Identification of lactic acid bacteria from spoiled, vacuum-packaged ‘gravad‘ rainbow 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 3ºC and 8ºC were characterised and identified using a molecular approach. The isolates were initially grouped according to their *Hin*dIII 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 3ºC. The relative proportion of *L. sakei* was higher in the samples stored at 8ºC.

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 ≤ 3ºC.

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 identifiaction 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

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

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

2. Material and methods

2.1. Bacterial strains

A total of 296 bacterial strains originating from spoiled, vacuum-packaged 'gravad' rainbow trout slices stored at 3ºC or 8ºC (Lyhs et al., 2000) were characterised. Determination of the spoilage had been based on both sensorial and microbiological analyses. At the time of spoilage the levels of LAB had been from 10^4-10^6 cfu/g and 10^5-10^7 cfu/g in the products stored 3ºC or 8ºC, respectively. The strains were considered as LAB since they all grew on MRS agar (Oxoid, Basingstoke, United Kingdom) and were Gram-positive and catalase-negative. Totals of 128 and 168 isolates originating from samples stored at 3ºC and 8ºC, respectively, were studied. All strains were stored at -70ºC in MRS broth (Difco, Detroit, Michigan, USA). Before use, they were subcultured overnight in 10ml MRS broth (Difco) at 25ºC and then plated on MRS agar (Oxoid). The plates were incubated anaerobically at 25ºC for 5 days in an anaerobic jar with a H_2+CO_2 generating kit (Oxoid).

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 (1996a) with a 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 *Hin*dIII (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 (1996a).

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

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 using the Gelcompar II 1.0 software package (Applied Maths, Kortrijk, Belgium), as recommended by the manufacturer. Based on internal controls, 1.8% position tolerance was allowed for the bands. 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. The 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

*Hin*dIII 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 3ºC and 8ºC. Most of the *L. curvatus* subsp. *curvatus* strains were found in the samples stored at 8ºC. *L. sakei* subsp. *sakei*/*carnosum* strains occurred mainly in the samples stored at 8ºC whereas most of the carnobacterial strains were found in the samples stored at 3ºC.

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

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

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

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

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

References

Baya, A.M., Toranzo, A.E., Lupiani, B., Li, T., Roberson, B.S., Hetrick, F.M., 1991. Biochemical and serological characterization of *Carnobacterium* spp. isolated from farmed and natural populations of stripped bass and catfish. Appl. Environ. Microbiol. 57, 3114-3120.

Björkroth, J., Korkeala H., 1996a. Evaluation of *Lactobacillus sake* contamination in vacuum-packaged sliced cooked meat products by ribotyping. J. Food Prot. 59, 398-401.

Björkroth, J., Korkeala H., 1996b. rRNA gene restriction patterns as a characterization tool for *Lactobacillus sake* strains producing ropy slime. Int. J. Food Microbiol. 30, 293-302.

Björkroth, J., Korkeala, H., 1997. Characterization of *Lactobacillus fructivorans* spoilage in ketchup. J. Food Prot. 60, 505-509.

Björkroth, J., Vandamme P., Korkeala, H., 1998. Identification and characterisation of *Leuconostoc carnosum*, associaoted with production and spoilage of vacuum-packaged, sliced, cooked ham. Appl. Environm. Bacteriol. 64, 3313-3319.

Björkroth, J., Geisen, R., Schillinger, U., Weiss, N., De Vos, P., Holzapfel, W.H., Korkeala, H., Vandamme, P., 2000. Characterization of *Leuconostoc gasicomitatum* sp. nov. associated with spoiled raw tomato-marinated broiler meat strips packaged under modified atmosphere conditions. Appl. Environ. Microbiol. 66, 3764-3772.

Civera, T., Parisi, E., Amerio, G.P., Giaccone, V., 1995. Shelf-life of vacuum-packed smoked salmon: microbiological and chemical changes during storage. Arch. Lebensmittelhyg. 46, 1-24.

Dykes, G.A., von Holy, A., 1994. Taxonomic status of atypical *Lactobacillus sake* and *Lactobacillus curvatus* strains associated with vacuum-packed meat spoilage. Curr. Microbiol. 28, 197-200.

Falsen, E., 1999. Personal communication, internet address http://www.ccug.gu.se (2001-05-15)

Gancel, F., Dzierszinski, F., Tailliez, R., 1997. Identification and characterization of *Lactobacillus* spp. isolated from fillets of vacuum-packed smoked and salted herring (*Clupea Hargenus*). J. Appl. Microbiol. 82, 722-728.

Hiu, S.F., Holt, R.A., Siranganathan, N., Seidler, R.J., Fryer, J. L., 1984. *Lactobacillus piscicola*, a new species from salmonid fish. Int. J. System. Bacteriol. 34, 393-400.

Holzapfel, W.H., Gerber, E.S., 1986. Predominance of *Lactobacillus curvatus* and *Lactobacillus sake* in the spoilage association of vacuum-packaged meat products. In: Abstracts of the 32nd European Meeting of Meat Research Workers, Ghent. p 26.

Jeppesen, V.T., Huss, H.H., 1993. Characteristic and antagonistic activity of lactic acid bacteria isolated from chilled fish products. Int. J. Food Microbiol. 18, 305-320.

Knøchel, S., 1983. Fermented fish products in Scandinavia. Kor. J. Appl. Microbiol. Bioeng. 11, 347-351.

Krüger, K.-E., 1973. Hygienische Probleme bei der Fischwarenherstellung. Feinkostwirtsch. 10, 186-190.

Leisner, J.J., 1992. Characterisation of lactic acid bacteria isolated from lightly preserved fish products and their ability to metabolise various carbohydrates and

amino acids. Ph.D.Thesis. Royal Veterinary and Agricultural University. Denmark.

