

Quality of chilled, vacuum packed cold-smoked salmon

by

Lisbeth Truelstrup Hansen

Industrial Ph.D.thesis, August 1995

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Lisbeth Truelstrup Hansen

This report has been submitted as an industrial Ph. D. thesis (EF-444) to the Royal Veterinary and Agricultural University, Copenhagen, Denmark and the Academy of Technical Sciences, Denmark.

August 1995

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THE HISTORY OF THE CITY OF BOSTON

BY
JOHN B. HENNING

THE HISTORY OF THE CITY OF BOSTON, FROM THE FIRST SETTLEMENT IN 1630 TO THE PRESENT TIME. BY JOHN B. HENNING. VOL. I. FROM THE FIRST SETTLEMENT TO 1700.

1850

THE HISTORY OF THE CITY OF BOSTON, FROM THE FIRST SETTLEMENT IN 1630 TO THE PRESENT TIME. BY JOHN B. HENNING. VOL. II. FROM 1700 TO 1850.

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THE HISTORY OF THE CITY OF BOSTON, FROM THE FIRST SETTLEMENT IN 1630 TO THE PRESENT TIME. BY JOHN B. HENNING. VOL. III. FROM 1850 TO THE PRESENT TIME.

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THE HISTORY OF THE CITY OF BOSTON, FROM THE FIRST SETTLEMENT IN 1630 TO THE PRESENT TIME. BY JOHN B. HENNING. VOL. V. FROM 1850 TO THE PRESENT TIME.

Abstract.

This thesis deals with quality and safety aspects in the modern production of vacuum packed cold-smoked salmon. These aspects are addressed through a discussion of available information in the literature and results from a number of experiments conducted during the present study.

The importance of autolysis and microbiological activity on spoilage of cold-smoked salmon was studied in a storage experiment using dry salted and injection brined, vacuum packed salmon with normal and reduced loads of microorganisms. The spoilage odours and flavours were only found in salmon supporting growth of bacteria in numbers of 10^6 - 10^8 cfu/g, and these spoilage characteristics therefore resulted from microbiological activity. However, the texture deteriorated regardless of microbiological loads, indicating the importance of autolytic activity for the quality of the texture.

It became clear through a number of storage experiments that the microflora on cold-smoked salmon is highly variable among production batches from the same plant and among different processing plants. Characterization of 59-60 bacteria isolated from spoiled salmon from each of 3 different smokehouses revealed significant differences in the representation of various lactic acid bacteria (LAB) species and *Enterobacteriaceae* species among the smokehouses. Use of Malthus methods calibrated to traditional agar methods revealed similar differences among the 3 processing plants.

A complex mixed microflora consisting of LAB, *Enterobacteriaceae* and/or bacteria belonging to the group of *Photobacterium* spp. and marine vibrios were shown to develop during chill storage of smoked salmon. The occurrence of bacteria from the latter group has not been reported previously, and further work is required to elucidate the importance of these bacteria with respect to quality. In the characterization of LAB, tentatively identified *Lactobacillus curvatus* was the most common species among the LAB. Other LAB groups included *Lactobacillus sake/bavaricus*, *Lactobacillus plantarum*, *Carnobacterium* spp., *Leuconostoc* spp. and an unidentified homofermentative *Lactobacillus* sp. Tentatively identified *Enterobacteriaceae* spp. included *Serratia liquefaciens*, *Enterobacter agglomerans* and *Hafnia alvei*.

Preliminary model experiments were aimed at evaluating the growth and spoilage potentials among selected isolates. Initial experiments were conducted in heat sterilised salmon juice, but to obtain a more realistic picture, inoculation experiments were performed on smoked salmon blocks produced with a reduced number of the indigenous flora. Results from these experiments indicated the existence of spoilage potentials among LAB,

Enterobacteriaceae and *Photobacterium phosphoreum* strains, all of which were isolated from spoiled cold-smoked salmon. It is concluded that further work is required to characterise the biochemistry of microbiological spoilage and to analyze the microbial interactions which seem to take place through the storage period.

The use of chemical quality indicators such as hypoxanthine, total volatile bases, trimethylamine, ethanol and acetic acid, is confounded by the fact that the spoilage microflora is highly variable. The production of these compounds was shown to be related to the metabolisms of the individual bacteria present, each group producing them at different rates and this without relation to occurrence of spoilage. It may be more promising to try and identify chemical compounds, which are essential for the development of spoilage odours and flavours, and hence use those as indicators.

Hazard analysis of factors (microbiological, biological and chemical), which may affect the quality and safety of the product, showed that it is possible under proper conditions to control all potential hazards except the risk of growth of *Listeria monocytogenes* to unacceptable levels in the final product during chill storage. It is therefore recommended that incorporation of additional hurdles into the product is considered.

Sammendrag.

Denne afhandling omhandler aspekter vedrørende kvalitet og sikkerhed i moderne produktion af vakuumpakket koldrøget laks. Disse aspekter er behandlet igennem en diskussion af tilgængelig information fra den videnskabelige litteratur og resultater fra en række eksperimenter udført i løbet af dette studium.

Betydning af autolytiske og mikrobiologiske aktiviteter i forbindelse med fordærv af koldrøget laks blev belyst i et lagringsforsøg med tørsaltet og stiksaltet vakuumpakket laks fremstillet med et normalt eller reduceret indhold af mikroorganismer. De typiske bilugte og bismage forbundet med fordærv fandtes kun i laks med et højt indhold af bakterier (10^6 - 10^8 kim/g), og forekomst af disse fordærvelseskarakteristika var således forbundet med mikrobiologisk aktivitet. Konsistensen blev imidlertid forringet uafhængigt af indholdet af mikroorganismer. Dette indicerede, at konsistensforandringer i koldrøget laks i høj grad skyldes aktivitet af autolytiske enzymer.

Det blev klart igennem en række lagringsforsøg, at mikrofloraen på koldrøget laks varierer mellem forskellige produktioner fra den samme virksomhed og mellem forskellige virksomheder. Karakterisering af 59-60 bakterier isoleret fra fordærvet laks fra hver af 3 røgerier viste således signifikante forskelle mellem de 3 producenter med hensyn til sammensætning af både mælkesyrebakteriefloraen og den Gram-negative *Enterobacteriaceae* flora. Kalibreringskurver mellem Malthus konduktansmetoder og traditionelle kimtal viste lignende forskelle mellem de 3 virksomheder.

En kompleks mikroflora bestående af mælkesyrebakterier, *Enterobacteriaceae* og/eller bakterier tilhørende gruppen af *Photobacterium*/marine vibrioer udviklede sig under lagring af røget laks. Forekomsten af bakterier fra den sidstnævnte gruppe er ikke tidligere rapporteret i koldrøget laks, og det vurderes, at yderligere forskning er nødvendig for at belyse betydningen af disse bakterier med hensyn til kvalitet af produktet. Tentative *Lactobacillus curvatus* var den mest almindelige species blandt de isolerede mælkesyrebakterier. Andre mælkesyrebakterier blev tentativt identificeret som *Lactobacillus saké/bavaricus*, *Lactobacillus plantarum*, *Carnobacterium* spp., *Leuconostoc* spp., mens en gruppe af homofermentative *Lactobacillus* sp. forblev uidentificeret. Blandt de Gram-negative *Enterobacteriaceae* kunne følgende species tentativt identificeres: *Serratia liquefaciens*, *Enterobacter agglomerans* og *Hafnia alvei*.

Preliminære modelforsøg blev udført for at belyse vækst- og fordærvelsespotentialer blandt isolater fra fordærvet koldrøget laks. De første eksperimenter blev udført i varmesteriliseret laksejuice, mens videre modelforsøg blev udført på røget laksestykker

produceret med et lavt indhold af bakterier og derfor under forhold tættere på dem i produktet. Resultaterne fra disse eksperimenter indicerede, at mælkesyrebakterier, *Enterobacteriaceae* og *Photobacterium phosphoreum* isoleret fra fordærvet laks var i stand til at frembringe nogle af de typiske fordærvelseskarakteristika fundet i produktet. Det kunne konkluderes, at videre arbejde bør fokuseres på en karakterisering af biokemien bag det mikrobiologiske fordærv og på en analyse af de mikrobiologiske interaktioner, som finder sted i produktet.

Brugen af kemiske kvalitetsindikatorer som hypoxanthin, totale flygtige baser (TVN), trimethylamin, ethanol og eddikesyre, er konfunderet som følge af den variable fordærvelsesflora. Det blev vist, at produktionen af disse stoffer var relateret til stofskiftet hos de enkelte almindeligt forekommende bakterier, men også at hver gruppe producerede dem med forskellig hastighed. Desuden havde ingen af de testede indikatorer direkte forbindelse med forekomsten af hverken den typiske lugt eller smag af fordærvet koldrøget laks. Det ville være bedre, hvis kemiske forbindelser med direkte forbindelse til det typiske fordærv blev identificeret og derefter brugt som indikatorer.

Analysen af de mikrobiologiske, biologiske og kemiske farer (hazards), som er forbundet med produktion af koldrøget laks og muligt kan påvirke kvaliteten og den sundhedsmæssige sikkerhed af produktet, viste, at det er muligt under ordentlige forhold at kontrollere alle potentielle farer med undtagelse af risikoen for opformering af *Listeria monocytogenes* til uacceptable niveauer under kølelagring. Det anbefales derfor, at ekstra konserverende parametre indarbejdes i produktionen af produktet.

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Lisbeth Truelstrup Hansen, August 1995.

Table of Contents.

	Page
Abstract	iii
Sammendrag (Abstract in Danish)	v
Acknowledgements	vii
Table of Contents	ix
1. Introduction.	1
1.1 Objectives, focus and strategy in experimental work	3
2. Cold-smoked salmon, quality and shelf life.	6
2.1 Definition of cold-smoked salmon	6
2.2 Production of cold-smoked salmon	7
2.2.1 Quality aspects of the raw material	7
2.2.1.1 Colour	8
2.2.1.2 Fat content and composition	9
2.2.1.3 Sexual maturity	10
2.2.1.4 Texture	10
2.2.1.5 Microbiology	10
2.2.2 Production methods	11
2.3 Cold-smoking as a preservation method	13
2.3.1 Salt	17
2.3.2 Cold-smoking	18
2.3.3 Packaging and storage temperature	19
2.4 Quality changes during storage	21
2.4.1 Sensory changes and importance of autolysis	21
2.4.2 Microbiological changes	24
2.4.2.1 Intra- and interplant variations and existence of one specific microflora for cold-smoked salmon	30
2.4.3 Potential chemical quality indicators for cold-smoked salmon	33
2.5 Conclusion	36
3. Characteristics of the microflora on cold-smoked Salmon.	38
3.1. Lactic acid bacteria	38
3.1.1 Occurrence of lactic acid bacteria in fish and lightly preserved fish products	38
3.1.2 Characteristics of lactic acid bacteria from cold- smoked salmon	38
3.1.3 Spoilage potential among lactic acid bacteria	40

3.2	<i>Enterobacteriaceae</i>	42
3.3	<i>Photobacterium phosphoreum</i> and marine vibrio	43
3.3.1	Occurrence of <i>Photobacterium phosphoreum</i> and other marine vibrio.	43
3.3.2	Spoilage potential among the <i>Photobacterium</i> /marine vibrio group.	44
3.4	<i>Brochothrix thermosphacta</i> and yeast	45
3.5	Conclusion	46
4.	Hazards related to production of cold-smoked salmon.	47
4.1	<i>Clostridium botulinum</i>	47
4.2	<i>Listeria monocytogenes</i>	48
4.2.1	Occurrence of <i>Listeria monocytogenes</i> in cold-smoked salmon	48
4.2.2	Sources of <i>Listeria monocytogenes</i>	49
4.2.3	Growth and survival during and after cold-smoking	50
4.3	Other pathogenic bacteria	52
4.3.1	<i>Aeromonas</i> spp.	52
4.3.2	Pathogenic <i>Vibrionaceae</i>	53
4.3.3	<i>Yersinia enterocolitica</i>	53
4.3.4	Non-indigenous bacteria	54
4.4	Parasites, viruses, biotoxins and chemicals	54
4.5	Biogenic amines	55
4.6	Conclusion	56
5.	Critical control points in production of cold -smoked salmon.	57
5.1	Additional hurdles	59
6.	Conclusions.	61

Papers, manuscripts and notes.	62
A. Effects of salt and storage temperature on chemical, microbiological and sensory changes in cold-smoked salmon. (Paper, published)	63
B. Importance of autolytic and microbiological activities on quality of cold-smoked salmon. (Paper, accepted)	77
C. Characteristics of the microflora isolated from spoiled cold-smoked salmon. (Manuscript)	93
D. Microbiological quality and shelf life of cold-smoked salmon from 3 different processing plants. (Manuscript)	117
E. Activity of potential spoilage bacteria from cold-smoked salmon. (Manuscript)	135
F. Occurrence of <i>Listeria</i> spp. in farmed salmon and during subsequent slaughter: Comparison of Listertest™ Lift and the USDA method. (Research note, collaborative work)	156
References.	164

The first part of the book is devoted to a general history of the United States from its discovery to the present time.

1875

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THE HISTORY OF THE UNITED STATES

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The tenth part of the book is devoted to a general history of the United States from its discovery to the present time.

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1. Introduction.

Production of cold-smoked salmon is of increasing importance in Denmark and several other traditional fish processing countries. The production in Denmark has increased from 6,000,000 kg in 1991 to 8,000,000 kg in 1993, which represented market values of 710 and 829 million Danish kroner in 1991 and 1993, respectively. For 1994, the total production was expected to be approximately 8,840,000 kg worth, 819 million Danish kroner and while the production still is increasing, the unit price is expected to go down from 102.56 Dkr./kg to 92.65 Dkr./kg (Source: Danmarks Fiskeindustri- og Eksportforening). The competition is fierce on the international market and the Danish producers are required to produce high quality cold-smoked salmon at the least cost to stay in business.

Cold-smoked salmon is a traditional lightly preserved fish product in Denmark, where it is produced by a large number of smokehouses using traditional or modern production methods. The trend in modern production has gone toward a more mildly cured product with less salt, more moisture and less smoke flavour. Practically all Danish cold-smoked salmon is packaged under vacuum in barrier materials and sold frozen or chilled (0-8°C). With the modern production methods, the price of sliced vacuum packed cold-smoked salmon has decreased and opened a market for cold-smoked salmon being a delicatessen on the weekend menu for a larger range of consumers. This is especially true in Northern-European countries, Switzerland, Italy and France.

It is surprising that with cold-smoked salmon being a traditional fish product, there is not much scientific knowledge about patterns and causes for quality changes during storage at chill temperatures. It is not known which biochemical changes take place in the flesh, nor whether the origin for these changes are microbiological or autolytic. In spite of numerous microbiological analyses being recommended for monitoring quality of cold-smoked salmon by the Danish authorities (and other countries') there are few reports dealing with development of the microflora during chill-storage, and little if any consensus on which organisms are "normal" and which are potential spoilage organisms.

Increased production and export of cold-smoked salmon has made producers aware of the lack of knowledge about quality changes during storage. All smoked salmon producers have had their products subjected to a number of so-called "quality criteria" by authorities and buyers. Use of quality criteria i.e. related to total bacterial numbers has been known to result in warnings being issued to producers by authorities.

The relatively recent entrance on the "hazard" scene of *Listeria monocytogenes* in 1981 (Schlech *et al.*, 1983) has resulted in focus on the occurrence of this pathogen on

ready-to-eat products, e.g. products that do not undergo a listericidal process before consumption. Cold-smoked salmon is an example of such a product and the occurrence, survival, growth and origin of *L. monocytogenes* on cold-smoked salmon has been the topic of several publications (Guyer & Jemmi, 1991; Farber, 1991; Rørvik *et al.*, 1991; Valenti *et al.*, 1991, Ben Embarek & Huss, 1992; Guyer & Jemmi, 1992; Hudson *et al.*, 1992; Hudson & Mott, 1993; Dillon *et al.*, 1994; Fuchs & Nicolaidis, 1994; Eklund *et al.*, 1995; Rørvik *et al.*, 1995). Fortunately, cold-smoked salmon has yet to be connected with an incidence of listeriosis, but the importance of the problem for the smoked salmon producers should not be underestimated. Other psychrotrophic pathogens have also been considered to pose a threat for the wholesomeness of cold-smoked salmon. Examples are *Yersinia enterocolitica*, *Aeromonas hydrophila* (Hudson & Mott, 1993) and *Clostridium botulinum*.

