 ^T
<i>Leuconostoc pseudomesenteroides</i>	DSM 20193 ^T
<i>Leuconostoc gelidum</i>	NCFB 2775 ^T
<i>Leuconostoc carnosum</i>	NCFB 2776 ^T
<i>Leuconostoc citreum</i>	LMG 9824 ^T
<i>Weissella viridescens</i>	ATCC 12706 ^T
<i>Weissella halotolerans</i>	ATCC 35410 ^T
<i>Weissella hellenica</i>	LMG 15125 ^T
<i>Weissella kandleri</i>	LMG 14471 ^T
<i>Weissella paramesenteroides</i>	DSM 20288 ^T
<i>Carnobacterium pisciola</i>	ATCC 35586
<i>Carnobacterium divergens</i>	ATCC 35677
<i>Lactobacillus sakei</i> subsp. <i>sakei</i>	ATCC 15521
<i>Lactobacillus sakei</i> subsp. <i>carnosum</i>	CCUG 31331
<i>Lactobacillus curvatus</i> subsp. <i>curvatus</i>	ATCC 25061
<i>Lactobacillus curvatus</i> subsp. <i>melibiosus</i>	CCUG 34545
<i>Lactobacillus plantarum</i>	ATCC 14971

¹ATCC: American Type Culture Collection; BCCMTM/LMG: Belgian Coordinated Collection of Microorganisms; CCUG: Culture Collection of University of Gothenburg; DSM: Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); NCFB: National Collection of Food Bacteria.

Table 2. Number of isolates, REA and ribotypes originating from spoiled, vacuum-packaged, cold-smoked rainbow trout fillets by *HindIII* and *EcoRI* digestion

No. of isolates	REA type	Ribopattern		Ribotype
		<i>HindIII</i>	<i>EcoRI</i>	
84	R1	H1	E1	I
49	R2	H1	E1	I
1	R3	H1	E1	I
2	R4	H1	E1	I
94	R5	H2	E2	II
45	R6	H3	E3	III
11	R7	H3	E3	III
26	R8	H3	E3	III
1	R9	H3	E3	III
2	R10	H4	E4	IV
3	R11	H5a	E5a	V
31	R12	H5a	E5a	V
1	R13	H5a	E5b	V
10	R14	H5b	E5b	V
2	R15	H6	E6	VI
11	R16	H7	E7	VII
2	R17	H8	E8a	VIII
3	R18	H8	E8b	VIII
1	R19	H8	E8b	VIII
1	R20	H9	E9	IX
12	R21	H10	E10	X
7	R22	H11	E11	XI
3	R23	H12	E12	XII
2	R24	H13	E13	XIII
1	R25	H14	E14	XIV

Table 3. Distribution of the lactic acid bacteria spp. isolated from spoiled vacuum-packaged, cold-smoked rainbow trout fillets produced using different curing methods and stored at 4° C and 8° C

Curing method ^a	Storage temperature (°C)	Number of isolates	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>	<i>Leuconostoc citreum</i>	<i>Lactobacillus sakei</i> subsp. <i>sakei/carnosum</i>	<i>Lactobacillus curvatus</i> subsp. <i>curvatus/melibiosus</i>
NaCl	4	44	30 (66%) ^b	4 (9%)	6 (13%)	0
NaCl and KNO ₃	4	91	43 (47%)	0	43 (47%)	2 (2%)
NaCl and NaNO ₂	4	77	38 (49%)	4 (5%)	25 (32%)	2 (2%)
NaCl ^a	8	45	12 (27%)	19 (42%)	3 (7%)	1 (2%)
NaCl and KNO ₃	8	61	4 (6%)	4 (6%)	41 (67%)	2 (2%)
NaCl and NaNO ₂	8	87	8 (9%)	63 (70%)	12 (14%)	1 (1%)

^a Concentration of NaCl: 2.2%, KNO₃: 686 ppm and NaNO₂: 166 ppm.

^b Percentage of all strains in isolated different curing method at each storage temperature.

1 Fig. 1. Dendrogram based on HindIII ribopatterns. The similarity between all pairs was expressed by Dice coefficient correlation, and the unweighted
 2 pair-group method with arithmetic averages (UPGMA) was used for the construction of the dendrogram.
 3
 4
 5
 6

^a Number of isolates

1 Fig. 2. Dendrogram based on EcoRI ribopatterns. The similarity between all pairs was expressed by Dice coefficient correlation, and the
 2 unweighted pair-group method with arithmetic averages (UPGMA) was used for the construction of the dendrogram.
 3
 4
 5
 6

^a Number of isolates