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Origin and spoilage potential of the microbiota dominating genus *Psychrobacter* in sterile rehydrated salt-cured and dried salt-cured cod (*Gadus morhua*)

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Abstract

Salt-cured and dried salt-cured cod rehydrated using sterile water and equipment have a short shelf life at 4 °C due to high bacterial counts. The microbiota develops off-odours which partly can be described as musty, causing sensory rejection within 7–10 days of chilled storage. The microbiota composition was studied in a total of 38 samples obtained from 10 different, both commercial and laboratory produced, salt-cured and dried salt-cured cod products. The dominating bacterium, representing at least 90% of the total viable count in all products studied, was identified as belonging to the genus *Psychrobacter*; a Gram-negative, oxidase- and catalase-positive, nonpigmented, halotolerant, psychrotolerant, facultative aerobe and nonmotile bacterium. The morphology of the bacterium resembles coccobacilli and the cells occur most often in pairs. The bacterium was able to hydrolyze lipids, but not proteins. It did not produce H_2S or TMA and the spoilage in rehydrated salt-cured and dried salt-cured cod is therefor different from what is observed in fresh cod. However, samples inoculated with *Psychrobacter immobilis* gave the same musty odour as spoiled control samples but earlier in the storage period and of a stronger intensity. In a field experiment, carried out to investigate the origin of the dominating bacterium, it was found that the microbiota in both sterile rehydrated commercially produced and laboratory (aseptically) produced salt-cured cod was dominated by this same bacterium. The bacterium was also isolated from cod skin mucus immediately after capture. The bacterium survived NaCl concentrations up to 25% (w/v) NaCl, stating its ability to survive during the salt-curing process. The dominating bacterium in rehydrated salt-cured and dried salt-cured cod seems to mainly originate from the fresh fish itself and not from contamination during processing.
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Keywords: Rehydrated salt-cured and dried salt-cured cod; Bacalao; *Psychrobacter*; Origin; Spoilage potential

1. Introduction

Salt-cured or heavily salted cod (*Gadus morhua*) is a highly appreciated product due to the high nutritional value and long shelf life as well as its taste. The Basques were the first to introduce salt-cured cod in Europe and by year 1000 A.D. they had developed an

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international trade with the product. Today, it is an important product from the North Atlantic fisheries and primarily consumed in the Latin American and the Mediterranean countries under the name bachalau or bacalao. Salt-cured cod is produced from thoroughly washed, split or filleted, fresh or thawed cod. The fish are most often pickle salted or brine cured for a week and then salt ripened by kench curing in stacks (dry-salting) for at least 10 days. This product has a salt and water content of approximately 20% (w/w) and 55% (w/w), respectively. The water content can be further reduced by drying and when it becomes less than 50%, so-called dried salt-cured cod (klipfish) is obtained.

Due to the high salt concentration in the fish muscle, salt-cured and dried salt-cured cod must be rehydrated for at least 24 h before consumption. This process has traditionally been carried out in the households, but the time consuming rehydration process harmonises poorly with modern "fast food" consumer trends. Accordingly, "ready to use" products of rehydrated salt-cured and dried salt-cured cod have been developed and sold both as frozen or chilled products. However, the shelf life of chilled products is very short due to a rapid growing microbiota (Skjerdal et al., 1997).

Until recently, reddening ("pink") caused by extremely halophilic Archaea (Pétursson, 1933; Bjarnason, 1986) and brown spots ("dun") caused by extremely xerophilic moulds (Farlow, 1880; Beatty and Fougère, 1957) have been considered the only microbes growing in salt-cured and dried salt-cured cod. However, moderate halophilic Gram-negative rods in relatively high numbers and several Gram-positive bacteria have also been isolated from cod in the early stages of salt curing and from salt-cured and dried salt-cured cod (Vilhelmsson et al., 1996, 1997; Skjerdal et al., 1997).

Commercial rehydration of salt-cured and dried salt-cured cod is a new process, and as far as known, limited work has been carried out on the microbiota in rehydrated products (Skjerdal et al., 1997; Vilhelmsson et al., 1996). After rehydration, the conditions for bacterial growth are favourable, due to a high water content (approximately 70% w/w) and a low salt concentration (2–4% w/w NaCl) in the product. In the present work, the overall dominating genus of the microbiota growing in 38 sterile rehydrated product samples of salt-cured and dried salt-cured cod has been characterized. The origin has also been investigated in a field experiment by processing freshly caught cod to rehydrated salt-cured cod under aseptic conditions. In addition, the spoilage potential of the bacterium has been investigated.

2. Materials and methods

2.1. Raw materials

Salt-cured and dried salt-cured cod products were obtained from five different commercial sources, three in Norway and two in Spain and Portugal (Table 1). One salt-cured and one dried salt-cured product made in the laboratory at the Norwegian Institute of Fisheries and Aquaculture Research were included in the study. In total, 38 samples of commercial or laboratory produced salt-cured and dried salt-cured cod products were analysed. In two experiments, six

Table 1
Salt-cured and dried salt-cured product characteristics and number of products analysed

	Type of product	Producer	Number of products
A	dried salt-cured cod	Andreas Bjørge	4
B	salt-cured cod	Andreas Bjørge	4
C	salted fillet of cod packed in traditional cardboard box	West Fish	4
D	salted fillet of cod packed in sealed plastic box		4
E	salted fillet of cod packed in sealed plastic box with M[a]P		4
F	salt-cured cod	Tromvik Fiskeindustri	3
G	salt-cured cod (field experiment)	Fiskeriforskning	6
H	dried salt-cured cod	Fiskeriforskning	3
I	dried salt-cured cod	Unknown, Lisbon, Portugal	3
J	salted fillet of cod	Unknown, Valencia, Spain	3

Products A–E are obtained from producers of salt-cured and dried salt-cured cod situated on the west coast of Norway and products F–G are produced in Northern Norway. Product G and H are produced at our institute while I and J were obtained from supermarkets in Portugal and Spain.