As part of the free market and harmonization of the regulations affecting the production of foods in the European Union (EU), all processors of fish products must implement a quality assurance programme based on the concept of Hazard Analysis of Critical Control Points (HACCP) in their production plants (EEC, 1991a; EEC, 1991b). As a result of these directives the Danish Ministry of Fisheries (now Danish Ministry of Agriculture and Fisheries) required that all producers of fish products had established quality assurance programmes based on the HACCP concept effective January 1., 1993. Needless to say, it is not an easy task to perform a hazard analysis, when the necessary information is limited. Without more knowledge, more money and energy will continuously be spent on measuring "quality criteria" of unknown relevance by both the producers themselves, buyers and public authorities.

To actively deal with these problems faced by the industry, a group of Danish producers (Norlax A/S, Royal Greenland Seafood A/S and Skagen Laks A/S) decided to collaborate on a research project, which aimed at solving some of the questions raised concerning the quality standards of cold-smoked salmon. With financial support from the Danish Academy of Technical Sciences the research project was carried out as an industrial Ph.D. project and was performed in collaboration among the 3 smoked salmon producers, the Danish Institute of Fisheries Research, Department of Seafood Research of the Danish Ministry of Agriculture and Fisheries¹, the Royal Veterinary and Agricultural University and Nordlab Aps., Skagen.

This thesis is the result of this research project and aimed at giving considerations and recommendations for a HACCP-based quality assurance programme for production of cold-

¹ Formerly Technological Laboratory, Danish Ministry of Fisheries.

smoked salmon based on results from this research project and data from the scientific literature. The thesis is divided into two parts. The first part reviews and discusses own original research and the experimental results of others with relevance to the quality of cold-smoked salmon. Included in the first part, is a discussion of the hazards related to production of cold-smoked salmon, critical control points and recommendations for a HACCP-based quality assurance. The second section is a presentation of the experimental work carried out during the research project.

1.1 Objectives, focus and strategy in experimental work.

Quality changes and spoilage mechanisms.

The main objective and focus of the experimental work has been to describe microbiological, chemical and sensory changes in vacuum packed cold-smoked salmon processed under different conditions and by different producers during chill storage to identify causes of spoilage, objective quality parameters and potential spoilage organisms. The hypothesis was that vacuum packed cold-smoked salmon during chill storage spoils as a result of microbiological activity by a narrow group of specific spoilage organisms.

The experimental work was divided into 4 different phases:

1) Study of quality changes and evaluation of potential quality indicators in vacuum packed cold-smoked salmon.

Chemical, microbiological and sensory changes were studied in a storage experiment with a factorial experimental design with two salt levels (2.2% and 4.6% salt in water phase (SWP)) and two storage temperatures (5°C and 10°C). **(Paper A)**

2) Analysis of the relative importance of autolysis and microbiological activities in deterioration of cold-smoked salmon.

A storage experiment was performed, where the characteristics of smoked salmon produced by two salting methods (dry salted and injection brined) and with two microbial loads (reduced e.g. less than 10^3 cfu/g throughout storage and normal) were compared. **(Paper B)**

3) *Description of microflora and comparison of the microbiological quality and shelf life of cold-smoked salmon from 3 different processing plants.*

The microflora of spoiled salmon was studied through description of the microflora during storage followed by tentative identification of representative isolates from spoiled salmon produced by 3 different smokehouses. (Manuscript C)

In another experiment the microbiological quality and shelf life of cold-smoked salmon from different production batches, degrees of processing and smokehouses were compared with sensory analysis, traditional microbiological plate counts and Malthus conductance methods. (Manuscript D)

4) *Evaluation of the activity of potential spoilage bacteria from cold-smoked salmon.*

Several model experiments were conducted with spoiled salmon isolates inoculated in salmon juice and/or cold-smoked salmon blocks produced with a reduced load of microorganisms. The production of off-odours, growth and metabolism of the bacteria were monitored during anaerobic (in 100% N₂ or vacuum packaging) incubation at 5°C, and their relative activities were calculated (yield factors). (Manuscript E)

Occurrence of *Listeria monocytogenes*.

Most of the research effort directed toward quality of cold-smoked salmon has been focused on the occurrence and growth potential of *Listeria monocytogenes* and a few other pathogens in cold-smoked salmon. However, it was felt that knowledge about quality changes and the developing microflora was too scarce although of great importance. All microorganisms compete for the same nutrients, and pathogens present in low numbers may be significantly inhibited by the indigenous microflora with better growth potentials. Also, some lactic acid bacteria are known to produce bacteriocins with listeriocidal effects (Holzapfel *et al.*, 1995). At present it is not known, whether such lactic acid bacteria are naturally present on cold-smoked salmon.

From the industrial perspective, it is of great importance to identify the source of *L. monocytogenes* and other potential pathogens. If the bacteria arrive to the smokehouse with the fish, producers may have to change their production methods in order to eliminate or inhibit growth, whereas in-house contaminations may be dealt with from a hygienic point of view. In real life, a combination of these approaches may be necessary.

Based on these considerations a minor study was undertaken in collaboration with a

fellow Ph.D. student (P. K. Ben Embarek) to survey the occurrence of *Listeria monocytogenes* and *Listeria* spp. in a Norwegian fish farm and in the attached slaughterhouse. This study included a comparison of two detection techniques one based upon growth on selective agars (USDA method) and the other on immunological detection of *Listeria monocytogenes* (Listertest™ Lift). (Note F)

2. Cold-smoked salmon, quality and shelf life.

2.1 Definition of cold-smoked salmon.

Cold-smoked salmon belongs to the group of traditional lightly preserved fish products, which according to the Danish law is defined by having less than 6% salt in water phase (SWP), pH values higher than 5 and having undergone a process giving a slightly better shelf life than what is found for fresh fish (FM, 1993a). The product undergoes a mild salt cure followed by drying and smoking at temperatures below 28°C, and this cold-smoking process results in a product with salt levels ranging between 3.5 to 5% SWP, moisture content between 65-70% and pH between 5.8-6.2.

Production and quality control of cold-smoked salmon is regulated by directives no. 175 and 649 of the Danish Ministry of Fisheries (FM, 1993a; FM, 1993b). The European Union (EU, formerly European Economic Community [EEC]) directives concerning quality control and health aspects in the production and sale of seafood (EEC, 1991a; EEC, 1991b) are implemented in the above mentioned Danish directives. Every seafood processing plant is according to these national and EU-directives required to establish a quality assurance programme based on the hazard analysis of critical control points (HACCP) concept (EEC, 1991a; FM, 1993a).

In addition to general good manufacturing considerations in the Danish directive dealing with wholesome (quality and safety) production of seafood (FM, 1993a), the following requirements are explicitly specified for smoked fish products:

a) Conditions for smoking:

- Physical separation of the smoking process from other processes (§ 83).
- Sufficient ventilation for smokeovens and smokegenerators, the latter placed in a separate room (§ 84).
- Hygienic storage of wood for smoke generation and prohibition of the use of painted, glued or otherwise chemically contaminated wood (§ 85).

b) Cleaning and sanitizing:

- Regularly cleaning of the steel mesh racks used for smoking (§ 86).
- Frequent cleaning of slicing equipment, at least in conjunction with the personnel's break periods (§ 90).

c) Temperature and storage regimes:

- Rapid chilling after smoking and before packing (§ 87).
- Immediate chilling of slicing scraps if they are to be used for human consumption (§ 90).

- The finished product must be packed for sale within 3 days of smoking and stored at maximum 5°C (§ 97)

d) Parasite control:

- Obligatory freezing of wild salmon (Atlantic and Pacific) at min. -20°C for 24 h for the control of parasites (§ 88).

The value of these recommendations will be evaluated later in the thesis with the general consideration for appropriate production procedures to obtain good quality cold-smoked salmon.

Sensory quality of cold-smoked salmon is very much in the eye of the beholder with regard to smoke flavour, saltiness, colour etc., but all consumers would agree on the basic requirements for the product to be wholesome and not pose a public health risk. In principle, it is the responsibility of the producer to supply the consumer with a satisfactory quality product. This goal can be achieved through optimisation of production methods, plant organisation, implementation of a quality assurance programme and motivation and education of the staff at all levels. In the text below, different technical aspects of production of cold-smoked salmon will be discussed with emphasis on their importance for the quality of the product.

2.2 Production of cold-smoked salmon.

Danish cold-smoked salmon is mainly produced from farmed Atlantic salmon (*Salmo salar*). There are no salmon farms in Denmark, and the fish are therefore imported from countries like Norway, the Faroe Islands, Scotland, Iceland and Chile. Norwegian farmed salmon is by far the most common source, maybe accounting for 90-95% of the raw material. Apart from Atlantic salmon (*Salmo salar*) there is a smaller production of cold-smoked salmonids from imported Pacific *Oncorhynchus* species and from Danish saltwater farmed steelhead (rainbow trout, *Oncorhynchus mykiss*). This thesis will, however, only be concerned with quality of cold-smoked salmon produced from *Salmo salar*, but as the production methods are the same, many of the considerations will be useful for cold-smoked products made from the other salmon species.

2.2.1 Quality aspects of the raw material.

Only salmon of the best quality should be used in the production of cold-smoked salmon. Among the parameters of technological importance apart from the obvious of

freshness, are colour, fat content, fat composition and texture.

2.2.1.1 Colour.

The typical pink salmon colour originates from consumption of fish and crustaceans containing carotenoids, especially astaxanthin (Simpson, 1982). In order to achieve a desirable colour in farmed salmon it is therefore necessary to add carotenoids to the feed, and this is typically done in the form of astaxanthin and/or canthaxanthin (Skrede & Storebakken, 1986a & b; Storebakken *et al.*, 1986; Torrissen, 1986; Bjerkeng *et al.*, 1992; Choubert *et al.*, 1992; Sigurgisladóttir *et al.*, 1994). The characteristics of the colour in the salmon flesh will depend on a number of factors including the chemistry of the added pigment with canthaxanthin giving a more yellow/orange and astaxanthin a more reddish/pink colour, the concentration of the pigment in the feed, the size of the fish and stage of sexual maturity (Aknes *et al.*, 1986; Skrede & Storebakken, 1986a; Storebakken *et al.*, 1986; Skrede *et al.*, 1989; Choubert *et al.*, 1992; Bjerkeng *et al.*, 1992). The colour of smoked salmon was found to be directly related to the colour of the raw material (Skrede & Storebakken, 1986a). While consumer's preference for colours may vary between a light pink in one survey (Gormley, 1992) and a deep red/orange colour in another survey (Blokhus, 1986), there is no doubt of the importance of colour.

Depigmentation problems in farmed salmon have been reported in recent years, and smoked products prepared from such fish will also contain depigmented areas (Johnsen & Bjerkeng, 1991; Bjerkeng *et al.*, 1992b). The reasons for these pigment problems are unknown but are believed to be related to factors in the rearing of salmonids including feed composition, growth rate, vitamin balance (C and E), infectious diseases and stress (Bjerkeng *et al.*, 1992b). This problem does not only exist in Norway and Europe, as Canadian salmon growers in the Atlantic region have encountered the same depigmentation problems (Ives, 1995).

Frozen storage can change the colour significantly toward a lighter and less intensive colour, where the red and/or yellow colour can become more dominant (Yu *et al.*, 1973; No & Storebakken, 1991). There seems to be two factors influencing the colour loss. Firstly, carotenoids are bound to the actomyosin complex with weak hydrophobic interactions (Henmi *et al.*, 1989; Henmi *et al.*, 1990) and frozen storage could disturb these interactions and thereby the colour because of alterations in the protein structure. Secondly, carotenoids have been found to have a scavenging effect against oxygen radicals produced from oxidised lipids, but this protective property concurrently causes loss of colour in the tissue (Andersen

et al., 1990; Ingemansson *et al.*, 1993). Colour stability during frozen and chill storage will therefore depend on factors related to the oxidation stability of the lipids i.e. the intensity of surrounding light, storage temperature, oxygen permeability of packaging material as well as factors related to the stability of the flesh proteins.

2.2.1.2 Fat content and composition.

The fat content in Atlantic salmon is related to the amount of fat in the feed, growth rate, amount of exercise, age, etc. and as a rule, farmed fish contains more fat than wild fish (Haard, 1992). Farmed salmon can contain as much as 14% (w/w) fat in the muscle tissue and feeding experiments have shown that additional fat will be stored in the viscera (Lie *et al.*, 1988). The fat in the muscle tissue is not evenly distributed throughout the fish, as there is a tendency toward a higher fat content under the skin and in the belly flaps (Mohr, 1986; Haard, 1992). The composition of lipid in salmon reflects the lipid composition in the feed (Lie *et al.*, 1988; Polvi & Ackman, 1992; Waagbø *et al.*, 1993). The higher content of polyunsaturated fatty acid, the more susceptible the lipid will be toward oxidation if not protected. Levels of tocopherols between 200 and 1400 mg tocopherols/kg in the feed have been found to protect the unsaturated lipid in salmonids from oxidation (Frigg *et al.*, 1990; Waagbø *et al.*, 1993; Sigurgisladóttir *et al.* 1994).

The technological significance of the oxidative stability of the fat content has not been examined in depth, but it is probably of great importance as off-flavours related to oxidation and rancidity are regularly encountered in cold-smoked salmon (Nieper, 1986; Hildebrandt & Erol, 1988; Manuscript D). Waagbø *et al.* (1993) showed that cold-smoking can mask the rancidity and off-flavours found in oxidation-sensitive salmon to some extent, but numerous factors including packaging material, storage temperatures, temperature fluctuations, exposure to light, length of time in storage and processing methods will undoubtedly influence the probability of oxidation.

The fat content in raw salmon will also influence the texture of both the raw and the cold-smoked fish (Mohr, 1986). The taste panellists specifically noted in one storage experiment with cold-smoked salmon that the fat content was too high, and the texture was consequently described as being oily, greasy and very soft (Manuscript D). The fat content was not analyzed in that experiment, but it was noted that the fillets were difficult to slice.

2.2.1.3 Sexual maturity.

During sexual maturation farmed salmon undergo major physiological changes. The water content has been found to increase at the expense of decreasing protein and fat contents, which ultimately caused significant deterioration of the odour, flavour and texture of both raw and processed meat (Aknes *et al.*, 1986; Blokhus, 1986; Reid & Durance, 1992). Also the colour changed as the flesh was depigmented during the later stages of maturation (Aknes *et al.*, 1986; Bjerkgeng *et al.*, 1992a). These changes render sexually maturing and spawning salmon unsuitable for cold-smoking.

2.2.1.4 Texture.

Gaping is a textural problem occurring seasonally, where the connective tissue in the myocommata is unable to hold the muscle segments (myotomes) together (Mohr, 1986; Lavéty *et al.*, 1988). This degradation of the connective tissue makes it almost impossible to slice the cold-smoked salmon (Lavéty *et al.*, 1988). The exact reason for gapping is unknown, but it has been shown to be a seasonal problem occurring in the hotter summer months of June and July and to coincide with a fall in the post-mortem pH (Lavéty *et al.*, 1988). In cod, it has been shown that the collagen fibrils lose their mechanical strength when pH is lowered (Mohr, 1986) and this may also be the case in salmon.

2.2.1.5 Microbiology.

There are very few reports on the composition of the microflora on freshly-caught salmon. Horsley (1973) examined the microflora on Scottish salmon caught in rivers, estuarine and coastal waters, and found that the principal bacterial genera on skin and gills reflected the microflora of the water. *Moraxella*, *Flavobacterium*, *Cytophaga* and *Pseudomonas* dominated in numbers ranging between 10^1 and 10^3 cfu/cm³ skin, while *Acinetobacter*, *Bacillus*, *Aeromonas*, *Vibrio*, *Enterobacteriaceae*, *Micrococcaceae* and some coryneforms were present in smaller numbers. The coryneforms described could include lactic acid bacteria (LAB), which have been found in smaller numbers on fish (Blood, 1975; Schrøder *et al.*, 1980; Knøchel, 1981; Mauguin & Novel, 1994). No accounts on the presence of luminous bacteria frequently associated with marine environments and the intestines of some fish species have been reported (van Spreekens, 1974; Woodland Hastings & Neilson, 1981; Baumann & Baumann, 1984; Dalgaard *et al.*, 1993), but this may be due

to the recovery methods applied, as discussed later. This flora composition matched the generalised description of the microflora of fish from temperate water as being dominated by psychrotrophic Gram-negative bacteria with Gram-positives predominantly cocci occurring in lower numbers (Liston, 1980).

It is generally assumed that the bacterial flora on freshly caught fish solely reflects the microflora in the surrounding environment (Shewan, 1977; Liston, 1980), and that the microflora on fish is therefore transient.

The occurrence of pathogenic bacteria and parasites in the raw material will be discussed in Chapter 4.

2.2.2 Production methods.

There are a multitude of different methods to produce cold-smoked salmon. As mentioned previously, the trend has gone from a cold-smoking process which often lasted for a week, toward milder cures where the total process from filleting to vacuum packing can be completed in 1-2 days.

