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The spoilage flora of vacuum-packaged, sodium nitrite or potassium nitrate treated, cold-smoked rainbow trout stored at 4 °C or 8 °C

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Abstract

The spoilage flora 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, was studied. Of 620 isolates, lactic acid bacteria were the major fraction (76%), predominating in all samples of spoiled product. However, the phenotypical tests used were insufficient to identify the lactic acid bacteria to the species level. Gram-positive, catalase-positive cocci, Gram-negative, oxidase-negative rods and Gram-negative, oxidase-positive rods were found in 6%, 16% and 2% of the samples, respectively. Of 39 Gram-positive, catalase-positive cocci, 29 were identified as staphylococci and 10 as micrococci. Eighty-five isolates were found to belong to the family *Enterobacteriaceae*, with 45 of those being *Serratia plymuthica*. Eleven isolates from the nitrate treated samples stored at 8°C were identified as *Pseudomonas aeruginosa*. The occurrence of *P. aeruginosa* and staphylococci in the nitrate-containing samples, stored at 8°C, may cause problems with respect to the safety of the product. The types of lactic acid and other bacteria in the spoilage flora were generally reduced by the addition of nitrate or nitrite to fillets.

Keywords: Fish; Spoilage; Vacuum-packaging; Cold-smoking; Nitrite; Nitrate; NaCl;

1. Introduction

Vacuum-packaged, cold-smoked fish products are highly perishable foods. Previous studies with cold-smoked salmon have shown that increasing the salt concentration and decreasing the storage temperature extend their storage life (Hildebrandt and Erol, 1988; Civera et al., 1995; Truelstrup Hansen et al., 1995). During their storage at chill temperatures, a complex microflora develops which is dominating by lactic acid bacteria at the end of the storage period along with lower numbers of other bacteria like *Enterobacteriaceae*, *Pseudomonas* spp., enterococci, micrococci and yeasts (Magnússon and Traustadóttir, 1982; Schneider and Hildebrandt, 1984; Shimasaki et al., 1994; Civera et al., 1995). There was variation in the composition of the microflora described by the authors, probably due to the different processes applied and differing smokehouse production environment (Truelstrup Hansen, 1995). However, only limited work has been carried out about the exact composition and characterisation of the lactic acid bacteria and other bacteria as part of the spoilage flora of vacuum-packaged, cold-smoked fish products.

Nitrate (NO_3) has been added to the curing salt mixture of certain semi-preserved pickled fish products in order to delay spoilage and to control microbial activity during storage (Pedersen and Meyland, 1981; Knøchel and Huss, 1984). It may also act as a reservoir of nitrite if nitrate-reducing bacteria are present (Skovgaard, 1992). Nitrite is an important antimicrobial agent. It has shown to have an inhibitory effect on bacterial spoilage and *Clostridium botulinum* growth and toxin production also in fish (Sofos et al., 1979; Pierson and Smoot, 1987; Hyytiä et al., 1997). Combinations of sodium chloride (NaCl) and sodium nitrite ($NaNO_2$) or potassium nitrate (KNO_3) have been used as a preservative in hot-smoked fish products (Pelroy et al., 1982) and cold-smoked rainbow trout (Hyytiä et al., 1997).

However, no reports been published about the changes in the bacterial groups causing spoilage after adding of nitrate or nitrite to cold-smoked fish product.

As the use of nitrate or nitrite in cold-smoked fish might be advantageous, this study was undertaken to characterise the spoilage flora of vacuum-packaged, cold-smoked rainbow trout stored at $4^{O}C$ or $8^{O}C$ and to determine the effect of nitrate and nitrite on the spoilage flora of the product.