[a] Modified atmosphere packaging (30-50% CO_2).

cod, three in April 1998 (seawater temperature–salinity: 4 to 6 °C–3.5%) and three in August 1998 (seawater temperature–salinity: 12 to 14 °C–3.5%), were caught in coastal waters close to Ålesund on the west coast of Norway by a hand line. After stunning the fish with a blow to the head immediately after coming out of the water and before the body of the fish had been in contact with any objects in the boat, skin mucus samples (approximately 200 μl) were obtained by carefully scraping the skin surface 10 cm along the lateral line with a sterile scalpel. The samples were immediately after transferred to 1.0 ml sterile tubes for microbiological analysis. Glycerol (20%) was included when the mucus samples were frozen at − 80 °C. The fish were then eviscerated, split with a sterile knife, washed with sterile water and processed to salt-cured cod under aseptic conditions. The fish were pickle salted for 2 days at 4 °C in a clean, lid-covered plastic box previously washed with 70% ethanol. After 2 days, the fish were cut into smaller pieces (3×4 cm) with sterile equipment and transferred to sterile glass jars for further salt-curing for 4 weeks at 4 °C. The laboratory produced dried salt-cured cod was dry-salted in stacks for several weeks at 4 °C before air-drying the fish indoors until the water content was below 50%. The muscle NaCl content in salt-cured, dried salt-cured, as well as in rehydrated salt-cured cod samples, was measured according to Volhard's Method (AOAC, 1990a).

2.2. Rehydration trials and bacterial counts

Pieces (20–30 g) of both salt-cured and dried salt-cured cod were rehydrated in 200 ml water at 4 °C for 24 h. The water was changed once (after 12 h) during the rehydration period. As with the other experiments, the rehydration trials were carried out using sterile water and equipment. Rehydrated fish muscle in sterile plastic bags were stored for 10 days at 4 °C. Samples for determination of total viable counts were obtained after 0, 3, 5, 7 and 10 days of storage. From each sample approximately 5 g muscle was diluted in 10-folds with saline (0.9% or 3.0% NaCl) containing 0.1% peptone. Homogenates were obtained after 1 min of homogenisation in a Lab-Blender 400 Stomacher (Seward Medical UAC House, London, UK). The total viable count development during the storage period was determined by using standard plate count agar (PCA) (Oxoid, Basingstoke, UK) containing 0.9% or 3.0% (w/v) NaCl. Duplicates were made of each sample. Plates were incubated at 37, 20, 12 or 4 °C, and colonies were counted after 2, 4, 7 or 14 days, respectively. Strains for further studies were isolated from the samples taken after 10 days of storage. From all samples at least five isolates of each of the dominating colony types found were isolated. The slime mucus samples were incubated on PCA with 3% (w/v) NaCl at 12 °C and from each fish at least eight colonies similar to the ones dominating in the rehydrated samples were isolated and characterized.

2.3. Characterization

The colonies growing on PCA were described morphologically. Colonies were further characterized using an indirect Gram-test employing the KOH-method (Gregersen, 1978). The presence of catalase and oxidase were determined using the 3% H_2O_2-method as described by Gram (1991) and the cytochrome-oxidase test by Kovacs (1956). The morphology of the isolated bacteria was observed by using a phase-contrast microscope (Nikon HFX-II A), with a Nikon FX-35 WA camera attached (Nikon, Tokyo, Japan).

Glucose fermentation, aerobic and anaerobic growth, gas production and motility were investigated by using the Zofa-method (Hansen and Sørheim, 1991). The Zofa-medium contains per litre: 2.5 g yeast extract (Difco Laboratories, Detroit, MI, USA), 5.0 g casitone (Difco), 3.0 g bacto agar (Difco), 0.5 g tris hydroxymethyl aminomethane (tris base), 5.0 g glucose, 10 ml of 0.2% phenol red solution and 0.9 or 3.0% (w/v) NaCl. The phenol red solution was prepared by dissolving in 96% ethanol and diluting five times in distilled water. The pH of the media was adjusted to 7.6 with HCl/NaOH. The medium was heated until the agar melted, aliquots of 10 ml were transferred to test tubes, and autoclaved. The bacteria were inoculated by introducing an inoculation loop half way into the sloppy agar in the test tubes. Inoculated tubes were incubated at 12 °C and checked daily. Glucose fermentation is regarded positive if the medium changes colour from red to yellow while aerobic or anaerobic growth is detected by bacterial growth in, respectively, the upper or lower part of the test tube.

Gas production and motility are indicated by the formation of, respectively, bubbles and turbidity.