Briefly, the cold-smoking process consists of a preparatory step, where the fish are thawed, filleted and cleaned as required. The fillets are not skinned at this time and the degree of trimming varies between processing plants. The next step is salting, including dry salting (crystalline salt on flesh side), brining and/or injection brining. After salting, the fish are placed on steel mesh racks and left to equilibrate for an appropriate number of hours, during which excess salt on dry salted fillets is removed by washing. The cold-smoking process is divided into an initial drying step followed by the cold-smoking step, where the temperatures never exceed 30°C. It has been shown in our laboratory that it is possible to cold-smoke salmon at temperatures up to 30°C without heat denaturation of the proteins (Jensen & Ladefoged, 1983). However, the salmon producers prefer lower temperatures, which in their experience gives better texture and less smoke sheen. After smoking, the fish are chilled and in some cases frozen. This processing step serves as a storage buffer in most smokehouses, and depending on the capacity of the skinning and slicing lines, smoked fillets can be stored here up to several days (see also Table 2.1). The fillets are skinned and sliced, and the slices are manually laid onto gold laminated cardboard and the units weighed. Shortly thereafter, the smoked fish are vacuum packed in barrier material of variable quality. Further distribution, storage conditions etc. varies. A flow diagram of the whole process is presented in Figure 2.1.

Table 2.1 gives some examples of production methods including approximate

temperatures (T), times (t) and regimes (T x t). It is obvious from this table that the procedures are highly variable. The weight losses obtained during smoking have consequently been minimised, and while a loss of 20% was common 20 years ago, today's producers aim at less than 10% and preferably around 0%, if injection brining is used (Horner, 1992).

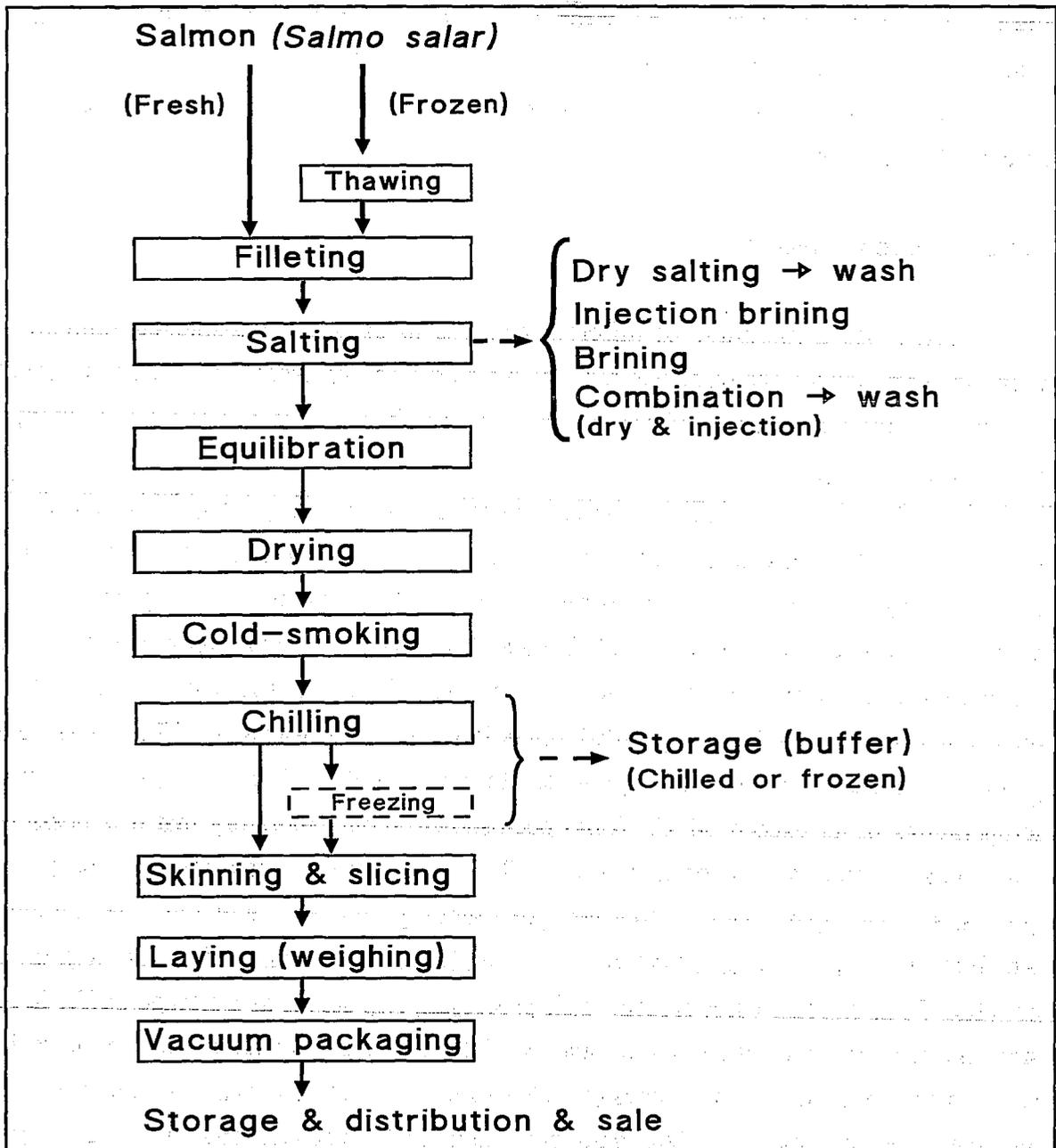


Figure 2.1 Flow diagram of the production of cold-smoked salmon. Processes in boxes take place in the smokehouse. Dashed box and arrows show variations in production methods.

Table 2.1 Examples of production recipes for cold-smoked salmon with typical composition of 3.5-5% SWP and 60-65% moisture.

Method	Dry salting	Brined (salt)	Brined (salt & sugar)	Injection brined
Process				
Salting	Ca. 250 g crystalline salt on meat side for 3 h at <4°C + 16 h equilibration at <4°C	10% (w/v) brine for 18 h at 5°C, fish:brine 1:1	10% (w/v) salt and 3% (w/v) sugar for 5 d at <5°C	23% (w/v) brine injected to approximately 10% weight gain
Drying	2-3 h at 20-25°C, 55% rh ^b	6 h at 26°C, from 80 to 50% rh	24 h at 22°C	1.5-3 h at 20-25°C, 55-75% rh
Smoking	4-5 h at 18-25°C, 60-70% rh	6 h at 26°C, 63% rh	24 h at 22°C, rh 85%	3-4 h at 18-26°C, 60-70% rh
Processing time ^a	1.5-2 d	2-3 d	7-8 d	2-5 d
Time at temperatures above 4°C	6-8 h	12 h	48 h	4.5-7 h
Sources	Choubert <i>et al.</i> , 1992; Paper B	Paper A	Skrede & Storebakken, 1986a	Paper B, Manuscript D

a) Processing time includes the time spent from filleting to vacuum packaging, where especially the chilling/storage time between cold-smoking and skinning varies; b) rh - relative humidity.

2.3 Cold-smoking as a preservation method.

The modern cold-smoking process has become so mild that the drying and smoking processes are of minor importance for the preservation of the product. Horner (1992) suggested that cold-smoking should be considered a smoke-flavouring rather than smoke-preserving process. The main preserving factors are the salt level (in water phase), the packaging material (permeability) and storage temperature. No single preservation parameter will determine the shelf life as it clearly can be seen from Tables 2.2 & 2.3, where a number of interacting factors are shown to be related to the shelf life. Shelf life of cold-smoked salmon seems to be a rather complex function of storage temperature, salt, moisture content, microflora, packaging material as well as factors not easy to control or measure i.e. quality of raw material, processing method, time and temperature history, etc.

Table 2.2 Shelf life and spoilage characteristics of vacuum packed cold-smoked salmon. Data from the literature.

Shelf life (max. days)	Temp. °C	Salt (%SWP)	Dry matter (%)	Packaging Material & permeability	Chemical indices		Microflora (total) Log ₁₀ (cfu/g)	Microflora (specific counts Log ₁₀ (cfu/g)	Sensory of spoilage	Reference
					TVN/TMA (mgN/100g)	Other				
30	0	2.8	38.8	Nylon 11	40/- ^a	TBA, 1.1 ^b	4.4(TSA ^c , 22°C)	-	Putrid	Declerck & Vyncke (1972)
25	0	4.1	41.8	Vacuum	44/-	Hx, 3.5 ^d	6.7 (20°C)	LAB ^e , 4.5; H ₂ S ^f , 4.0; Br.t ^g , 5.0	Rancid, bitter sour	Cann et al. (1984)
21	2	2.1	42.7	Vacuum	40/40(NH ₃)	TBA, 5 Vol. acids, 100 ^h	6.2 (TSA, 22°C)	Predominantly Gram neg.	Colour changes, viscous	Declerck (1976)
21	5	4.1	41.8	Vacuum	48/-	Hx, 5.4	6.6 (20°C)	LAB, 6.0; H ₂ S, 3.0; Br.t, 5.0	Rancid, bitter sour	Cann et al. (1984)
10-13	5	4.4	-	PE; O ₂ , 3250; CO ₂ , 16300 ⁱ	27/10	-	8.4 (IA ^j , 25°C)	LAB, 5.6; H ₂ S < 5.0; Br.t, 5.9; Yeast, 5.9	Stringent, acid sour, putrid	From (1987)
27	5	4.4	-	Riloten X; O ₂ , 4; CO ₂ , 16	40/20	-	9.0 (IA, 25°C)	LAB, 6.0; H ₂ S, 7.0;	Putrid, fruity sour, bitter, fishy	From (1987)
20-23	5	4.7	-	Amilon; O ₂ , 60; CO ₂ , 250	32/12	-	8.3 (IA, 25°C)	LAB, 7.2; H ₂ S, 6.1; Br.t, 4.6; Yeast, 4.3	Sour, nauseating, stringent, putrid, bitter	From (1987)
20	6	5.3	38.4	Vacuum	30/-	D-lactate, 1.1 μmol/g	6.0 (TSA, 30°C)	LAB, 5.0; Ent. ^k , 2.0	Triangle test, no description	Schneider & Hildebrandt (1984)
14	10	4.1	41.8	Vacuum	70/-	Hx, 5.6	7.3 (20°C)	LAB, 6.8; H ₂ S, 4.0; Br.t, 5.0	Rancid, bitter sour	Cann et al. (1984)
10	10	4.3	-	PE; O ₂ , 3250; CO ₂ , 16300	55/25	-	8.9 (IA, 25°C)	LAB, 6.7; H ₂ S, 5.0; Br.t, 6.3; Yeast, 6.2	Putrid, stringent, H ₂ S	From (1987)
13	10	4.3	41.8	Riloten X; O ₂ , 4; CO ₂ , 16	34/12	-	7.9 (IA, 25°C)	LAB, 6.9; H ₂ S, 7.0	Fruity sour, slightly putrid	From (1987)
16-20	10	4.5	-	Amilon; O ₂ , 60; CO ₂ , 250	70/24	-	8.4 (IA, 25°C)	LAB, 7.0; H ₂ S, 6.2; Br.t, 5.5	Putrid, rotten, ammonia, sour	From (1987)
6-7	12	5.3	38.4	Vacuum	30/-	D-lactate, 1.1 μmol/g	6,2 (TSA, 30°C)	LAB, 6.0; Ent., 4.8	Triangle test, no description	Schneider & Hildebrandt (1984)

a) Not tested; b) TBA - thiobarbiturate acid (mg malone aldehyde/kg); c) TSA - Tryptone Soy Agar; d) Hx - Hypoxanthine (μmol/g); e) LAB - lactic acid bacteria; f) H₂S - H₂S producers; g) Br. t. - *Brochoitrix thermosphacta*; h) Volatile acids (ml NaOH [0.001 N]/100 g); i) Permeability for O₂ and CO₂ in cm³/m² x 24 h x atm (20-25°C, rh 75%); j) IA - Iron Agar (Lyngby, Oxoid); k) Ent. - *Enterobacteriaceae*.

Table 2.3 Shelf life and spoilage characteristics of vacuum packed cold-smoked salmon. Data from this study.

Shelf life (max. days)	Temp. °C	Salt (% SWP)	Dry matter (%)	Packaging (material & permeability)	Chemical indices		Microflora (total) Log ₁₀ (cfu/g)	Microflora (specific counts) Log ₁₀ (cfu/g)	Sensory of spoilage	Reference
					TVN/TMA (mgN/100g)	Other (μmol/g)				
35	5	2.2 ± 0.6	39.4	Nylon/PE; O ₂ , 29 ^a	- ^b / 13.5	Hx ^c , 8.0; Acetate, 19.6; EtOH ^d , 3.4	8.7 (TSA ^e , 25°C)	TPC ^f , 8.7; LAB ^g , 8.7; Ent. ^h , 5.0	Bitter, faecal, putrid, rancid, soft	Paper A
21	5	3.9 ± 0.9	39.4	Riloten/X; O ₂ , 1; CO ₂ , 4 ⁱ	38/23	Hx, 4.4; Acetate, 14.0; EtOH, 1.4; TBA 1.1 ^j	6.9 (TPC, 10°C)	TVC, 4.0; LAB, 4.0; Ent. < 2.0	Putrid, stringent, sour, faecal, soft	Paper B (Dry salted)
36	5	4.1 ± 0.4	35.7	PETX/PE & PETM/PE; O ₂ , 10; CO ₂ , 40	-	-	8.2 (TSA, 25°C)	TPC, 8.3, LAB, 7.8; Ent., 6.8	Spoiled, bitter, sweet, sour, soft, mushy	Manuscript D, Plant 2, 1st batch
28	5	4.2 ± 0.5	30.4	PETX/PE; O ₂ , 10; CO ₂ , 40	-	-	7.1 (IA, 25°C)	TPC, 7.1; LAB, 7.0; Ent., 6.3	Faecal, sour, cabbage, stringent, putrid, rancid, bitter, soft	Manuscript C, Plant 2
21	5	4.4 ± 0.4	34.8	Riloten/X; O ₂ , 1; CO ₂ , 4	28/14	Hx, 3.8; Acetate, 6.0; EtOH, 1.1; TBA 1.2	7.8 (TSA, 21°C)	TPC, 7.8; LAB, 7.8; Ent., 6.9	Sour, bitter ammonia, fruity, stringent, soft	Paper B (Injection salted)
29	5	4.4 ± 0.3	33.7	PETX/PE, PETM/PE; O ₂ , 10; CO ₂ , 40	-	-	6.8 (TPC, 10°C)	TSA, 6.7; LAB, 7.1; Ent., 4.4	Rancid, spoiled, sweet, amine, cabbage, soft, mushy	Manuscript D, Plant 2, 2nd batch
56	5	4.6 ± 0.5	42.6	Nylon/PE; O ₂ , 29	-/14.6	Hx, 7.2; Acetate, 11.6; EtOH, 2.8	8.8 (TSA, 25°C)	TPC, 8.8; LAB, 8.8; Ent., 5.0	-	Paper A
42	5	4.8 ± 0.3	35.3	PET/PVdC/ PE; O ₂ , 8; CO ₂ , 40	-	-	7.2 (TPC, 10°C)	TSA, 7.1; LAB, 7.0; Ent., 6.8	Sweet, stringent, fruity, oily, greasy, soft	Manuscript C, Plant 1

To be continued.

