Department of Food and Environmental Hygiene
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

Lactic acid bacteria associated with the spoilage of fish products

Ulrike Lyhs

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Veterinary Medicine, University of Helsinki, for public examination in Auditorium Maximum, Hämeentie 57, Helsinki, on 24th May 2002, at 12 noon.

Helsinki 2002

ISBN 952-91-4642-6 (Print)
ISBN 952-10-0532-7 (PDF)

Helsinki 2002
Yliopistopaino

Cover photo: Maria Stark

CONTENTS

ACKNOWLEDGEMENTS

This study was carried out during 1995 and 2001 at the Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki. The work was made possible by financial support from the following organisations, which are gratefully acknowledged: the Faculty of Veterinary Medicine, Helsinki University Research Fund, Jenny and Antti Wihuri Foundation, the Finnish Veterinary Foundation, the Finnish Food Research Foundation, the Finnish Centre for International Mobility (CIMO), the Science Foundation for Women and the Iceland-Finland Culture Foundation.

I wish to express my warmest thanks to Prof. Timo Pekkanen, former Head of the department, for his confidence in me and for giving me the opportunity to start with this thesis.

I am deeply grateful to my supervisors Prof. Johanna Björkroth and Prof. Hannu Korkeala, Head of the department, for introducing me to scientific work and for their kind guidance and support throughout whole process. For helping me during this work, I would like to thank the following individuals from the Department of Food and Environmental Hygiene: Jari Aho, Mirja Eerikäinen, Dr. Sebastian Hielm, Jouni Hirvonen, Anneli Luoti, Elsa Mikkonen, Kirsi Ristkari and Johanna Seppälä. My particular gratitude to Maria Miettinen, Dr. Maria Fredriksson-Ahomaa, Dr. Eija Hyytiä-Trees and Sirkku Ekström for their assistance, useful discussions, listening and many encouraging words.

I would like to express my sincere appreciation to Dr. Satu Sankari, Dr. Peter Vandamme, Kai Elfving and Liisa Palmu for their kind help and support, Prof. Eero Puolanne, Dr. Marita Ruusunen and Dr. Esko Petäjä for constructive criticism of the thesis manuscript, Carol Pelli and Stephen Skate for correcting the English text of my manuscripts and summary and Prof. Alexander von Holy and Prof. Wilhelm Holzapfel for reviewing the thesis manuscript. Maria Stark is acknowledged for creating the picture used on the cover page.

My dearest thanks to my husband Janne Lahtinen, my parents Ellen and Lothar and all my friends for having faith in me and for encouraging me to continue during difficult moments.

Ulrike Lyhs

ABBREVIATIONS

AFLP, amplification fragment length polymorphism

ATCC, American Type Culture Collection

AMV-RT, avian myeloblastosis virus reverse transcriptase

APC, aerobic plate count

BCCM, Belgian Coordinated Collection of Microorganisms

bp, base pair

CCUG, Culture Collection of the University of Gothenburg

cDNA, complementary DNA

CFU, colony forming units

DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen

H_2S-TC, H_2S-producing bacteria count

kb, kilobase(pair)

LAB, lactic acid bacteria

LMG, Laboratory of Microbiology

MAP, modified atmosphere packaging

MVC, mesophilic viable count

NCFB, National Collection of Food Bacteria

PCR, polymerase chain reaction

ppm, parts per million

PVC, psychrotrophic viable count

RAPD, random amplified polymorphic DNA

REA, restriction endonuclease analysis

RFLP, restriction fragment length polymorphism

rRNA, ribosomal RNA

SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis

SMRICC, Swedish Meat Research Institute Culture Collection

TAE, Tris-Acetate with EDTA

TE, Tris-EDTA

ABSTRACT

Lactic acid bacteria (LAB) associated with three types of spoiled fish products, vacuum-packaged cold-smoked and 'gravad' rainbow trout and semi-preserved marinated herring, were enumerated and identified. Traditional phenotypic methods were used for initial genus-level identification followed by species identification using a 16+23S rRNA gene restriction pattern- (ribotyping) based database. Furthermore, a study was made of the effect of two preservatives, potassium nitrate (KNO_3) and sodium nitrite ($NaNO_2$), on the LAB isolated from vacuum-packaged cold-smoked rainbow trout.

LAB were the major fraction predominating in all samples of spoiled vacuum-packaged cold-smoked rainbow trout, with or without the addition of nitrate or nitrite, stored at 4°C and 8°C. In spoiled vacuum-packaged 'gravad' rainbow trout stored at 3°C and 8°C, LAB were found in lower numbers than the other bacterial groups were. H_2S-producing bacteria constituted a high proportion of the psychrotrophic counts. In all spoiled herring samples, LAB were the only microorganisms detected. The slight rise in pH value together with the gas production and growth of a facultatively heterofermentative organism suggested a rare LAB spoilage type called 'protein swell'.

The LAB isolates in all fish products studied were characterised by phenotypical key tests enabling genus-level identification. For ribotyping, they were initially grouped according to their restriction endonucleases profiles using *Eco*RI and/or *Hin*dIII restriction enzymes. One strain representing each group was further ribotyped. *Lactobacillus sakei* and *Lactobacillus curvatus* strains were detected in both types of rainbow trout products, whereas *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuconostoc citreum* were detected only in the cold-smoked product. On the other hand, carnobacteria were detected only in the 'gravad' product. One *Lactobacillus alimentarius* clone was considered as the specific spoilage organism in marinated herring samples with 'protein swell'.

When compared to the identification results obtained using the ribotyping database, two of the phenotypic tests, the production of gas from glucose and the growth on Rogosa SL agar, caused problems resulting in genus-level misclassification

of leuconostocs and lactobacilli. Altogether 224 out of 469 LAB isolates from vacuum-packaged cold-smoked rainbow trout were not assigned with phenotypic tests to the corresponding genera detected by ribotyping. Only a few isolates of the cold-smoked fish product could not be identified by ribotyping with the aid of the reference strains used. All isolates could be identified from the vacuum-packaged ‘gravad’ fish and the marinated herring.

The proportion of LAB in the spoilage microflora of vacuum-packaged cold-smoked rainbow trout was higher in the nitrite- and nitrate-treated samples than in the samples containing NaCl only. This was seen at both storage temperatures. The high proportion of LAB was considered to be owing to resistance to nitrate and nitrite, favoured by the anaerobic conditions.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers referred to in the text by the Roman numerals I to V:

I. Lyhs, U., Björkroth, J., Hyytiä, E. and 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.

II. Lyhs, U., Lahtinen, J., Fredriksson-Ahomaa, M., Hyytiä-Trees, E., Elfing, K. and 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. 70, 189-199.

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

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

V. Lyhs, U., Korkeala, H. and Björkroth, J. (2002) Characterization of lactic acid bacteria from spoiled, vacuum-packaged 'gravad' rainbow trout using ribotyping. Int. J. Food Microbiol. 72, 147-153.

1. INTRODUCTION

Fish has been one of the main foods for humans for many centuries and still constitutes an important part of the diet in many countries. The advantages of fish as a food are its easy digestibility and high nutritional value. Since 70% of the Earth's surface is covered by water, there are plenty of sources to harvest fish. The range of fish products is very large and includes foods prepared using a broad spectrum of both traditional and modern food technologies.

In Finland, some 39 million kg of fish were processed in 1997. Domestic fish processed accounted for about 33 million kg, of which about 10 million kg was rainbow trout. Of this amount, 48% were filleted and 36% were hot- or cold-smoked (Anonymous, 2000). In 1998, a total of some 70 million kg of fish was eaten, equivalent to 14 kg of fish per person. In 1999, the consumption of domestic rainbow trout (by filleted weight) was 1.6 kg per person (Anonymous, 2000).

Vacuum packaging is used to extend the shelf-life and to keep the quality of fresh and processed fish. However, strict temperature control and storage at refrigeration is required to ensure food safety and to guarantee high product quality. During the storage favoured by anaerobic conditions, psychrotrophic LAB usually become the dominant bacterial group or occur in very high numbers. Earlier studies have shown LAB to be relevant in the spoilage flora of chill stored vacuum-packaged cold-smoked (Truelstrup Hansen, 1995; Leroi et al., 1998; Jørgensen et al., 2001) and 'gravad' (Knøchel, 1983; Leisner et al., 1994) fish products. The increasing popularity of vacuum-packaged fish products calls for research into the role of LAB in the spoilage microflora in order to ensure continued quality and a continuous supply of shelf-stable choice products. Only few papers have been published dealing with the composition of species in LAB spoilage flora in cold-smoked (Truelstrup Hansen, 1995; Leroi et al., 1998) and 'gravad' (Leisner, 1992) fish. Thus, there is a need for further knowledge about the LAB species and their exact division in order to understand more about their role in the spoilage process of vacuum-packaged fish products.

In semi-preserved marinated fish, the nature of the marinating process, i.e. the treatment of the fish with acetic acid and NaCl, inhibits the Gram-negative spoilage

bacteria, which are sensitive to this kind of preservation. LAB may spoil a product if it is not manufactured and stored correctly (Meyer, 1956b; Kreuzer, 1957; Lerche, 1960; Reuter, 1965).

The identification of LAB in vacuum-packaged fish products has been mainly based on traditional biochemical and physiological tests. However, these tests are often time-consuming and sometimes entail difficulties resulting in the conflicting identification of LAB (Dykes and von Holy, 1994; Schleifer et al., 1995; Björkroth et al., 2000). Nowadays, genotypic methods provide a powerful tool in bacterial characterisation and identification. Computer-assisted numerical analysis of resulting macromolecular patterns and the identification of patterns of an unknown isolate against libraries containing corresponding patterns of known species have been successfully used for identification of LAB in spoiled foods (Björkroth and Korkeala, 1996b; Björkroth and Korkeala, 1997; Björkroth et al., 1998; Björkroth et al., 2000). Ribotyping has been used for the taxonomic approach (Grimont and Grimont, 1986) and later for the typing of epidemiological associated strains (Tenover et al., 1997). In spoiled vacuum-packaged cold-smoked and ‘gravad’ fish products, reliable identification of the different LAB species helps focus on the specific spoilage organisms of those products. In spoilage cases, it is particularly important to be able to rapidly identify the microorganisms causing spoilage so as to prevent further problems with the same kind of product.

2. REVIEW OF LITERATURE

2.1. Lactic acid bacteria (LAB)

2.1.1. General properties of LAB

A group of Gram-positive, non-spore-forming strictly fermentative bacteria producing lactic acid as the major end product of sugar fermentation are called LAB. They are devoid of cytochromes and are of a nonaerobic nature but tolerate air. LAB are catalase-negative, although pseudo-catalase can be found in rare cases. Phylogenetically, they are a very diverse group of organisms and belong to the clostridial branch of Gram-positive bacteria.

Two major pathways of hexose (e.g., glucose) fermentation can be distinguished among LAB. Glycolysis (Embden-Meyer pathway) results in almost exclusively lactic acid as the end product (homofermentative). The 6-phosphogluconate/phosphoketolase pathway results in significant amounts of other end products, such as ethanol, acetic acid and CO_2 in addition to lactic acid (heterofermentative). Based on the fermentative characteristics, lactobacilli can be divided into three groups: obligatory homofermentative, obligatory heterofermentative and facultatively heterofermentative. Obligatory homofermentative lactobacilli degrade hexoses exclusively to lactic acid and do not ferment pentoses or gluconate. Obligatory heterofermentative lactobacilli degrade hexoses to lactic acid and additional products such as acetic acid, ethanol and CO_2 and pentoses to lactic and acetic acid. Facultatively heterofermentative lactobacilli ferment hexoses to lactic acid and may produce CO_2 from gluconate but not from glucose. They also ferment pentoses to produce lactic and acetic acid.

The LAB of importance for foods belong to the genera of *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Paralactobacillus*, *Pediococcus*, *Streptococcus* and *Weissella* (Sharpe, 1979; Kandler and Weiss, 1986; Axelsson, 1993; Vandamme et al., 1996; Stiles and Holzapfel, 1997; Leisner et al., 2000).

2.1.2. Identification of LAB

Mainly classical phenotypical methods have been used to identify LAB. Specific DNA primers or probes and numerical analysis of macromolecular banding patterns have also been applied.

Classical phenotypic criteria for genus-level identification of LAB

Bacteria that are Gram-positive, catalase-negative and grow on MRS agar can be considered LAB. Traditional phenotypical tests constituted the basis for the description, differentiation and identification of LAB. The first attempt to classify LAB was made by Orla-Jensen (1919). This work still has a major impact on the systematics of LAB, although species-level identification has changed much in recent years. Different key tests are being widely adopted and nowadays the morphology as well as the physiological, metabolic/biochemical and chemotaxonomic methods are mostly used. Physiological methods include, for example, growth at certain temperatures and salt levels. Metabolic/biochemical methods include the production of gas from glucose, the production of acids from carbohydrates, the hydrolysis of arginine and the determination of the lactic acid configuration. Chemotaxonomic methods, for example, include the detection of m-DPA in the peptidoglycan. Table 1 shows a list of identification of LAB genera with classical genus-level tests. There are phenotypic overlaps between the genera and exception to the rules outlined in Table 1 can be found. With the increasing importance of LAB in the spoilage of modern packed and chill stored foods, including fish products, their identification is becoming dependent on more sophisticated methods.

Table 1. The most important genus-level phenotypic tests for identifying LAB[a].

	Lactobacillus	*Carnobacterium*	*Leuconostoc*	*Weissella*	*Enterococcus*	*Lactococcus*	*Pediococcus*	*Streptococcus*	*Paralactobacillus*
Morphology	Rods	Rods	Oval cocci	Cocci/rods	Cocci	Cocci	Cocci	Cocci	Rods
Gas from glucose	+/-	-	+	+	-	-	-	-	-
Growth at 10°C	+/-	+	+	+/-	+	+	+/-	-	-
45°C	+/-	-	-	+/-	+	-	+/-	+	-
Lactic acid isomere	D, L, DL	L	D	DL, D	L	L	L, DL	L	DL
Hydrolysis of arginine	+/-	+	-	+/-	+/-	-	+/-	+/-	-
m-DPA	+/-	+	-	-	-	-	-	-	ND[b]
Growth on Rogosa SL agar	+	-	-	+/-	-	-	-	-	-

[a] Derived from Shaw and Harding, 1984; Holzapfel and Schillinger, 1992; Axelsson, 1993; Wood and Holzapfel, 1995; Leisner et al., 2000.
[b] ND, not determined.

Specific primers and probes in the identification of LAB

Molecular methods based on the knowledge of species-specific sequences, such as hybridisation or PCR-amplification, require the use of specific probes and primers and offer new opportunities for the identification of bacterial species. A probe is a fragment of a single-stranded nucleic acid that will specifically bind (hybridise) to complementary regions of a target nucleic acid. There are three different approaches to the design of nucleic acid probes, randomly cloned probes, probes complementary to specific genes and gene fragments (16S or 23S rRNA targeted oligonucleotide probes) and rRNA target probes (Schleifer et al., 1995). In addition to DNA hybridisation techniques, specific areas of the genes encoding rRNA can be used as templates for primer design for the detection of nucleic acid hybridisation (Van Belkum, 1994).

Numerical analysis of macromolecular banding patterns as a means for lactic acid bacterial identification

Species-specific macromolecular patterns, such as lipopolysaccharides, fatty acids, proteins or DNA, have been used for bacterial identification. Strains belonging to the same species share characteristic bands and can be compared to type or reference strains in a database. Computer-assisted numerical comparison enables large numbers of strains to be compared. Data matrices show the degree of similarity between the strain profiles or patterns and the reference strains. Large-scale grouping analysis (species-specific cluster analysis) involves the creation of dendrograms or other means to reveal groups of related organisms. Patterns of an unknown isolate can be identified against libraries containing corresponding patterns of known species.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Among the methods for species-level identification the SDS-PAGE of whole-cell proteins or cell envelope proteins plays an important role and has been applied with varying degrees of success for the discrimination of microbial strains (Vauterin et al.,

1993). SDS-PAGE has been frequently applied to taxonomic studies with success in LAB (Dicks and van Vuuren, 1987; Pot et al., 1993; Dykes and von Holy, 1994a; Björkroth et al., 2001). In a study of LAB associated with the spoilage of modified atmosphere-packaged raw tomato-marinated broiler meat strips Björkroth et al. (2000a) successfully used both SDS-PAGE and ribotyping to identify *Leuconostoc* species. Paludan-Müller et al. (1998), studying the role of *Carnobacterium* species in the spoilage of vacuum- and modified atmosphere-packaged cold-smoked salmon, used SDS-PAGE to identify the LAB as carnobacteria.

Restriction endonuclease analysis (REA) and 16+23S rRNA gene restriction patterns (ribotyping)

Chromosomal DNA restriction endonucleases have been employed to identify lactobacilli. Simple REA of DNA (Mancini and Parini, 1983; Ståhl et al., 1990; Johansson et al., 1995) has been used as a tool for separating different species of lactobacilli. Each restriction endonuclease enzymatically cuts ("digests") DNA in a particular ("restricted") nucleotide recognition sequence. This might be repeated numerous times around the chromosome. The number and size of the restriction fragments generated by digestion of a given DNA piece reflect the frequency and distribution of the restriction sites. The fragments, ranging from approximately 1000 to 20 000 bp, can be separated by agarose gel electrophoresis and then visualised by ethidium bromide staining using standard methods (Sambrook et al., 1989; Maslow et al., 1993; Bingen et al., 1994; Tenover et al., 1997). The result of the agarose gel electrophoresis is represented by a pattern of bands on the gel. The banding patterns of the various DNA fragments are called a DNA fingerprint or profile.

All isolates are typeable by REA, however, it is very difficult to interpret the complex profiles due to the consistence of hundreds of bands (Maslow et al., 1993; Tenover et al., 1997). A probe, a specific chemically or radioactive labelled piece of DNA or RNA, can be used to obtain stable and more easily interpretable patterns. Complex DNA patterns generated after restriction enzyme digestion can be transferred to a membrane (Southern blot) and then hybridised with the probe that binds to only a limited number of DNA fragments with complimentary nucleic acid sequences. The

locations of various restriction enzyme recognition sites within a particular genetic locus can be polymorphic from strain to strain, resulting in bands of differing sizes between unlike strains. Thus, restriction fragment length polymorphism (RFLP) refers to the polymorphic nature of the locations of restriction enzyme sites within defined genetic regions. Only the genomic DNA fragments that hybridise to the probes are visible in RFLP analysis (Olive and Bean, 1999).