Optimum NaCl concentration for growth of *Psychrobacter immobilis* CCUG 21770 (see Section 2.4) was detected by measuring OD at 600nm with a Spectrophotometer (VERSAmax Tuneable Microplate Reader, Göteborgs Termometerfabrik, Göteborg, Sweden). To standard plate count broth (PCB) (Oxoid), containing NaCl concentrations from 0% to 15.0% (w/v) as shown in Fig. 4, a *P. immobilis* culture (approximately 10^6 cells/ml in the wells) was added. The culture had previously been cultivated in PCB medium with 0.9% NaCl for 24 h at 20–22 °C. During OD measurements, the bacteria suspensions were stored in Costar Cell Culture Clusters (96 wells) (Corning, Corning, NY, USA) at 20–22 °C for 6 h. OD values obtained during the logarithmic growth phase were used in estimation of growth rate. Measurements were carried out on eight parallels (wells) for each NaCl concentration.

For detection of salt tolerance, eight strains obtained from rehydrated salt- and dried salt-cured cod and eight strains from skin mucus, grown on PCA plates (3.0% (w/v) NaCl) at 4 °C, were pre-cultured at 4 °C in PCB containing 3.0% NaCl. Approximately 10^6 cells in mid-exponential growth phase were transferred to tubes containing 20 ml PCB with 0%, 1.5%, 3%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, 22.5% or 25% (w/v) NaCl. The tubes were stored at 4 °C and samples taken after 30 min and then at regular intervals for 1 week. The number of viable bacteria was determined using PCA containing 3.0% (w/v) NaCl and incubation of plates at 4 °C for 14 days.

Temperature tolerance were tested by incubation on PCA containing 3.0% (w/v) NaCl at temperatures 4, 12, 20 and 37 °C. Required colony formation time (colony size of 2–4 mm) was used as an indicative measure of the temperature preference for growth. The tolerance for penicillin was investigated by incubation on PCA agar (3.0% NaCl) with penicillin strips attached to the agar surface. The Minimum Inhibitory Concentration (MIC-value) was determined according to the manufacturer instructions (Etest™, AB BIODISK, Solna, Sweden). Subinhibitory concentrations were used to differentiate between rod and coccoid cell forms (Bøvre and Hagen, 1981).

A total of 20 randomly chosen strains from the products analysed, as well as from cod skin mucus, were tested for urease and phenlyalanine deaminase activity on respectively Urea Agar Base (Oxoid) according to Christensen and Phenylalanine agar (Britania Laboratories, Buenos Aires, Argentina). Acid production from D-xylose, L-arabinose, fructose, sucrose and maltose was detected by using the Zofa-method exchanging glucose with the respective sugars tested. Utilization of sole energy and carbon sources for growth was investigated as described by Bowman et al. (1996). Ethanol, acetate, L-arginine and phenol were added at a 0.2% (w/v) level to the mineral salts medium.

Bacteria isolated from rehydrated salt-cured cod (1 strain), dried salt-cured cod (1 strain) and isolates (3 strains) from cod skin mucus were sent to The Department of Clinical Bacteriology, Göteborg, Sweden for Phenotypic Probabilistic Identification with CCUG NFX-Database and Fatty Acid Methyl Ester profiling (FAME).

2.4. Reference strain

P. immobilis CCUG 21770 (Culture Collection University of Göteborg) was included as a reference strain and compared to the isolates from rehydrated salt-cured and dried salt-cured cod and cod skin mucus. This strain was chosen because it was isolated from Arctic cod skin (Torry Research Station, Scotland).

2.5. Spoilage potential analysis

Of a total of 50 pieces rehydrated salt-cured cod (Product C, Table 1), approximately 30 g each, 25 were inoculated with *P. immobilis* CCUG 21770 by dipping the pieces for 1 min in a culture containing 10^8 cells/ml. Final inoculum in the fish was 6.2×10^5 cells/g muscle. The remaining 25 pieces were untreated controls. Inoculated (I) and control (C) samples were stored at 4 °C and samples for analysis obtained after 0, 3, 7, 10 or 14 days of storage. At each sampling day for both inoculated and control 2 samples were analysed sensorially. The sensorial analysis was carried out at our institute by a panel of five persons all experienced with rehydrated salt-cured cod quality. The properties evaluated were rancid, musty, putrid, sulphur, ammonium, sour and fresh odour. In duplicate, one piece was analysed at each sampling

day for each of the subsequent analysis; total viable base nitrogen (TVN) (Conway and Byrne, 1933; AOAC, 1990b), rancidity by measuring thiobarbituric acid reactive substances (TBAR) (Tarlagdis et al., 1954; Dulavik et al., 1998) and total bacterial count using PCA with 3.0% (w/v) NaCl. Total amount of moulds and yeast were also analysed in the same pieces as for the total viable count analysis, using respectively Dichloran-Glycerol Agar Base (DG18) (Hocking and Pitt, 1980) and Yeast Extract Agar (Microbiology Manual, 1994, Darmstadt, Germany).

Growth medium broth agar (GMB) was used as an indicative method for detection of TMAO-reducing bacteria (Dalgaard et al., 1994). The medium contains trimethylamineoxide and positive strains produce the characteristic smell of TMA on GMB. H_2S producing bacteria were identified using Iron Agar, Lyngby (Oxoid) (Gram et al., 1987). Extracellular proteolytic activity and extracellular lipolytic activity were identified employing Standards Methods Agar with caseinate (Martley et al., 1970) and Basal Medium Agar with 1% (v/v) Tween 80 (Lányi, 1987), respectively.