Table 2.3 Shelf life and spoilage characteristics of vacuum packed cold-smoked salmon. Data from this study. *Continued.*

Shelf life (max. days)	Temp. °C	Salt (% SWP)	Dry matter (%)	Packaging (material & permeability)	Chemical indices		Microflora (total) Log ₁₀ (cfu/g)	Microflora (specific counts) Log ₁₀ (cfu/g)	Sensory of spoilage	Reference
					TVN/TMA (mgN/100g)	Other (µmol/g)				
28	5	4.8 ± 0.3	33.1	OPA/PE & PA/PE; O ₂ , 30-35; CO ₂ , 140	-	-	7.5 (TPC, 10°C)	TSA, 7.3; LAB, 7.2; Ent., 5.4	Sour, putrid, faecal, ammonia, soft/mushy	Manuscript D, Plant 1, 2nd batch
29	5	5.2 ± 0.3	36.9	PA/PE; O ₂ , 44; CO ₂ , 175	-	-	6.0 (TSA, 25°C)	TPC, <4.0; LAB, 5.5; Ent., 2.0	Bitter, faecal, putrid, rancid, soft, pasta	Manuscript D, Plant 3, 1st batch
26	5	5.2 ± 0.3	32.9	OPA/PE & PA/PE; O ₂ , 30-35, CO ₂ , 140	-	-	6.1 (TPC, 10°C)	TSA, 5.9; LAB, 5.9; Ent., 1.8	Bitter, sweet, sour, stringent, soft/mushy	Manuscript D, Plant 1, 1st batch
24	5	5.7 ± 0.3	36.0	CCP/PA/PE; O ₂ , 10; CO ₂ , 40	-	-	6.9 (IA ^k , 25°C)	TPC, 6.6; LAB, 6.7; Ent., 5.5	Bitter, sweet, plastic, ammonia, stringent, rancid, soft	Manuscript C, Plant 3
28	5	6.1 ± 0.3	37.5	PA/PE; O ₂ , 44; CO ₂ , 175	-	-	8.0 (TSA, 25°C)	TPC, 7.0; LAB, 8.0; Ent., 6.3	Bitter, faecal, stringent, rancid, soft/mushy, pasta	Manuscript D, Plant 3, 2nd batch
14	10	2.2 ± 0.6	39.4	Nylon/PE; O ₂ , 29	-/10.3	Hx - 8.0 Acetate - 16.6 EtOH - 3.4	8.7 (TSA, 25°C)	TPC, 8.7; LAB, 8.7; Ent., 6.9	Bitter, faecal, putrid, ammonia, soft	Paper A
42	10	4.6 ± 0.5	42.6	Nylon/PE; O ₂ , 29	-/12.0	Hx - 7.2 Acetate - 23.0 EtOH - 2.2	8.8 (TSA, 25°C)	TPC, 8.8; LAB, 8.8; Ent., 6.0	Bitter, faecal, putrid, oxidised, soft	Paper A

a) Permeability for O₂ in cm³/m² x 24 h x atm (20-23°C, rh 75%); b) Not tested; c) Hx - Hypoxanthine; d) EtOH - Ethanol; e) TSA - Tryptone Soy Agar; f) TPC - Total psychrotrophic count (TSA, surface, 10°C); g) LAB - lactic acid bacteria; h) Ent. - *Enterobacteriaceae*; i) Permeability for CO₂ in cm³/m² x 24 h x atm (20-23°C, rh 75%); j) TBA - thiobarbiturate acid (mg malone aldehyde/kg); k) IA -Iron Agar (Lyngby; Oxoid).

2.3.1 Salt.

It is generally recognized that salt has an inhibitory effect on bacterial growth, which is partially caused by a decrease in water activity (a_w) (Jay, 1986). The importance of salt in cold-smoked fish was shown in an experiment, where unsalted vacuum packed cold-smoked cod stored at 15°C was found to spoil at a much faster rate than cold-smoked cod with 5% salt in the water phase (SWP) stored under the same conditions (Huss & Larsen, 1980). Comparison of brined cold-smoked salmon produced under exactly the same conditions with low (2.2% SWP) and high (4.6% SWP) salt contents revealed that this difference resulted in the shelf life of high salt salmon being at least 3 weeks longer than that for the low salt salmon (Table 2.3; Paper A). The same effect of salt levels was, however, not seen among sliced cold-smoked salmon from 3 different smokehouses, which obtained shelf lives of 21-26 to 30-36 days at 5°C with no systematic relation to salt levels ranging between 4.1-6.1% SWP (Manuscript D). It may be that the effect of varying salt levels in the range of 3.5-5% usually found in cold-smoked salmon is minor compared to the other variable factors, but that markedly lower levels like 2.2% SWP will make a significant difference.

It is common belief in the industry that the method of salting affects the quality and shelf life of cold-smoked salmon. Differences between dry salted and injection brined cold-smoked salmon manufactured from the same raw material and same cold-smoking process were analyzed by taste panellists, and they found the initial texture to be firmer in the dry salted salmon (Table 2.3; Paper B). This may also have been related to the higher dry matter content found in dry salted salmon although no difference was found in shelf lives. Brined salmon with 4.6% SWP kept for up to 7-8 weeks when stored at 5°C (Paper A), which is long compared to a shelf life of 3-4 weeks obtained by injection brined salmon with 4.1-6.1% SWP (Manuscript D). The brined salmon had higher dry matter content (39.4-42.6%) and had undergone a considerably longer drying and smoking process totalling 12 h as compared to the injection brined salmon, which was produced using cold-smoking procedures lasting 4.5-7 h and with dry matter contents ranging between 32.9-37.5% SWP (Tables 2.1 & 2.3). These differences make it impossible to conclude that brining is better than injection brining with respect to shelf life extension. It is felt that more systematic experimental work is necessary to document the advantages and disadvantages for the different salting methods.

Salting plays an important function for the safety of vacuum packed, chilled cold-smoked salmon, because it is the only critical control point (CCP1) in the process which will

prevent growth and toxin production by *Clostridium botulinum* during chill storage. This will be discussed in detail in Chapter 4.

2.3.2 Cold-smoking.

The smoke-flavouring process involves deposition of vapour and droplets from wood-smoke pyrolysing at temperatures between 400-600°C. The deposition is dependant upon the temperature, relative humidity as well as the solubility of the individual compounds in salmon flesh (Daun, 1979). Table 2.4 gives an overview of compounds found in wood smoke and their desirable and undesirable effects. The typical smoke flavour results from a number of chemicals, but the smoke-phenols syringol, guaiacol, vanillin, isoeugenol and derivatives have all been shown to contribute significantly to the typical smoked flavour (Tóth & Potthast, 1984).

Table 2.4 Compounds found in wood smoke and their presumed functions. (Compiled from Olsen [1976], Tóth & Potthast [1984]).

Compounds	Number of identified compounds	Desirable properties	Undesirable properties
Phenols	85	Flavour, antioxidant, colour, antimicrobial	Co-carcinogenic
Carbonyles (aldehydes & ketones)	49	Colour, surface texture, antimicrobial (HCOH)	Denaturation of essential amino acids
Organic acids	33	Flavour, colour, antimicrobial	-
Furans & other heterocyclic compounds	11	Flavour, colour	-
Alcohols & esters	17	Flavour	-
Lactones	13	Flavour enhancer	-
Polycyclic aromatic hydrocarbons	47	-	Carcinogenic
Nitrogen oxides	3	Colour	N-nitroso compounds
Terpenes	?	Flavour	?

Several of the smoke compounds including phenols, organic acids and formaldehyde exhibit antimicrobial properties when analyzed in model systems (Table 2.4, Olsen, 1977; Daun, 1979). Gram-positive bacteria are in general, less sensitive to the smoke's antimicrobial compounds than Gram-negatives. Donnelly *et al.* (1982) found that addition of liquid smoke to sausage emulsions had very little effect on starter cultures of *Lb.*

plantarum and *Pediococcus cerevisiae*. *Staphylococcus aureus* was similarly found to be resistant toward a number of smoke-concentrates prepared from different woods, while *Aeromonas hydrophila* and *Pseudomonas fragi* were sensitive toward several of the concentrates (Boyle *et al.*, 1988). Gram (1991) found some effect of liquid smoke on *Aeromonas* spp. isolated from spoiled fish. This effect was, however, dependent on the initial levels of organisms. The cold-smoking process may therefore reduce the number of organisms on the surface of the salmon fillets. Repeated experiments with salmon produced with short cold-smoking processes (Table 2.1), showed that freshly produced salmon, vacuum packed immediately after smoking, still would develop a mixed Gram-positive and Gram-negative microflora during subsequent storage (Manuscript D). The size of the initial microflora on these fillets were similar to that found on day 1 on sliced salmon from the same production batch.

The smoke compounds are deposited on the surface and the amount depends on the length of the process, temperature, humidity etc. It has also been shown that the phenols appear initially only in the 1-2 mm top layer of the tissue and only diffuse slowly into the muscle (Moini & Storey, 1980; Eklund *et al.*, 1982, Sikorski, 1988). When further processing steps including skinning and slicing (Figure 2.1) are considered, it is not likely that the smoke compounds will be evenly distributed on the salmon slice and exert any inhibiting effect on the microorganisms during chill storage.

Cold-smoking could theoretically eliminate or decrease initial contamination of psychrotrophic pathogens present on the fillets before smoking, as smoke compounds have been found to have some effect against *Listeria monocytogenes*, *Clostridium botulinum* and *Aeromonas hydrophila* (Eklund *et al.*, 1982; Boyle *et al.*, 1988, Messina *et al.*, 1988), but there is ample evidence that *Listeria monocytogenes* present on the raw fish survives the modern mild process (Chapter 4.2).

Production of nitrosamines and polycyclic aromatic hydrocarbons have been of concern in smoked fish because of their carcinogenic properties. These compounds are found in much lower levels in cold-smoked fish than in hot-smoked parallels because of the lower processing temperatures (Röper *et al.*, 1981; Sikorski, 1988; Simko *et al.*, 1991). Intake of cold-smoked salmon is therefore of no concern to the consumers with regard to smoke carcinogens, especially when the limited consumption is taken into consideration.

2.3.3 Packaging and storage temperature.

The majority of Danish cold-smoked salmon is sold vacuum packed today. Vacuum

packaging of cold-smoked salmon changes the microflora from an initial mixture of Gram-negative and -positive bacteria at levels of 10^2 - 10^4 cfu/g, to be dominated by LAB at levels of 10^7 - 10^8 cfu/g. Also, *Enterobacteriaceae* in numbers of 10^5 - 10^7 cfu/g, psychrotrophs of the *Photobacterium* spp. and marine vibrio types in levels of 10^6 cfu/g (Paper B; Manuscripts C & D) are found in the vacuum packed product. *Shewanella putrefaciens* and *Pseudomonas* spp., which dominate spoilage of fresh iced fish from temperate waters (Herbert *et al.*, 1971; Liston, 1980; Gram *et al.*, 1987), were not found among the 428 microorganisms isolated and characterized from spoiled vacuum packed cold-smoked salmon (Manuscript C). But as *Shewanella putrefaciens* is found on vacuum packed fresh cod (Ravn Jørgensen *et al.*, 1988; Dalgaard *et al.*, 1993), the inhibition of this bacteria on smoked salmon is probably a combined result of the salt content and vacuum packing.

In many ways this picture resembles the effect vacuum packaging has on red meat products, where LAB also thrive at the expense of *Enterobacteriaceae* and *Pseudomonaceae* (Dainty *et al.*, 1992). The selection for LAB on vacuum packed red meat is generally recognized as being beneficial for the shelf life, because the spoilage potential of most LAB is relatively small and their growth can inhibit more potent spoilers through antagonistic activities. This may very well be the case for cold-smoked salmon as well. However, the parallels are limited by the fact that psychrotrophs of the *Photobacterium* spp. and marine vibrio types have been found to dominate the flora occasionally.

The permeability with respect to O₂ and CO₂ of the packaging material no doubt has an impact on the microbiological development and thereby shelf life of the product. From (1987) stored cold-smoked salmon vacuum packed in material with 3 varying permeability properties for O₂ and CO₂ and found the shelf life (sensory acceptable) at 5°C increased from 10-13 to 27 days with decreasing permeability ranging from 3,250 to 4 cm³/m² x 24 h and from 16,300 to 16 cm³/m² x 24 h for O₂ and CO₂, respectively (Table 2.2). A similar picture was found at 10°C, except salmon packed in Amilon (medium permeability) had a slightly longer shelf life as compared to salmon packed in Riloten X (low permeability) (Table 2.2). These differences were partially explained by the high numbers of yeasts found on salmon packed in the highly permeable polyethylene (PE), but other than that it was difficult to identify the microbiological reason for the result. Modified atmosphere packaging (MAP) of cold-smoked salmon in 60/40:CO₂/N₂ was found to give no shelf life extension compared to vacuum packed reference samples (Cann *et al.*, 1984), indicating that no improvements may be achieved. Other gas mixtures of CO₂, N₂ and O₂ in various proportions have been applied experimentally to cold-smoked salmon (Civera *et al.*, 1993). These workers found shelf lives of 60 days for MAP salmon, but they gave no information

on the salt content, included no vacuum packed reference samples for comparison and only described the sensory characteristics very superficially.

Other aspects in relation to quality are the role of packaging materials in development of rancidity during storage and diffusion of contaminants from the material into the salmon resulting in off-flavours (See also manuscripts C & D).

When cold-smoked salmon produced with a high and low salt content were stored at 5 or 10°C, the shelf lives increased with decreasing temperatures for the salmon (Table 2.3; Paper A). The same observation was made by Cann *et al.* (1984), who stored vacuum smoked salmon at 0, 5 and 10°C and achieved shelf lives of 25, 21 and 14 days, respectively. Schneider & Hildebrandt (1984) found a similar effect of temperature when storage at 6 and 12°C was compared (Table 2.2). From (1987) found that salmon stored at 5°C had longer shelf lives than salmon packed in the same material stored at 10°C regardless of the packaging permeability (Table 2.2).

2.4 Quality changes during storage.

During chill storage of cold-smoked salmon quality changes of both microbiological and autolytic origin can take place, as there is no heat inactivation of native enzymes in the smoking process. The quality changes will ultimately cause the product to become unacceptable to the consumer and therefore determine the shelf life of the product. In the following discussion, typical quality changes will be described and aspects of their origin and measurement discussed.

2.4.1 Sensory changes and importance of autolysis.

Cold-smoked salmon gradually loses the sensory quality during storage and typically this quality loss happens in the following 3 steps:

a) Fresh cold-smoked salmon has characteristic flavours and odours, where the salt and smoky flavours are balanced by the "salmon" itself. The texture varies between relative firm and softer depending on the salting method and dry matter content. Salmon not previously frozen can also have a tender texture.

b) Borderline cold-smoked salmon is still acceptable but has lost the initial characteristic salmon flavours and odours. Instead the fish is neutral and bland. The texture has gone significantly softer. Slightly sour, bitter and faecal flavours and odours are occasionally described.

c) Spoiled cold-smoked salmon is characterized by objectionable off-odours and -flavours and the texture has sincerely deteriorated.

(Descriptions are adapted from Paper A and Manuscript D).

An overview of the sensory characteristics described for spoiled cold-smoked salmon is presented in Table 2.4. There seem to be some general trends as the sour, bitter, sweet and rancid flavours often are described, and the texture seem to be judged as unacceptably soft at spoilage.

Table 2.4 Sensory characteristics in spoiled vacuum packed cold-smoked salmon (Odour, flavour and texture).

Spoilage characteristics	Analytical methods	Comments	Source
Sour, bitter and rancid.	Descriptive scoring by taste panel during storage experiment.	Same characteristics at 0, 5 and 10°C. Permeability of packaging material unknown.	Cann <i>et al.</i> (1984)
Fruity, sour, bitter, fishy, putrid, nauseous, pungent.	Description by taste panel during storage experiment.	Data for Amilon and Riloten X stored at 5 and 10°C (Table 2.2).	From (1987)
Sour and rancid. Soft texture.	9-point hedonic descriptive scale.	Permeability of packaging material unknown. Storage at 2-3°C for 2 months, only sampling every 4 weeks.	Hammed & El-Mangy (1992)
Faecal, putrid, rancid, stale. Soft, mushy texture.	Description of appearance, texture and flavour after storage of commercial products until expire of declared minimal shelf life at 4-7°C.	Commented on discoloration of the flesh. Permeability of packaging material unknown.	Nieper (1986)
Soft, gluey texture. Caustic bitter, sweet, sour, faecal, soapy and rancid.	Description of appearance, texture and flavour after storage of commercial products until expire of declared minimal shelf life at 4-7°C.	Commented on the findings of pale, yellowish and purple off-colours, remains of bones, skin and blood spots. Permeability of packaging material unknown.	Hildebrandt & Erol (1988)
Bitter, faecal, putrid, stringent, sour, sweet, cabbage, fruity, ammonia, amine, rancid and oxidised. Soft mushy, pasta-like, oily and greasy texture.	5 or 7 point hedonic smiley scale and descriptions by taste panel during storage experiments.	Different packaging materials, salt levels between 2.2-6.1 % SWP and storage temperatures between 5 and 10°C included.	This study (Table 2.3, Papers A & B, Manuscripts C & D)

Most of the described off-odours and/or -flavours are typical of microbiological activity and it is therefore tempting to conclude that spoilage of cold-smoked salmon is microbiological.

Tissue softening and development of rancid and oxidised off-flavours may be caused by autolytic activities. Rancidity and oxidised flavours may additionally be influenced by the permeability of the packaging material, a discussion of this is, however, beyond the scope of this thesis.

The relative importance of autolysis on the quality of chill-stored cold-smoked salmon has been elucidated in a storage experiment, where dry salted and injection brined salmon was prepared with normal and reduced loads of microorganisms and then followed during the subsequent storage with chemical, microbiological and sensory techniques (Paper B).