A typical example of these developments is the determination of 16+23S rRNA gene restriction patterns (ribotyping) (Grimont and Grimont, 1986) that is based on patterns generated from the ribosomal RNA genes only. It has also been successfully applied to identify lactobacilli (Rodtong and Tannock, 1993; Le Jeune and Lonvaud-Funel, 1994; Johansson et al., 1995; Björkroth and Korkeala, 1996a; Björkroth and Korkeala, 1996b). In ribotyping, nucleic acid probes are used to recognise fragments containing all or parts of the genes' coding for rRNA. rRNA operons contain three highly conserved genes' coding for 5S, 16S and 23S rRNA - all "housekeeping genes" essential for cell division and reproduction. These operons are usually present in 2 to 11 copies per bacterial chromosome to safeguard against DNA damage. Consequently, several restriction fragments carrying rRNA sequences can be observed (Grimont and Grimont, 1986). All bacteria carry these operons and are therefore typeable. Theoretically, ribotyping is done by Southern transfer of the REA gel onto a nylon membrane, which is hybridised with a labelled probe complementary to ribosomal DNA. After probing, each fragment of bacterial DNA containing a ribosomal gene will be highlighted, creating a fingerprint pattern containing approximately 1 to 15 bands that can be easily compared among isolates (Farber, 1996). With a commercially available system, all stages of ribotyping can be performed and the basic protocol takes at least 5 days (Grimont and Grimont, 1986) manually. However, the development of an automated ribotyping system, the RiboPrinter®, (Qualicon Inc., Wilmington, Del., USA) made it possible to shorten the procedure to 8 hours (deBoer and Beumer, 1999). Originally, ribotyping was intended for taxonomic use (Grimont and Grimont, 1986) but it was also later applied for the typing of epidemiologically associated strains (Maslow et al., 1993; Bingen et al., 1994; Tenover et al., 1997). However, due to its weak discriminatory power as a typing method other techniques have replaced it (Maslow et al., 1993; Farber, 1996).

It has been successfully used for at least the identification of the genera of *Lactococcus* (Rodrigues et al., 1991), *Enterococcus* (Hall et al., 1992), *Leuconostoc* (Björkroth et al., 1998), *Lactobacillus sakei* (Sanz et al., 1998), *Corynebacterium* (Björkroth et al., 1999), *Clostridium botulinum* (Hielm et al., 1999), *Leuconostoc* (Björkroth et al., 2000) and *Weissella* (Björkroth et al., 2001).

PCR-based methods

Randomly amplified polymorphic DNA (RAPD) analysis and amplified fragment length polymorphism (AFLP) are techniques based on the numerical analysis of PCR and used for identification.

In RAPD assay, also referred to as arbitrary primed PCR, patterns are generated by the amplification of random DNA segments with single short (typically 10 bp) primers of arbitrary nucleotide sequence. The primer is not targeted to amplify any specific bacterial sequences and will hybridise at multiple random chromosome locations and initiate DNA synthesis. After separation of the amplified DNA fragments by agarose gel electrophoresis, a pattern of bands results which is characteristic of the particular bacterial strain (Williams et al., 1990; Welsh and McClelland, 1992; Maunier and Grimont, 1993). The RAPD assay has been shown to be suitable for different bacteria (Mazurier and Wernars, 1992; Stephan et al., 1994) including *Lactobacillus* spp. (DuPlessis and Dicks, 1995). It allowed discrimination at species level (van der Vossen and Hofstra, 1996). However, the use of empirically designed primers makes standardisation of the method difficult and the discriminatory power largely depends on the primers used (Welsh and McClelland, 1992; Maunier and Grimont, 1993; Kerr, 1994).

AFLP is a genome fingerprinting technique based on the selective amplification of a subset of DNA fragments generated by restriction enzyme digestion and visualised by agarose gel electrophoresis (Vos et al., 1995). AFLP has been more recently applied to bacterial characterisation (Janssen et al., 1996; Koeleman et al., 1998; Kunene et al., 2000) and it has been reported to generate species-specific patterns that may be useful in identifying bacteria.

2.2. LAB associated with fish and fish products

2.2.1. LAB in fresh fish

LAB are not considered as belonging to aquatic environments, but certain species (i.e. *Carnobacterium*, *Vagococcus*, *Lactobacillus*, *Enterococcus*, *Lactococcus*) have been found in freshwater fish and their surrounding environment (Austin and Austin, 1992; Huss, 1995; Stiles and Holzapfel, 1997; González et al., 1999, 2000; Ringø et al., 2000). Carnobacteria, *C. piscicola* in majority and lower numbers *C. divergens*, have been reported as a part of the normal intestinal microbial population of many fish species, for example, Atlantic salmon (*Salmo salar*), wild pike (*Esox lucius*) and wild brown trout (*Salmo trutta*) (Ringø and Gatesoupe, 1998; González et al., 1999; González et al., 2000; Ringø et al., 2000). On the other hand, *C. piscicola* (Hiu et al., 1984; Baya et al., 1991) and *Lactobacillus* sp. (Cone, 1982) have been associated with fish diseases. *C. piscicola* appears to have a low virulence to fish and it seems to be most susceptible when the fish is exposed to stress conditions, such as handling (Starliper et al., 1992). However, the importance of carnobacteria as a fish pathogen cannot be accurately assessed (Schillinger and Holzapfel, 1995).

2.2.2. LAB in fish products

Vacuum-packaged hot-smoked fish products

The few studies dealing with vacuum-packaged hot-smoked fish that have been undertaken concentrate on the influence of processing and on the microbiological quality. At the end of storage at different chilled temperatures, LAB were found in numbers ranging from 10^6 to 10^8 CFU/g (Schulze, 1985; Jöckel et al., 1986; Paleari et al., 1990; Zorn et al., 1993). Zorn et al. (1993) reported carnobacteria dominating in vacuum-packaged hot-smoked rainbow trout at the end of the storage period at 8ºC and 20ºC.

Vacuum-packaged cold-smoked fish products

Earlier studies of vacuum-packaged cold-smoked fish have concentrated on the hygienic quality, with the shelf-life at storage temperature and salt concentration being the main factors studied. At the end of shelf-life a variable microflora with respect to proportions of LAB, *Enterobacteriaceae* and other bacteria has been observed in the products stored at chilled temperatures. In vacuum-packaged cold-smoked fish products, the spoilage microflora has usually been dominated by LAB at varying levels ranging from 10^6 to 10^8 CFU/g (Magnússon and Traustadóttir, 1982; Schneider and Hildebrandt, 1984; Hildebrandt and Erol, 1988; Beltrán et al., 1989; Shimasaki et al., 1994; Civera et al., 1995; Jørgensen et al., 2001; Joffraud et al., 2001). In some cases LAB counts of between 10^6 and 10^7 CFU/g together with counts of Gram-negative bacteria from 10^5 to 10^7 CFU/g were detected (Paludan-Müller et al., 1998). Truelstrup Hansen (1995) concluded that in vacuum-packaged cold-smoked salmon at the time of sensorial rejection, three different types of microflora were present, dominated by LAB, LAB and *Enterobacteriaceae* or LAB and *Photobacterium phosphoreum*. Also Leroi et al. (2001) distinguished at the time of spoilage for the same type of fish product three different types of microflora: lactobacilli, a mixture of lactobacilli and *Enterobacteriaceae* or a mixture of LAB and *Brochothrix thermospacta*. Testing the effect of different salt additions on the spoilage characteristics, Truelstrup Hansen et al. (1996) found marine vibrio dominating in normal dry salted salmon and a mixture of LAB and *Enterobacteriaceae* in injected-brined salmon at the time of spoilage. Furthermore, differences in the bacterial counts can be seen between fillets and sliced vacuum-packaged cold-smoked salmon (Truelstrup Hansen et al., 1998). Several factors influence the spoilage flora in vacuum-packaged cold-smoked fish products. Size and composition of the developing microflora depend on the quality of the raw material, the in-house flora of the processing plant and the production method (Truelstrup Hansen, 1995).

LAB dominating in spoiled vacuum-packaged cold-smoked fish products include the genera of *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Carnobacterium*. Magnússon and Traustadóttir (1982) reported the complete dominance of homofermentative lactobacilli in vacuum-packaged cold-smoked herring. In vacuum-

packaged cold-smoked salmon and herring, *Lactobacillus curvatus* has been found in majority together with lower numbers of *Lactobacillus sakei*, *Lactobacillus plantarum*, *Lactococcus* spp. and *Leuconostoc mesenteroides* (Truelstrup Hansen, 1995; Gancel et al., 1997; Leroi et al., 1998; Paludan-Müller et al., 1998). Paludan-Müller et al. (1998) reported carnobacteria as the major genus of the LAB microflora in spoiled vacuum-packaged cold-smoked salmon dominated by *Carnobacteria piscicola*. In the same product type, Leroi et al. (1998) detected carnobacteria during the first 2 weeks of storage and then observed a change with the appearance of *Lactobacillus farciminis*, *Lactobacillus sakei* and *Lactobacillus alimentarius* at the end of storage.

Vacuum-packaged 'gravad' fish products

Limited research has been conducted into the development and composition of the spoilage microflora in vacuum-packaged 'gravad' fish products stored at chilled temperatures. Usually LAB dominated the microflora at the end of the storage period (Knøchel, 1983; Leisner, 1992, Leisner et al., 1994) and different genera in different proportions were identified. The majority of them were assigned to carnobacteria (Leisner, 1992; Leisner et al., 1994) or *L. sakei* (Jeppesen and Huss, 1993) besides lower numbers of *Leuconostoc* spp., *L. curvatus* and *Weissella viridescens* (Leisner et al., 1994).

Semi-preserved marinated fish products

Spoilage of semi-preserved marinated fish products, seen as gas formation after some weeks of storage, may occur due to the growth of non-putrefactive organisms such as acetic acid-tolerant LAB (Blood, 1975). Thus, the nature of the marinating process, i.e. the treatment of the fish with acetic acid and sodium chloride, is responsible for the type of bacterial spoilage (Blood, 1975). Meyer (1956a) reported a type of lactic acid bacterium spoilage in blown canned semi-preserved marinated fish and called it 'protein swell'. The swelling is caused by production of CO_2 by LAB through the decarboxylation of amino acids in the absence of fermentative carbohydrates.

Ammonia production by bacterial deamination of amino acids has been assumed to be the reason for the increase of pH observed in the spoiled product. It has been suggested that the acetic acid provides an acidic environment suitable for the action of proteolytic enzymes present in fish muscle. The products of proteolysis, i.e. amino acids, provide an energy source for the growth of acetic acid-tolerant LAB, leading to CO_2 production (Meyer, 1962a; Stamner, 1976). 'Protein swell' caused by LAB growth has also been associated with anchovy-stuffed olives (Harmon et al., 1987). *L. plantarum* and *L. brevis* have been identified, and enzymes originating from the anchovy were considered to be the reason for initial proteolysis. In spoiled modified-atmosphere-packaged raw tomato-marinated broiler meat strips, 'protein swell' was caused by a mixed LAB population dominated by *Leuconostoc* species (Björkroth et al., 2000a).

In contrast to 'protein swell', CO_2 formation during 'carbohydrate swell', results from the heterofermentative utilisation of glucose and so very low pH values can be observed. In a case of *L. fructivorans* spoilage in tomato ketchup, pH values of 3.3 to 3.4 were detected (Björkroth and Korkeala, 1997).

Lactobacillus spp. have been identified as the specific spoilage organisms in unspoiled and spoiled marinated herring (Meyer, 1956b, 1962b; Kreuzer, 1957; Lerche, 1960; Reuter, 1965; Erichsen, 1967; Sharpe and Pettipher, 1983). Heterofermentative *Lactobacillus* spp. as *L. brevis*, *L. buchneri* and *L. fermentum* have predominated in different kinds of spoiled herring types (Meyer, 1956b; Kreuzer, 1957; Lerche, 1960; Meyer, 1962b; Reuter, 1965; Erichsen, 1967; Sharpe and Pettipher, 1983). *L. plantarum* and *L. delbruckii* subsp. *lactis* (*L. leichmannii*), possessing homofermentative glucose metabolism have been detected in some spoilage cases (Lerche, 1960; Meyer, 1962b; Sharpe and Pettipher, 1983). In unspoiled marinades, *L. buchneri* and *L. delbruckii* subsp. *lactis* have been identified (Reuter, 1965).

Impact of LAB in the spoilage of fish products

The role of LAB in the spoilage of vacuum-packaged cold-smoked and 'gravad' fish products is still not clear. Various studies reported testing the spoilage potential of

lactobacilli and carnobacteria on sterile or semi-sterile models and inoculated vacuum-packaged cold-smoked fish. Some LAB strains were found to be able to produce some characteristic off-odours associated with the spoilage of cold-smoked salmon (Truelstrup Hansen, 1995) or they did not produce any spoilage off-odour (Leroi et al., 1996; Leroi et al., 1998; Paludan-Müller et al., 1998). Leisner (1992) showed that no or very faint off-odours were produced by LAB compared with the strong off-odours produced by the Gram-negative spoilers. Joffraud et al. (2001) reported *Lactobacillus* spp. that released large amounts of volatile compounds and therefore was possibly responsible for the off-odours perceived on spoiled vacuum-packaged cold-smoked salmon. According to the same authors, the strong butter odour produced from *C. piscicola* did not affect the sensory quality of the product. The spoilage process called 'protein swell' in semi-preserved marinated fish products caused by LAB is better understood (Meyer, 1956a; Meyer, 1962b; Stamner, 1976).

2.3. Processing factors predisposing to LAB spoilage in fish products

In the past, preservation of fish, such as air-drying, smoking, salting and pickling in brine permitted greater availability and use of fish as food. Nowadays, the preservation of fish and fish products, as applies to all food, is based on a combination of several factors to delay or prevent the growth of food spoilage microorganisms so as to extend shelf-life and secure microbial safety, stability and sensory quality (Gould, 2000). Reducing the growth of fast-growing spoilage bacteria through the application of different preservation hurdles results in the selection of LAB as the main group of spoilage microflora. The main hurdles applied in fish and fish products are a low storage temperature, the use of salt, nitrite and acids, smoking or packaging in vacuum or under modified atmosphere.

2.3.1. Storage temperature

Storage temperature is the most important environmental parameter influencing the growth rate and type of spoilage microorganisms of highly perishable foods such as fish products. Storage at chilled temperature, one of the most widely practised methods of controlling microbial growth, reduces contaminating microorganisms that cause spoilage and prolongs the shelf-life of the fish. Storage temperatures below the growth optimum lead to extended generation times and lag times, and the growth rate decreases. Storage temperatures below the growth minimum result in a continued extension of the lag-phase until multiplication ceases and the growth of the microorganisms stops (Doyle et al., 1997). In the temperature range between 0 and 10°C, minor changes have an enormous effect on bacteria growth (Huss, 1988; Korkeala et al., 1989). However, refrigeration cannot kill or completely eliminate spoilage bacteria but will limit the spoilage to psychrotrophic microorganisms, which can grow in products chilled at a temperature of below 7°C (Ashie et al., 1996; Huis in´t Veld, 1996).

LAB tend to grow slowly at refrigeration temperatures (Huis in´t Veld, 1996). Under anaerobic conditions at temperatures below 20°C, psychrotrophic LAB, which are capable of growth at 5°C or below, enable successful competition with other psychrotrophic spoilage microoganisms (Doyle et al., 1997). Psychrotrophic LAB include carnobacteria, leuconostocs and *Weissella* spp., but also some facultatively heterofermentative lactobacilli, such as *L. sakei* and *L. curvatus* (Lücke, 1996a).

2.3.2. Effect of preservatives

Salt

The preservation of fish products by rendering the fish unsuitable for microbial proliferation has long been realised by reducing the water activity (a_w) through drying or the addition of solutes. During curing, which includes the processes of salting,

brining or sugar curing, the concentration of soluble substances increases either by abstracting water or by causing substances to diffuse (Horner, 1997). In addition to the preservation effect, salting is applied as a presmoking treatment in order to provide flavour, to prevent discolouration and to firm the fish flesh (Southcott and Razzell, 1973; Deng et al., 1974). The most commonly used salt is sodium chloride (NaCl), which is added in two different ways; dry salting or wet salting (brining). In dry salting the fish is covered with an amount of salt corresponding to 5-10% of the weight of the fish and the brine formed is allowed to drain away. In brining, the product is immersed in a prepared salt solution of a particular degree of salt saturation (Dillon et al., 1994; Horner, 1997). NaCl is more effective than other safe, common and cheap food solutes, such as sugar (Horner, 1997). Besides age and type of the brine and curing duration, the effect of salt on bacterial activity is concentration-dependent (Sofos, 1984; Leroi et al., 2000). In smoked fish products, the salting process, mostly injection brining, plays an important role. The salt denatures the surface protein and, with drying, the denatured protein forms a skin-like layer on the product surface, known as pellicle. It protects the inner part of the fish and keeps the smoke aroma inside the fish (Horner, 1992; Dillon et al., 1994).

Curing typically results in a shift from a population in raw freshwater fish dominated by Gram-negative bacteria to one in which Gram-positive organisms dominate (ICMSF, 1998). Gram-negative bacteria are considered to be more salt sensitive than Gram-positive bacteria (Salama and Khalafalla, 1993; Leroi et al., 2000). This might be because of the inhibition of enzymatic systems of some microorganisms due to the toxicity of chloride ions (Leroi et al., 2000). LAB are able to grow in relatively high NaCl concentrations (Castellani and Niven, 1955; Deloquis, 1988; Korkeala et al., 1992). Of the psychrotrophic LAB, *Leuconostoc* and *Carnobacterium* spp. tolerate less salt (Lücke, 1996b). Gancel et al. (1997), characterising lactobacilli from vacuum-packaged salted and smoked herring, found 66 out of a total of 77 strains to be resistant to 10% NaCl. However, studying the effect of smoke and salt on the microflora of vacuum-packaged cold-smoked salmon stored at 5°C for 5 weeks, Leroi et al. (2000) reported that total viable and LAB counts were mainly inhibited by a 5% salt concentration.