2. Materials and methods

2.1. Samples

Rainbow trout (*Oncorhyncus mykiss*) from two Finnish fish farms was used. Before brining, the trout were deheaded and filleted at a processing plant. The fillets had an average weight of 600-900 g. Brining was carried out by the injection method. The pressure used in the brine injection equipment (Fomaco 44/176, Fomaco Food Machinery Company A/S, Køge, Denmark) was 1.6 bar. The brine concentration was 21%, producing a NaCl concentration of 2.2% (w/w) in the final product. The $NaNO_2$ and KNO_3 (Riedel-deHaën AG, Seelze, Germany) concentrations of the curing solutions were 3 g/l and 13 g/l respectively, producing nitrite and nitrate concentrations of 166 ppm and 686 ppm in the product after preparation (Hyytiä et al., 1997). The fillets were cold-smoked overnight at 18-$21^{O}C$, at the processing plant, in an electronically controlled, electrially heated smoke house equipped with an external smoke generator (Alpas, Alpas GmbH, Bremen, Germany). After the smoking process, the fillets were vacuum-packaged, using a Multivac R 7000 1976 packaging machine (Multivac Verpackungsmaschinen, Wolfertschwenden,

Germany), in a polyethylene / polyamide film (Suomen Union Verpackungs Ltd, Helsinki, Finland) with an oxygen permeability of 29-45 ml O_2/m^2/ 24 h /atm (23^oC, 50% RH) and a water vapour permeability of 10-15 g/m^2/ 24 h (38^oC, 90% RH). Immediately after processing the samples were transported to the laboratory and stored at either 4^oC or 8^oC.

2.2. Sensory evaluation

Sensory evaluation was performed once a week in order to determine when the samples studied were spoiled. The sensory evaluation panel consisted of 9 or 10 trained panelists. The samples were evaluated for aroma and taste using the method described by Amerine et al. (1965) on a scale from zero to five, in which a score of two points or less indicated unacceptable product. The sample was deemed spoiled if at least two judges considered it unfit. The colonies were selected from samples which were deemed spoiled.

2.3. Microbiological analyses

Each 10 g cold-smoked, rainbow trout sample was homogenized with 90 ml of 0.1% (w/v) peptone water and 10-fold serial dilutions were used for microbiological analyses. The aerobic plate count (APC) was determined by the method of the Nordic Committee on Food Analysis (1986) using Plate Count Agar (Difco, Detroit, Michigan, USA). At least 10 colonies from the highest dilutions that yielded colonies were selected at random from the APC plates when the total bacteria count was > 10^7 cfu/g.

For samples stored at 4^{O}C, totals of 99, 110 or 100 isolates were obtained from the flora from samples containing NaCl only, NaCl and KNO_3 or NaCl and $NaNO_2$, respectively. For samples stored at 8^{O}C, totals of 104, 108 or 99 isolates were obtained from the flora from samples containing NaCl only, NaCl and KNO_3 or NaCl and $NaNO_2$, respectively.

All isolates were Gram-stained and were tested for haemolytic activity (Columbia agar base, GIBCO BRL, Paisley, UK). Also, all organisms were grown on brain heart infusion (BHI) agar (Difco) at 25^{O}C to test for catalase production (Baird-Parker, 1979).

2.4. Characterisation tests

Gram-positive, catalase-negative bacteria were tested for growth in de Man, Rogosa and Sharpe (MRS) broth (Difco) incubated at 25^{O}C for 3 days. Isolates showing growth were further plated on Rogosa selective *Lactobacillus* (SL) agar (Orion Diagnostica, Espoo, Finland). The plates were incubated anaerobically at 25^{O}C for 5 days using a model BR 38 gas-generating kit (Oxoid, Basingstoke, UK) in an anaerobic jar. Growth on Slanetz and Bartley agar (Orion Diagnostica) was observed after 2 days' incubation at 37^{O}C. Production of gas from glucose was studied by the method of Schillinger and Lücke (1987). Acetoin production was detected using the Voges-Proskauer test after 3 days incubation. Hydrolysis of arginine was examined as described by Reuter (1970). Lactic acid configuration was determined enzymatically using a UV method kit (Boehringer Mannheim GmbH, Mannheim, Germany), according to the manufacturer`s instructions. The presence of

m-DPA in the cell walls was determined by the two - dimensional - thin layer paper chromatography method of Harper and Davis (1979).