Table 2.5 Sensory characteristics of vacuum packaged cold-smoked salmon with a normal load (10^6 - 10^8 cfu/g) and a reduced load (less than 10^3 cfu/g) of microorganisms after storage for 28 days at 5°C^a .

Salting method & microbiological load	Salt level (% SWP) ^b	Dry matter (%)	Sensory characteristics	
			Texture	Flavour & Odour
Dry salted, normal	3.9 (0.9) ^c	39.4 (2.9) ^c	Soft, sticky	Putrid, astringent, sour, faecal
Dry salted, reduced	4.0 (0.9)	40.3 (2.4)	Soft, mushy, oily	Slightly bitter, neutral, bland
Injection brined, normal	4.4 (0.4)	34.8 (2.6)	Soft, mushy, creamy	Bitter, sour, ammonia, fruity, astringent, sharp
Injection brined, reduced	3.6 (0.5)	38.7 (3.6)	Soft, mushy, crumbling	Slightly sour, bitter, neutral

a) Adapted after Paper B; b) SWP, salt in water phase; c) Standard deviation (n-1).

Salmon with less than 10^3 cfu/g was not rejected by the taste panellists after 28 days of storage at 5°C , whereas the corresponding salmon with normal loads were rejected after only 21 days of storage. The taste panellists found, however, that the textural deterioration in salmon with reduced loads were of the same magnitude as those found in normal salmon (Table 2.5). This strongly indicated that the textural changes found during chill storage of cold-smoked salmon were mainly caused by autolysis. In experiments with sterile muscle blocks of cod stored anaerobically and aerobically at 0 - 2°C for up to 24 weeks Herbert *et al.* (1971) found some textural deterioration, which they also attributed to autolysis. Different fish muscle cathepsins have been held responsible for tissue softening in fish (Gill, 1995) and the enzymes have been found to be active at salt levels relevant to smoked salmon (Makinodan *et al.*, 1991). In another experiment cold-smoked salmon fillets vacuum packed immediately after smoking were found to have shelf lives 4 to 16 days longer than sliced salmon from the same production batch when stored at 5°C , but these extended shelf lives

were always associated with more pronounced textural deterioration. This may be explained by the extended storage time giving the native enzymes in the salmon a longer incubation time to be active (Manuscript D).

Another important aspect of the storage experiment with reduced and normal loads of microorganisms was the fact that the typical spoilage off-odours and -flavours were only produced in the normal salmon harbouring 10^6 - 10^8 cfu/g at spoilage (Table 2.5, Paper B). This pattern was repeated in another experiment, where vacuum packed salmon blocks with less than 1000 cfu/g served as reference samples to inoculated samples and no production of off-odours and flavours was detected after 21 days at 5°C (Manuscript E). This suggests that overt spoilage only occurs as a result of microbiological activity. However, as indicated above, it is also a fact that the textural deterioration of autolytic origin may ultimately render the product unacceptable.

Prolonged storage of sterile muscle blocks made from cod (*Gadus morhua*) under anaerobic and aerobic conditions at 0-2°C similarly failed to procure any spoilage off-odours (Herbert *et al.*, 1971; Dalgaard *et al.*, 1993), whereas other researchers found identical spoilage characteristics regardless of the absence or presence of microorganisms during chill storage of yellow-eyed mullet (*Aldrichetta forsteri*) (Fletcher & Statham, 1988). The relative importance of microbiological and autolytic activities on development of spoilage may therefore be dependant on the individual fish species. However, in the case of vacuum packed cold-smoked salmon (*Salmo salar*) it may be concluded that the spoilage during chill storage primarily is due to microbiological activity with autolytic deterioration of the tissue playing a secondary role under normal conditions.

2.4.2 Microbiological changes

What kind of microorganisms develop during chill storage of cold-smoked salmon and ultimately causes spoilage?

According to the initial hypothesis, spoilage is caused by activity of a narrow group of specific spoilage organisms. However, a thorough review of the available literature as well as results from this study have revealed that it is difficult to generalise about the microflora on chilled cold-smoked salmon and to compare results from different sources. This in part results from inadequate descriptions of the microbiological methods, the lack of sufficient information about how the salmon were processed and different criteria for assessing onset of spoilage. Tables 2.2 and 2.3 give an overview of, what has been described in the literature and in this study with regard to the size and composition of the

microflora found on cold-smoked salmon at the time of rejection.

In the storage experiment of Declerck & Vyncke (1972) only 3×10^4 cfu/g were found on spoiled cold-smoked salmon with 2.8% SWP after 30 days storage at 0°C. There were no further details on the microflora, but the total viable count (TVC) was obtained using the pour plate technique in Tryptic Soy Agar (TSA) incubated at 22°C. Declerck (1976) found in a later experiment that smoked salmon with 2.1% SWP spoiled after 21 days at 2°C with 4×10^6 cfu/g (TSA, pour plate). A number of isolates from this experiment were tentatively characterized and *Enterobacteriaceae* and *Achromobacter* were found to constitute the major part of the microflora with minor numbers of *Alcaligenes* and *Vibrio* spp. present. No lactic acid bacteria (LAB) were isolated from the vacuum packed salmon.

Schneider & Hildebrandt (1984) studied the effect of storage temperatures (6 and 12°C) on shelf life and found that the salmon with 5.3% SWP in both cases contained approximately 10^6 cfu/g at spoilage after 21 and 6-7 days, respectively. Selective and/or elective agar substrates showed that the flora was dominated by LAB in salmon stored at 12°C, where LAB and micrococci were prevalent on salmon stored at 6°C. Gram-negatives (tentative *Enterobacteriaceae* and *Pseudomonas* spp.) and yeasts were not found in numbers of any significance (2 or more log cycles below).

Cann *et al.* (1984) described the microflora on vacuum packed cold-smoked salmon with 4.1% SWP with TVC at 20 and 37°C and specific counts for LAB, *Brochothrix thermosphacta*, H₂S-producing bacteria and *Alteromonas putrefaciens* (*Shewanella putrefaciens*). Vacuum packed salmon stored at 5°C spoiled after 25 days with TVC of 4×10^6 cfu/g, the majority of which were LAB (10^6 cfu/g) while *B. thermosphacta*, H₂S-producing bacteria and *Shewanella putrefaciens* were found at levels 2 or more log cycles below. Similar microfloras were found on salmon stored at 0 and 10°C, indicating the temperature did not change the composition of the microflora. Another interesting aspect of this experiment was that the microflora on salmon from the same production batch stored in modified atmosphere (MAP, 60/40:CO₂/N₂) were identical to the flora found on the vacuum packed.

Much higher numbers of 8×10^7 to 10^9 cfu/g (TVC, pour plates, Iron Agar (IA)) were found by From (1987) in her study on the influence of permeability of packaging material on the shelf life of cold-smoked salmon. Salmon packed in medium and low oxygen-permeable material contained a LAB dominated flora while H₂S-producing bacteria (black colonies in IA), *B. thermosphacta* and yeasts were never present in numbers where they could contribute to spoilage. Subsequent isolation of microorganisms revealed that LAB together with non-H₂S-producing *Enterobacteriaceae* (*Serratia* spp. and *Enterobacter* spp.)

and *Vibrionaceae* dominated the flora (From & Huss, 1991).

Another study where LAB seemed to dominate the flora completely was that of Parisi *et al.* (1992). Unfortunately these workers gave no information on the salt levels in the cold-smoked salmon, which were found to be shelf stable for 40 days at 2°C.

In the present study it seems that one of 3 situations occurred upon spoilage at chill temperatures (5-10°C) with regard the size and composition of the microflora:

- a) Domination by LAB at levels of 10^7 - 10^9 cfu/g.
- b) Domination by a mixture of LAB and *Enterobacteriaceae* at levels of 10^7 - 10^8 cfu/g.
- c) Domination by temperature sensitive bacteria of the *Photobacterium*/marine vibrio type at levels of 10^6 - 10^7 cfu/g. Occasionally high levels of LAB were found at the same time.

Examples of cold-smoked salmon from the present study with dominance of LAB at the time of spoilage are shown in Figure 2.2.

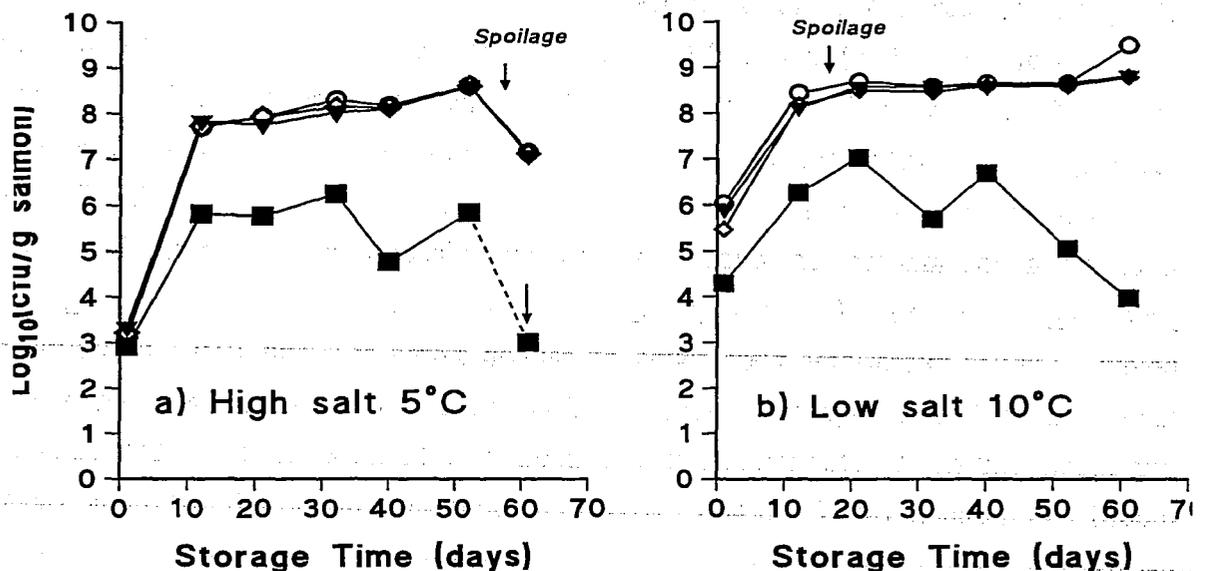


Figure 2.2 Microflora on chilled cold-smoked salmon with a) 4.6% and b) 2.2% SWP. ○, TVC; ▼, TPC; ◇, LAB; ■, *Enterobacteriaceae*; ↓, below detection. (From Paper A)

Initial levels of 10^3 - 10^6 cfu/g rapidly increased to levels of 10^7 - 10^8 cfu/g salmon for TVC, Total Psychrotrophic Counts (TPC, spread plate, TSA, 10°C) and tentative LAB, whereas the numbers of tentative *Enterobacteriaceae* were at least 2 orders of magnitude lower during the entire storage period and tended to decrease below the detection limit of 10^4

cfu/g at the end of the experiment. The microflora of salmon with high and low salt levels stored at 5 or 10°C, were remarkably similar despite the fact that the high salt salmon had a shelf life nearly 4 times that of the low salt salmon (Figure 2.2; Paper A). Chemical parameters suggested that salt and low storage temperatures influenced the bacteria's production of metabolites, indicating the metabolism of bacteria in high salt samples stored at 5°C were inhibited (Paper A).

It is perhaps interesting to note that the high salt salmon harboured high numbers of microorganisms (LAB, 10^8 cfu/g) for extended periods of time without any adverse effect on the sensory properties. A similar observation has been made during prolonged storage of vacuum packed cold-smoked herring (5.4% SWP) at 10°C, where homofermentative LAB developed to final levels of 10^8 cfu/g (Magnússon & Traustadóttir, 1982). This product remained edible for up to 3 months and the authors suggested that this was due to the biopreserving effect of LAB. It is tempting to speculate that this was also the case in this experiment, but unfortunately the microflora was not further characterized.

Other examples in the present study of LAB dominated microflora were found in storage experiments with commercially processed cold-smoked salmon from 3 different processing plants (Batches 1.2S, 2.1S and 2.2S, Manuscript D). However, in this case extended shelf lives were not achieved (only 4-5 weeks at 5°C) when compared to production batches with different microflora from the same study. Several factors may have contributed to these discrepancies but one certainly is the fact that the spoilage potential among LAB is highly variable. This will be further discussed in Chapter 3.

Examples of the occurrence of mixed LAB and *Enterobacteriaceae* were found in a study of the microflora on salmon produced by 3 smokehouses. The flora developed from initial counts of 10^2 - 10^4 cfu/g to final counts of 10^7 - 10^8 cfu/g at spoilage, which occurred after 24, 28 and 42 days for plant No. 3, No. 2 and No. 1, respectively (Manuscript C). At the time of spoilage, the selective LAB counts on pH 6.7 Nitrite Actidion Polymyxin agar (NAP) and Violet Red Bile Glucose agar (VRBG) for *Enterobacteriaceae*, were almost identical.

Table 2.6 Composition of the microflora at point of sensory rejection on individual vacuum packs of sliced, cold-smoked salmon produced by 3 smokehouses and stored at 5°C.

Bacteria groups ^a	Plant No. 1			Plant No. 2			Plant No. 3		
	Vacuum packs	1	2	3	1	2	3	1	2
No. of lactic acid bacteria (LAB) isolates	12	10	14	14	20	15	9	20	18
LAB cluster IA <i>Lb. curvatus</i> ^b	1	10	14	-	-	-	8	20	18
IB <i>Lb. saké/bavaricus</i>	6	-	-	-	-	-	1	-	-
II <i>Lactobacillus</i> spp.	-	-	-	10	9	5	-	-	-
III <i>Carnobacterium</i> spp.	-	-	-	3	7	1	-	-	-
IV <i>Lb. plantarum</i>	-	-	-	1	4	9	-	-	-
V <i>Leuc. mesenteroides</i>	5	-	-	-	-	-	-	-	-
No. of <i>Enterobacteriaceae</i> isolates	8	10	6	6	0	5	10	0	2
Ent. cluster I <i>Enterobacter agglomerans</i>	8	-	2	5	-	5	-	-	-
IIA <i>Hafnia alvei</i>	-	9	4	-	-	-	-	-	-
IIB <i>Serratia liquefaciens</i>	-	-	-	1	-	-	10	-	2
III <i>Enterobacteriaceae</i> spp.	-	1	-	-	-	-	-	-	-

a) Bacteria groups and clusters according to results from biochemical characterization (18 tests for lactic acid bacteria and 20 for *Enterobacteriaceae*) and numerical cluster analysis using S_{SM} ; b) Tentative identity. (From Manuscript C).

Isolation and biochemical characterization of 59-60 representative colonies from TPC plates from each plant showed that the flora consisted of 60%, 82% and 80% LAB and 40%, 18% and 20% *Enterobacteriaceae* on salmon from plants No. 1, No. 2 and No. 3, respectively (Table 2.6, Manuscript C). One hundred and thirty-two Gram-positive and catalase-negative isolates were further divided into 6 main groups of LAB and tentatively identified as reported above (Table 2.6). Apart from cluster II, which did not resemble any known species isolated from fish or meat products (see discussion in Manuscript C), all other clusters could be assigned to species previously reported in fish products (Jeppesen & Huss, 1993; Mauguin & Novel, 1994; Leisner *et al.*, 1994). There are no other reports on the identity of the LAB flora on cold-smoked salmon to the author's knowledge. Although Bersani & Cantoni (1989) reported that between 33 and 65% of the LAB on vacuum packed smoked salmon were non-aciduric and further went on to characterize 81 salmon isolates with the API 50 CHL kit, it was impossible to extract any further information on the identity or characteristics of these isolates, as the results were mixed with strains from chicken and meat

products.

The remaining 27 isolates (Table 2.6) all belonged to *Enterobacteriaceae*, which are ubiquitous and known as spoilage bacteria in vacuum packed meat products (McMeekin, 1982; Dainty & Mackey, 1992). Similar species have previously been described on cold-smoked salmon (Nieper, 1986; Cantoni *et al.*, 1993) and From & Huss (1991) also reported presence of *Serratia* spp. and *Enterobacter* spp. in their analysis of the flora on smoked salmon. In fact, it seemed that the flora composition found in the latter report may have resembled the mixed LAB and *Enterobacteriaceae* flora just described (Table 2.6; From, 1987; Manuscript C). The biochemical and phenotypical characterization also revealed that the composition of the microflora varies among different salmon processing plants and even among vacuum packs from the same production batch, the aspects of which will be discussed later (see section 2.4.2.1).