3. Results

3.1. Colony characterization and bacterial counts

A total of 38 product samples from five commercial producers of salt-cured and dried salt-cured cod and laboratory productions of salt-cured and dried salt-cured cod products were rehydrated under sterile conditions and analysed (Table 1). The salt content in the muscle of salt-cured and dried salt-cured cod were 20–22% (w/w) and 22–25% (w/w). After rehydration, both types of products had a salt content of 2–4% (w/w).

Table 2
Colony characterization of the three dominating colony types in rehydrated salt-cured and dried salt-cured cod

	Type 1	Type 2	Type 3
Appearance	smooth and wet	smooth and wet	rough and dry
Shape	circular	circular	non-circular
Diameter	4–8 mm	2–5 mm	2–8 mm
Colour	dark beige, tone of yellow	light beige	light to dark beige

Growth incubation on PCA containing 0.9% (w/v) and 3.0% (w/v) NaCl and incubation temperatures 4, 12 and 20 °C.

The bacterial counts in rehydrated samples stored at 4 °C were almost identical when plates, both PCA with 0.9 and 3.0% NaCl, where incubated at 4, 12 or 20 °C while incubation at 37 °C gave considerably lower counts. The development in total counts incubated at 4, 20 and 37 °C is shown in Fig. 1. Incubation at 12 °C is not shown due to identical counts with 20 °C incubation. The microbiota growing at 20 °C or below were dominated by three colony types (Table 2). The three colony types always constituted at least 90% of the total viable count in all samples analysed. The distribution of these colony types varied from product to

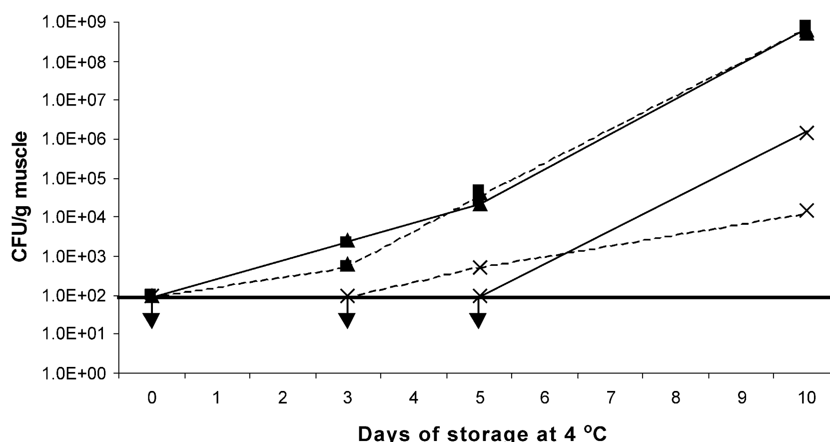

Fig. 1. Bacterial counts in rehydrated salt-cured cod (Tromvik Fiskeindustri, Tromsø, Norway, Product F) stored at 4 °C. Growth incubation on PCA containing 0.9% (w/v) (broken line) and 3.0 % (w/v) NaCl (unbroken line) at incubation temperatures 4 (■), 20 (▲) and 37 °C (×). Horizontal line at CFU = 100 shows detection level, arrows indicate CFU values below detectable level.

Table 3
Distribution of *Psychrobacter* colony types and bacterial counts in rehydrated products A–J stored at 4 °C

Product	Bacterial counts (CFU/g)		*Psychrobacter* (%) of total count	Distribution of *Psychrobacter* colony types (%)		
	12 °C	37 °C		Type 1	Type 2	Type 3
A	4.7×10^6	< 100	95	50–80	10–40	10–20
B	4.4×10^6	< 100	95	70–80	10–20	0–10
C	$7.0 \times 10^{7*}$	–	98	20–40	50–70	0–20
D	$4.1 \times 10^{7*}$	–	100	30–50	40–50	10–20
E	$4.2 \times 10^{7*}$	–	96	10–40	40–80	0–10
F	$4.2 \times 10^{4*}$	5×10^2	90	90	10	–
G	6.0×10^6	–	100	0–60	40–100	0–10
H	4.7×10^6	< 100	100	–	100	–
I	2.4×10^6	4×10^2	95	–	100	–
J	3.7×10^6	< 100	95	20–80	20–80	–

Samples taken after 7 or 5* days of storage. Plates (PCA containing 0.9% or 3.0% NaCl) were incubated at 12 or 37 °C.

product (Table 3), Type 1 and Type 2 being the dominant. The remaining part of the microbiota, less than 10%, consisted of a large variety of colony types. These colonies differed in size, colour (often pigmented) and shape, also varying considerably from product to product. Only the three dominating colony types were studied further in this work.