The Gram-positive, catalase-positive cocci were examined for acid production from glycerol in the presence of erythromycin (Schleifer and Kloos, 1975) and sensitivity to lysostaphin by the method of Kloos et al. (1974). For further identification, API Staph (bio Mérieux, Marcy l`Etoile, France) was used and the results were recorded after incubation at 25^{o}C for 24 - 48 h.

Gram-negative microorganisms were examined for oxidase production using Kovàcs reagent (Kovàcs, 1956). For further identification, API 20 E (bio Mérieux) and API 20 NE (bio Mérieux) were used and the results were recorded after incubation at 25^{o}C for 24 - 48 h.

Yeasts were isolated on Sabouraud-Dextrose-Medium (Oxoid) and Rose-Bengal-Medium (Difco) after 3 days' incubation at 25^{o}C.

3. Results

Of 620 isolates, 469 Gram-positive, catalase-negative cocci or rods, which grew on MRS were classified as lactic acid bacteria; 39 were Gram-positive, catalase-positive cocci; 98 were Gram-negative, oxidase-negative rods; and 12 isolates were Gram-negative, oxidase-positive rods (Table 1).

Lactic acid bacteria predominated in the spoilage flora of all samples. The isolated lactic acid bacteria were divisable into seven subgroups on the basis of cell morphology, the formation of gas from glucose, the growth on Rogosa selective *Lactobacillus* (SL) agar, the lactic acid isomers produced, the hydrolysis of arginine,

the production of acetoin and the presence of diaminopimelic acid in the cell walls (Table 2). The 189 isolates of subgroup 1 were homofermentative rods which grew well on SL agar. The second subgroup of 50 isolates were heterofermentative oval cocci which did not grow on SL agar. The three isolates forming subgroup 3 were heterofermentative rods with diaminopimelic acid in their cell walls. The 182 isolates in subgroup 4 were heterofermentative oval cocci that grew on SL agar. The 3 isolates in subgroup 5 were cocci which formed colonies with a red-pink centre on Slanetz and Bartley agar but did not grow on SL agar. They produced predominantly L(+) - lactic acid. They were isolated only from the samples without KNO_3 or $NaNO_2$ which were stored at 8^{o}C. Subgroup 6 contained heterofermentative and subgroup 7 homofermentative cocci or oval cocci. Table 3 presents the distributions of the lactic acid bacteria subgroups in the differently cured samples at either storage temperature.

Based on the production of acid from glycerol in the presence of erythromycin and lysostaphin sensitivity, 29 of the 39 Gram-positive, catalase-positive cocci were identified as staphylococci and 10 as micrococci. From the API Staph tests, most of the staphycoccal isolates from the Gram-positive, catalase-positive cocci were classifiable as *Staphylococcus epidermidis* or *Staphylococcus hominis*. Other isolates were *Staphylococcus sciuri*, *Staphylococcus capitis*, *Staphylococcus warneri*, *Staphylococcus intermedius* or *Staphylococcus lentus*. Three of the ten micrococcal isolates were identified as *Micrococcus kristinae*. Micrococci were mainly isolated from the nitrate-containing samples stored at 4^{o}C. Nine staphylococci originated from the nitrate-containing samples stored at 8^{o}C.

The API 20 E tests placed 85 of the Gram-negative, oxidase-negative rods in the family *Enterobacteriaceae*, 9 in the genus *Xanthomonas* and one in the genus *Acinetobacter*. Most of the *Enterobacteriaceae* were identified as *Serratia*

plymuthica, *Serratia liquefaciens*, *Hafnia alvei* or *Enterobacter* spp. (Table 4). Of the 45 *S. plymuthica* isolates, 32 originated from the samples containing NaCl only and stored at 4°C. Of the nine *H. alvei*, six were recovered from the nitrate-containing samples and two from the samples which contained NaCl only and were stored at 8°C. All eight *Enterobacter amnigenus* isolates were isolated from nitrate-containing samples stored at 8°C and all five *Enterobacter sakazakii* isolates from the samples which contained NaCl only and which were stored at 8°C.