Microbiological patterns similar to the ones shown in Figure 2.3 were also found on cold-smoked salmon. The microflora were apparently dominated by bacteria which were unable to survive the pour plating technique involving exposure to 45°C warm agar. Characterization of the colonies found on the TPC plates and TVC plates from dry salted salmon (Figure 2.3a) revealed that these organisms were temperature sensitive and never found on TVC plates. The bacteria were large Gram-negative coccobacilli (3-5µm) with a negative or very slow oxidase reaction and a smaller proportion were bioluminescent (*ca.* 20-25%). Further characterization of 18 strains resulted in a tentative identification as *Photobacterium phosphoreum* (Paper B). This identification has later been confirmed by Dalgaard (1994). Similar organisms have later been found on injection brined salmon, which were stored as whole fillets or as sliced salmon (Figure 2.3b; Manuscript D [sample codes 1.1S, 2.2S & 2.2W]) and these organisms were tentatively assigned to a *Photobacterium*/marine vibrio group.

In the latter experiment the presence of psychrotrophic bacteria was only monitored through observation of the difference between TVC (pour plates) and TPC (spread plates) and screening for presence of bioluminescent colonies on TPC plates. This is a very insensitive technique considering the analytical uncertainties connected with traditional agar methods and the fact that dark non-luminescent mutants are common among the *Photobacterium* spp. and the marine vibrio group (Baumann & Baumann, 1984; Dalgaard, 1995). Due to the lack of specific detection methods, it is quite possible that this group of organisms has been overlooked in the past. Also, most routine laboratories do not include a psychrotrophic count prepared with the spread plate technique and refrigerated diluents and spatulas, when analyzing fish products.

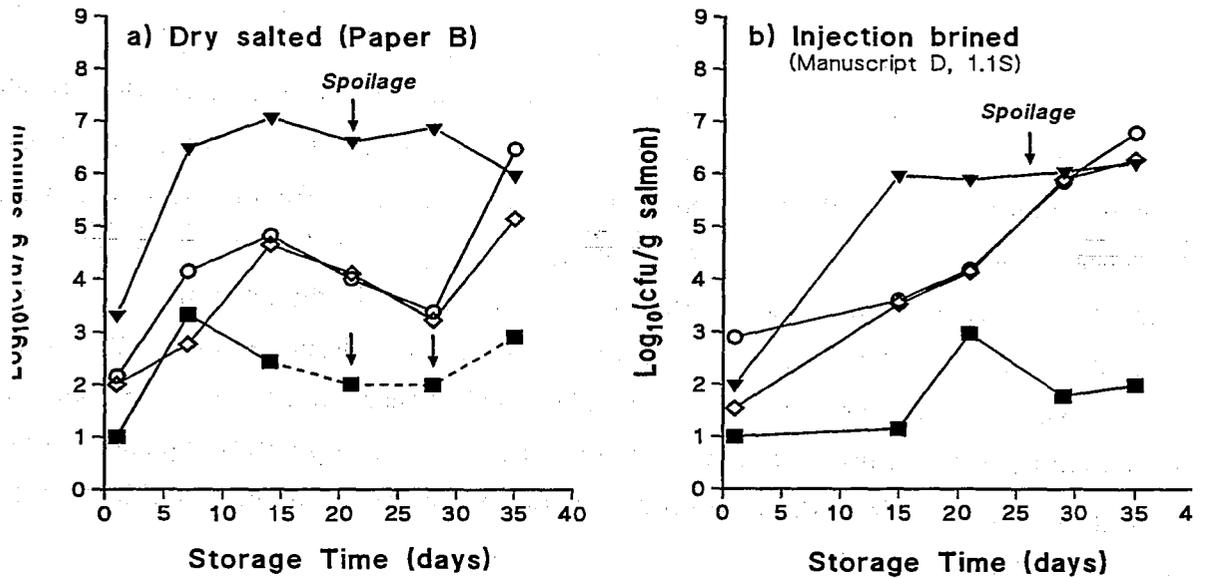


Figure 2.3 Development of a psychrophilic microflora during storage of cold-smoked salmon at 5°C. ○, TVC; ▼, TPC; ◇, LAB; ■, *Enterobacteriaceae*; ↓, below detection.

Presence of the *Photobacterium*/marine vibrio group has not previously been reported in cold-smoked salmon, but van Spreekens (1987) has found similar organisms on vacuum packed lightly salted matjes herring, indicating this group of organisms may occur on this type of product. Thus when Declerck & Vyncke (1972) reported vacuum packed cold-smoked salmon to spoil with pour plate counts of only 4.4 Log₁₀(cfu/g) after 30 days at 0°C, it may be that in reality the bacterial numbers were much higher (due to psychrophiles) but not detected due to the analytical technique applied.

Dalgaard *et al.* (1993) concluded in their work on MAP cod, that *Photobacterium phosphoreum* was the dominating specific spoilage organism for this product. In the two examples shown in Figure 2.3, it also seemed that bacteria of this type were logically the only potential spoilage organisms. Aspects of this will be discussed in Chapter 3.

Direct microscopy of the 10⁻¹-dilution of salmon samples was performed routinely in the experiment described in Paper B, Manuscript C and E. The microscopy always agreed well with the findings on the agar plates and the presence of obligate anaerobic sporeforming spoilage bacteria similar to those previously described by Dainty *et al.* (1989b), Segner (1992), Lawson *et al.* (1994) and Ben Embarek (1994b), was never suspected.

2.4.2.1 Intra- and interplant variations and the existence of one specific microflora for cold-smoked salmon.

The different microbiological patterns, which were reported in the literature and

obtained in this study, raised the question as to whether there exists one typical microflora for this product as initially proposed.

This question was answered through 2 experimental studies, one which involved characterization and tentative identification of a larger number of organisms from spoiled salmon and another which involved repeated storage experiments and characterization of the microflora using traditional plate count methods and Malthus conductance methods.

In the first experiment cold-smoked salmon from 3 different smokehouses were stored at 5°C until rejection by the taste panellists. At that point 19-20 colonies were selected from TPC plates of the highest countable dilution from each of the 3 vacuum packs sampled from each of the 3 smokehouses (Manuscript C). The results from the subsequent characterization are shown in Table 2.6 and they clearly show considerable variations in the microflora not only among different plants but also among the 3 vacuum packs from the same plant. Examples of the interplant variations were the findings of LAB cluster II, III, and IV only on salmon from plant No. 2 and the isolation of *Hafnia alvei* from plant No 1 only. One vacuum pack from plant No. 1 contained *Leuconostoc mesenteroides* not found on the 2 other vacuum packs and the numbers of *Enterobacteriaceae* varied between 0 and 10 on vacuum packs from plant No 2, showing the intraplant variation (Table 2.6, Manuscript C).

Similar differences in the LAB flora have been observed on vacuum packed meat products from different sources, i.e. interplant variations, by Morishita & Shiromizu (1986) and Hugas *et al.* (1993). Hitchener *et al.* (1982), Borch *et al.* (1988) and Samelis *et al.* (1994) all found batch to batch variations in the LAB flora on vacuum packed beef, vacuum packed emulsion sausages and naturally fermented salami, respectively, from the same processing plants, indicating the existence of intraplant variations.

The other approach for analysis of the uniformity of the microflora used correlation of traditional plate count methods with Malthus detection times (DT) for vacuum packed cold-smoked salmon samples stored at 5°C from 3 different salmon processing plants. Theoretically the regression lines obtained for each of these processing plants should be identical if the composition of the microflora was similar. However, the regression line between TPC and DT from the Malthus Total Viable Count (TVC-Malthus) obtained for plant No. 1 was significantly ($P < 0.001$) different from the regression lines obtained for plants No. 2 and No. 3 (Figure 2.4a; Manuscript D). Regression lines between VRBG counts and DT from the Malthus *Enterobacteriaceae* method were similar for plants No. 1 and No. 3, whereas the regression line for plant No. 2 was significantly different ($P < 0.001$) from the others (Figure 2.4b; Manuscript D). These results strongly supported the findings from the characterization study that the microflora on salmon from the 3

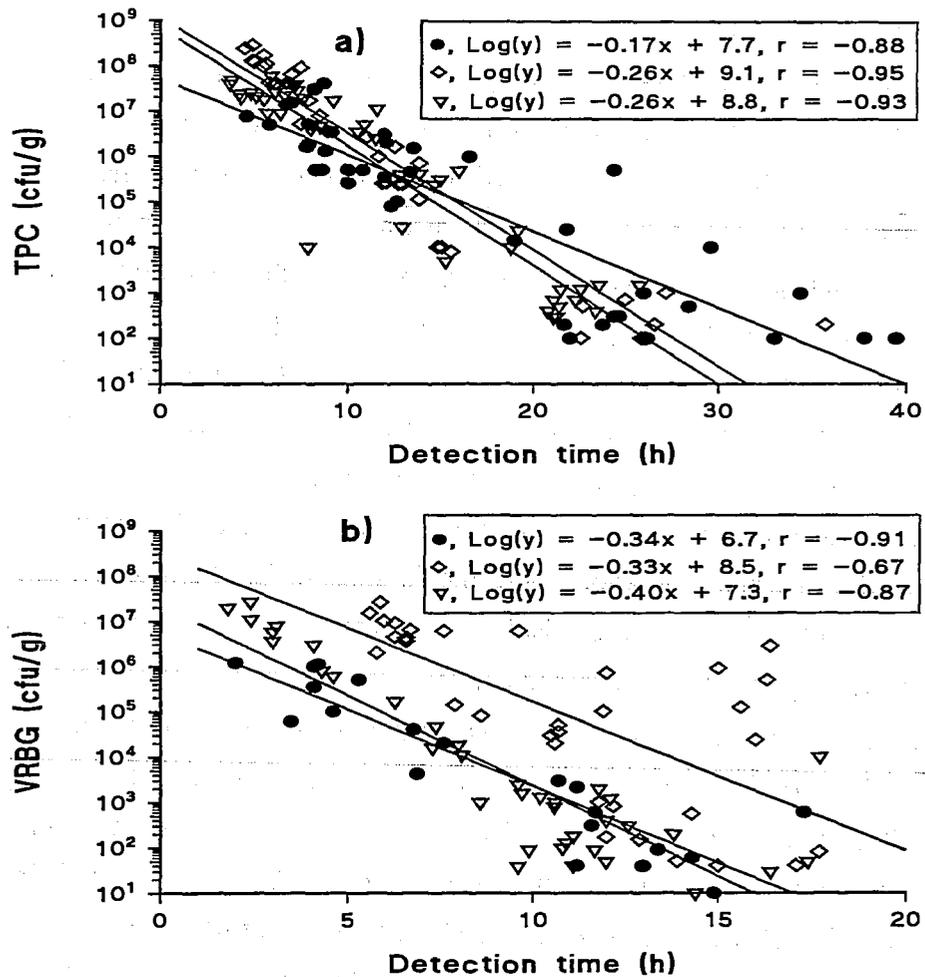


Figure 2.4 Calibration curves between a) Malthus detection time (DT) and TPC or b) DT and VRBG in cold-smoked salmon. ●, Plant No. 1; ◇, Plant No. 2; ▽, Plant No. 3. (From Manuscript D).

smokehouses were different from one-another. Gibson and Ogden (1987) found variable regression lines for the same fish products packed under different conditions and they also suggested that this phenomenon was caused by the prevalence of different microorganisms.

When regression lines between TPC and TVC-Malthus from whole smoked salmon fillets vacuum packed immediately after smoking were compared with the regression lines obtained for sliced vacuum packed salmon from the same smokerack from plants No. 1 and No. 3, it became clear that the microflora on sliced salmon differed from that on whole fillets. It was speculated that the additional handling and exposure to the in-house flora had altered the flora (Manuscript D). This is similar to observations done with emulsion sausages sampled at different points of the processing line (Borch *et al.*, 1988).

Based on the above mentioned results and available data in the literature it is not

likely that there exists one specific spoilage microflora on cold-smoked salmon. The product rather represents a frame where a number of different organisms can grow and thrive depending on whether they are successfully introduced either through the raw material or from the production environment. Spoilage occurs as a result of microbiological activity and will therefore be influenced by the spoilage potential among the prevalent microorganisms.

2.4.3 Potential chemical quality indicators for cold-smoked salmon.

Chemical quality indicators should ideally reflect the edibility or sensory quality of the product. The indicators must therefore be either directly involved in the production of the spoilage characteristics, related to growth of the bacteria responsible for spoilage or preferable both. For a chemical quality indicator to be applicable in quality control laboratories in industry and the public domain the analytical procedure should be simple, fast, cheap and not require complicated analytical equipment.

A number of different quality indicators, which would meet some of these requirements, have been evaluated for a number of fish products including Hx, total nitrogen volatile bases (TVB), trimethylamine (TMA), ammonia (NH₃), ethanol (EtOH) and non-volatile amines including putrescine, cadaverine, tyramine and histamine (Hebard *et al.*, 1982; Lindsay *et al.*, 1986; Gill, 1990; Gill, 1992; Oehlenschläger, 1992; Rehbein, 1993). Some of these quality indicators including the thiobarbiturate acid value (TBA) as a measurement for oxidative changes in the lipid, have been applied in storage studies of cold-smoked salmon and the results of this are presented in Table 2.7. The biogenic amines will be discussed in Chapter 4 as some of them pose a health hazard in relation to the product.

Hypoxanthine is a breakdown product of adenosine triphosphate (ATP) and while the conversion of ATP to inosine (Ino) mainly is thought to be autolytic (Ehira & Uchiyama, 1987; Gill, 1990), the conversion of Ino to Hx has been shown to be caused by both bacterial and autolytic enzymes (Ravn Jørgensen *et al.*, 1988; Surrence *et al.*, 1988; Gram, 1989; Dalgaard, 1993; Paper B; Manuscript E). During the comparative storage study of cold-smoked salmon with reduced bacterial loads (less than 10³ cfu/g) and normal loads (10⁶-10⁸ cfu/g), it was found that 68% of the Hx in normal samples originated from microbiological activity (Paper B). Representatives from the *Enterobacteriaceae* and *Photobacterium*/marine vibrio group have further been shown to convert inosine monophosphate (IMP) into Ino (van Sprekens, 1987; Manuscript E). Hx is thought to contribute to bitter off-flavours developed in spoiling fish (Jones *et al.*, 1964; Jones, 1965; Hughes & Jones, 1966, Fletcher *et al.*, 1990), though it is still not clear whether this is related to the disappearance of IMP (Tarr,

1966; Bremner *et al.*, 1988). In cold-smoked salmon, however, IMP usually disappears within the first week of storage in products with a remaining shelf life of 2 to 6 weeks (own unpublished data).

Table 2.7 Concentrations of various chemical compounds in spoiled vacuum packed cold-smoked salmon.

Hx ^a ($\mu\text{mol/g}$)	Acetic acid ($\mu\text{mol/g}$)	TVB ^b (mgN/100 g)	TMA ^c (mgN/100 g)	EtOH ^d ($\mu\text{mol/g}$)	TBA ^e (MA mg/kg)	Comments (incl. storage temp.)	Sources
-	-	40	-	-	1.1	0°C	Declerck & Vyncke (1972)
-	100 (ml NaOH (0.001N)/100 g volatile acids)	40 (40 NH ₃)	-	-	5.0	2°C	Declerck (1976)
3.5	-	44	-	-	-	0°C	Cann <i>et al.</i> (1984)
5.4	-	48	-	-	-	5°C	Cann <i>et al.</i> (1984)
5.6	-	70	-	-	-	10°C	Cann <i>et al.</i> (1984)
-	-	40	20	-	-	5°C, Riloten X	From (1987)
-	-	32	12	-	-	5°C, Amilon	From (1987)
-	-	34	12	-	-	10°C, Riloten X	From (1987)
-	-	70	24	-	-	10°C, Amilon	From (1987)
-	D-lactate, 1.1	30	-	-	-	6°C	Schneider & Hildebrandt (1984)
-	D-lactate, 1.1	30	-	-	-	12°C	Schneider & Hildebrandt (1984)
-	-	75	13	-	0.2	0-2°C, salt?	Parisi <i>et al.</i> (1992)
8.0	19.6	-	13.5	3.4	-	5°C, low salt	Paper A
7.2	11.6	-	14.6	2.8	-	5°C, high salt	Paper A
8.0	16.6	-	10.3	3.4	-	10°C, low salt	Paper A
7.2	23.0	-	12.0	2.2	-	10°C, high salt	Paper A
4.4	14.0	38	23	1.4	1.1	5°C, dry salted	Paper B
3.8	6.0	28	14	1.1	1.2	5°C, injection brined	Paper B

a) Hx - hypoxanthine; b) TVB - total volatile nitrogen bases; c) TMA - trimethylamine; d) EtOH - ethanol; e) TBA - thiobarbiturate values (MA - malonaldehyde).