Nitrate, Nitrite

Nitrite and nitrate have been used as preservatives in several meat, fish and cheese products (Ingram, 1973; Kolbein Dahle, 1979; Skovgaard, 1992). Both components, commonly used together with NaCl, are considered curing salts. Sodium nitrite ($NaNO_2$) in combination with NaCl is responsible for the colour and for most of the texture, flavour, safety and storage stability characteristics in cured meat products (Kolbein Dahle, 1979; Sofos et al., 1979; Dodds and Collins-Thompson, 1984; Sebranek and Fox, 1985; Skovgaard, 1992). Nitrite is an important antimicrobial agent. It has been 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). Nitrite can form undissociated nitrous acid (nitric oxide, NO). Nitric oxide reacts with iron-sulphur complexes to form iron-nitrosyl complexes that disrupt the function of iron- and sulphur- containing enzymes, such as ferredoxin, in the bacterial cells (Woods et al., 1981; Woods and Wood, 1982; Pierson and Smoot, 1987; Reddy et al., 1992). Because LAB lack ferredoxin, they are resistant to nitrite (Castellani and Niven, 1955; Nielsen, 1983; Skovgaard, 1992). However, due to its capability under certain conditions to react with amines forming carcinogenic nitrosamine compounds, many European countries restrict or prohibit the use of nitrite (Pelroy et al., 1982; Walters, 1992; European Parliament and the Commission of the European Communities, 1995).

Nitrate is widely used as an additive in the production of cured meat products and, to a lesser extent, in the preservation of fish and cheese products (Leistner, 1973; Poulsen, 1980; Massey and Lees, 1992; Meah et al., 1994; Sanz et al., 1997). In the Scandinavian countries it has been added to the curing salt mixture of certain pickled fish products to control microbial activity during storage in barrels (Pederson and Meyland, 1981; Knøchel and Huss, 1984). Nitrate has been permitted in pickled herring and sprats in Finland and other EU Member States (European Parliament and the Commission of the European Communities, 1995; Ministry of Trade and Industry, Finland, 1999). It acts as a reservoir of nitrite in the presence of nitrate-reducing bacteria, but exerts a little or no bacteriostatic or bacteriocidal effect *per se* in the concentrations used (Skovgaard, 1992). After reduction to nitrite an activity in foods

may be expected (Dahle, 1979). Micrococci, staphylococci and pseudomonads are able to use nitrate as an alternative electron acceptor to oxygen under vacuum (Yamanaka et al., 1959; Taylor and Shaw, 1975; Doelle, 1975).

Acids

Since pH influences the susceptibility to microbial growth, acidification, for example, by the addition of acids, is used in the preservation of many foods as well as in fish products (Lund and Eklund, 2000). Acid-curing is performed in marinated fish by salt, vinegar (acetic acid), sugar and preservatives, resulting in a pH below 5.0. The acid and/or salt-treatment is applied to inhibit spoilage or to reduce the activity of salt- and acid-sensitive putrefactive Gram-negative bacteria (Blood, 1975). *Lactobacillus* species tolerate high acid concentrations and can therefore be important spoilage organisms in acetic acid preserves, such as salad dressings, conserves, pickled vegetables and marinated fish products. *L. fructivorans* caused spoilage of tomato ketchup containing acetic and benzoic acid, which reduced the initial pH of 3.8 to 3.3 – 3.4 (Björkroth and Korkeala, 1997).

2.3.3. Smoking

Smoking, one of the oldest methods of preserving fish, combines the effects of salting, drying, heating and smoke (Blight et al., 1988). The smoke contains a wide variety of organic constituents such as phenolic and carbonyl compounds and organic acids (Shewan, 1949; Shewan, 1961; Daun, 1979; Asita and Campbell, 1990; Horner, 1997). These bacteriostatic and bactericidal compounds, together with the concerted action of low water activity, the applied heat and enzymatic protein and lipid changes, inactivate autolytic enzymes and retard spoilage microorganisms (Gilbert and Knowles, 1975; Asita and Campbell, 1990; Salama and Khalafalla, 1993; Dillon et al., 1994; Sirkorski, 1994; Ashie et al., 1996). Usually the smoke compounds adhere to the surface of smoked fish and accumulate not deeper than 1 mm under the skin during storage (Sirkorski, 1994; Truelstrup Hansen, 1995; Marc et al., 1998). Smoke

has an effect on odour and taste (Southcott and Razzell, 1973; Karnop, 1980). It may mask spoilage changes and therefore affect the relationship between sensory and microbiological analyses (Ristiniemi and Korkeala, 1998). A strong correlation between sensory results and the total aerobic plate count has been observed at the end of the shelf-life of untreated modified atmosphere- and vacuum-packaged fish products (Scott et al., 1984). No clear association between microbial levels and sensory changes has been established for vacuum-packaged cold-smoked fish products (Magnússon and Traustadóttir, 1982; Leroi et al., 1998; Truelstrup Hansen et al., 1995; Truelstrup Hansen et al., 1998).

It has been shown that smoking effects a change from Gram-negative to Gram-positive microflora, probably due to the greater sensitivity of the Gram-negative bacteria to the antimicrobial compounds of the smoke (Liston, 1980; Salama and Khalafalla, 1993; Truelstrup Hansen, 1995; Efiuvwevwere and Ajiboye, 1996). Gancel et al. (1997) studied lactobacilli from vacuum-packaged salted and smoked herring and found all isolated strains to be resistant to liquid smoke. However, Leroi et al. (2000) did not find any difference in sensitivity to smoke directly after the process between Gram-negative bacteria, (*Vibrio* spp., *Pseudomonas* spp., *Shewanella putrefaciens*, and *Photobacterium* spp.) and Gram-positive bacteria (LAB and *Brochothrix* spp).

There has been a change in production from traditional smoke-preserved fish towards lightly smoke-flavoured fish products (Horner, 1992). In industrialised countries, smoking is primarily required to produce a lightly salted, lightly smoked product of attractive appearance, odour and flavour. Seafood producers use two types of smoking processes, hot-smoking and cold-smoking.

Hot-smoking

Hot-smoking is a process in which the fish is exposed to a temperature of at least 70°C in order to cook the flesh in addition to smoking. During the process, a brine-soluble protein pellicle is formed on the surface. This pellicle takes up most of the antioxidant and bacteriostatic substances from the smoke. After hardening, it represents a barrier against the further invasion of bacteria (Horner, 1992; Dillon et

al., 1994). After the hot-smoking process, most of the competitive bacteria present in the raw material are eliminated (Horner, 1992). Therefore, hot-smoked fish products are susceptible to contamination by pathogenes such as *Listeria monocytogenes*, because, unlike cold-smoked fish, there is no competitive background flora in hot-smoked fish and post-processing contamination might more easily lead to the multiplication of pathogens during storage (Jemmi and Keusch, 1992). Thus, a hot-smoked product has to be refrigerated prior to further packaging (Dillon et al., 1994).

Cold-smoking

Cold-smoked fish is processed at smoking temperatures not higher than 28°C (Horner, 1992). The formation of a pellicle, a result of denaturation of the surface protein during salting, is essential in the same way as in hot-smoking (Horner, 1992; Dillon et al., 1994). The products are preserved by salt levels of 2% to 3% NaCl (w/w) and are often vacuum-packaged. Several fish species are used as raw material, including salmon (*Salmo salar*), herring (*Clupea harengus*) and rainbow trout (*Oncorhyncus mykiss*). The cold-smoking process does not destroy the natural microbial population or psychrotrophic pathogens of the raw fish, nor does it decrease the water activity enough to inhibit post-process microbial growth (Horner, 1992; ICMSF, 1998). Therefore, ready products must be stored at chilled temperatures (ICMSF, 1998; Gram and Huss, 2000). Cold-smoked fish comes under lightly preserved fish products and is typically consumed as ready-to-eat products without heat treatment.

2.3.4. ‘Gravad’ process

Salting is the most important step in processing ‘gravad’ fish. The Swedish term ‘gravad’ originates from the original way of preparing the fish wrapped in leaves and buried in sand or peat (From and Huss, 1988). Additionally, the fish is filleted and salted and spices are added. This processing method has a long tradition in fish preparation in the Nordic countries. Dry salting and injection brining are used in

industrially produced ‘gravad’ fish because the salting process can be better controlled (From and Huss, 1988).

Nowadays, fish is filleted and salt, sugar, dill, sometimes pepper and fennel are added. The fillets go through a maturing process of 1-3 days. The ready product is sold sliced or as a fillet, packed in cling film or under vacuum and stored at chilled temperatures. Salmon (*Salmo salar*), herring (*Clupea harengus*), rainbow trout (*Oncorhyncus mykiss*), mackerel (*Scomber scombrus*) and Greenland halibut (*Reinhardtius hippoglossoides*) are used as raw material. Fish products of this type come under lightly preserved and ready-to-eat fish products, characterised by a salt content of 3%-6% NaCl (w/w) and a pH of > 5, and are typically consumed without heat treatment.

2.3.5. Semi-preserved marinated fish products

Marinating means curing raw or cooked fish in a low pH (below 5.0) solution containing acetic acid, salt and sometimes spices. Cold marinated-products of this type come under the category of semi-preserves. Semi-preserves such as marinated herring are not heat-treated, are therefore not sterile and have a limited shelf-life of up to six to twelve months in chilled storage (<10°C) (Borgström, 1953; McLay and Pirie, 1971; Blood, 1975; Gram and Huss, 2000). Extended shelf-life is achieved by the use of preservatives such as benzoic acid (Somers, 1975). *Lactobacillus* spp. have been identified as the specific spoilage organisms in unspoiled and spoiled herring marinades (Meyer, 1956b, 1962b; Kreuzer, 1957; Lerche, 1960; Reuter, 1965; Erichsen, 1967; Sharpe and Pettipher, 1983). Besides LAB, yeasts were also found in the marinating liquids or in the fresh herring (Lerche, 1960), also causing spoilage of the herring marinades (Somers, 1975).

2.3.6. Packaging

Different forms of packaging, such as modified atmosphere- and vacuum packaging, are used to extend the shelf-life and to keep the microbiological quality of fresh and processed fish as high as possible (Scott et al., 1984; Bremner and Statham, 1987; Pellegrino et al., 1990). Furthermore, packaging protects the fish product from contamination, fat oxidation, shrinkage and colour deterioration.

The most important factor in the microbiology of packaged perishable foods like fish is the relative permeability of the packaging material to O_2, CO_2 and water vapour (Cavett, 1968; Adams and Moss, 2000). Each gas has a specific effect on the shelf-life of the fish by inhibiting or slowing down the physico-chemical changes and the respiration rates of the spoilage microorganisms and by reducing their growth as well as retarding enzymatic spoilage (Finne, 1982; Wilhelm, 1982).

Modified atmosphere packaging

In MAP, normal air is replaced with gases such as CO_2, O_2 and N_2. Various amounts in different combinations are used. Bacteria initially present in the product show different responses to CO_2 and O_2 contents of the atmosphere. Therefore, selection of the appropriate levels of these gases makes it is possible to alter the flora developing during chilled storage (Finne, 1982). The proportion of every gas component is fixed when the mixture is introduced into the package and no further control is made during the storage (Flick et al., 1991, Reddy et al., 1992; Davies, 1997). The composition of the gas mixture changes during storage from its initial composition as a result of the chemical and enzymatic activity of the product and microbial activity (Hintlian and Hotchkiss, 1986; Young et al., 1988). The effectiveness of the MAP system in the preservation of fish lies in the combined inhibitory effect of low storage temperature and the carbon dioxide atmosphere in the package on microbial activity (Gray et al., 1983).

Vacuum packaging

Vacuum packaging can be defined as a form of MAP since the removal of air is, in itself, a modification of the atmosphere (Wilhelm, 1982; Flick et al., 1991; Davies, 1997). In vacuum packages, slow diffusion of atmospheric O_2 through the high-oxygen barrier film leaves only residual O_2 (1%). This amount is absorbed through chemical reactions with compounds in the product, through any residual respiratory activity in the product and microbial activity (Adams and Moss, 2000). The residual levels of CO_2 (10% to 20%) in the packages are due to the metabolism of the product tissue and microorganisms (Silliker and Wolfe, 1980; Smith et al., 1990). Aside from the gases in the package, the initial quality of the fish and proper temperature control throughout storage are important for the success of vacuum packaging (Wilhelm, 1982; Clingman and Hooper, 1986). Recent years have seen vacuum packaging increasingly used for the retail packaging of different kinds of products, including fish (Adams and Moss, 2000).

Effect of the gases on the microflora

CO_2, O_2 and N_2 have different effects on the different bacteria species. CO_2, the most inhibitory gas, has a strong antimicrobial action, which varies between different microorganisms (Gould, 2000). It is responsible for extending the lag phase of bacterial growth and for decreasing the growth rate during the log phase (Farber, 1991). The bacteriostatic effect of CO_2 depends on the gas concentration, the initial bacterial count and the type of fish product (Finne, 1982; Gray et al., 1983). CO_2 is highly soluble in water, forming carbonic acid that may lower the pH (Smith et al., 1990). The antimicrobial efficacy of CO_2 is greatly enhanced as the storage temperature of the product is reduced due to its increasing solubility with decreasing temperature (Gould, 2000). Thus, to guarantee maximum antimicrobial effect, the storage temperature should be kept as low as possible (Finne, 1982; Farber, 1991). Aerobic spoilage bacteria such as Gram-negative bacteria like *Pseudomonas* and *Flavobacterium,* Gram-positive bacteria like *Micrococcus* as well as moulds and yeasts are very CO_2 sensitive, whereas facultatively anaerobes, such as

Enterobacteriaceae, are less sensitive. Gram-positive bacteria, including LAB and *Brochothrix thermospacta* and obligatory anaerobic bacteria, are usually resistant to CO_2 (Silliker and Wolfe, 1980; Molin, 1983; Clingman and Hooper, 1986; Ward and Baj, 1988; Pellegrino et al., 1990; Smith et al., 1990; Flick et al., 1991; Farber, 1991; Adams and Moss, 2000).

The main effect of O_2 is to maintain the organoleptic properties, such as the colour and taste, of the fish product and to reduce the drip (Pellegrino et al., 1990; Stammen et al., 1990). O_2 generally stimulates the growth of aerobic bacteria and can inhibit strictly anaerobic bacteria (Davies, 1997), but suppresses certain psychrotrophic Gram-negative bacteria such as *Moraxella* spp. and *Acinetobacter* spp. (Pellegrino et al., 1990). LAB tolerate the ambient partial pressure of molecular O_2 (Lücke, 1996a).

N_2 is used in MAP as an inert filler to balance the gas, displace oxygen and to keep the package from collapsing. It has a negligible effect on bacterial growth and shelf-life (Davies, 1997).

During modified atmosphere- and vacuum packaging, storage results in a shift from an initial Gram-negative aerobic microflora to a predominantly Gram-positive facultatively anaerobic spoilage microflora and usually psychrotrophic LAB become the dominating spoilage flora (Stenström, 1985; Pellegrino et al., 1990; Farber, 1991). The LAB selection is generally recognised as beneficial for shelf-life because their growth can inhibit more potent spoilers due to antagonistic activities such as the production of organic acids, hydrogen peroxide and CO_2, and the synthesis of antibiotics and bacteriocins (Daeschel, 1989; Lindgren and Dobrogosz, 1990).

3. OBJECTIVES OF THE STUDY

The main objectives of the study dealing with LAB in spoiled vacuum-packaged cold-smoked and ‘gravad’ rainbow trout and semi-preserved marinated herring were to

1) study the proportions of LAB in the microbial flora of spoiled vacuum-packaged cold-smoked rainbow trout fillets and ‘gravad’ rainbow trout slices stored at chilled temperatures and semi-preserved marinated herring showing clear bulging of lids and gas formation when stored at chilled temperatures,

2) identify the LAB species associated with spoiled vacuum-packaged cold-smoked and ‘gravad’ rainbow trout and marinated herring to genus level using phenotypical tests and to species level using restriction endonuclease analysis- and rRNA gene restriction analysis (ribotyping) -based database,

3) compare the results from phenotypical genus-level identification of the LAB to the corresponding results obtained using ribotyping,

4) evaluate if the fish product type has an effect on the developing spoilage LAB species division,

5) evaluate the effect of $NaNO_2$ and KNO_3 on the LAB of vacuum-packaged cold-smoked rainbow trout stored at 4°C and 8°C.

4. MATERIAL AND METHODS

4.1. Samples (I, II, III)

Immediately after processing, samples of vacuum-packaged cold-smoked (I) and 'gravad' (II) Finnish rainbow trout (*Oncorhyncus mykiss*) were sent to the laboratory and stored at either 4°C or 8°C (I) and at either 3°C or 8°C (II). Microbiological analyses and sensory evaluation of the samples of vacuum-packaged cold-smoked rainbow trout were carried out once a week (I). Sample units of three vacuum-packaged 'gravad' rainbow trout packages were withdrawn from both temperatures for further analyses (II). Samples were studied microbiologically after 3, 6, 8, 10, 13, 15, 17, 18, 20, 22, 24, 27, 29 and 31 days and sensorially after 8, 15, 18, 20, 22, 27 and 31 days after production (II).

Twelve glass jars containing three types of marinated herring (*Clupea hargenus harengus*) from different lots produced by a single company were sampled for analysis (III). Ten of these showed clear bulging of lids and gas formation indicating spoilage, and two jars were of normal appearance. At the time of analysis, there were seven months of the expected shelf-life still remaining. All the glass jars contained herring cut into pieces, water, saccharose, NaCl, onions, vinegar, spices and sodium benzoic acid as a preservative. The two normal (nos. 1 and 2) and six of the spoiled (nos. 3 to 8) samples contained only the basic ingredients described above. Three spoiled samples (nos. 9 to 11) also contained garlic and one spoiled sample (no. 12) additional onion to the basic marinade. According to the manufacturer, the salt concentration of each sample was 3%.