From the API 20 NE tests 11 of the Gram-negative, oxidase-positive rods were identified as *Pseudomonas aeruginosa*. They originated from the nitrate-containing samples stored at 8°C. The remaining one isolate was identified as *Ochrobacterium* and it originated from a nitrite-containing sample which was stored at 4°C. The isolates originating from the samples which contained NaCl only did not include Gram-negative, oxidase-positive rods.

Two colonies obtained from nitrite-containing samples which were stored at 8°C grew both on Sabouraud-Dextrose-Medium and Rose-Bengal-Medium.

4. Discussion

The spoilage flora in all samples mainly consisted of lactic acid bacteria. The dominance of lactic acid bacteria in vacuum-packaged, lightly preserved fish products after a few weeks' storage at chilled temperatures has been reported previously. Magnússon and Traustadóttir (1982) found lactic acid bacteria dominating in vacuum-packaged cold-smoked herring, as did Schneider and Hildebrandt (1984), Hildebrandt and Erol (1988), Shimasaki et al. (1994), Truelstrup Hansen (1995) and

Civera et al. (1995) in vacuum-packaged cold-smoked salmon and Jeppesen and Huss (1993) in vacuum-packaged sugar-salted ('gravad') fish.

The relative proportion of lactic acid bacteria in microflora was higher in the nitrite- and nitrate-containing samples than in the samples which contained NaCl only at both storage temperatures. Lactic acid bacteria have previously been reported to be resistant to nitrite (Doods and Collins-Thompson, 1984; Skovgaard, 1992). Their nitrite resistance may explain the high proportion of lactic acid bacteria found in the nitrite-containing samples in this study.

Based on the characteristics of subgroup 1 of lactic acid bacteria, these bacteria could be considered as homofermentative or facultatively heterofermentative lactobacilli. The occurrence of lactobacilli with homofermentative glucose metabolism has also been reported by Magnússon and Traustadóttir (1982) in vacuum-packaged cold-smoked herring fillets stored for 12 weeks at chill temperatures. In the present study, their proportion in the nitrate-containing samples was higher than in the nitrite-containing samples and in the samples which contained NaCl only (Table 3).

The isolates in subgroup 2 could be considered to belong to *Leuconostoc/Weissella*-species. The dominance of leuconostocs has been reported previously. Jeppesen and Huss (1993) studied the lactic acid bacteria from vacuum-packaged, minced herring and identified all isolated lactic acid bacteria as *Leuconostoc* spp. However, Mauguin and Novel (1994) found that only eight out of 86 lactic acid bacteria isolated from various samples of seafood belonged to the genus *Leuconostoc*. In the present study, high numbers of heterofermentative lactobacilli and *Leuconostoc* spp. occurred in the nitrite treated samples stored at 8^{o}C (Table 3).

The bacteria in subgroup 3 posessing m-DPA in their cell walls appeared to belong to the genus *Carnobacterium*. Carnobacteria have earlier been found in vacuum-packaged 'gravad' fish (Leisner et al., 1994) and some other vacuum-packaged fish products (Mauguin and Novel, 1994). However, Gancel et al. (1997) did not find any carnobacteria in fillets of vacuum-packaged smoked and salted herring and proposed smoking the fish to be the reason.

The other lactic acid bacterium groups formed could not be identified to the species level by the phenotypical methods used. Subgroup 4, forming the second largest group, could be classified as leuconostocs because of their cell morphology, oval cocci, and their formation of gas from glucose. On the other hand, they grew on SL agar as do heterofermentative lactobacilli. Most of them were found in the nitrite-containing samples stored at either 4^{o}C or 8^{o}C.

The three isolates in subgroup 5 seemed to belong to the genus *Enterococcus*. The occurrence of enterococci in vacuum-packaged cold-smoked salmon has also been reported previously (Schneider and Hildebrandt, 1984; Hildebrandt and Erol, 1988). Ben Embarek et al. (1994) isolated enterococci during studies of bacterial survivors in *sous-vide* cooked fish fillets. The fractions of lactic acid bacteria in subgroups 6 and 7 decreased after the addition of nitrite and nitrate, indicating possible sensitivity to this kind of treatment. The phenotypical tests were insufficient to characterise accurately the dominant lactic acid genera of these bacterial groups. Species level identification of these above named bacterial groups warrants further analysis such as genotyping.