Leisner, J.J., Millan, J.C., Huss, H.H., Larsen, C.M., 1994. Production of histamine and tyramine by lactic acid bacteria isolated from vacuum-packaged sugar-salted fish. J. Appl. Bacteriol. 76, 417-423.

Leroi, F. Joffraud, J., Chevalier, F., Cardinal, M., 1998. Study of the microbial ecology of cold-smoked salmon during storage at 8ºC. Int. J. Food Microbiol. 39, 111-121.

Leroi, F. Joffraud, J., Chevalier, F., 2000. Effect of salt and smoke on the microbiological quality of cold-smoked salmon during storage at 5ºC as estimated by the factorial design method. J. Food Prot. 63, 502-508.

Lücke, K.F., 1996. Indigenous lactic acid bacteria of various commodities and factors affecting their growth and survival. In: Bozoglu, T. F. and Ray, B. (Eds.), Lactic acid bacteria: Current advantages in metabolism, genetic and applications. NATO ASI Series, vol. H 98, Springer-Verlag Berlin, p. 262.

Lyhs, U., Björkroth, J., Hyytiä E., Korkeala, H., 1998. The spoilage flora of vacuum-packaged, sodium nitrite or potassium nitrate treated, cold-smoked rainbow trout stored at 4°C or 8°C. Int. J. Food Microbiol. 45, 135-142.

Lyhs, U., Björkroth, J., Korkeala, H., 1999. Characterisation of lactic acid bacteria from spoiled, vacuum-packaged, cold-smoked rainbow trout using ribotyping. Int. J. Food Microbiol. 52, 77-84.

Lyhs, U., Lahtinen, J., Fredriksson-Ahomaa, M., Hyytiä-Trees, E., Elfing, K., Korkeala, H., 2001. Microbiological quality and shelf-life of vacuum-packaged ’gravad’ rainbow trout stored at 3°C and 8°C. Int. J. Food Microbiol. Submitted.

Lyhs, U., Korkeala, H., Vandamme, P., Björkroth, J., 2001. *Lactobacillus alimentarius* – a specific spoilage organism in marinated herring. Int. J. Food Microbiol. 64, 355-360.

Mäkelä, P., Korkeala, H., 1987. *Lactobacillus* contamination of cooked ring sausages at sausage processing plants. Int. J. Food Microbiol. 5, 323-330.

Mäkelä, P.M., Schillinger, U., Korkeala, H., Holzapfel, W.H., 1992. Classification of ropy slime-producing lactic acid bacteria based on DNA-DNA homology, and identification of *Lactobacillus sakei* and *Leuconostoc amelibiosum* as dominant spoilage organisms in meat products. Int. J. Food Microbiol. 16, 167-172.

Paludan-Müller, C., Dalgaard, P., Huss, H.H., Gram, L., 1998. Evaluation of the role of *Carnobacterium piscicola* in spoilage of vacuum- and modified-atmosphere-packed cold-smoked salmon at 5ºC. Int. J. Food Microbiol. 39, 155-166.

Pitcher, D.G., Saunders, N.A., Owen, R.J., 1989. Rapid extraction of bacteria genomic DNA with guanidium thiocynate. Lett. Appl. Microbiol. 8, 151-156.

Ringo, E., Bendiksen, H.R., Wesmajervi, M.S, Olsen, R.E:, Jansen, P.A., Mikkelsen, H., 2000. Lactic acid bacteria associated with the digestive tract of Attlantic salmon (*Salmon salar L.*). J. Appl. Microbiol. 89, 317-322.

Schillinger, U., Lücke, K.F., 1986. Milchsäurebakterien-Flora aus vakuumverpacktem Fleisch und ihr Einfluss auf die Haltbarkeit. Fleischwirtsch. 66, 1515-1520.

Schillinger, U., Lücke, K.F., 1987. Identification of lactobacilli from meat and meat products. Food Microbiol. 4, 199-208.

Starliper, C. E., Shotts, E. B., Brown, J., 1992. Isolation of *Carnobacterium piscicola* and an unidentified Gram-positive *Bacillus* from sexually mature and post-spawning rainbow trout *Oncorhynchus mykiss*. Diseases Aquatic Organisms 13, 181-187.

Stiles, M.E., Holzapfel, W.H., 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36, 1-29.

Stoffels, G., Nes, I. F., Gudmundsdottir, A., 1992. Isolation and properties of a bacteriocin-producing *Carnobacterium piscicola* isolated from fish. J. Appl.Bacteriol. 73, 309-316.

Truelstrup Hansen L., 1995. Quality of chilled, vacuum-packed cold-smoked salmon. Ph.D.Thesis. Danish Institute of Fisheries Research, Department of Seafood Research, Technical University, Denmark.

Table 1

Number of isolates, types obtained by *Hin*dIII restriction endonuclease analysis and ribotypes of LAB from spoiled, vacuum-packaged 'gravad' rainbow trout slices stored at 3ºC and 8ºC

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

Table 2

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

Storage temperature (°C)	No. of isolates	*Lactobacillus curvatus* subsp. *curvatus*	*Lactobacillus curvatus* subsp. *melibiosus*[a]	*Lactobacillus sakei* subsp. *sakei*	*Lactobacillus sakei* subsp. *carnosum*	*Carnobacterium piscicola*	*Carnobacterium divergens*
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

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

[b] Percentage of all strains included in the study at each storage temperature.

Legend to the figure:

Fig. 1. Dendrogram and schematic banding patterns based on *Hin*dIII ribopatterns. H1 to H11 represent restriction endonuclease analysis based groups of lactic acid bacterium isolates from spoiled, vacuum-packaged 'gravad' rainbow trout slices stored at 3°C or 8°C.