4.2. Bacterial strains (I, III, IV, V)

In study I, at least 10 colonies of the highest dilutions that yielded colonies were selected at random from the APC plates (Difco, Detroit, Michigan, USA) when the total bacteria count was $>10^7$CFU/g. For samples stored at 4°C, totals of 99, 110 and 100 isolates were obtained from the spoilage flora from cold-smoked fish containing

NaCl only, NaCl together with KNO_3 and NaCl together with $NaNO_2$, respectively. For samples stored at 8°C, totals of 104, 108 and 99 isolates were obtained from the flora from samples containing NaCl only, NaCl together with KNO_3 and NaCl together with $NaNO_2$, respectively.

Following the initial grouping of the microbial flora, a total of 405 LAB were identified from spoiled cold-smoked rainbow trout (IV). For samples stored at 4°C, totals of 44, 91 and 77 isolates were obtained from the flora from samples containing NaCl only, NaCl together with KNO_3 and NaCl together with $NaNO_2$, respectively (IV). For samples stored at 8°C, totals of 45, 61 or 87 isolates were obtained from the flora from samples containing NaCl only, NaCl together with KNO_3 and NaCl together with $NaNO_2$, respectively (IV).

In study III, the isolation of LAB from the unspoiled and spoiled herring product resulted in selection of six and 200 isolates, respectively. All six isolates growing on MRS agar (Oxoid, Basingstoke, UK) from the unspoiled samples were included. Of all spoiled samples, half of the isolates analysed originated from MRS agar (Oxoid), and the other half from Rogosa SL agar (Orion Diagnostica, Espoo, Finland).

A total of 296 LAB isolates from the spoiled 'gravad' rainbow trout stored at 3ºC or 8ºC were identified (V), 128 and 168 isolates originated from samples stored at 3ºC and 8ºC, respectively.

All LAB strains (III, IV, V) were stored at -70ºC in MRS broth (Difco). Before use, they were subcultured overnight in 10 ml MRS broth at 30°C, plated on MRS agar (Oxoid) and incubated anaerobically at 25°C for 5 days using a model BR 38 gas-generating kit (Oxoid) in an anaerobic jar.

The following LAB type strains were included in the studies (III, IV, V): from the ATCC *Weissella viridescens* ATCC 12706, *Weissella halotolerans* ATCC 35410, *Carnobacterium piscicola* ATCC 35586, *Carnobacterium divergens* ATCC 35677, *Lactobacillus sakei* subsp. *sakei* ATCC 15521, *Lactobacillus curvatus* subsp. *curvatus* ATCC 25061, *Lactobacillus plantarum* ATCC 14971, *Lactobacillus hilgardii* ATCC 8290, *Lactobacillus collinoides* ATCC 27612, *Lactobacillus brevis* ATCC 14869, *Lactobacillus buchneri* ATCC 4005, *Lactobacillus fructivorans* ATCC 8288, from the

BCCM/LMG *Leuconostoc citreum* LMG 9824, *Weissella hellenica* LMG 15125, *Weissella kandleri* LMG 14471, from the CCUG *Leuconostoc mesenteroides* subsp. *cremoris* CCUG 21965, *Lactobacillus sakei* subsp. *carnosum* CCUG 31331, *Lactobacillus curvatus* subsp. *melibiosus* CCUG 34545, *Lactobacillus fermentum* CCUG 30138, from the DSM *Leuconostoc mesenteroides* subsp. *mesenteroides* DSM 20343, *Leuconostoc mesenteroides* subsp. *dextranicum* DSM 20484, *Leuconostoc pseudomesenteroides* DSM 20193, *Weissella paramesenteroides* DSM 20288, *Lactobacillus alimentarius* DSM 20249, *Lactobacillus curvatus* subsp. *curvatus* DSM 20019 and from the NCFB *Leuconostoc gelidum* NCFB 2775 and *Leuconostoc carnosum* NCFB 2776. In addition to the type strains, the reference strain of *Carnobacterium piscicola* SMRICC 192, was used.

4.3. Microbiological analyses (I, II, III)

In studies I, II and III, a 10 g portion of the sample was aseptically weighed in 90 ml of 0.9% NaCl (w/v) and 0.1% (w/v) peptone water in a sterile plastic bag, and then blended in a Stomacher 400 Lab Blender (Seward Medical, London, UK) for 30 seconds. All slices in a 'gravad' rainbow trout package (II) were chopped up in a Moulinex Food Processor (La Moulinette, type D 56, Paris, France) before weighing. Ten-fold serial dilutions were used for microbiological analyses. In study I, the APC was determined by the method of the Nordic Committee on Food Analysis (1986) using Plate Count Agar (Difco). In study II, MVC and H_2S–TC were performed in pour plates with Iron agar (IA) according to the method of the Nordic Committee on Food Analysis (1994). The Iron agar plates were counted after 3 days' aerobic incubation at 21ºC. PVC was carried out using the spread plate method on TSA agar (Tryptic soy agar, Merck, Darmstadt, Germany) according to Truelstrup Hansen et al. (1998). The TSA plates were counted after 5 days' aerobic incubation at 10ºC.

LAB counts in studies I, II and III were determined by the method of the Nordic Committee on Food Analysis (1991) using MRS agar (Oxoid). In study III, Rogosa SL agar (Orion Diagnostica) was cultured parallel to MRS agar (Oxoid). All strains were stored at -70ºC in MRS broth (Difco). Before use, they were subcultured

overnight in 10ml MRS broth (Difco) at 25ºC and then plated on MRS agar (Oxoid). All plates were incubated anaerobically at 25ºC for 5 days in an anaerobic jar with a H_2+CO_2 generating kit (Oxoid).

Yeasts in study III were determined by the method of the Nordic Committee on Food Analysis (1993) using OGYE agar (Oxoid).

4.4. Phenotypical criteria for distinguishing LAB from other bacterial groups (I, II, III)

All isolates were Gram-stained, tested for haemolytic activity (Columbia agar base, GIBCO BRL, Paisley, UK) and for catalase production (Baird-Parker, 1979). Furthermore, the bacteria were examined for oxidase production using Kovàcs reagent (Kovàcs, 1956).

All LAB isolates were tested for growth on MRS agar (Oxoid) (I, II, III) and on Rogosa SL agar (Orion Diagnostica) (I, III). In order to determine enterococci growth, isolates were cultured on Slanetz and Bartley agar (Orion Diagnostica) for 2 days' incubation at 37ºC (I). Production of gas from glucose was studied using the method of Schillinger and Lücke (1987) (I, III). Acetoin production was detected using the Voges-Proskauer test after 3 days' incubation (I). Hydrolysis of arginine was examined as described by Reuter (1970a) (I). Lactic acid configuration was determined enzymatically using a UV method kit (Boehringer Mannheim GmbH, Mannheim, Germany), according to the manufacturer`s instructions (I). 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) (I).

In study I, for further identification of the Gram-negative, oxidase-negative bacteria as *Enterobacteriaceae*, API 20 E (bio Mérieux, Marcy l`Etoile, France) and of the Gram-negative, oxidase-positive bacteria as *Pseudomonas* spp. API 20 NE (bio Mérieux) were used. For further identification of the other Gram-positive, catalase-positive cocci as S*taphylococcus* spp. and *Micrococcus* spp. (I), acid production from glycerol in the presence of erythromycin was determined according to Schleifer and

Kloos (1975), sensitivity to lysostaphin by the method of Kloos et al. (1974) and API Staph (bio Mérieux) was used.

4.5. 16+23S rRNA gene restriction patterns (ribotyping) (III, IV, V)

Isolation of DNA (III, IV, V)

The cultures were grown overnight in 10 ml MRS broth (Difco) at 25ºC. Cells were harvested by centrifuging for 2 minutes at full speed (13 000 rpm, about 15 000 x g) in a Biofuge A bench centrifuge (Heraeus Sephatec GmbH, Osterode am Kalkberg, Germany). DNA was isolated according to the guanidium thiocyanate method of Pitcher et al. (1989) modified by Björkroth and Korkeala (1996b). The cells were suspended in a TE (Tris-HCl 10 mM, EDTA 1 mM) solution containing mutanolysin 200 U/ml (Sigma Chemical Company, St. Louis, MO, USA) and lysozyme 25 mg/ml (Sigma). The mixture was incubated at 37ºC for 2 h.

Restriction endonuclease analysis and 16+23S rRNA gene restriction patterns (ribotyping) (III, IV, V)

Restriction endonuclease digestion of 6 µg of DNA was carried out according to the manufacturer's instructions with the restriction endonuclease *Hin*dIII (New England Biolabs, Beverly, MA, USA) in the studies III and V or with *Eco*RI (New England Biolabs) and *Hin*dIII (New England Biolabs) in study IV. The digested DNA was separated in 0.8% agarose gels (SeaKem IDNA agarose, FMC, Rockland, ME, USA) at 25 V for 16 h in a 1 × TAE buffer (Amresco, Solon, OH, USA) containing 0.5 mg of ethidium bromide in a GNA 200 apparatus (Pharmacia, Uppsala, Sweden). Digoxigenin labelled phage lambda DNA cleaved with *Hin*dIII (Roche Molecular Biochemicals, Mannheim, Germany) was used as a fragment size marker. Photodocumentation of the ethidium bromide stained gels was carried out using a Polaroid camera or digital imaging with an Alpha Imager 2000 documentation system (Alpha Innotech, San Leandro, CA, USA).

The cDNA probe was prepared from *E. coli* 16 and 23S rRNA (Boehringer Mannheim GmbH) by reserve transcription. The probe was labelled by incorporating digoxigenin-modified dUTP (Roche Molecular Biochemicals) by avian myeloblastosis virus reverse transcriptase (AMV-RT; Promega, Madison, WI, USA) (Blumberg et al., 1991).

The similarity between all isolates was initially checked by the naked eye from *Eco*RI and *Hin*dIII REA gels. One strain representing each REA group was further subjected to ribotyping. After electrophoresis, southern transfer and hybridisation were performed as previously described by Björkroth and Korkeala (1996b). The DNA was transferred from the gels to MSI Magnagraph membranes (MSI, Westboro, MA, USA) by a VacuGene XL blotting system (Pharmacia) according to the manufacturer's protocol. The DNA was fixed by UV irradiation in optimal crosslink mode in a Spectrolinker XL 1000 (Spectronics Corporation, New York, NY, USA). The membranes were hybridised in a Techne Hybridiser (Techne, Cambridge, UK) at 68ºC. Solutions for hybridisation, washes and development of the digoxigenin label were performed as described in the instructions for DIG DNA Labelling and Detection Kit (Boehringer Mannheim GmbH).

Numerical analysis of 16+23S rRNA gene restriction patterns (III, IV, V)

The membranes were scanned with a Hewlett-Packard ScanJet 4c/T tabletop scanner (Boise, Idaho, USA). Numerical analysis of the ribopatterns was performed using a Gelcompar II 1.0 software package (Applied Maths, Kortrijk, Belgium). Based on internal controls, a 1% (III) to 1.8% (V) 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 to construct 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. This database comprises patterns of all relevant spoilage LAB in the genera of *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Weissella* (Björkroth and Korkeala, 1996b; Björkroth and Korkeala, 1997; Björkroth et al., 1998; Björkroth et al., 2000a; Björkroth et al., 2000b).

Identification of the isolates was made on the basis of locations of the type and reference strains in the clusters formed.

4.6. Sensory analyses and visual examination (I, II, III)

Sensory analysis of the cold-smoked products (I) was performed once a week in order to detect the time of spoilage. The products 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 an unacceptable product. A sample was deemed spoiled if at least two judges out of 8 to 10 trained panellists considered it unfit for human consumption.

The 'gravad' products (II) were evaluated after 8, 15, 18, 20, 22, 27 and 31 days of production for appearance, texture, odour and taste in order to indicate the sensorial changes during storage, including the time of spoilage. The method described by Meilgaard et al. (1987) was used and adapted to the present study. For appearance and texture, a numerical scale from zero to five, and for odour and taste, from zero to ten, was used. The description used for the sensory characteristics had been developed during the training sessions. All samples were coded by a letter and were presented to the judges in random order. The judges were asked to comment on all four sensory properties, including any colour changes, and to state the reason for rejecting a particular sample. A sample was deemed spoiled if at least three judges out of 8 to 10 trained panellists considered it unfit for human consumption.

Visual examination of the herring flesh (III), the marinade and the gas production was performed in order to determine changes between both unspoiled and spoiled samples.

4.7. Chemical and physical analyses (II, III)

The pH was determined using first dilution by a WTW-530 Digital-pH-meter (Wissenschaftliche-Technische Werkstätten, Weilheim, Germany) (II, III).

Waterphase NaCl concentrations were determined according to the method of the Nordic Committee on Food Analysis (1974) (II).

4.8. Statistical analyses (II)

The bacterial counts for TVC, TPC, H_2S-TC and LAB at both storage temperatures were analysed as a function of time. The growth curve was modelled using the four-parameter Gompertz model as described by Jeffries and Brain (1984). A typical bacterial growth curve could not be obtained for TVC and TPC at an 8°C storage temperature due to the very short lag phase. The equation of the Gompertz growth curve is:

$$L(t) = A + C \cdot e^{-e^{-B \cdot (t-M)}}, \qquad (1)$$

where $L(t)$ is the $\log_{10}$ bacteria count/g in time t (days), A is the asymptotic $\log_{10}$-count as time decreases infinitely, C is the asymptotic amount of growth as time increases indefinitely, M is the time at which the absolute growth rate is at a maximum, and B is the relative growth rate at that point. The parameters were estimated using a Matlab programme with a Statistics Toolbox on a personal computer.

5. RESULTS

5.1. Bacterial levels and the main bacterial groups associated with spoiled vacuum-packaged cold-smoked and 'gravad' rainbow trout and marinated herring (I, II, III)

Bacterial levels at the time of spoilage and association with sensory changes

In study I, samples of vacuum-packaged cold-smoked rainbow trout were deemed spoiled when the total bacteria count was $>10^7$ CFU/g.

In study II, at the time of spoilage, after 27 and 20 days of storage at 3°C and 8°C, respectively, both MVC and PVC reached 10^6–10^7CFU/g at 3°C and 10^7–10^8 CFU/g at 8°C and most of the samples were deemed sensorially unfit for human consumption. The main reasons for sensory rejection at both storage temperatures were a lack of typical product odour or an ammonia off-odour and colour change to dark violet. At spoilage, H_2S-producing bacteria counts varied from 10^5-10^7 CFU/g and from 10^6-10^7 CFU/g in samples stored at 3°C and at 8°C, respectively. The corresponding LAB counts reached 10^4-10^6 CFU/g and 10^5-10^7 CFU/g (II, Table 1). Figs 1 and 2 (II) show the growth of MVC, PVC, H_2S–TC and LAB as a function of storage time at 3°C and 8°C, respectively.

In all herring samples (III), LAB counts were very similar on MRS and Rogosa SL media. In the spoiled samples, counts reached 10^6–10^7 CFU/g (III, Table 1). No yeasts were detected in any samples. Visual examination of the spoiled herring showed soft herring flesh, cloudy marinade and strong gas production that were not seen in the unspoiled samples. The pH values in the spoiled samples ranged from pH 3.7 to 3.9, whereas in the samples possessing normal appearance the pH ranged from 3.6 to 3.7.

Main bacterial groups at the time of spoilage

Of 620 isolates obtained from spoiled vacuum-packaged cold-smoked rainbow trout, 469 Gram-positive, catalase-negative cocci or rods growing on MRS agar were considered as LAB; 39 were Gram-positive, catalase-positive cocci; 98 were Gram-negative, oxidase-negative rods; and 12 isolates were Gram-negative, oxidase-positive rods (I, Table 1).

In the vacuum-packaged 'gravad' rainbow trout at the time of spoilage, after 27 and 20 days of storage at 3°C and 8°C, respectively 10^6–10^7 CFU/g in samples stored at 3°C and 10^7–10^8 CFU/g in samples stored at 8°C were detected in both MVC and PVC. H_2S-producing bacteria counts varied from 10^5-10^7 CFU/g and from 10^6-10^7 CFU/g in samples stored at 3°C and at 8°C, respectively. The corresponding LAB counts reached 10^4-10^6 CFU/g and 10^5-10^7 CFU/g (II, Table 1).

In all spoiled herring samples, bacterial counts were very similar on MRS and Rogosa SL media, suggesting *Lactobacillus* spp. growth (III, Table 1). A total of 206 colonies were cultured pure. All six isolates growing on MRS agar from the unspoiled samples were included. With respect to all spoiled samples, half of the isolates analysed originated from MRS agar, and the other half from Rogosa SL agar.

5.2. LAB associated with spoiled vacuum-packaged cold-smoked and 'gravad' rainbow trout and marinated herring (I, III, IV, V)

5.2.1. Vacuum-packaged cold-smoked rainbow trout (I, IV)

Based on phenotypic tests, LAB in the vacuum-packaged cold-smoked rainbow trout were divided into seven subgroups (I, Table 2). Subgroup 1, forming the largest group (40%), contained 189 isolates of homofermentative rods which grew well on Rogosa SL agar and could be considered as homofermentative or facultatively heterofermentative lactobacilli. The 50 isolates (11%) of the second subgroup were heterofermentative oval cocci, which did not grow on Rogosa SL agar. They could be considered as belonging to *Leuconostoc/Weissella*-species. The three isolates forming

subgroup 3 were heterofermentative rods possessing m-DPA in their cell walls and appeared to belong to the genus *Carnobacterium*. The 182 isolates (39%) of subgroup 4 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 Rogosa SL agar as heterofermentative lactobacilli do. The 3 isolates in subgroup 5 were cocci that formed colonies with a red-pink centre on Slanetz and Bartley agar but did not grow on Rogosa SL agar. They produced predominantly L(+)-lactic acid. They seemed to belong to the genus *Enterococcus*. Subgroup 6 contained heterofermentative and subgroup 7 homofermentative cocci or oval cocci. The isolates of these subgroups could not be assigned to genera with the used phenotypic tests.