Characterization of at least 10 isolates from each of the three colony types showed large conformity, differing only by colony appearance. In all the phe-

Table 4
Characterization of the reference strain *P. immobilis* CCUG 21770, the dominating strains isolated from rehydrated salt-cured and dried salt-cured cod (Types 1, 2 and 3) and strains isolated from cod skin mucus

	Strains		
	P. immobilis reference strain	Rehydrated salt-cured and dried salt-cured strains (isolated)	Skin mucus strains (isolated)
Phenotypic tests			
Gram-test	negative	negative	negative
Catalase-test	positive	positive	positive
Oxidase-test	positive	positive	positive
Motility			
microscope	no	no	no
Zofa-test	no	no	no
Morphology	coccobacilli, often in pairs	coccobacilli, often in pairs	coccobacilli, often in pairs
Respiration	facultative aerobe	facultative aerobe	facultative aerobe
Gas production	no	no	no
Growth response			
Salt tolerance	0–25% NaCl	0–25% NaCl	0–25% NaCl
Temperature adaption	psychrotolerant	psychrotolerant	psychrotolerant
MIC[a]-value	= 0.1 μg/ml	= 0.1–0.5 μg/ml	= 0.2 μg/ml
Spoilage properties			
Extracellular lipolytic activity	yes	yes	yes
Extracellular proteolytic activity	no	no	no
TMA-production on GMB[b]	no	no	no
H_2S production on IA[c]	no	no	no

[a] Minimal inhibitory concentration of penicillin.
[b] Growth medium broth.
[c] Iron agar (Lyngby).

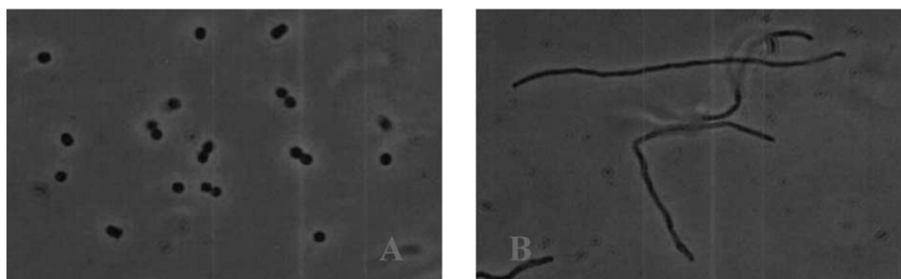

Fig. 2. Phase-contrast micrographs of dominating strain isolated from rehydrated salt-cured cod (A) and elongated bacteria isolated from skin mucus incubated with subinhibitory concentrations of penicillin (B). Cells shown at 1000 × magnification.

notypic tests carried out, as well as growth response and spoilage potential analysis (Table 4), the three types gave identical results. Based on these results, the three colony types were in the further work defined as the dominating bacterium in rehydrated salt-cured and dried salt-cured cod. The development in bacterial counts for product F is shown in Fig. 1. The numbers for products A, B, G, H, I and J were similar to those of F. Products C, D and E differed from the other products by containing the same bacterial load after 5 days, as the other products contained after 7–8 days of storage at 4 °C (Table 3). By visually determining the colony formation time, the dominating bacterium was found to form colonies faster at 20 °C than at 12 and 4 °C. The dominating bacterium formed no colonies at 37 °C and the total count at this temperature was low (Table 3).

3.2. Characterization of dominating microbes

The dominating bacterium was Gram-negative, catalase- and oxidase-positive, non gas producing, facultative aerobe and nonmotile. When grown on standard medium, the bacterium formed coccobacilli most often occurring in pairs (Fig. 2A). Cell elongation was registered after growth incubation in subinhibitory concentrations of penicillin (Fig. 2B). These results indicated that the dominating bacterium was similar to the genus *Psychrobacter*. The strain was therefor compared with the reference strain *P. immobilis* CCUG 21770. The results from the characterization tests were almost identical for the dominating bacterium from rehydrated salt-cured and dried salt-cured cod (products A–I), strains isolated from skin mucus and the reference strain (Table 4).

Isolates from sterile rehydrated commercial obtained salt-cured and dried salt-cured cod products and three isolates from cod skin mucus were identified as belonging to the *P. immobilis* complex (CCUG 42946–42950) at the Department of Clinical Bacteriology based on the Phenotypic Probabilistic Identification with CCUG NFX-Database (data not shown) and Fatty Acid Methyl Ester profiling (FAME) shown in Table 5. High amounts of oleic acid, 18:1ω9c (44–

Table 5
Fatty Acid Metyl Ester profile (FAME) of strains isolated from rehydrated salt-cured and dried salt-cured cod and skin mucus of cod

Compound	Fatty acid composition (%)		
	42 948	42 949	42 950
10:0	3.5	4.5	4.3
12:0	3.1	4.3	4.2
3-OH12:0	4.1	5.4	5.5
3-OH13:0/15:1[a]		1.3	
3-OH14:0/i16:1 ω5[b]	1.6	1.8	2.2
16:1ω7c	10.7	11.2	11.1
16:0	2.9	5.2	2.7
17:0		1.7	
i17:0	2.4		
17:1[c]	11.9		8.6
18:2ω6c	1.7		
18:1ω9c	51.9	43.6	54.7
18:1ω7c[d]		1.5	
18:0	2.3	3.9	3.6
Unidentified	3.9	5.7	1.7

Analysis carried out at the Department of Clinical Bacteriology, Göteborg, Sweden (strains shown are CCUG 42948, 42949 and 42950).
[a] Identified as 15:1ω5 or 15:1ω6.
[b] Identified as 3-OH14:0 or i16:1ω5.
[c] Possibly 17:1ω14.
[d] Alternative identification 18:1ω12t or 18:1ω9t.