Hx was found to be the best suitable quality indicator in a study of spoilage patterns in cold-smoked salmon with high and low salt levels stored at 5 and 10°C, where levels of 5-7 $\mu\text{mol/g}$ indicated the limit of sensory acceptability (Table 2.7, Paper A). These data were not reproducible in a subsequent study, where dry salted and injection brined salmon spoiled with levels of 4.4 and 3.8 $\mu\text{mol/g}$, respectively (Table 2.7, Paper B). Cann *et al.* (1984) found in their study levels of Hx ranging between 5.4 and 5.6 $\mu\text{mol/g}$ for salmon stored at 5 and 10°C, agreeing with the original levels suggested in Paper A. However, only 3.5 $\mu\text{mol/g}$ Hx was found in spoiled salmon stored at 0°C from the same batch (Table 2.7).

Hx may influence the occurrence of bitter off-flavours in smoked salmon but these flavours may also occur as a result of proteolysis. It has been shown that various peptides containing hydrophobic amino acids exhibit very bitter flavours in pure solutions (Ishibashi *et al.*, 1987; Ishibashi *et al.*, 1988).

Acetic acid, TVB, TMA and EtOH are all characterised by being end products from bacterial metabolism i.e. acetic acid from degradation of amino acids and carbohydrates, TMA (included in TVB) from TMAO in anaerobic respiration, NH₃ (included in TVB) from deamination of amino acids and ethanol from various fermentations of carbohydrates, amino acids and short chained acids (Baumann & Baumann, 1984; Gottschalk, 1986; Axelsson, 1993). TMA contributes to the fishy odours found on spoiling fish, but as discussed by Dalgaard (1993) there is a considerable difference in the levels found in aerobically spoiled iced cod (10-15 mg-N/100 g) and levels found in spoiled MAP cod (*ca.* 30 mg-N/100 g). This indicated that TMA alone is not responsible for perceived spoilage off-odours. The TMAO content in *Salmo salar* is relatively low, ranging from 24 ± 10 mg-N/100 g according to Hebard *et al.* (1982) to 15 ± 5 mg-N/100 g in Norwegian farmed salmon (own unpublished observations). In spoiled salmon, values for TMA and TVB have been found to vary between 10 to 24 mg-N/100 g for TMA and between 28 and 75 mg-N/100 g, respectively (Table 2.7). Cantoni *et al.*'s (1993) suggestion of an upper acceptable limit of 40 mg-N/100 for TVB does not seem to have any bearing on 6 out of 14 experimental data sets, where spoilage occurred with TVB levels lower than 40 mg-N/100. Acetic acid may contribute to the sour off-flavours found in spoiled salmon, though it has not been analyzed whether the actual concentrations of 6.0 to 23 µmol/g found in smoked salmon would attribute to such sensory impressions (Table 2.7). Ethanol is only produced in small concentrations with 3.4 µmol/g being the highest (Table 2.7). Rehbein (1993) found similar low values in smoked salmon stored at 6-8°C, whereas significantly higher concentrations (up to 34 µmol/g) were produced when the product was temperature abused at room temperature for 7 days. The number of samples was limited in this survey, and the same vacuum packs were repeatedly sampled throughout the storage and the vacuum seal thus broken. However, it seems that EtOH may be usable as an indicator of temperature abuse in the distribution steps and retail shops.

Storage experiments with salmon muscle blocks with reduced microbiological loads (less than 10³ cfu/g) showed that acetic acid and TMA exclusively are produced by microorganisms, while a smaller proportion of the TVB is produced by autolytic enzymes (Paper B; Manuscript E).

Considering the variable nature of the microflora on vacuum packed cold-smoked

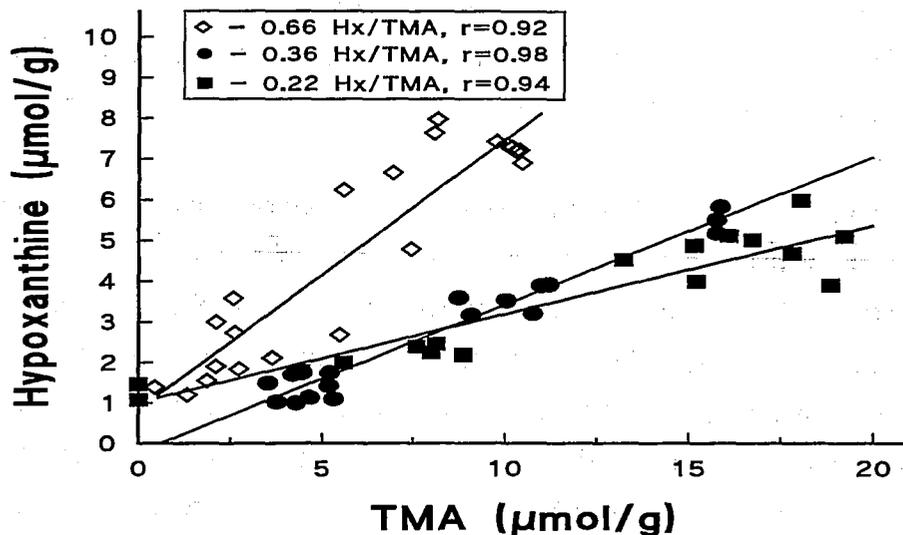


Figure 2.5 Relation between Hx and TMA in cold-smoked salmon. ◇ , Brined and sliced (4.6% NaCl); ● , Dry salted (3.9% NaCl); ■ , Injection brined (4.4% NaCl); r , Correlation coefficient. (From Paper B).

salmon and the bacterial origin of the tested compounds, it is therefore not surprising that none of the quality indicators can be used universally to measure quality of the product. This is also clear from Table 2.7 and it is caused by the different bacteria producing these indicators at different rates if at all.

In Figure 2.5 the ratios of Hx and TMA produced by the microorganisms from 3 different storage experiments are shown. The spoilage flora and other characteristics were distinctly different and the chemical patterns were also significantly ($P < 0.001$) different from one-another (Paper B).

In conclusion, the variable mixed microflora found on cold-smoked salmon makes the use of chemical indicators only related to the bacteria's metabolism but not directly related to production of spoilage characteristics, impossible. Instead spoilage indicators may be found through screening of compounds, probably of volatile character including sulphur compounds, developing in smoked salmon during spoilage with the range of different microflora, in the hope that one general compound essential to characteristic "salmon spoilage" can be found.

2.5 Conclusion.

A number of different factors involving the raw material determine the quality of the final cold-smoked products. These include appropriate colouring of the muscle tissue, fat content, oxidative stability of the lipid, texture (physiological state of muscle cells) and the

absence of pathogens and excessive loads of microorganisms.

The trend in cold-smoking has gone toward lighter cures, which has decreased the importance of the smoking and drying processes. Preservation and shelf life rely therefore on the salt content, storage temperature and quality of the packaging material.

Spoilage of cold-smoked salmon is caused by microbiological activity, while textural softening results from autolysis.

At spoilage, the microflora on cold-smoked salmon was found to consist of a) LAB only, b) a mixture of LAB and *Enterobacteriaceae* or c) psychrotrophic organisms of the *Photobacterium*/marine vibrio group in levels of 10^7 - 10^9 cfu/g, 10^7 - 10^8 cfu/g and 10^6 - 10^7 cfu/g, respectively. Characterization of the microflora from 3 different smokehouses revealed significant batch to batch as well as plant to plant differences. Further work is required on determining the background for the highly variable microflora.

Most of the known quality indicators for fresh fish have been shown to be of limited use, because they are related to bacterial growth and activity without being essential parts of the a "spoiled cold-smoked salmon flavour or odour". The ideal quality indicator should be related to such flavours and odours, so passing a certain threshold value coincides with overt spoilage of the product.

3. Characteristics of the microflora on cold-smoked salmon.

3.1. Lactic acid bacteria.

3.1.1 Occurrence of lactic acid bacteria in fish and lightly preserved fish products.

Lactic acid bacteria (LAB) are readily recovered from vacuum packed lightly preserved fish products such as cold-smoked herring, cold-smoked salmon, sugar-salted (gravad) fish (salmon, Greenlandic halibut and mackerel), brined prawn, shellfish salads, fish paté and minced herring with 4.6% NaCl and 5% sucrose (Magnússon & Traustadóttir, 1982; Schneider & Hildebrandt, 1984; Jeppesen & Huss, 1993; Leisner *et al.*, 1994, Manuscript C).

Although LAB never dominate the microflora on fresh fish, these bacteria have been isolated from fresh herring (Kraus, 1961), various finfish from the Gulf of Mexico (Nickelson II *et al.*, 1980), the intestines of cod, saithe and capelin (Schröder *et al.*, 1980), North and Baltic Sea fish (cod, herring, mackerel and plaice) (Knøchel, 1981) and commercial fillets of cod, salmon and pollock (Mauguin & Novel, 1994). Two LAB species *Carnobacterium piscicola* and *Vagococcus salmoninarum*, have specifically been associated with fish and diseased farmed rainbow trout (Hiu *et al.*, 1984; Collins *et al.*, 1987; Wallbanks *et al.*, 1990).

The origin of LAB on cold-smoked salmon may therefore be the raw material, although the in-house flora in the smokehouses are also likely to contribute or alter the flora as previously discussed (see section 2.4.2.1; Manuscripts C & D). Borch and coworkers (1988) tried to identify the major sources of post process contamination of emulsion sausages, and one of their findings was the colonization of sausages with a *Lactobacillus* sp. only found in the cold storage room.

Production of vacuum packed cold-smoked salmon involves many manual processing steps (see Figure 2.1) and when this exposure to the production environment is considered, the in-house is most likely to play a major role in the composition and size of the microflora on the final products.

3.1.2 Characteristics of lactic acid bacteria from cold-smoked salmon.

A total of 286 LAB strains have been isolated from spoiled cold-smoked salmon, of which only 11 strains or 3.9% were obligate heterofermenters (Manuscript C). Magnússon

& Traustadóttir (1982) also reported complete dominance of homofermentative *Lactobacillus* spp. on vacuum packed cold-smoked herring, while Leisner *et al.* (1994) found dominance of *Carnobacterium* spp. on vacuum packed sugar-salted salmon. But as just indicated, these generalisations may be of little value considering the variability of the flora on these products.

Further biochemical and phenotypical characterization of 168 strains enabled tentative identification of most of the groups or clusters (Manuscript C). *Lb. curvatus* was the most frequent occurring LAB accounting for approximately 50 to 55% of the isolates from spoiled salmon. This species resembles *Lb. saké/bavaricus* but is unable to produce acid from melibiose and NH₃ from arginine (Schillinger & Lücke, 1987a). The profiles of the salmon *Lb. curvatus* were similar to strains found on red meat products by Grant & Patterson (1991, cluster 3), Samelis *et al.* (1994, group 1 & 2) and Hugas *et al.* (1993) but not with that reported by Dykes *et al.* (1994, cluster IV), where 33% did not grow on acetate agar and only between 17 and 50% produced acid from cellulose, salicin and trehalose.

Seventeen strains were tentatively identified as *Lb. saké* or the closely related *Lb. bavaricus* as described by the keys of Schillinger & Lücke (1987a) and Döring *et al.* (1988). These strains combined, represented 30% and 6% of the LAB from spoiled salmon in the screening and storage experiment, respectively. The biochemical and phenotypical profile for salmon isolates was in good agreement with profiles previously reported for *Lb. saké* from a variety of meat products (Kandler & Weiss, 1986; Borch & Molin, 1988; Grant & Patterson, 1991; Dykes *et al.*, 1994; Samelis *et al.*, 1994). *Lb. bavaricus* is different from *Lb. saké*, which is arginine negative (Döring *et al.*, 1988), and 65% of the strains most likely belonged to this species based on this property.

Jeppesen & Huss (1993) characterised 61 LAB strains isolated from a variety of fish products and found 25-26 strains to be tentative *Lb. saké*. They did, however, not include a test for acid production from melibiose and *Lb. curvatus* strains may be included in this group. Mauguin & Novel (1994) found no *Lb. saké/bavaricus* nor any *Lb. curvatus* in their screening of seafoods.

Tentative *Carnobacterium* spp. only accounted for approximately 9% of the LAB flora from spoiled cold-smoked salmon and none of these strains produced gas from glucose in the media of Schillinger & Lücke (1987a). Collins *et al.* (1987) reported difficulties in detection of gas from glucose, and the bacteria were found to be homofermentative by Hiu *et al.* (1984) and de Bruyn *et al.* (1987), indicating some variability among strains. The *Carnobacterium* group do not seem to be specifically associated with lightly preserved fish products as suggested by Leisner *et al.* (1994). This was supported by Mauguin & Novel

(1994) and Jeppesen & Huss (1993), who found 16 of 86 strains and 2-7 of 61 strains to belong to *Carnobacterium* spp., respectively.

Leuconostoc strains tentatively identified as *Leuco. carnosum* and *Leuco. mesenteroides* only accounted for 3.3-4.5% of the total LAB flora on spoiled salmon. *Leuconostoc* spp. dominated a minced herring product with 5% sugar and they are occasionally found in brined prawn products in connection with slime production and spoilage (From, 1991), but they have not been found to cause similar problems in smoked salmon.

Fourteen strains (12% of LAB in storage experiment) were tentatively identified as *Lb. plantarum*. This LAB species is often found in seafoods (Schrøder *et al.*, 1980; From & Huss, 1993; Mauguin & Novel, 1994) and in meat products (Morishita & Shiromizo, 1986; Ferusu & Jones, 1988; Hugas *et al.*, 1993; Samelis *et al.*, 1994), but reports on the biochemical properties vary, indicating this group of organisms is quite heterogenous.

One LAB cluster containing 24 strains from the storage experiment could not be assigned to any known species or group of LAB. This cluster was characterised by inability to grow on acetate agar, no production of acid from sucrose, lactose, melezitose and production of acid from rhamnose.

The characterization of LAB isolated from spoiled cold-smoked salmon revealed that the majority of the salmon strains belonged to LAB species frequently associated with meat and fish products.

3.1.3 Spoilage potential among lactic acid bacteria.

Traditionally the spoilage potential among LAB has been considered to be very low to non-existent. There are however, several reports in the literature referring to the potential role of LAB in spoilage of vacuum packed meat products (Egan, 1983). As discussed in Chapter 2, it seemed that in several storage experiments LAB were the only bacteria present to spoil the product (Paper A; Manuscript C & D). It was therefore decided to perform model experiments with selected LAB strains isolated from spoiled salmon in order to study their growth, metabolism and production of off-odours in pure culture.

In sterile salmon juice with 4.3% SWP, *Lb. sake* 1T18, *Lb. curvatus* 3N6 and *Carnobacterium* 2T2 grew to final levels of 10^8 cfu/g during anaerobic incubation for 15 days at 5°C with production of sour, astringent, fruity and sweet/burnt odours (Manuscript E). This indicated that the LAB were able to produce some of the characteristic off-odours found on cold-smoked salmon (Table 2.4). The odours were not as intense, as those found in smoked salmon, in spite of the high cell concentrations, but this may be related to differences

between the composition of the heat sterilised salmon juice and cold-smoked salmon. It was therefore decided use a more realistic model system consisting of vacuum packed smoked salmon blocks produced with a reduced load of microorganisms following the procedure described in Paper B. This was, however, not without problems, because approximately 50% of the salmon blocks were not sterile but contained low levels of indigenous bacteria, which predominantly belonged to the *Photobacterium*/marine vibrio group and careful data interpretation was therefore necessary.

When *Lb. saké* 1T18 and *Lb. saké/bavaricus* S1.11 were inoculated on vacuum packed salmon blocks and incubated for 20-21 days at 5°C, the bacteria grew to levels of 10⁸ cfu/g with the production of sour, cabbagey and sulphurous off-odours (Manuscript E). It was subsequently shown that *Lb. saké* 1T18 produced H₂S during growth on smoked salmon. Shay & Egan (1981) isolated and described an H₂S-producing LAB from meats and this strain L13 was later identified as *Lb. saké* (Egan & Shay, 1989). Borch & Agerhem (1992) found H₂S production by a homofermentative *Lactobacillus* sp. during anaerobic growth on beef at 4°C, whereas Hanna *et al.* (1983) detected sulphide-like odours in beef inoculated with *Lb. viridescens*, *Lb. coryneformis*, *Lb. dextranicum* and *Lb. paramesenteroides*. These results indicate that the ability to produce H₂S frequently occurs among certain fish and meat LAB species.

Off-odours produced by the homofermentative *Lactobacillus* R1.12 and *Lb. curvatus* N1.4 were different and described as slightly acidic, burnt and buttermilk-like after 16 days, and these odours evolved into sour, astringent and almost faecal odours after 21 days (Manuscript E). These odours have previously been described in spoiled cold-smoked salmon (Table 2.4) and reported to be produced by LAB inoculated in meat (Patterson & Gibbs, 1977; Hanna *et al.*, 1983; Borch & Agerhem, 1992).