REA of the 405 LAB isolates of vacuum-packaged cold-smoked rainbow trout (IV) resulted in the formation of 25 groups. When one isolate from each group was further ribotyped, 15 (H1 – H14) and 16 (E1 – E14) patterns were obtained with *Hin*dIII and *Eco*RI, respectively (IV, Table 2). The similarity between the clusters of isolates and some of the reference strains used is shown in Figs 1 and 2 (IV), for *Hin*dIII and *Eco*RI, respectively. Using *Hin*dIII, ten clusters (I – X) were defined at a similarity level of 65%. Cluster I contained the type strain of *Lactobacillus plantarum* (ATCC 14971) and cluster IV the *Lactobacillus curvatus* subsp. *curvatus* type strain (ATCC 25061). Cluster VIII contained the type strains of *Lactobacillus sakei* subsp. *carnosum* (CCUG 31331), *Lactobacillus sakei* subsp. *sakei* (ATCC 15521) and *Lactobacillus curvatus* subsp. *melibiosus* (CCUG 34545). Two clusters of leuconostocs were formed, one containing the *Leuconostoc mesenteroides* subsp. *mesenteroides* (DSM 20343) (cluster X) and the other the *Leuconostoc citreum* (LMG 9824) (cluster VI) type strain. Using *Eco*RI, nine clusters (I – IX) were defined at a similarity level of 50%. Two distinct subclusters, subcluster IIIa and IIIb, were identified; subcluster IIIa containing the type strains of *Lactobacillus sakei* subsp. *carnosum* (CCUG 31331), *Lactobacillus curvatus* subsp. *melibiosus* (CCUG 34545) and *Lactobacillus sakei* subsp. *sakei* (ATCC 15521) and subcluster IIIb the type strain of *Lactobacillus curvatus* subsp. *curvatus* (ATCC 25061). Cluster V included the type strain of *Lactobacillus plantarum* (ATCC 14971). Two clusters contained leuconostocs, one the *Leuconostoc mesenteroides* subsp. *mesenteroides* (DSM 20343) (cluster IX), and the other the *Leuconostoc citreum* (LMG 9824) (cluster VII)

type strain. Table 2 shows the species distribution detected using ribotyping within the seven phenotypically determined groups of LAB in order to show the differences obtained in LAB identification using the described methods. The majority of *L. mesenteroides* subsp. *mesenteroides* were found in the samples stored at 4ºC. Most of the *L. citreum* were isolated from the samples stored at 8ºC (Table 3).

Table 2. Species distribution within the 7 phenotypically determined groups of LAB from spoiled, vacuum-packaged cold-smoked rainbow trout in order of detection frequency[a] by 16+23S rRNA gene restriction patterns (ribotyping) based database.

	Phenotypically determined subgroups						
	1	2	3	4	5	6	7
	Lactobacillus	*Leuconostoc/ Weissella*	*Carnobacterium*	*Leuconostoc/ Lactobacillus*	*Enterococcus*	NI[b]	NI[b]
Species identified by ribotyping	*Leuconostoc*[a] *mesenteroides* subsp. *mesenteroides* *Lactobacillus sakei* subspp. *sakei/carnosum* *Lactobacillus curvatus* subspp. *curvatus/melibiosus* *Leuconostoc citreum* Unknown	*Leuconostoc mesenteroides* subsp. *mesenteroides* *Leuconostoc citreum* *Lactobacillus sakei* subspp. *sakei/carnosum*	Unknown	*Leuconostoc mesenteroides* subsp. *mesenteroides* *Leuconostoc citreum*	Unknown	*Leuconostoc citreum* *Leuconostoc mesenteroides* subsp. *mesenteroides* Unknown	*Leuconostoc mesenteroides* subsp. *mesenteroides* *Lactobacillus sakei* subspp. *sakei/carnosum* *Leuconostoc citreum* Unknown

[a] Most frequently detected species is placed first.
[b] NI, not identified.

5.2.2. Vacuum-packaged ‘gravad’ rainbow trout (V)

*Hin*dIII REA of the 296 LAB isolates resulted in the formation of 37 groups possessing group-specific REA patterns (V, Table 1). Eleven different ribopatterns (H1- H11) were obtained when one strain from each group was further ribotyped. A dendrogram (V, Figure 1) comprises the different clusters containing the type and reference strains and the ribopatterns of the LAB isolates. One cluster included the *Lactobacillus sakei* subsp. *sakei* (ATCC 15521), *Lactobacillus curvatus* subsp. *melibiosus* (CCUG 34545) and *Lactobacillus sakei* subsp. *carnosum* (CCUG 31331) type strains, the second cluster the *Lactobacillus curvatus* subsp. *curvatus* (DSM 20019) type strain, the third cluster the *Carnobacterium divergens* (ATCC 35677) type strain and the fourth cluster the reference strain (SMRICC 192) and *Carnobacterium piscicola* type strain (ATCC 35586). 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 (Table 3).

Table 3. LAB identified from spoiled, vacuum-packaged rainbow trout products using different curing methods and stored at different storage temperatures using 16+23S rRNA gene restriction patterns (ribotyping) based database.

Product	Storage temperature (°C)	Number of isolates	*L. mesenteroides* subsp. *mesenteroides*	*L. citreum*	*L. sakei* subspp. *sakei/ carnosum*	*L. curvatus* subspp. *curvatus/melibiosus*[a]	*L. plantarum*	*C. piscicola*	*C. divergens*	Unidentified
Cold-smoked	4	44	30	4	6	0	0	0	0	4
Cold-smoked and KNO_3	4	91	43	0	43	2	0	0	0	3
Cold-smoked and $NaNO_2$	4	77	38	4	25	2	0	0	0	8
Cold-smoked	8	45	12	19	3	1	0	0	0	10
Cold-smoked and KNO_3	8	61	4	4	41	2	0	0	0	10
Cold-smoked and $NaNO_2$	8	87	8	63	12	1	1	0	0	2
'Gravad'	3	128	0	0	7	83	0	37	1	0
'Gravad'	8	168	0	0	31	119	0	18	0	0
Total		701	135	94	168	210	1	55	1	37

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

5.2.3. Marinated herring (III)

All LAB in the marinated herring (III) were short, thick or long, filamentous rods. A total 139 of the 206 isolates showed gas production in the media used (Schillinger and Lücke, 1987).

The isolates of both unspoiled and spoiled samples shared the same *Hin*dIII restriction endonuclease type (III, Figure 1). Identical patterns were also obtained when two isolates from each herring jar were further ribotyped. The ribotype of the spoilage isolates was compared with corresponding patterns of LAB type strains known from literature as a spoiler of marinated herring products. The *L. alimentarius* type strain (DSM 20249) possessed very similar *Hin*dIII ribotypes to the pattern of the herring isolates, merging in the dendrogram at a similarity level of 89% (III, Figure 2). The *L. alimentarius* cluster was clearly distinct from the other LAB species.

5.3. Effect of nitrite and nitrate on the LAB of vacuum-packaged cold-smoked rainbow trout (I)

The relative proportion of LAB in the spoilage microflora was higher in the nitrite- and nitrate-containing samples than in the samples containing NaCl only at both storage temperatures. High numbers of *L. sakei* subsp. *sakei* and *L. sakei* subsp. *carnosum* occurred in the nitrate-treated samples stored at both storage temperatures. Most of the *L. citreum* were found in the nitrite-treated samples stored at 8ºC. The relative proportion of *L. mesenteroides* subsp. *mesenteroides* was higher in all kinds of samples stored at 4ºC (Table 3).

6. DISCUSSION

6.1. Main bacterial groups and the proportion of LAB associated with spoiled vacuum-packaged cold-smoked and 'gravad' rainbow trout and marinated herring (I, II, III)

The spoilage flora in all vacuum-packaged cold-smoked rainbow trout samples consisted mainly of LAB (76%). *Enterobacteriaceae* formed the second largest group (16%), besides lower numbers of micrococci, staphylococci and pseudomonads. Earlier studies of the same type of fish product have shown that at the end of chilled storage, different bacterial mixtures, such as LAB and *Enterobacteriaceae* (Truelstrup Hansen, 1995; Leroi et al., 2001) or LAB and *Photobacterium phosphoreum* (Truelstrup Hansen, 1995) or LAB and *Brochothrix thermosphacta* (Leroi et al., 2001), may develop. However, LAB have usually been found predominating or occurring in high numbers in spoiled vacuum-packaged cold-smoked fish (Truelstrup Hansen, 1995; Leroi et al., 1998; Leroi et al., 2001; Jørgensen et al., 2001; Joffraud et al., 2001). Studying the effect of salt and smoke on different bacteria in chilled stored vacuum-packaged cold-smoked salmon, Leroi et al. (2000) observed that salting produced a marked linear inhibition of lactobacilli growth in weeks 2 and 3 of storage. After 6 weeks' storage, lactobacilli counts were enhanced in the samples, which had been smoked in addition to high salting levels. In the spoiled cold-smoked rainbow trout, it seems that LAB tolerate combined salt and smoke preservation better than the other bacteria and thus dominate towards the end of the storage period.

In the vacuum-packaged 'gravad' rainbow trout, LAB counts were lower than the other bacterial counts determined at the time of spoilage (II, Table 1). These results were clearly different from the findings in other studies dealing with the same fish product type. Knøchel (1983) found the microflora of vacuum-packaged 'gravad' salmon dominated by LAB after 2 weeks storage at 4°C, as did Leisner et al. (1994) after 18 days storage at 5°C. Jeppesen and Huss (1993) also reported LAB as a dominant microbial group in vacuum-packaged 'gravad' fish products stored 2-4 weeks at chilled temperatures. In 'gravad' rainbow trout, the H_2S-producing bacteria constituted a larger proportion than lactobacilli. In the fresh product, both the H_2S-

producing bacteria counts and the LAB counts were below 100 CFU/g. H_2S-producing bacteria are known spoilage bacteria in aerobically stored and in vacuum-packaged fresh fish stored at chilled temperatures (Jensen et al., 1979; Sumner and Gorczyca, 1981; Scott et al., 1984; Jørgensen et al., 1988; Huss, 1995). Different bacteria such as *Shewanella putrefaciens*, *Aeromonas* spp., some *Enterobacteriaceae*, some *Vibrionaceae* and some lactobacilli as *L. sakei* are able to produce hydrogen sulphide (Gram et al., 1987; Truelstrup Hansen, 1995; Gram and Huss, 1996; Joffraud et al., 2001; Leroi et al., 2001). H_2S-producing bacteria are favoured by a low O_2 level, but inhibited in environments with increasing CO_2 levels (Jensen et al., 1979; Jørgensen et al., 1988). In vacuum-packages, CO_2 levels increase inside the package due to bacterial metabolism (Silliker and Wolfe, 1980), thereby also reducing the growth of some H_2S-producing bacteria during the later storage phase. However, during the storage of 'gravad' rainbow trout they grew faster than the LAB, thus representing another important part of the spoilage flora.

Several factors influence the development of a specific spoilage microflora in vacuum-packaged fish products. During production, the fish undergoes many processing steps enabling microbial contamination in all these steps. Truelstrup Hansen et al. (1998) showed that hygiene conditions in the smokehouse have a great effect on the amount and composition of the microflora in the final cold-smoked product. LAB do not dominate in the microflora of the raw material, and only certain species have been found in freshwater fish and their surrounding environment (Stiles and Holzapfel, 1997; González et al., 1999; González et al., 2000; Ringø et al., 2000). In the spoiled fish products, the selection of LAB results from the combination of the characteristic processing factors together with the growth competition of the bacteria and contamination of the production environment.

LAB were the only microorganisms detected in all the samples of spoiled marinated herring (III). Spoilage of semi-preserved marinated fish products due to the growth of LAB was reported earlier (Meyer, 1956a; Lerche, 1960; Reuter, 1965; Erichsen, 1967; Blood, 1975; Sharpe and Pettipher, 1983). In marinated herring, the acid and salt-treatment of the marinade inhibits Gram-negative spoilage bacteria, which are sensitive to this kind of preservation. Therefore, salt- and acetic acid-tolerant LAB, already occurring in the unspoiled product in low numbers (Reuter,

1965), can easily predominate in the spoiled product. Besides LAB, yeasts have been found in the marinating liquids or in the fresh herring (Lerche, 1960), also causing spoilage of the herring marinades (Somers, 1975). However, no yeasts were detected in this spoilage case.

6.2. Comparison of results from phenotypic genus-level identification and species identification by ribotyping (I, III, IV, V)

Phenotypical identification tests may sometimes cause difficulties resulting in the conflicting identification of LAB. In this study, two of the tests, glucose fermentation type determination and the selectivity of Rogosa SL agar, produced controversial genus-level identification results when compared to species identification by ribotyping. Table 2 shows the species distribution detected using ribotyping within the seven phenotypically determined groups of LAB in study I. The growth on Rogosa SL agar was used to differentiate between lactobacilli and other LAB. Isolates of subgroup 4 were considered leuconostocs because of their morphology, oval cocci and their ability to form gas from glucose. However, they grew also on Rogosa SL agar indicating that they are heterofermentative lactobacilli. This LAB group includes species such as *L. brevis*, *L. buchneri*, *L. collinoides*, *L. fructivorans* or *L. sanfranciscensis* which, however, have usually been associated with milk, cheese, vinegar preserves, fermenting plant material or sour dough, but never with spoiled cold-smoked fish. Without a sound experience of determining the morphology, it is sometimes difficult to distinguish between oval cocci (leuconostocs) and rods (heterofermentative lactobacilli). This misclassification resulted in the false identification of LAB in the spoiled cold-smoked fish product and lower numbers of leuconostocs (39%) were detected in study I compared to the results from ribotyping (57%) in study IV. The isolates of subgroup 5 were considered enterococci because their colonies showed a red-pink centre on Slanetz and Bartley agar and they did not grow on Rogosa SL agar. The isolates of subgroup 3 were considered as carnobacteria due to the presence of meso-DPA in their cell walls. However, they were able to form gas. Carnobacteria possess predominantly a homofermentative glucose metabolism

with small amounts of CO_2 being produced not coming from glucose (De Bruyn et al., 1987). The results of both subgroups could not be confirmed by ribotyping. With the phenotypic tests used, a total of 224 out of 469 isolates from vacuum-packaged cold-smoked rainbow trout could not be assigned to the correct genera.

In study III, 139 of the 206 *L. alimentarius* isolates showed gas production in the test by Schillinger and Lücke (1987). Facultative heterofermentative organisms such as *L. alimentarius* should not produce CO_2 from glucose. These controversial reactions obtained from testing facultatively heterofermentative *Lactobacillus* spp. using the same test have also been reported earlier (Kunene et al., 2000). Under certain circumstances, LAB of this fermentation type are able to produce gas from gluconate. Since this test medium does not contain gluconate or citrate, the reason for gas production is unclear. The test for gas production has also caused problems in other studies. Mauguin and Novel (1994) isolated LAB from certain vacuum-packaged fish products and grouped them using different phenotypic tests and a DNA hybridisation method. They concluded that the gas production test from glucose was not distinctive enough to differentiate between *Carnobacterium* and *Lactococcus* because *Lactococcus* cultures can also produce small amounts of gas in the stationary phase. It is known that some properties have a tendency to vary as a result of changes in growth conditions, growth phase, environmental conditions and spontaneous mutations due to the involved gene expression in phenotypic tests (Vandamme et al., 1996). This may result in the non-reproducibility of the tests or difficulties in interpretation and therefore limits the use of traditional methods.

Ribotyping was a great help in identifying LAB in all three types of spoiled fish products studied. Only 37 out of 405 isolates of the cold-smoked fish product (IV) could not be identified with the set of reference strains used. All isolates could be identified in the vacuum-packaged ‘gravad’ product (V). In the case of spoiled marinated herring (III), it was possible to accurately identify the specific spoilage organism.

6.3. LAB associated with spoiled fish products (I, III, IV, V)

6.3.1. LAB in vacuum-packaged cold-smoked rainbow trout (I, IV)

Two main groups of LAB were defined: leuconostocs and lactobacilli. The largest group, 57% of all isolates, was leuconostocs; 33% *L. mesenteroides* and 23% *L. citreum*. Leuconostocs in various numbers have also been found previously in vacuum-packaged cold-smoked fish products (Jeppesen and Huss, 1993; Mauguin and Novel, 1994; Truelstrup Hansen, 1995). Jeppesen and Huss (1993) identified all LAB from vacuum-packaged, minced herring as *Leuconostoc* spp. Mauguin and Novel (1994) found that only eight of the 86 LAB from various samples of seafood belonged to the genus *Leuconostoc*. Moreover, Truelstrup Hansen (1995) characterised 168 LAB from spoiled vacuum-packaged cold-smoked salmon and identified only 4.5% as *L. mesenteroides*. *L. 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, it has not previously been reported as being associated with vacuum-packaged cold-smoked fish products.

The second largest group, 39% of the isolates, was identified as *L. sakei/curvatus*. The occurrence of *L. sakei/curvatus* in vacuum-packaged fish products has also been reported previously (Jeppesen and Huss, 1993; Truelstrup Hansen, 1995; Leroi et al., 1998). In a study of LAB in different, lightly preserved, chilled fish products, Jeppesen and Huss (1993) identified the majority of the 37 isolates in vacuum-packaged chilled fish products as *L. sakei*. Studying vacuum-packaged cold-smoked salmon, from 168 LAB Truelstrup Hansen (1995) identified 10% as *L. sakei* and 50% as *L. curvatus* species. Leroi et al. (1998), phenotyping LAB isolated from the same type of fish product, assigned only 6% of 155 isolates as *L. sakei*. However, Mauguin and Novel (1994) did not detect any *L. sakei* in various samples of seafood. Since phenotypic tests have been found insufficient in *L. curvatus* and *L.sakei* identification (Dykes and von Holy, 1994b), the proportions of these two species reported in earlier studies may not have been totally correct.