Table 6
Characters of *Psychrobacter* strains isolated from rehydrated salt-cured and dried salt-cured cod products and cod skin mucus samples

Groups	A	B	C	*P. immobilis* (CCUG 21770)
Number of strains	11	8	1	
Urease	0	100	0	100
Phenylalanine deaminase	18	80	100	100
Acid from:				
Xylose	100	0	100	0
Arabinose	50	0	0	0
Fructose	0	0	0	0
Sucrose	0	0	0	0
Maltose	0	0	0	0
Growth on carbon sources:				
Acetate	100	100	0	100
Ethanol	91	100	0	100
L-Arginine	64	0	0	0
Phenol	0	0	0	0

Numbers shown are % positive strains.

55%), and moderate amounts of palmitoleic acid, 16:1ω7c (11%), were found in all isolates, and the presence of i17:0 or 17:0 was noticed in two out of the three isolates analysed. In order to investigate whether the isolated *Psychrobacter* strains were *P. immobilis* or other *Psychrobacter* strains, further phenotypic analysis on 20 randomly chosen strains from the products analysed were carried out. These strains formed three groups shown in Table 6. Group A (11 strains), B (8 strains) and C (1 strain) were separated by urease and phenylanaline deaminase activity as well as acid production from sugars and carbon utilization.

3.3. Spoilage potential of dominating strain

To investigate the spoilage potential of the dominating bacterium in rehydrated salt-cured and dried salt-cured cod a comparison was carried out between a sample inoculated with a *P. immobilis* solution and a control sample during storage at 4 °C. The inoculated sample had a bacterial count evolving from 6.2×10^5/g at day 0 to 5.0×10^8/g after 14 days of storage. The control sample had a development in bacterial load as shown for Product C in Table 3. Throughout the storage time, the microbiota was in both samples dominated by the same type of bacterium as described earlier.

In the sensory analysis off-odours in the control sample were noticed in the rehydrated product after 7–10 days. The odour was described as musty with a putrid, ammonium and sour smell (Table 7). For both control sample and sample inoculated with *P. immobilis* prior to storage, a rancid odour with low and rather constant intensity was found throughout the storage. This rancid smell was dominating together with the fresh smell up to 3 and 7 days of storage in respectively inoculated and control sample. For the inoculated samples, the same musty odour as registered in the control sample was detected, but at an earlier stage in the storage and of a stronger intensity. After 7 days this odour was intense. For the 10 samples analysed, only small traces of moulds or yeast ($< 10^3$ CFU/g) were detected when incubated on, respectively, DG18 and Yeast Extract Agar. The

Table 7
Development in odour characteristics in rehydrated salt-cured cod (West Fish, Product C) during 14 days of storage at 4 °C

Sample	Odour characteristics							
	Rancid	Musty	Putrid	Sulphur	Ammonium	Sour	Fresh	Total score
C-D0	0.8	0.0	0.2	0.0	0.0	0.0	1.8	2.0
C-D3	1.2	0.0	0.2	0.0	0.2	0.8	1.4	1.6
C-D7	1.0	0.0	0.2	0.0	0.0	0.2	1.6	1.6
C-D10	0.8	0.4	0.2	0.0	0.0	0.4	0.8	0.2
C-D14	0.6	0.8	2.0	0.4	1.4	0.4	0.4	0.2
I-D0	0.6	0.0	0.0	0.0	0.2	0.2	1.4	2.0
I-D3	0.6	0.2	0.6	0.0	0.0	0.2	1.0	1.6
I-D7	0.6	1.6	0.6	0.0	0.2	0.6	0.0	0.2
I-D10	0.4	1.6	0.6	0.0	0.8	0.4	0.0	0.0
I-D14	0.4	2.2	0.8	0.0	1.0	0.4	0.0	0.0

Product odours evaluated after 0, 3, 7, 10 and 14 days of storage (D0–D14). Sample I is inoculated with *P. immobilis* CCUG 21770 by dipping pieces for 1 min in a solution containing 10^8 cells/ml after rehydration. Sample C is untreated control. Score 0 defines absent odour and 3 defines dominant odour. Total score 3 indicates optimum rehydrated salt-cured cod odour.

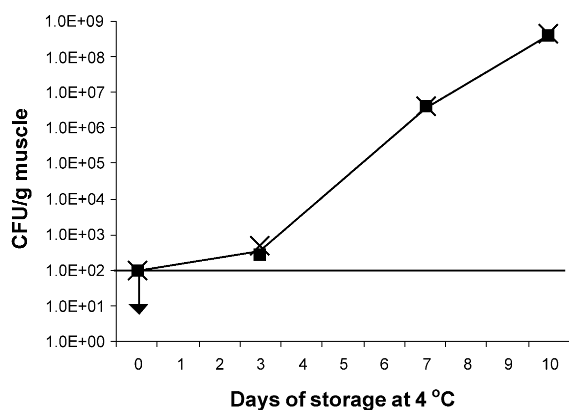

Fig. 3. Development in bacterial counts for laboratory produced (Fiskeriforskning, Product G) (■) and commercial produced (×) salt-cured cod (Andreas Bjørge, Product B) after sterile rehydration and storage at 4 °C. The bacterial counts were determined on PCA containing 3.0% (w/v) NaCl, incubation temperature 12 °C. Horizontal line at CFU = 100 indicates detection level, arrow indicates CFU values below detectable level.