The species identification of *Enterobacteriaceae* in this study generally agrees with the results of Truelstrup Hansen (1995), who studied spoiled vacuum-packaged cold-smoked salmon. However, there are no previous reports about the high

prevalence of *S. plymuthica* in fish products. This can be due to the fact that the fish of the present study were originated in farms located in brackish water. *S. plymuthica* strains isolated from water have been isolated frequently from fresh water (Nieto, et al., 1990).

Micrococci were mainly isolated from the nitrate treated samples stored at 4^{o}C. It is possible that nitrate might facilitate the growth of these bacteria. Micrococci, as strict aerobic organisms, are presumed to use nitrate as an alternative electron acceptor to oxygen under vacuum (Taylor and Shaw, 1975). The highest prevalence of staphylococci was deteced in the nitrate treated samples stored at 8^{o}C. The anaerobic respiration of nitrate appears to be widespread among facultatively anaerobic bacteria, such as staphylococci (Doelle, 1975). This may explain why staphylococci were found in the nitrate treated samples. Of the nitrate treated samples stored at 8°C 11 isolates, were classified as *P. aeruginosa.* Since it is well known, that *P. aeruginosa* can utilize nitrate as an electron acceptor it is able to grow under anaerobic conditions if nitrate is present (Yamanaka et al., 1959*).* No *Pseudomonas* spp. were isolated from any of the samples containing NaCl only, indicating the attribution of vacuum packaging and sensitivity of *Pseudomonas* spp. to low oxygen and high carbon dioxide levels (Clark and Takacs, 1980; Flick et al., 1991). Therefore, the growth of *P. aeruginosa* may cause food hygienie problems in vacuum-packed fish products treated with nitrate in the case of temperature abuse.

The composition of the spoilage flora was found to be affected by the nitrate and nitrite treatment. Insensitivity to nitrate and nitrite, favoured by the anaerobic conditions, resulted in lactic acid bacteria to constitute the major proportion of the total flora in the nitrate- and nitrite-containing samples. However, the types of lactic acid and other bacteria in the spoilage flora were generally reduced by the

addition of nitrate or nitrite to the product. The occurrence of *P. aeruginosa* and staphylococci in the nitrate-containing samples stored at 8°C may cause problems with respect to the safety of the product. Therefore, nitrate is not recommended as a preservative in this type of fish product and the maintenance of the chill chain under the temperature of 4°C should be ensured.

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Table 1. The four main bacterial groups obtained from spoiled vacuum-packed cold-smoked rainbow trout fillets produced using different curing methods and stored at 4^{o} C and 8^{o} C

Curing method [a]	Storage temperature (°C)	Number of colonies isolated	Microbial group				
			Lactic acid bacteria	Gram-positive, catalase-positive cocci	Gram-negative, oxidase-negative rods	Gram-negative, oxidase-positive rods	Yeast
NaCl	4	99	50 (50%)[b]	12 (12%)	37 (37%)	0	0
NaCl and KNO_3	4	110	94 (85%)	9 (9%)	7 (6%)	0	0
NaCl and $NaNO_2$	4	100	79 (79%)	1 (1%)	19 (19%)	1 (1%)	0
NaCl	8	104	81 (80%)	5 (5%)	18 (17%)	0	0

NaCl and KNO_3	8	108	68 (63%)	12 (11%)	17 (16%)	11 (10%)	0
NaCl and $NaNO_2$	8	99	97 (97%)	0	0	0	2 (2%)

[a] Concentration of NaCl: 2.2%, KNO_3: 686 ppm and $NaNO_2$: 166 ppm.
[b] Proportion of strains isolated from samples with specified curing method and storage temperature.