In studies IV and V, the *L. curvatus* subsp. *melibiosus* ribotypes clustered together with the ribotypes of the two *L. sakei* subspecies. The *L. curvatus* subsp. *curvatus* cluster was clearly distinguished by both enzymes, *Hin*dIII and *Eco*RI (IV, Figs 1, 2; V, Fig 1) from the clusters containing the type strains of the three other subspecies. Current taxonomy of *L. sakei* and *L. curvatus* divides these two species into 4 subspecies (Klein et al., 1996; Torriani et al., 1996). The correctness of this subspecies level division is doubtful. When meat-associated *L. sakei* strains were characterised by *Eco*RI and *Hin*dIII ribotyping (Björkroth and Korkeala, 1996b), strain A210, possessing 84% homology in DNA-DNA reassociation with *L. sakei* subsp. *sakei* type strain (Mäkelä et al., 1992), gave exactly the same *Eco*RI and *Hin*dIII ribotypes as the "new" *L. curvatus* subsp. *melibiosus* type strain. 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).

With ribotyping, no *Carnobacterium* strains were detected in the vacuum-packaged cold-smoked rainbow trout fillets studied. Gancel et al. (1997) did not find any carnobacteria in fillets of vacuum-packaged, smoked and salted herring and suggested that this may be due to the smoking process. However, other authors have reported variable proportions of carnobacteria in vacuum-packaged cold-smoked fish products (Leroi et al., 1998; Leroi et al., 2000; Paludan-Müller et al., 1998).

6.3.2. LAB in vacuum-packaged 'gravad' rainbow trout (V)

In the spoiled vacuum-packaged 'gravad' rainbow trout (V), two main groups were detected: lactobacilli and carnobacteria. The largest group, 54% of all isolates, was identified as *L. sakei.* Based on phenotypic identification, this has also been observed earlier, either as a main organism or with other LAB dominating in vacuum-packaged 'gravad' fish products (Jeppesen and Huss, 1993; Leisner et al., 1994). Considering *L. curvatus* subsp. *melibiosus* as *L. sakei*, *L. curvatus* subsp. *curvatus* strains formed the second largest group, 27% of all isolates studied. In vacuum-packaged 'gravad' fish products, the occurrence of *L. curvatus* in such high numbers has not previously

been reported. Leisner et al. (1994) identified only one out of a total of 18 LAB strains as *L. curvatus*.

The third major LAB group consisted of carnobacteria, 19% of all isolates, with 55 *C. piscicola* isolates and one *C. divergens* isolate. *C. piscicola* has been associated with fresh fish and with packaged chill-stored fresh fish (González et al., 1999; González et al., 2000; Ringø et al., 2000). In contrast to the present findings, earlier studies have assigned carnobacteria as the largest group of the predominating LAB in vacuum-packaged 'gravad' fish products stored at chilled temperatures (Leisner, 1992; Leisner et al., 1994).

6.3.3. LAB species division between different types of vacuum-packaged fish products (IV, V)

There was a difference in the proportion of spoilage LAB species in vacuum-packaged cold-smoked and 'gravad' rainbow trout. Lactobacilli, in both fish products mainly consisting of *L. sakei* spp. and *L. curvatus* spp., dominated the LAB flora in the 'gravad' fish and lower numbers were detected in the cold-smoked product. Since both species are able to multiply at low temperatures (Lücke, 1996a), they can establish themselves on chilled stored fish. *L. sakei* and *L. curvatus* are already known as typical spoilage organisms in different meat products (Holzapfel and Gerber, 1986; Mäkelä and Korkeala, 1987; Mäkelä et al., 1992; Björkroth and Korkeala, 1996a; Björkroth and Korkeala, 1996b; Stiles and Holzapfel, 1997). For example, in cured and smoked meat products containing less water and a higher salt concentration than boiled whole meats, *L. sakei*/*curvatus* predominated together with *Weissella* spp. (Shaw and Harding, 1984; Samelis and Georgiadou, 2000; Samelis et al., 2000). It seems that *L. sakei*/*curvatus* tolerate the preservation factors characteristic for cold-smoked and 'gravad' fish, such as salt and smoke. They are therefore likely to play an important role also in the spoilage of both types of vacuum-packaged fish products.

The discovery of leuconostocs in the cold-smoked and their absence in the 'gravad' rainbow trout contrasts with studies dealing with meat products. In the cold-smoked fish, leuconostocs formed the largest group and thus seem to represent an

important part of the lactic acid flora in the spoiled product. No leuconostocs were found in the 'gravad' fish. Several studies have shown that in cooked, non-smoked meat products with a low salt content, *Leuconostoc* spp. have a better chance of predominating than in smoked, salted meat products (Borch et al., 1996; Samelis et al., 1994; Samelis et al. 2000; Björkroth et al., 1998; Samelis and Georgiadou, 2000). Borch and Agerhem (1992) reported that leuconostocs appear to grow fastest on chilled fresh meat. Lücke (1996a) suggested that heavy salting tends to suppress the growth of leuconostocs in the manufacture of raw meat products. The present 'gravad' fish had a higher salt concentration in the water phase (3.1%-7.2%) than the cold-smoked fish (3.4%). The sensitivity to salt might explain the absence of leuconostocs in the 'gravad' product.

In the 'gravad' fish, carnobacteria were detected in a relatively large proportion whereas in cold-smoked fish no carnobacteria were discovered at all. In a study of vacuum-packaged cold-smoked salmon stored for 5 weeks at 5°C, carnobacteria were found to be more sensitive to the combination of low salt concentrations and smoke treatment than lactobacilli (Leroi et al., 2000). When salt or smoke treatments were studied separately, the sensitivity of both genera was identical. Gancel et al. (1997) suggested that the smoking process was the reason they did not find any carnobacteria in vacuum-packaged smoked and salted herring. It might be that in cold-smoked fish the combination of salt and smoke creates a growth hurdle that affects carnobacteria more than lactobacilli. Leroi et al. (1998, 2000) observed in vacuum-packaged cold-smoked salmon that carnobacteria dominated the lactic acid bacterial flora during the first 2 to 3 weeks of storage and lactobacilli at the end of the storage. On the other hand, lactobacilli may recover faster from the processing stress and overgrow carnobacteria in the later storage phase in fish products as Schillinger and Lücke (1986) have indicated takes place in meat processing.

6.3.4. *Lactobacillus alimentarius* spoilage in marinated herring (III)

Ribotyping was used to identify *L. alimentarius* as the specific spoilage organism in marinated herring. It has not been previously associated with herring spoilage. The

slight pH rise together with marked gas production observed in the product suggested a rare LAB spoilage type called ‘protein swell’ (Meyer, 1956a). The rise in pH has been attributed to the production of ammonia by deamination of amino acids, whereas CO_2 production is due to the decarboxylation of amino acids. In the absence of fermentable carbohydrates both reactions can be caused by LAB (Meyer, 1964; Stamner, 1976). *L. alimentarius*, as a facultatively heterofermentative *Lactobacillus* spp., possesses homofermentative glucose metabolism, but may produce CO_2 while utilising gluconate or citrate without amino acid decarboxylation. Gluconate or citrate are not of metabolic importance in fish and therefore play no role in the ready product. The gaseous spoilage described here might therefore represent a typical form of ‘protein swell’ even when the change in pH values between spoiled and unspoiled samples was only nominal.

All isolates originating from various lots of different types of marinated herring were of the same clonal type suggesting contamination of the processing facilities or the raw material by an organism possessing strong spoilage potential. To restrict LAB growth, a pH value no higher than 4.0, a salt concentration of the marinade not much under 6% and a low storage temperature has been recommended for the marinating process (Meyer, 1956b). Nowadays, the salt concentrations used in herring are much lower than 6% and it is therefore difficult to prevent the growth of lactobacilli.

6.4. Effect of nitrate and nitrite on LAB associated with spoiled vacuum-packaged cold-smoked rainbow trout (I)

The proportion of LAB in the spoilage microflora of vacuum-packaged cold-smoked rainbow trout was higher in the nitrite- and nitrate-treated samples than in the samples containing NaCl only at both storage temperatures. These results are consistent with those of Hyytiä-Trees (1999). Nitrite is, as a hurdle effect, together with common salt and low temperature, partly responsible for arresting the growth of psychrotrophic spoilage organisms (Skovgaard, 1992). In vacuum-packaged meat products low concentrations of nitrite together with NaCl have been earlier reported to be selective for LAB growth (Korkeala et al., 1992; Skovgaard, 1992; Dodds and Collins-

Thompson, 1984). The same mechanisms might explain the high proportion of LAB in the present nitrite-treated samples.

7. CONCLUSIONS

1) LAB occurred in different proportions in spoiled vacuum-packaged cold-smoked and 'gravad' rainbow trout and in marinated herring stored at chilled temperatures. In vacuum-packaged cold-smoked rainbow trout, LAB formed the dominating proportion of the spoilage microflora, along with lower numbers of *Enterobacteriaceae*, micrococci, staphylococci and pseudomonads. Among the predominant psychrotrophic microorganisms in vacuum-packaged 'gravad' rainbow trout, LAB counts were lower than H_2S-producing bacteria. In the spoiled samples of marinated herring characterised by bulging lids and gas formation, LAB were the only microoragnisms determined at the time of the spoilage.

2) Phenotypic key tests indicate that the LAB isolated in spoiled vacuum-packaged cold-smoked rainbow trout could be considered as belonging to the genera of *Lactobacillus*, *Leuconostoc/Weissella*, *Carnobacterium* and *Enterococcus*. Using ribotyping, it was concluded that *L. mesenteroides*, *L. citreum*, *L. sakei* and *L. curvatus* were the major species associated with the spoilage of vacuum-packaged cold-smoked rainbow trout and *L. sakei*, *L. curvatus* and *C. piscicola* of vacuum-packaged 'gravad' rainbow trout.

3) Phenotypical methods may cause problems in genus-level identification of LAB in spoiled fish products. The controversial results of two key tests, glucose fermentation type determination and the selectivity of Rogosa SL agar, resulted in misidentification at the genus-level and in the wrong results of the leuconostocs and lactobacilli numbers from vacuum-packaged cold-smoked rainbow trout. The identification of *Carnobacterium* and *Enterococcus* could not be confirmed by ribotyping. A total of 224 out of 469 LAB isolates could not be assigned to the correct genera with the phenotypic tests used. Ribotyping has been used with good results for LAB species-level identification in all three spoiled products. Only a few isolates from the cold-smoked fish could not be identified with the aid of the reference strains used.

All isolates could be identified from the vacuum-packaged ‘gravad’ product. In the case of spoiled marinated herring, it was possible to identify the specific spoilage organism.

4) Lactobacilli, mainly consisting of *L. sakei* spp. and *L. curvatus* spp., dominated the LAB flora in the spoiled vacuum-packaged ‘gravad’ rainbow trout and were found in high numbers in the spoiled vacuum-packaged cold-smoked rainbow trout. Leuconostocs formed the largest group in the cold-smoked fish but were absent in the ‘gravad’ product. In the ‘gravad’ product, carnobacteria formed the third major group but were not detected in the cold-smoked product. A single *L. alimentarius* clone was considered to be the cause of gaseous spoilage in all three different types of marinated herring.

5) The proportion of LAB in the spoilage microflora was higher in the samples of vacuum-packaged cold-smoked rainbow trout treated with $NaNO_2$ or KNO_3 than in the samples containing NaCl only at both storage temperatures. Resistance to nitrite resulted in LAB constituting the major proportion of the total spoilage flora in the samples containing these preservatives.

6) The findings of this study (LAB species in spoiled fish products) have a basic research value. In the future, it would be of interest to test which role the different species play in the fish products, especially concerning sensorial and biochemical changes and how the spoilage associated species could be controlled. Since *L. sakei/curvatus* and *Leuconostoc* spp. often dominated the LAB flora in the spoiled products, it is of importance to focus on specific hurdles against these species. The results of this thesis are also important from the pathogen-study aspect. Only after identifying the main bacterial groups in an ecosystem can the competitive ability and survival of pathogenic organisms within this population be modelled.

8. REFERENCES

Adams, M.R. and Moss, M.O., 2000. Food Microbiology. 2nd ed., Royal Society of Chemistry, 448 pp.

Amerine, M.A., Pangborn, R.M. and Roessler, E.B., 1965. Principles of Sensory Evaluation of Food. Academic Press, New York and London, pp. 354-366.

Anonymous, Finnish Fisheries Statistics, 2000. Finnish Game and Fisheries Research Institute, 27 pp.

Ashie I.N.A., Smith, J.P. and Simpson, B.K., 1996. Spoilage and shelf-life extension of fresh fish and shell fish. Crit. Rev. Food Sci. Nutr. 36, 87-121.

Asita, A.O. and Campbell, I.A., 1990. Anti-microbial activity of smoke from different woods. Lett. Appl. Microbiol. 10, 93-95.

Austin, B. and Austin, D.A., 1992. The sanitary quality of waters associated with fresh water aquaculture. Microbiol. Aliment. Nutr. 10, 303-311.

Axelsson, L.T., 1993. Lactic acid bacteria: Classification and physiology. In: Salminen, S. and von Wright, A. (Eds.), Lactic acid bacteria. Marcel Dekkers Inc., New York, pp.1-65.

Baird-Parker, A.C., 1979. Methods for identifying staphylococci and micrococci. In: Skinner, F.A. and Lovelock, D.W. (Eds.), Identification Methods for Microbiologists. Academic Press, London, pp. 201-210.

Baranyi, J., Roberts, T.A. and McClure, P., 1993. A non-autonomous differential equation to model bacterial growth. Food Microbiol. 10, 43-59.

Baya, A.M., Toranzo, A.E., Lupiani, B., Li, T., Roberson, B.S. and 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.

Beltrán, A., Peláez, C. and Moral, A., 1989. Keeping quality of vacuum-packed smoked sardine filets: microbiological aspects. Z. Lebensm. Unters. Forsch. 188, 232-236.

Bingen, E.H., Denamur, E. and Elion, J., 1994. Use of ribotyping in epidemiological surveillance of nosocomial outbreaks. Clin. Microbiol. Rev. 7, 311-327.

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

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

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

Björkroth, K.J., Vandamme, P. and Korkeala, H.J., 1998. Identification and characterization of *Leuconostoc carnosum*, associated with production and spoilage of vacuum-packaged, sliced, cooked ham. Appl. Environ. Microbiol. 64, 3313-3319.

Björkroth, J., Korkeala, H. and Funke, G., 1999. rRNA gene RFLP as an identification tool for *Corynebacterium* species. Int. J. Syst. Bacteriol. 49, 983-989.

Björkroth, K.J., Geisen, R., Schillinger, U., Weiss, N., De Vos, P., Holzapfel, W.H., Korkeala, H.J. and 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.

Björkroth, J., Geisen, R., Schillinger, U., Weiss, N., Hoste, B., Holzapfel, W.H., Korkeala, H. and Vandamme, P., 2001. Taxonomic study of *Weissella confusa* and description of *Weissella cibaria* sp. nov., a novel species in food and clinical samples. Int. J. Syst. Evol. Microbiol. In press.

Blight, E.G., Shaw, S.J. and Woyewoda, A.D., 1988. Effects of drying and smoking on lipids of fish. In: Burt, J.R. (Ed.), Fish smoking and drying. Elsevier Applied Science, pp. 41-52.

Blood, R.M., 1975. Lactic acid bacteria in beverages and food. In: Proc. 4th Long Ashton Symposium 1973, Academic Press, London, pp. 195.

Blumberg, H.M., Kielbauch, J.A. and Wachsmuth, K., 1991. Molecular epidemiology of *Yersinia enterocolitica* O:3 infection: use of chromosomal DNA restriction fragment length polymorphism of rRNA gene. J. Clin. Microbiol. 20, 2368-2374.

de Boer, E. and Beumer, R.R., 1999. Methodology for detection and typing of foodborne microorganisms. Int. J. Food Microbiol. 50, 119-130.

Borch, E. and Agerhem, H., 1992. Chemical, microbial and sensory changes during the anaerobic cold storage of beef inoculated with a homofermentative *Lactobacillus* sp. or a *Leuconostoc* sp. Int. J. Food Microbiol. 15, 99-108.

Borch, E., Kant-Muermans, M.-L. and Blixt, Y., 1996. Bacterial spoilage of meat and cured meat products. Int. J. Food Microbiol. 33, 103-120.

Borgström, G., 1953. Herring delicatessen and marinated products. In: Jul, M., Kondrup, M. (Eds.), The technology of herring utilization. Boktrykkeri Griegs, Bergen, pp. 243-256.

De Bruyn, I.N., Louw, A.I., Visser, L. and Holzapfel, W.H., 1987. *Lactobacillus divergens* is a homofermentative organism. System. Appl. Micobiol. 9, 173-175.

Bremner, H.A. and Statham, J.A., 1987. Packaging in CO_2 extends shelf-life of scallops. Food Technol. Australia 39, 177-179.

Castellani, A.G. and Niven, C.F., 1955. Factors affecting the bacteriostatic action of sodium nitrite. Appl. Microbiol. 3, 154-159.

Cavett, J.J., 1968. The effects of newer forms of packaging on the microbiology and storage life of meats, poultry and fish. Prog. Ind. Microbiol. 7, 77-123.

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

Clingman, C. D. and Hooper, A.J., 1986. The bacterial quality of vacuum-packaged fresh fish. Dairy Food Sanit. 6, 194-197.

Cone, D.K., 1982. A *Lactobacillus* sp. from diseased female rainbow trout, *Salmo gairdneri* Richardson, in Newfoundland, Canada. J. Fish Diseas. 5, 479-458.

Daeschel, M.A., 1989. Antimicrobial substances from lactic acid bacteria for the use as food preservatives. Food Technol. 43, 164-167.

Dahle Kohlbein, H., 1979. Nitrite as a food additive. NIPH Annals 2, 17-24.

Daun, H., 1979. Interaction of woodsmoke components and foods. Food Technol. 33, 66-71.

Davies, A.R., 1997. Modified-atmosphere-packaging of fish and fish products. In: Hall, G.M. (Ed.), Fish processing technology. 2nd ed., Blackie Academic & Professional, London, pp. 200-223.

Deloquis, P.J., 1988. The effect of salt on growth of attached and unattached cells of *Lactobacillus viridescens*. J. Can. Inst. Food Sci. Technol. 21, 106-108.

Deng, J., Toledo, R.T. and Lillard, D.A., 1974. Effect of smoking temperatures on acceptability and storage stability of smoked Spanish mackerel. J. Food Sci. 39, 596-601.