TVN analysis showed an increase from 6.3 to 11.7 and from 6.9 to 12.3 mg N/100 g from day 0 to day 14, in respectively the control and inoculated sample. No changes in chemically measured rancidity (TBAR) were observed during the storage period in the samples (results not shown). The muscle was firm with no visual signs of deterioration even after 14 days of storage.

The dominating bacterium failed to produce H_2S and TMA on, respectively, Iron Agar and GMB agar. When grown on Caseinate Agar, no excretion of extracellular proteolytic enzymes were observed. However, the dominating bacterium excreted lipolytic enzymes as shown by the formation of turbidity in Basal Medium Agar with 1% Tween 80.

3.4. Origin of dominating strain

In order to investigate the origin of the dominating bacterium, laboratory produced salt-cured cod (product G), which were handled aseptically during the catch and the salt curing, were rehydrated with sterile water and equipment. The total viable count was very similar to the majority of commercial samples analysed (Fig. 3). The skin mucus isolates appeared identical to the dominating bacterium in both the laboratory produced and the commercially obtained rehydrated salt-cured cod, and to *P. immobilis* CCUG 21770 (Table 4).

Relative growth rate for *P. immobilis* CCUG 21770 in PCB with NaCl concentrations from 0% to 15% are shown in Fig. 4. Growth medium containing 3.5% NaCl gave the highest growth rate at 20–22 °C. Slow bacterial growth was detected at 10%, but no growth was detected at 15% NaCl. However, the maximum salt concentration for survival was higher. Incubation in standard medium broths containing NaCl concen-

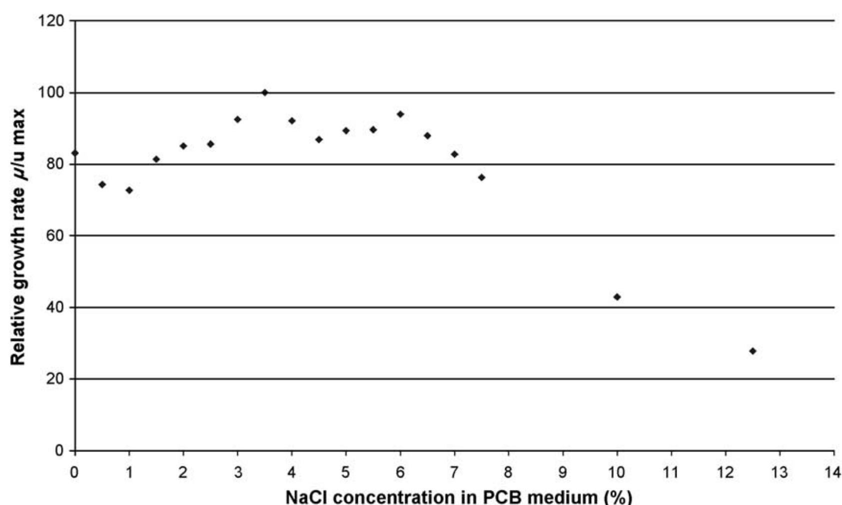

Fig. 4. Relative growth rate μ/μ_{max} for *P. immobilis* CCUG 21770 cultivated in PCB containing NaCl concentrations from 0% to 15% (w/v) NaCl. The growth rate was determined by measuring OD at 600 nm. The incubation temperature was 20–22 °C, $n = 8$.

trations ranging from 0% to 25%, showed that all the strains tested (Table 4) survived well in 25% NaCl. After 7 days, almost 100% of the cells could be cultivated on PCA with 3.0% NaCl.

4. Discussion

4.1. Characterization

The dominating bacteria, characterized in Section 3.2, are grouped in the family *Neisseriaceae* (Bøvre, 1984). The two main genus in this family, *Moraxella* (short rod) and *Neisseria* (coccoid), can be separated by their growth response in subinhibitory concentrations of penicillin, as *Moraxella* form elongated cells, while *Neisseria* do not (Bøvre and Hagen, 1981). The dominating bacteria formed elongated cells with penicillin, indicating that the bacteria was a *Moraxella* (subgenus *Moraxella)*. However, according to Bøvre (1984), all *Moraxella* species grow at 37 °C. In addition, *Moraxella* species are not halotolerant (Henriksen and Bøvre, 1968). The only exception, *Moraxella phenylpyruvica*, has been renamed *Psychrobacter phenylpyruvicus* (Bowman et al., 1996). The dominating bacterium in rehydrated salt-cured and dried salt-cured cod was extremely salt tolerant and did not grow at 37 °C, and could therefor not be a true *Moraxella*.