Table 2. Characterisation of lactic acid bacteria isolated from spoiled vacuum-packed cold-smoked rainbow trout fillets produced using different curing methods and stored at 4^{o} C and 8^{o} C

Characteristic	Subgroups and number of isolates in parenthesis						
	1 (189)	2 (50)	3 (3)	4 (182)	5 (3)	6 (17)	7 (25)
Morphology	Rods	Oval cocci	Rods	Oval cocci	Cocci	Cocci or oval cocci	Cocci or oval cocci
Production of gas from glucose	-	+	+	+	-	+	-
Growth on SL agar	+	-	-	+	-	-	-
Lactic acid isomere [a]	DL /D(L)[b]	D(L)	D	DL /D(L)	L	DL	DL /L(D)
Hydrolysis of arginine	+/-[c]	-	+	+/-	+/-	+/-	+/-

Production of acetoin	-	-	+	-	+/-	+/-	+/-
m-DPA [a]	+/-	-	+	-	-	-	-

[a] Ten or all strains analysed from each group.
[b] Parenthesized isomers indicate < 15% of total lactic acid.
[c] +/- : positive or negative reactions.

Table 3. Distribution of the lactic acid bacteria isolated from spoiled vacuum-packed cold-smoked rainbow trout fillets produced using different curing methods and stored at 4^{o} C and 8^{o} C

Subgroup of lactic acid bacteria	No. of isolates	Storage temperature (°C)					
		4			8		
		NaCl[a]	NaCl and KNO_3[a]	NaCl and $NaNO_2$[a]	NaCl[a]	NaCl and KNO_3[a]	NaCl and $NaNO_2$[a]
1	189	8 (2%)[b]	56 (12%)	37 (8%)	9 (2%)	59 (12%)	20 (4%)
2	50	11 (2%)	2 (0.5%)	1 (0.5%)	8 (2%)	2 (0.5%)	26 (5%)
3	3	0	0	1 (0.5%)	1 (0.5%)	1 (0.5%)	0
4	182	27 (6%)	36 (7%)	40 (9%)	24 (5%)	5 (1%)	50 (11%)
5	3	0	0	0	3 (1%)	0	0
6	17	1 (0.5%)	0	1(0.5%)	13 (3%)	1 (0.5%)	1(0.5%)
7	25	3 (1%)	0	0	22 (5%)	0	0

[a] Concentration of NaCl: 2.2%, KNO_3: 686 ppm and $NaNO_2$: 166 ppm.
[b] Percentage of all strains in isolated different curing method at each storage temperature.

Table 4. Distribution of the Gram-negative, oxidase-negative rods isolated from spoiled vacuum-packed cold-smoked rainbow trout fillets produced using different curing methods and stored at 4^{o} C and 8^{o} C

Microorganisms	No. of isolates	Storage temperature (°C)					
		4			8		
		NaCl [a]	NaCl and KNO_3 [a]	NaCl and $NaNO_2$ [a]	NaCl [a]	NaCl and KNO_3 [a]	NaCl and $NaNO_2$ [a]
Serratia plymuthica	42	32	3	0	5	2	0
Hafnia alvei	9	0	0	1	2	6	0
Enterobacter amnigenus	8	0	0	0	0	8	0
Xanthomonas maltophilia	9	0	0	9	0	0	0
Serratia liquefaciens	7	3	0	0	4	0	0
Enterobacter sakazakii	5	0	0	0	5	0	0
Morganelli morganii	4	0	0	4	0	0	0
Rahnella aquatilis	4	0	3	0	0	1	0
Citrobacter freundii	3	0	0	3	0	0	0
Serratia odofera	1	0	0	0	1	0	0
Enterobacter agglomerans	1	1	0	0	0	0	0
Escherichia fergusonii	1	0	0	0	1	0	0
Erwinia sp.	1	1	0	0	0	0	0
Acinetobacter sp.	1	0	0	1	0	0	0

Unidentified	2	0	1	1	0	0	0

[a] Concentration of NaCl: 2.2%, KNO_3: 686 ppm and $NaNO_2$: 166 ppm.