Dicks, L.M.T. and van Vuuren, H.J.J., 1987. Relatedness of heterofermentative *Lactobacillus* species revealed by numerical analysis of total soluble cell protein patterns. Int. J. System. Bacteriol. 37, 437-440.

Dillon, R., Patel, T.R. and Martin, A.M., 1994. Microbiological control for fish smoking operations. In: Martin, A.M. (Ed.), Fisheries processing: biotechnological application. Chapman & Hall, London, pp. 51-75.

Doelle H.W., 1975. Bacterial metabolism. 2nd ed. Academic Press, London, pp. 157-192.

Dodds, K.L. and Collins-Thompson, D.L., 1984. Nitrite tolerance and nitrite reduction in lactic acid bacteria associated with cured meat products. Int. J. Food Microbiol. 1, 163-170.

Doyle, M.P., Beuchat, L.R. and Montville, T.J. (Eds.), 1997. Food Microbiology: Fundamentals and Frontiers, ASM Press, Washington D.C., p. 94.

Duffner, F., Fitzsimons, A., O' Kiely, P. and O' Connell, M., 1993. Monitoring of potential silage inoculants using molecular biology techniques. FEMS Microbiol. Rev. 12, 152.

DuPlessis, E.M. and Dicks, L.M.T., 1995. Evaluation of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus acidophilus*, *Lactobacillus crispatus*, *Lactobacillus amylovorus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, and *Lactobacillus johnsonii*. Curr. Microbiol. 31, 114-118.

Dykes, G.A. and von Holy, A., 1994a. Strain typing in the genus *Lactobacillus*. Lett. Appl. Microbiol. 19, 63-66.

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

Dykes, G.A., Cloete, T.E. and von Holy, A., 1994. Identification of *Leuconostoc* species associated with the spoilage of vacuum-packaged Vienna sausages by DNA-DNA hybridisation. Food Microbiol. 11, 271-274.

Efiuvwevwere, B.J.O. and Ajiboye, M.O., 1996. Control of microbiological quality and shelf-life of catfish (*Clarias gariepinus*) by chemical preservation smoking. J. Appl. Bacteriol. 80, 465-470.

Erichsen, I., 1967. The microflora of semi-preserved fish products III. Principal groups of bacteria occurring in tibits. Antonie van Leeuwenhoek 33, 107-112.

European Parliament and the Commission of the European Communities, 1995. European Parliament and Commission Directive 95/2/EC. Official Journal of the European Communities 61, 1-40.

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

Farber, J.M., 1991. Microbiological aspects of modified-atmosphere packaging technology – a review. J. Food Prot. 54, 58-70.

Farber, J.M., 1996. An introduction to the hows and whys of molecular typing. J. Food Prot. 59, 1091-1101.

Finne, G., 1982. Modified- and controlled-atmosphere storage of muscle foods. Food Technol. 36, 128-133.

Flick, G.J., Hong, G.P. and Knobl, G.M., 1991. Non-traditional methods of seafood preservation. J. Mar. Technol. Soc. 25, 35-43

From, V. and Huss, H.H., 1988. Gravad fisk. Fremstilling og holdbarhed. Report. Technology Laboratory, Ministry of Fisheries. University of Technology, Denmark, 39 pp.

Gancel, F., Dzierszinski, F. and 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.

Gibson, A.M., Bratchell, N. and Roberts, T.A., 1988. Predicting microbial growth: growth responses of salmonellae in a laboratory medium as affected by pH, sodium chloride and storage temperature. Int. J. Food Microbiol. 6, 155-178.

Gilbert, J. and Knowles, M.E., 1975. The chemistry of smoked foods: a review. J. Food Technol. 10, 245-261.

González, C.J., López-Díaz, T.M., García-López, M.L., Prieto, M. and Otero, A., 1999. Bacterial microflora of Wild brown trout (*Salmo trutta*), Wild pike (*Esox hucius*) and aquacultured rainbow trout (*Oncorhynchus mykiss*). J. Food Prot. 62, 1270-1277.

González, C.J., Encinas, J.P., García-López, M.L. and Otero, A., 2000. Characterisation and identification of lactic acid bacteria from freshwater fishes. Food Microbiol. 17, 383-391.

Gould, G.W., 2000. Strategies for food preservation. In: Lund, B.M., Baird-Parker, T.C., Gould, G.W. (Eds.), Microbiological safety and quality of food. Vol. 1, Aspen Publishers, Inc., Gaithersburg, Maryland, p.25.

Gram, L., Trolle, G. and Huss, H.H., 1987. Detection of specific spoilage bacteria from fish stored at low (0°C) and high (20°C) temperatures. Int. J. Food Microbiol. 4, 65-72.

Gram, L. and Huss, H.H., 1996. Microbiological spoilage of fish and fish products. Int. J. Food Microbiol. 33, 121-137.

Gram, L. and Huss, H.H., 2000. Fresh and processed fish and shellfish. In: Lund, B.M., Baird-Parker, T.C., Gould, G.W. (Eds.), Microbiological safety and quality of food. Vol. 1, Aspen Publishers, Inc., Gaithersburg, Maryland, p. 488.

Gray, R.J.H., Hooner, D.G. and Muir, A.M., 1983. Attenuation of microbial growth on modified-packaged fish. J. Food Prot. 46, 610-613.

Grimont, F. and Grimont, P.A.D., 1986. Ribosomal ribonucleic acid gene restriction as potential taxonomic tools. Annales de l'Institute Pasteur/ Microbiologie 137 B, 165-175.

Hall, L.M.C., Duke, B., Guiney, M. and Williams, R., 1992. Typing of *Enterococcus* species by DNA restriction fragment analysis. J. Clin. Microbiol. 30, 915-919.

Harmon, S.M., Kautter, D.A. and McKee, C., 1987. Spoilage of anchovy-stuffed olives by heterofermentative lactobacilli. J. Food Safety 8, 205-210.

Harper, J.J. and Davis, G.H.G., 1979. Two dimensional thin-layer chromatography for the amino-acid analysis of bacterial cell-walls. Int. J. Syst. Bacteriol. 29, 56-58.

Hielm, S., Björkroth, J., Hyytiä, E. and Korkeala, H., 1999. Ribotyping as an identification tool for *Clostridium botulinum* strains causing human botulism. Int. J. Food Microbiol. 47, 121-131.

Hildebrandt, G. and Erol, I., 1988. Sensorische und mikrobiologische Untersuchung an vakuumverpacktem Räucherlachs in Scheiben. Arch. Lebensmittelhyg. 39, 109-132.

Hintlian, C.B. and Hotchkiss, J.H., 1986. The safety of MAP – a review. Food Technol. 40, 70-76.

Holzapfel W.H. and Schillinger, U. 1992. The genus Leuconostoc. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., Schleifer, K.H. (Eds.), The Prokaryotes. Vol. 2, Springer Verlag, New York, pp. 1508-1534.

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

Horner, B., 1992. Fish smoking: ancient and modern. In: Proc. Food Science and Technology Today 6, 166-171.

Horner, W.F.A., 1997. Preservation fish by curing (drying, salting and smoking). In: Hall, G.M. (Ed.), Fish processing technology. 2nd ed., Blackie Academic & Professional, London, p.32.

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

Huis in´t Veld, J.H.J., 1996. Microbial and biochemical spoilage of foods: an overview. Int. J. Food Microbiol. 33, 1-18.

Huss, H.H., 1988. Fresh fish - quality and quality changes in. In: Huss, H.H. (Ed.), FAO Fisheries Series no. 29, FAO Rome, Italy, pp. 81.

Huss, H.H., 1995. Quality and quality changes in fresh fish. In: Huss, H.H. (Ed.), FAO Fish Tech. Pap. 348, FAO Rome, Italy, pp. 51.

Hyytiä, E., Eerola, S., Hielm, S. and Korkeala, H., 1997. Sodium nitrite and potassium nitrate in control of nonproteolytic *Clostridium botulinum* outgrowth and toxigenesis in vacuum-packed cold-smoked rainbow trout. Int. J. Food Microbiol. 37, 63-72.

Hyytiä-Trees, E., 1999. Prevalence, molecular epidemiology and growth of *Clostridium botulinum* type E in fish and fishery products. Ph.D. Thesis, Faculty of Veterinary Medicine, Department of Food and Environmental Hygiene, University of Helsinki, Helsinki, Finland.

Ingram, M., 1973. The microbiological effects of nitrite. In: Proc. International Symposium Nitrite Meat Products, Zeist, 1973. Pudoc, Wageningen, 63-75.

International Commission on Microbiological Specifications for Food (ICMSF), 1998. Fish and fish products. VIII. Cured, smoked and dried seafood. Microorganisms in food 6. Microbial ecology of food commodities. 1st ed., Blackie Academic and Professional, London, p. 174.

Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M. and Kerstens K., 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology 142, 1881-1893.

Jeffries, C.J. and Brain, P., 1984. A mathematical model of pollen-tube penetration in apple styles. Planta 160, 52-58.

Jemmi, T. and Keusch, A., 1992. Behaviuor of *Listeria monocytogenes* during processing and storage of experimentally contaminated hot-smoked trout. Int. J. Food Microbiol. 15, 339-346.

Jensen, M.H., Petersen, A., Røge, E.H. and Jepsen, A., 1979. Chilled and frozen storage. Storage of chilled cod under vacuum and at various concentrations of carbon dioxide. In: Proc. International Conference On Fish Science and Technology, Aberdeen, 294-297.

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

Jöckel, J., Kirschfeld, R. and Dickertmann, D., 1986. Vakuumverpackter Räucherfisch - Mikrobiologischer Status von Handelsproben und technologische Modellversuche zur Histaminbildung bei Makrelenfilets. Arch. Lebensmittelhyg. 37, 109-128.

Joffraud, J.J., Leroi, F., Roy, C. and Berdagué, J.L., 2001. Characterisation of volatile compounds produced by bacteria isolated from the spoilage flora of cold-smoked salmon. Int. J. Food Microbiol. 66, 175-184.

Johansson, M.-L., Quednau, M., Molin, G. and Ahrné, S., 1995. Characterisation and species recognition of *Lactobacillus plantarum* strains by fragment length polymorphism (RFLP) of the 16S RNA gene. J. Appl. Bacteriol. 79, 536-541.

Jørgensen, B.R., Gibson, D.M. and Huss, H.H., 1988. Microbiological quality and shelf-life prediction of chilled fish. Int. J. Food Microbiol. 6, 295-307.

Jørgensen, L.V., Dalgaard, P. and Huss, H.H., 2001. Multiple compound quality index for cold-smoked salmon (*Salmon salar*) developed by multivariate regression of biogenic amines and pH. J. Agric. Food Chem. 48, 2448-2453.

Kandler, O. and Weiss, O., 1986. Regular, nonsporeforming Gram-positive rods. In: Sneath, P.H.A., Mair, N.S., Sharpe, M.E., Holz, J.G. (Eds.), Bergeys Manual of Systematic Bacteriology. Vol. 2, Williams and Wilkins, Baltimore, MD, pp. 1208-1260.

Karnop, G., 1980. Qualität und Lagerverhalten heiss geräucherter Fischereiprodukte. I. Sensorische und mikrobiologische Beschaffenheit von rauchfrischem Heilbutt, Bückling und Aal. Deutsche Lebensmittel Rundschau 2, 42-47.

Kerr, K.G., 1994. The rap of REP-PCR-based typing systems. Rev. Med. Microbiol. 5, 233-244.

Klein, G., Dicks, L.M.T., Pack, A., Hack, B., Zimmermann, K., Dellaglio, F. and Reuter, G., 1996. Emended description of *Lactobacillus sakei* (Katagiri, Kitahara and Fukami) and *Lactobacillus curvatus* (Abo-Elnaga and Kandler): Numerical classification revealed by protein fingerprinting and identification based on biochemical patterns and DNA-DNA hybridizations. Int. J. Syst. Bacteriol. 46, 367-376.

Kloos W.E., Schleifer, K.H. and Tornabene, T.G., 1974. Isolation and characterization of micrococci from human skin, including 2 new species: *Micrococcus lyae* and *Micrococcus kristinae*. Int. J. Syst. Bacteriol. 24, 79-101.

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

Knøchel, S. and Huss, H.H., 1984. Ripening and spoilage of sugar salted herring with and without nitrate. II. Effect of nitrate. J. Food Technol. 19, 215-224.

Koeleman, J.G.M., Stoof, J., Biesmans, D.J., Savelkoul, P.H.M. and Vandenbroucke-Grauls, C.M.J.E., 1998. Comparison of amplified ribosomal DNA restriction analysis, randomly amplified polymorphic DNA analysis, and amplified fragment length polymorphism fingerprinting for identification of *Acinetobacter* genomic species and typing of *Acinetobacter baumannii.* J. Clin. Microbiol. 36, 1522-2529.

Kolbein Dahle, H., 1979. Nitrite as a food additive. NIPH Annala 2, 18-24.

Korkeala, H., Alanko, T., Mäkelä, P. and Lindroth, S., 1989. Shelf-life of vacuum-packed cooked ring sausages at different chill temperatures. Int. J. Food Microbiol. 9, 237-247.

Korkeala, H., Alanko, T. and Tiusanen, T., 1992. Effect of sodium nitrite and sodium chloride on growth of lactic acid bacteria. Acta. Vet. Scand. 33, 27-32.

Kovàcs, N., 1956. Identification of *Pseudomonas pyogenea* by the oxidase reaction. Nature 178, 703.

Kreuzer, R., 1957. Untersuchungen über den biologisch bedingten Verderb von Fischwaren und seine Verhinderung. I. Kaltmarinaden-Organismem und Milieu. Arch. Fisch Wiss. 8, 104 -139.

Kunene, N.F., Geornaras, I., von Holy, A. and Hastings, J.W., 2000. Characterization and determination of origin of lactic acid bacteria from a sorghum-based fermented weaning food by analysis of soluble proteins and amplified fragment length polymorphism fingerprinting. Appl. Environ. Microbiol. 66, 1084-1092.

Le Jeune and C., Lonvaud-Funel, A., 1994. *Lactobacillus hilgardii* and *Lactobacillus brevis* DNA analysis by restriction fragment length polymorphism (RFLP). Food Microbiol. 11, 195-202.

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. and 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.

Leisner, J.J., Vancanneyt, M., Goris, J., Christensen, H. and Rusul, G., 2000. Description of *Paralactobacillus selangorensis* gen. Nov., sp. nov., a new lactic acid bacterium isolated from chili bo, a Malaysian food ingredient. Int. J. Syst. Evol. Microbiol. 50, 19-24.

Leistner, L., 1973. Welche Konsequenzen hätte ein Verbot oder eine Reduzierung des Zusatzes von Nitrat und Nitritpökelsalz zu Fleischerzeugnissen? Fleischwirtsch. 3, 371-378.

Lerche, M., 1960. Bombage-Ursache in Fischpräserven. Berl. Münch. Tierärztl. Wochenschr. 75, 12-14.

Leroi, F., Arbey, N., Joffraud, J. and Chevalier, F., 1996. Effect of inoculation with lactic acid bacteria omn extending shelf-life of vacuum-packed cold-smoked salmon. Int. J. Sci. Technol. 31, 497-504.

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

Leroi, F., Joffraud, J.J., Chevalier, F. and Cardinal, M., 2001. Research of quality indices for cold-smoked salmon using a stepwise multiple regression of microbiological counts and physico-chemical parameters. J. Appl. Microbiol. 90, 578-587.

Liston, J., 1980. Microbiology in fishery science. In: Conell, J.J. (Ed.), Advances in fish science and technology. Fishing New (Books) Ltd., London, pp. 138-57.

Lindgren, S.E. and Dobrogosz, W.J., 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev. 87, 149-164.

Lücke, F.-K., 1996a. Indigenous lactic acid bacteria of various food commodities and factors affecting their growth and survival. In: Bozoglu, T. F. and Bibek, R. (Eds.), Lactic acid bacteria: Current advances in metabolism, genetics and applications. NATO ASI Series. Vol. H 98, Springer, Berlin, pp. 253-267.

Lücke, F.-K., 1996b. Lactic acid bacteria involved in food fermentation and their present and future use in food industry. In: Bozoglu, T. F. and Bibek, R. (Eds.), Lactic acid bacteria: Current advances in metabolism, genetics and applications. NATO ASI Series. Vol. H 98, Springer, Berlin, pp.81-101.

Lund, B.M. and Eklund, T., 2000. Control of pH and use of organic acids. In: Lund, B.M., Baird-Parker, T.C., Gould, G.W. (Eds.), Microbiological safety and quality of food. Vol. 1, Aspen Publishers, Inc., Gaithersburg, Maryland, p.175.

Magnússon, H. and Traustadóttir, K., 1982. The microbial flora of vacuum-packed smoked herring fillets. J. Food Technol. 17, 695-702.

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

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

Mancini, P.L. and Parini, C., 1983. DNA restriction endonuclease patterns, DNA sequence similarity and phenotypical characteristics in some strains of *Lactobacillus helveticus* and *Lactobacillus jugurti*. Antonie van Leeuwenhoek 49, 143-152.

Marc, C., Kaakeh, R. and Mbofung, C.M.F., 1998. Effect of salting and smoking method on the stability of lipid and microbiological quality of Nile Perch (*Lates niloticus*). J. Food Quality 22, 517-528.

Maslow, J.N., Mulligan, M.E. and Arbeit, R.D., 1993. Molecular epidemiology: Application of contemporary techniques to the typing of microorganisms. Clin. Infect. Diseas. 17, 153-164.

Massey, R.C. and Lees, D., 1992. Surveillance of preservatives and their interactions in foodstuffs. Food Addit. Contam. 9, 435-440.

Mauguin, S. and Novel, G., 1994. Characterisation of lactic acid bacteria isolated from seafood. J. Appl. Bacteriol. 76, 616-625.

Maunier, J.R. and Grimont, P.A.D., 1993. Factors affecting reproduction of randomly amplified polymorphic DNA fingerprinting. Res. Microbiol. 144, 373-379.

Mazurier, S.S. and Wernars, K., 1992. Typing of *Listeria* strains by randomly amplified DNA. Res. Microbiol. 143, 499-505.