Juni and Heym (1986) described the new psychrotolerant and halotolerant genus, *Psychrobacter*, which belongs to the family *Neisseriaceae*, and is genetically closely related to *Moraxella.* The *Psychrobacter* genus consists of the species *P. immobilis* (Juni and Heym, 1986), *Psychrobacter frigidicola*, *P. phenylpyruvicus* and *Psychrobacter urativorans* (Bowman et al., 1996), *Psychrobacter glacincola* (Bowman et al., 1997) and *Psychrobacter pacificensis* (Maruyama et al., 2000). As shown in Bowman et al. (1996) and García-López and Maradona (2000), the fatty acid profiles of the *Psychrobacter* species are very similar. The fatty acid profiles of the three isolates obtained from the CCUG did not indicate a specific specimen of the *Psychrobacter* genus. However, only some of the Psychrobacters contain the fatty acid i17:0 or 17:0. Based on this, two of the three isolates analysed by CCUG could not be *P. phenylpyruvicus* or *P. frigidicola*.

According to Bowman et al. (1997), *P. immobilis* has been isolated from skin of cod. It is therefor tempting to assume, when also taking into account the strains identified at The Department of Clinical Bacteriology, that the bacteria dominating in the products studied is *P. immobilis*. However, only a limited number of strains were analysed. Additionally, the distinction between the *Moraxella* and *Psychrobacter* genus, and within the genus *Psychrobacter*, is still uncertain, due to lack of successful criteria to obtain sufficient clustering (Falsen, 2000, personal communication). The diversity among the *Psychrobacter* genus is also shown in the three groups of *Psychrobacter* strains in Table 6. None of these patterns are identical with the phenotypic groups reported by Bowman et al. (1996). Possible exceptions are group B and reference strain, since the ability to ferment sugars is not clear among *P. immobilis* (Gennari et al., 1989; García-López and Maradona, 2000). Group A being urease and phenlyalanine deaminase negative are more similar to *Moraxella*, but differing in acid production, salt tolerance and growth temperature. Group B has the same characters as the reference strain of *P. immobilis* being urease and deaminase positive and utilizing ethanol and acetate but not L-arginine and phenol. Extensive taxonomic studies of *Psychrobacter* and *Moraxella* are therefore required before the *Psychrobacter* in rehydrated salt-cured and dried salt-cured cod can be described on the species level.

4.2. Spoilage potential

Practical experience and our results show that the shelf life of rehydrated salt-cured cod is short. The musty off-odour noticed as more or less dominating in all products analysed was enhanced in the samples inoculated with *P. immobilis*. *Psychrobacter* is therefor the probable cause to this odour, since only small traces of moulds and yeast were detected. Previous work by Gennari et al. (1999) has also reported mushroom aroma off-odour production by *P. immobilis* in fish. It is beyond the frame of this work to identify which volatile compound that causes the musty odour. However, *Psychrobacter* and moulds have some similar metabolic pathways. For instance, both have similar mechanisms for degradation of phenylalanine (Juni and Heym, 1986; Gottschalk, 1986). The rancid smell found in all samples, regardless to storage time, is probably due to lipid oxidation,

which is known to occur during salt-curing of cod (Lauritzen et al., 1999).

The observation that the rehydrated salt-cured and dried salt-cured cod muscle remains firm even after prolonged storage supports the finding that no extracellular proteolytic activities are detected from the bacterium. In addition, our negative results with respect to spoilage properties such as extracellular proteolytic activity, TMA and H_2S production, are in accordance with several other authors reporting on *Moraxella*-like bacteria in fish (Gillespie and Macrae, 1975; Shewan and Murray, 1979; Gibbs et al., 1982; Gram et al., 1987).

Psychrobacter strains are dominant in the rehydrated salt-cured and dried salt-cured fish and not the usual spoilage biota of fresh fish. The survival of the fresh fish spoilage biota is probably limited by the high salt concentration in salt-cured and dried salt-cured cod products.

4.3. Origin of dominating bacteria

Based on temperature and salt preferences, the dominating bacterium in rehydrated salt-cured and dried salt-cured cod seemed to be well adapted to the marine environment. Findings referred to in Section 3.4 strongly suggest that one of the origins of the *Psychrobacter* in rehydrated salt-cured and dried salt-cured cod is the fresh fish itself, and that the bacterium originate from the normal skin microbiota of living cod or seawater. The *Psychrobacter* was present in all products, increasing the probability that the bacterium is endogenous to the cod.

4.4. Concluding remarks

Moraxella-like bacteria have been found in various amounts in different fish products (Colwell, 1962; Huss, 1983; Magnusson and Møller, 1985) and fish skin mucus (Georgala, 1958; Chai, 1981). Gennari et al. (1989) strongly suggests that *Moraxella*-like bacteria in fish products in most cases are the genus *Psychrobacter*. Our results are in accordance with this suggestion.

This work suggests that the dominating bacterium in rehydrated salt-cured and dried salt-cured cod products belongs to the genus *Psychrobacter* originating from the skin mucus of the live fish. This bacterium produces a spoilage characterised by a musty odour making the rehydrated salt-cured and dried salt-cured cod product sensorial unacceptable within 7–10 days or less, depending on the initial concentration of *Psychrobacter* in the sample.

The spoilage pattern is clearly different from what is observed during storage of fresh fish. Analysis showed that the dominating bacterium had maximal growth rate at rather high salt concentrations (3.5%) compared with normal spoilage bacteria in fresh fish. This can partly explain why *Psychrobacter* does not contribute significantly to the spoilage of fresh fish of cod which has a salt concentration of 1%. However, the main reason is probably that the spoilage by the biota in fresh fish overshadows the spoilage caused by *Psychrobacter*.

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