McLay, R. and Pirie, R., 1971. Development of marinated herring. J. Food Technol. 6, 29-38.

Meah M.N., Harrison, N. and Davies, A., 1994. Nitrate and nitrite in foods and diet. Food Addit. Contam. 11, 519-532.

Meilgaard, M., Civille, G.V. and Carr, B.T., 1987. Sensory evaluation techniques. CPR Press, Inc., Boca Raton, Fla., USA.

Meyer, V., 1956a. Die Bestimmung der Bombage-Arten bei Fischkonserven. Fischwirtsch. 8, 212-224.

Meyer, V., 1956b. Probleme des Verderbens von Fischkonserven in Dosen. II. Aminosäuredecarboxylase durch Organismen der *Betabacterium-Buchneri*-Gruppe als Ursache bombierter Marinaden. Veröff. Inst. Meeresforsch. Bremerhaven 4, 1-16.

Meyer, V., 1962a. Probleme des Verderbens von Fischkonserven in Dosen. VII. Untersuchung über die Entstehung von Aminosäuren beim Marinieren von Heringen. Veröff. Inst. Meeresforsch. Bremerhaven 8, 21.

Meyer, V., 1962b. Über Milchsäurebakterien in Fischmarinaden. Zentrbl. Bakt. Parastikde. 1, Orig. 184, 296-302.

Meyer, V., 1964. The biochemical and bacteriological cause of swelling of marinades and aspects of its preservation. 4th International Symposium of Food Microbiology, Gothenburg, Sweden.

Ministry of Trade and Industry, 1999. Kauppa- ja teollisuusministeriön päätös elintarvikkeissa ja alkoholijuomissa käytettävistä muista lisäaineista kuin makeutusaineista ja väreistä 9.7.1999/811. (Ministry of Trade and Industry Decision on food and alcohol additives other than sweeteners and colours 9.7.1999/811)

Molin, G., 1983. The resistance to carbon dioxide of some food related bacteria. Eur. J. Appl. Microbiol. Biotechnol. 18, 214-217.

Nielsen, H.-J.S., 1983. Influence of nitrite addition and gas permeability of packaging film on the microflora in a sliced vacuum-packed whole meat product under refrigerated storage. J. Food Technol. 18, 573-585.

Nordic Committee on Food Analysis, 1986. Aerobic micro-organisms. Enumeration at 30°C in meat and meat products. NCFA method no. 86, 2nd ed. Espoo, Finland.

Nordic Committee on Food Analysis, 1974. Chloride. Determination in meat and fish products. NCFA method no. 89, Espoo, Finland.

Nordic Committee on Food Analysis, 1991. Lactic acid bacteria. Determination in meat and meat products. NCFA method no. 140, Espoo, Finland.

Nordic Committee on Food Analysis, 1993. Yeasts. Detection in foods. NCFA method no. 149, Espoo, Finland.

Nordic Committee on Food Analysis, 1994. Bacteriological examination of fresh and frozen seafood. NCFA method no. 96, 2nd ed., Espoo, Finland.

Olive, D.M. and Bean, P., 1999. Principles and applications of methods for DNA-based typing of microbial organisms. J. Clin. Microbiol.37, 1661-1669.

Orla-Jensen, S. 1919. The lactic acid bacteria. Copenhagen: Anhr. Fred. Host and Son.

Paleari, M.A., Soncini, G. and Beretta, G., 1990. Smoked tuna, sliced and vacuum-packed, a relatively new product. Z. Lebensm. Unters. Forsch. 190, 118-120.

Paludan-Müller, C., Dalgaard, P., Huss, H.H. and 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.

Paludan-Müller, C., Huss, H.H. and Gram, L., 1999. Characterization of lactic acid bacteria isolated from a Thai low-salt fermented fish product and the role of garlic as substrate for fermentation. Int. J. Food Microbiol. 46, 219-229.

Pederson, E. and Meyland, I., 1981. Nitrate, nitrite and volatile nitrosamines in pickled fish prepared with addition of nitrate. Z. Lebensm. Unters. Forsch. 173, 359-361.

Pellegrino, C., Giaccone, V. and Parisi, E., 1990. Die Verpackung von Fischprodukten in modifizierten Atmosphären. Literaturübersicht und Ergebnisse eines Lagerungsversuches mit rehydratisierten Klippfischfillets. Fleischwirtsch. 70, 1340-1346.

Pelroy, G.A., Eklund, M.W., Paranjpye, R.N., Suzuki, E.M. and Peterson, M.E., 1982. Inhibition of *Clostridium botulinum* types A and E toxin formation by sodium nitrite and sodium chloride in hot-process (smoked) salmon. J. Food Prot. 45, 833-841.

Pierson, M.D. and Smoot, L.A., 1987. Nitrite, nitrite alternatives, and the control of *Clostridium botulinum* in cured meats. Crit. Rev. Food Sci. Nutr. 17, 141-187.

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

Pot, B., Hertel, C., Ludwig, W., Descheemaeker, P., Kersters, K. and Schleifer, K.-H., 1993. Identification and classification of *Lactobacillus acidophilus, L. gasseri* and *L. johnsonii* strains by SDS-PAGE and rRNA-targeted oligonucleotide probe hybridization. J. Gen. Microbiol. 139, 513-517.

Poulsen, E., 1980. Use of nitrates and nitrites as food aditives in Nordic countries. Oncology 37, 299-301.

Reddy, N.R., Armstrong, D.J., Rhodehamel, E.J. and Kautter, D.A., 1992. Shelf-life extension and safety concerns about fresh fishery products packaged under modified atmospheres: a review. J. Food Safety 12, 87-112.

Reuter, G., 1965. Das Vorkommen von Laktobazillen in Lebensmitteln und ihr Verhalten im menschlichen Intestinaltrakt. Zentrbl. Bakt. Parastikde. 1, Orig. 197, 468.

Reuter, G., 1970a. Laktobazillen und eng verwandte Mikroorganismen in Fleisch und Fleischerzeugnissen. 2. Mitteilung: Die Charakterisierung der isolierten Laktobazillenstämme. Fleischwirtsch. 7, 954-962.

Reuter, G., 1970b. Laktobazillen und eng verwandte Mikroorganismen in Fleisch und Fleischerzeugnissen. 3. Mitteilung: Abgrenzung der einzelnen Keimgruppen. Fleischwirtsch. 8, 1081-1084.

Ringø, E. and Gatesoupe, F.-J., 1998. Lactic acid bacteria in fish: a review. Aquaculture 160, 177-203.

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

Ristiniemi, M. and Korkeala, H., 1998. Shelf-life of smoked Baltic herrings stored at different temperatures. Arch. Lebensmittelhyg. 49, 103-106.

Rodtong, S. and Tannock, G.W., 1993. Differentation of *Lactobacillus* strains by ribotyping. Appl. Environ. Microbiol. 59, 3480-3484.

Rodrigues, U.M., Aguirre, M., Facklam, R.R. and Collins, M.D., 1991. Specific and intraspecific molecular typing of lactococci based on polymorphism of DNA encoding rRNA. J. Appl. Bacteriol. 71, 509-516.

Salama, N.A. and Khalafalla, G.M., 1993. Chemical, bacteriological and sensory changes in Eel fish (*Anguilla vulgaris*) during smoking and storage. Arch. Lebensmittelhyg. 44, 1-24.

Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor.

Samelis, J., Maurogenakis, F. and Metaxopoulos, J., 1994. Characterisation of lactic acid bacteria isolated from naturally fermented Greek dry salami. Int. J. Food Microbiol. 23, 179-196.

Samelis, J. and Georgiadou, K.G., 2000. The microbial association of Greek taverna sausage stored at 4 and 10ºC in air, vacuum or 100% carbon dioxide, and its spoilage potantial. J. Appl. Microbiol. 88, 58-68.

Samelis, J., Kakouri, A. and Rementzis, J., 2000. The spoilage microflora of cured, cooked turkey breasts prepared commercially with or without smoking. Int. J. Food Microbiol. 56, 133-143.

Sanz, Y., Vila, R., Toldrá, F., Nieto, P. and Flores, J., 1997. Effect of nitrate and nitrite curing salts on microbial changes and sensory quality of rapid ripened sausages. Int. J. Food Microbiol. 37, 225-229.

Sanz, Y., Herandez, M., Ferrus, M.A. and Herandez, J., 1998. Characterisation of *Lactobacillus sake* isolates from dry-cured sausages by restriction fragment length polymorphism analysis of the 16rRNA gene. J. Appl. Microbiol. 84, 600-606.

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

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

Schillinger, U. and Holzapfel, W.H., 1995. The genus *Carnobacterium*. In: Wood, B.J.B., Holzapfel, W.H. (Eds.), The genera of lactic acid bacteria. Blackie Academic and Professional, London. p. 315.

Schleifer, K.H. and Kloos, W.E., 1975. A simple test system for the separation of staphylococci from micrococci. J. Clin. Microbiol. 1, 337-338.

Schleifer, K.H., Ehrmann, M., Beimfohr, C., Brockmann, E., Ludwig, W. and Amann, R., 1995. Application of molecular methods for the classification and identification of lactic acid bacteria. Int. Dairy J. 5, 1081-1094.

Schneider, W. and Hildebrandt, G., 1984. Untersuchungen zur Lagerfähigkeit von vacuumverpacktem Räucherlachs. Arch. Lebensmittelhyg. 35, 49-72.

Schulze, K., 1985. Untersuchungen zur Mikrobiologie, Haltbarkeit und Zusammensetzung von Räucherforellen aus einer Aquakultur. Arch. Lebensmittelhyg. 36, 77-100.

Scott, D.N., Fletcher, G.C. and Summers, G., 1984. Modified atmosphere and vacuum packaging of snappers fillets. Food Technol. Austr. 36, 330-334

Sebranek, J.G. and Fox, Jr., J.B., 1985. A review of nitrite and chloride chemistry: interactions and implications for cured meats. J. Sci. Food Agr. 36, 1169-1182.

Sharpe, M.E., 1979. Identification of the lactic acid bacteria. In: Skinner, F.A. and Lovelock, D.W. (Eds.), Identification methods for microbiologists. Soc. Appl. Bacteriol. Technical Series no. 14, Academic Press, London, pp. 246-255.

Sharpe, M.E. and Pettipher, G.L., 1983. Food spoilage by lactic acid bacteria. Econ. Microbiol. 8, 199-223.

Shaw, B.G. and Harding, C.D., 1984. A numerical taxonomic study of lactic acid bacteria from vacuum-packed beef, pork, lamb and bacon. J. Appl. Bacteriol. 56, 25-40.

Shewan, J.M., 1949. The biological stability of smoked and salted fish. Chem. Ind. 501-505.

Shewan, J.M., 1961. In: Borgstrom, G. (Ed.), The microbiology of sea-water fish. Fish as food. Vol. 1, Academic Press, Inc., New York, pp. 487-560.

Shimasaki, T., Miake, K., Tsukamasa, Y., Sugiyama, M., Minegishi, Y. and Shinano, H., 1994. Effect of water activity and storage temperature on the quality and microflora of smoked salmon. Nippon Suisan Gakkaishi 60, 569-576.

Silliker, J.H. and Wolfe, S.K., 1980. Microbiological safety considerations in controlled atmosphere storage of meats. Food Technol. 34, 59.

Sirkorski, Z.E., 1994. The quality and shelf life of smoked fish. Pol. J. Food Nutr. Sci. 44, 5-13.

Skovgaard, N., 1992. Microbiological aspects and technological needs: technological needs for nitrates and nitrites. Food Add. Cont. 9, 391-397.

Smith, J.P., Ramaswamy, H.S. and Simpson, B.K., 1990. Developments in food packaging technology. Part II: Storage aspects. Trends in Food Science and Technology. Elsevier Science Publishers Ltd, pp. 111-118.

Sofos, J.N., Busta, F.F. and Allen, C.F., 1979. Botulism control by nitrite and sorbate in cured meats: a review. J. Food Prot. 42, 739-770.

Sofos, J.N., 1984. Antimicrobial effect of sodium and other ions in food. A review. J. Food Safety 6, 45-78.

Somers, J.M., 1975. Herring marinades. Food-Progress 2, 2-4.

Southcott, B.A. and Razzell, W.E., 1973. *Clostridium botulinum* control in cold-smoked salmon: a review. J. Fish. Res. Board Can. 30, 631-641.

Ståhl, M., Molin, G., Persson, A., Ahrné, S. and Ståhl, S., 1990. Restriction endonuclease patterns and multivariate analysis as a classification tool for *Lactobacillus* spp. Int. J. Syst. Bacteriol. 40, 189-193.

Stammen, K., Gerdes, D. and Caporaso, F., 1990. Modified atmosphere packaging of seafood. Crit. Rev. Food Sci. Nutr. 29, 301-331.

Stamner, J.R., 1976. Lactic acid bacteria. In: DeFiqueiredo, M.P. and Splittstoesser, D.F. (Eds.), Food Microbiology: Public health and spoilage aspects. AVI Publishing, Westport, CT, p. 413.

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

Stenström, I.- M., 1985. Microbial flora of cod fillets (*Gadus morhua*) stored at 2ºC in different mixtures of carbon dioxide and nitrogen/oxygen. J. Food Prot. 48, 585-589.

Stephan, R., Schraft, H. and Untermann, F., 1994. Characterisation of *Bacillus licheniformis* with the RAPD technique (randomly amplified polymorphic DNA). Lett. Appl.Microbiol. 18, 260-263.

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

Sumner, J.L. and Gorczyca, E., 1981. Effect of vacuum-packaging on the shelf-life of fish held either in ice or at 4-6°C. Advances in refrigerated treatment of fish. Paris, France 49, 365-370.

Taylor, A.A. and Shaw, B.G., 1975. Whiltshire curing with and without nitrate. J. Food Technol. 10, 157-167.

Tenover, F.C., Arbeit, R.D. and Goering, R.V., 1997. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. Infect. Control Hosp. Epidemiol. 18, 426-439.

Torriani, S., Van Reenen, C.A., Klein, G., Reuter, G., Dellaglio, F. and Dicks, L.M.T., 1996. *Lactobacillus curvatus* subsp. *curvatus* subsp. nov. and *Lactobacillus curvatus* subsp. *melibiosus* subsp. nov. and *Lactobacillus sakei* subsp. *sakei* subsp. nov and *Lactobacillus sakei* subsp. *carnosus* subsp. nov., new subspecies of *Lactobacillus curvatus* Abo-Elnaga and Kandler, 1965 and *Lactobacillus sakei* Katagiri, Kitahara and Fukami 1934 (Klein et al., 1996, emended description), respectively. Int. J. Syst. Bacteriol. 46, 1158-1163.

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.

Truelstrup Hansen, L., Gill, T. and Huss, H.H., 1995. Effects of salt and storage temperature on chemical, microbiological and sensory changes in cold-smoked salmon. Food Res. Int. 28, 123-130.

Truelstrup Hansen, L., Gill, T., Drewes Røntved, S. and Huss, H.H., 1996. Importance of autolysis and microbiological activity on quality of cold-smoked salmon. Food Res. Int. 29, 181-188.

Truelstrup Hansen, L., Drewes Røntved, S. and Huss, H.H., 1998. Microbiological quality and shelf-life of cold-smoked salmon from three different processing plants. Food Microbiol. 15, 137-150.

Van Belkum, A., 1994. DNA fingerprinting of medically important microorganisms by use of PCR. Clin. Microbiol. Rev. 7, 174-184.

Vandamme, P., Pot, B., Gillis, M., deVos, P., Kersters, K. and Swings, J., 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. 60, 407-438.

Van der Vossen, J.M.D.M. and Hofstra, H., 1996. DNA based typing, identification and detection systems for food spoilage microorganisms: development and implementations. Int. J. Food Microbiol. 33, 35-49.

Vauterin, L., Swings, J. and Kersters, K., 1993. Protein electrophoresis and classification. In: Goodfellow, M. and O´Donnell, A.G. (Eds), Handbook of new bacterial systematics. Academic Press Ltd., London, pp. 251-280.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, J., Pot, J., Peleman, J., Muiper, M. and Zabeau, M., 1995. AFLP: a new concept for DNA fingerprinting. Nucleic Acids Res. 18, 7213-7218.

Walters, C.L., 1992. Reactions of nitrate and nitrite in foods with special reference to the determination of N-nitroso compounds. Food Addit. Contam. 9, 441-447.

Ward, D.R. and Baj, N.J., 1988. Factors affecting microbiological quality of seafoods. Food Technol., 85-89.

Welsh, J., McClelland and M., 1992. PCR-amplified length polymorphisms in tRNA intergenic spacers for categorizing staphylococci. Mol. Microbiol. 6, 1673-1680.

Wilhelm, K.A., 1982. Extended fresh storage of fishery products with modified atmospheres: a survey. Mar. Fish. Rev. 44, 17-20.

Williams, J.G.K., Kubelik, A.E., Levak, K.J., Rafalski, J.A. and Tingey, S.C., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18, 6531-6535.

Wood, B.J.B. and Holzapfel, E.H. (Eds), 1995. The genera of lactic acid bacteria. Chapman and Hall, Glasgow, 398 pp.

Woods, L.F.J., Wood, J.M. and Gibbs, P.A., 1981. The involvement of nitric oxide in the inhibition of the phosphrorclastic system in *C. sporogenes* by sodium nitrite. J. Gen. Microbiol. 125, 399-406.

Woods, L.F.J. and Wood, J.M., 1982. A note on the effect of the nitrite inhibition on the metabolism of *C. botulinum*. J. Appl. Bacteriol. 52, 109-110.

Yamanka, T., Ota, A. and Okunuki, K., 1959. A nitrite reducing system reconstructed with purified cytochrome components of *Pseudomonas aeruginosa*. Biochem. Biophys. Acta 53, 294-308.

Young, L.L., Revere, R.D. and Cole, A.B., 1988. Fresh red meats: a place to apply modified atmosphere. Food Technol. 42, 65-69.

Zorn, W., Greuel, E. and Krämer, J., 1993. Beurteilung des Hygienestatus geräucherter, vakuumverpackter Forellenfillets. Arch. Lebensmittelhyg. 44, 81-104.