The LAB's production of acetic acid and Hx was also followed during these model experiments, but as discussed in Chapter 2, it was not possible to relate these compounds to production of spoilage characteristics.

It has been suggested that a microflora consisting of LAB is to prefer on fish and meat products, because spoilage often occurs weeks after the maximum cell concentrations has been reached (Egan & Shay, 1982; Schillinger & Lücke, 1987b; Paper A). In the model experiments on smoked salmon blocks off-odour production was detected after only 20 to 21 days at 5°C, which was approximately 10 days after the LAB strains reached their maximum levels (Manuscript E). Borch & Agerhem (1992) detected off-odours either before or as the maximum bacterial counts were obtained on beef inoculated with meat lactics. It may therefore be that isolates from spoiled fish and meat products have a higher spoilage potential

than normally found among fish and meat lactics. They may be selected as the storage progresses because of better abilities to adapt themselves to a situation with scarce resources of nutrients i.e semi starvation. Also, Borch *et al.* (1991) have shown that the homofermentative LAB can change their metabolism to become heterofermentative during situations of starvation. This shift may also involve other parts of the metabolism and explain off-odour production from otherwise harmless bacteria during prolonged chill storage.

3.2 *Enterobacteriaceae.*

Spoiled cold-smoked salmon contained variable levels of *Enterobacteriaceae* as discussed in Chapter 2. In the study of the composition of the microflora, 63 *Enterobacteriaceae* strains were isolated and further characterised using the API 20E system and among these strains, 24 were tentatively identified as *Serratia* spp including *S. liquefaciens*, 20 strains as *Enterobacter agglomerans*, 13 strains as *Hafnia alvei* and finally 8 strains could not be further identified (Manuscript C).

Similar species have previously been found on cold-smoked salmon (Nieper, 1986; From, 1987; Cantoni *et al.*, 1993). These organisms are found widespread in the environment (Krieg & Holt, 1984) and the above mentioned species have often been related to spoilage of vacuum packed meat products (Patterson & Gibbs, 1977; Gill & Newton, 1979; Hanna *et al.*, 1979; Stiles & Ng, 1981; McMeekin, 1982; Dainty *et al.*, 1989a).

Selected *Enterobacteriaceae* strains from spoiled salmon were screened for their production of off-odours during anaerobic growth in sterilised salmon juice with *ca.* 4.3% SWP at 15°C for 7 days (2 *Serratia* spp. & 2 *Enterobacteriaceae* spp.) and at 5°C for 15 days (*S. liquefaciens* 2R4) (Manuscript C & E). At 15°C faecal, acidic, pungent and nauseous off-odours were found in the juice, while *S. liquefaciens* 2R4 produced amine and ammonia-like odours at 5°C with a final cell concentration of 10⁹ cfu/ml. Similar strong off-odours were produced by *Enterobacteriaceae* from spoiled smoked salmon as reported by From & Huss (1991), indicating that these organisms have a high spoilage potential under optimal conditions.

In subsequent inoculation experiments on vacuum packed cold-smoked salmon blocks conducted at 5°C, *S. liquefaciens* 2R4 failed in colonizing the blocks. This may have been caused by competition from low levels of indigenous bacteria primarily from the *Photobacterium*/marine vibrio group (Manuscript E). In the same experiment, another *S. liquefaciens* (N1.6) and an *E. agglomerans* (R3.3) were similarly out competed, and only the *H. alvei* (S2.4) strain was successful in colonizing the salmon block reaching final levels of

10⁸ cfu/g. The latter strain produced stale, nauseous and slightly fruity off-odours, but no sign of sulphurous odours was detected. Hanna *et al.* (1979; 1983) and Dainty *et al.* (1989a) found production of H₂S by *H. alvei* strains in inoculation experiments on meat. Other sulphur compounds including dimethyldisulphide, were additionally detected by the latter research group. It was, however, indicated in experiments by Patterson & Gibbs (1977) that *H. alvei* may have greater potential for production of H₂S under aerobic conditions than under anaerobic, and this may explain the absence of sulphurous odours in salmon inoculated with *H. alvei* 2R4. Also the potential for H₂S production may vary among strains.

It is interesting to note that the *Enterobacteriaceae* originally isolated from vacuum packed salmon stored for 4-6 weeks at 5°C grew so poorly, when reinoculated on the salmon blocks and incubated under identical conditions. Decrease or apparent inhibition of *Enterobacteriaceae* has also been seen in storage experiments with cold-smoked salmon (Figure 2.2; Paper A). It may therefore be that *Enterobacteriaceae* spp. are inhibited by microbial interactions.

3.3 *Photobacterium phosphoreum* and marine vibrio.

3.3.1 Occurrence of *Photobacterium phosphoreum* and other marine vibrio.

Photobacterium phosphoreum is a Gram-negative marine bacteria belonging to the *Vibrionaceae* family and it can be readily isolated from fresh fish, including the intestines where they often are found in high levels of 10⁷-10⁸ cfu/g (van Spreekens, 1974; Woodland Hastings & Nealson, 1981; Baumann & Baumann, 1984; Dalgaard *et al.*, 1993). These bacteria are characterised by being large coccobacilli (1-5µm), oxidase negative, sensitive to Vibriostaticum O/129, able to grow at 5°C, producing gas from glucose under optimal conditions and strains are usually bioluminescent, although dark mutants are found (Hendrie *et al.*, 1970, van Spreekens, 1974; Baumann & Baumann, 1984; Dalgaard, 1995). As discussed by Dalgaard (1993) these bacteria are extremely heat labile and never found in pour plates, because the 45°C agar kills them. Dalgaard (1995) reported that only 23 of 50 strains from cod grew at 25°C, indicating this bacteria is a true psychrophile.

Based on the recent findings of the importance of *P. phosphoreum* in MAP cod (Dalgaard *et al.*, 1993), a total psychrotrophic count (TPC) was included using the spread plate technique and refrigerated diluents and utensils throughout this study to enable the detection of this bacteria on cold-smoked salmon, where it had not previously been found. As discussed in Chapter 2, the bacteria was found in some storage experiments and often on

salmon blocks, which were produced under conditions aimed at eliminating the contamination from the in-house flora (Paper A; Manuscript E). In some experiments *P. phosphoreum* and possibly other marine vibrio were the only organisms present at levels which could explain the spoilage of the salmon (Figure 2.3; Paper B; Manuscript D). Yet in other experiments no trace was found of their presence (Manuscript C). At this point there is no logical explanation for the apparently variable occurrence of *P. phosphoreum* (and other marine vibrio) on sliced, vacuum packed cold-smoked salmon, as this group was found on salmon produced by different plants, regardless of the degree or method of processing e.g sliced/unsliced, previously frozen/never frozen, dry salting/injection brining. Further research is needed to elucidate the occurrence and microbial ecology of these organisms in cold-smoked salmon and probably other lightly preserved fish products, such as sugar salted fish.

Species belonging to the marine vibrio group may also occur on cold-smoked salmon and be detected on the basis of their emission of light. These include *Vibrio splendidus* and *Vibrio logei*, both able to grow at low temperatures (4°C) and bioluminesce (Baumann & Baumann, 1984). These strains are oxidase positive, which distinguishes them readily from *P. phosphoreum*. Makarios-Laham & Levin (1984) isolated marine vibrio from haddock and these bacteria were described as psychrophilic, temperature labile, Gram-negative, oxidase positive, pleomorphic, TMA-producing bacteria, but unfortunately their ability to bioluminesce was not tested.

Further work is required to determine the exact identity of bioluminous bacteria on smoked salmon, though in one experiment it seemed that *P. phosphoreum* constituted 100% of this *Photobacterium*/marine vibrio group (Paper B).

3.3.2 Spoilage potential among the *Photobacteria*/marine vibrio group.

The spoilage potential of marine luminous bacteria has long been thought to be limited to non-existent as stated by Shewan (1971). This was based on the fact that these bacteria did not produce strong offensive off-odours compared to those produced by *Pseudomonas* spp. van Spreekens (1974, 1977) isolated *P. phosphoreum* in large numbers from spoiled whole iced cod. It was concluded that the spoilage potential was limited as the bacteria only produced buttermilk-like, aromatic, slightly sour and later musty, fishy and rubbery off-odours during inoculation experiments on cod. In later work, *Photobacterium* spp. were found on vacuum packed lightly salted matjes herring (2-3% salt), where they completely dominated the flora in levels of 6.0-6.5 Log₁₀(cfu/g) at the time of rejection by the sensory

panellists (van Spreekens, 1987). Unfortunately, the sensory characteristics of the spoiling matjes herring were not described. Also, Abgrall & Cleret (1990) concluded that although *P. phosphoreum* constituted 70-99% of the microflora on spoiling whiting fillets, they could not be responsible for spoilage. In the present study the ability of a *P. phosphoreum* strain (P66) to produce off-odours was assessed in sterile salmon juice and during inoculation experiments on vacuum packed smoked salmon blocks (Manuscript E). Production of sour, amine and ammonia-like off-odours were detected in both model systems, and odours described as fishy and stale were additionally found in salmon blocks after growth of *P. phosphoreum* P66 for 20-21 days at 5°C. Also, the organism grew well on cold-smoked salmon and was shown to be relatively more biochemically active than the other strains isolated from spoiled salmon (LAB and *Enterobacteriaceae*) (Manuscript E). This may indicate that these bacteria have a higher spoilage potential at lower cell concentrations than found for the other isolates. Dalgaard *et al.* (1993) and Dalgaard (1995) found *P. phosphoreum* to be responsible for the TMA found in spoiled vacuum and modified atmosphere packed cod stored at 0°C and concluded the organism most likely was responsible for spoilage of packed cod with production of amine and sour off-odours.

It therefore seems that the importance of this group of bacteria in fish spoilage has been underestimated in the past. More work is required to fully elucidate their role in the spoilage of vacuum packed lightly preserved fish products.

3.4 *Brochothrix thermosphacta* and yeast.

Yeasts and *Brochothrix thermosphacta* never occurred routinely at high levels during storage of vacuum packed smoked salmon (Paper B; Manuscript C).

Yeasts were found in high levels of up to 10^6 cfu/g on salmon stored in highly permeable polyethylene plastic in the experiment of From (1987, Table 2.2). The spoilage characteristics indicated that these organisms were partially responsible for spoilage. Two yeast strains isolated from spoiled salmon produced off-odours in salmon juice characterised as faecal, acidic, pungent and nauseous or aromatic (Manuscript C), indicating a spoilage potential. In that experiment the yeasts were with levels of 10^3 cfu/g present at levels 4 log cycles below the LAB (10^7 cfu/g) and not thought to have any impact on the spoilage. Growth and impact of yeasts is apparently controlled by the packaging material, and the use of oxygen impermeable plastic seems to eliminate them.

Low levels of *B. thermosphacta* have been detected in spoiled salmon, and in one experiment these bacteria were present at levels of $1.5-4.8 \times 10^4$ cfu/g in a microflora

dominated by LAB at levels of 3×10^7 cfu/g (Manuscript C). Although *B. thermosphacta* has been shown to produce off-odours during anaerobic growth in salmon juice (Manuscript C), their role as spoilage organisms on vacuum packed salmon seems to be limited based on their low numbers. High numbers of *B. thermosphacta* are occasionally found in vacuum packed red meat products, where they may contribute to spoilage (Dainty & Mackey, 1992). Occurrence of similar situations in cold-smoked salmon cannot be excluded.

3.5 Conclusion.

The microflora on cold-smoked salmon shares many characteristics with that reported in vacuum packed red meat products, except for the fact that psychrotrophic *Photobacterium* spp. and marine vibrios occur frequently on salmon. The composition of the microflora is highly variable and results from inoculation experiments with pure cultures of selected lactic acid bacteria, *Enterobacteriaceae* and a *P. phosphoreum* strain, indicated that all groups possessed the ability to produce off-odours resembling those found on spoiling cold-smoked salmon.

-Future research should aim at elucidating the occurrence and importance of bacteria from the *Photobacterium*/marine vibrio group, analysis of potential microbial interactions in the product and characterization of the chemistry in microbiological spoilage of cold-smoked salmon.

4. Hazards related to production of cold-smoked salmon.

In the following an evaluation of hazards with regard to safety in the production of cold-smoked salmon will be given based on published data in the literature.

4.1 *Clostridium botulinum*.

Clostridium botulinum is found widespread in the marine environment including the habitats of salmon, and *C. botulinum* type E seems to be the most prevalent (Eklund & Poysky, 1967; Craig *et al.*, 1968; Huss, 1980). Therefore cold-smoked salmon should be considered contaminated with this pathogen at very low levels. Fortunately outbreaks of botulism are very rare and vacuum packed cold-smoked salmon (Danish style, 3-5% SWP) has an excellent safety record (Huss, 1981; Huss, 1994).

It is primarily growth and toxin production of the non-proteolytic *C. botulinum* type B, E and F, which is of concern in lightly preserved ready-to-eat fish products receiving no cooking before consumption. This is related to their psychrotrophic nature (growth between 3.3-45°C) and the risk of toxin production before spoilage is overt (Gaze, 1994).

In model experiments no growth or toxin production was observed in vacuum packed hot-smoked trout with 3.0% SWP inoculated with 10^2 spores/g of *C. botulinum* types B, E and F during storage at 10°C for 30 days (Cann & Taylor, 1979). In a subsequent study of toxin production of *C. botulinum* types B and E (10^2 spores/g of each) in vacuum packed cold-smoked salmon with 4.1% SWP, Cann *et al.* (1984) similarly found no toxin production during storage at 10°C for 42 days. The same result was obtained with smoked salmon packed in modified atmosphere (60/40:CO₂/N₂). The shelf life of cold-smoked salmon was 24 days at 10°C indicating that there is very little risk of botulism in edible smoked salmon with an appropriate level of salt (more than 3.5% SWP) if stored at chill temperatures. However, as pointed out by Southcott & Razzell (1973) and as indicated by the results of Cann *et al.* (1984), temperature control is of utmost importance for control of this hazard.

In conclusion, the hazard of toxin production by *C. botulinum* is controlled by appropriate salting of the salmon to levels of 3.5% SWP or more in combination with storage at chill temperatures (preferably below 5°C). Both of these parameters thus become important critical control points (CCP1) in order to control this hazard.

4.2 *Listeria monocytogenes*.

Listeria monocytogenes is a psychrotrophic pathogen able to grow anaerobically and aerobically at temperatures down to 1°C (Juntilla *et al.*, 1988). The bacterium is causing listeriosis and is found widespread in the environment and has been isolated from a variety of different food products as reviewed by Farber & Peterkin (1991) and Ryser & Marth (1991). The occurrence and significance in seafoods has recently been extensively reviewed by Fuchs & Reilly (1992), Dillon & Patel (1992) and Ben Embarek (1994a). Most environmental *L. monocytogenes* strains are not likely to be pathogenic, but there are no techniques available to distinguish between apathogenic and pathogenic strains on a routine basis and the minimal infective doses for susceptible individuals are not known (Hof & Rocourt, 1992). Therefore the occurrence of this bacterium in ready-to-eat products must be treated with caution, but to date, no cases of listeriosis have been connected with cold-smoked salmon.

4.2.1 Occurrence of *Listeria monocytogenes* in cold-smoked salmon.

Several surveys of the occurrence of *L. monocytogenes* and other *Listeria* species on vacuum packed cold-smoked salmon have been published and these results are summarised in Table 4.1. It seems that *Listeria* may be isolated from this product regardless of country of origin. According to Table 4.1, positive samples vary from 0 to 79%, but some of these data may be biased (i.e. plants with problems were surveyed). Jemmi (1993) estimated in larger survey that approximately 10% of samples would be positive for *L. monocytogenes*.

The natural levels of contamination on freshly produced cold-smoked salmon are normally reported to be low. Guyer & Jemmi (1990) reported positive samples contained less than 1 MPN/g and Rørvik *et al.* (1995) isolated *L. monocytogenes* only after selective enrichment, indicating the presence of less than 100 cfu/g, while Eklund *et al.* (1995) found an average of 6.2 *L. monocytogenes* per g positive sample.

Table 4.1 Occurrence of *Listeria* spp. and *L. monocytogenes* in cold-smoked salmon (*Salmo salar* and *Oncorhynchus* spp).

Country	Samples (number)	Positives (%)		Source
		<i>Listeria</i> spp. ^a	<i>Listeria monocytogenes</i>	
Switzerland	64	12.6	6.3	Guyer & Jemmi (1990)
Switzerland	278	^b	12.2	Jemmi (1990a)
Switzerland	100	-	24	Jemmi (1990b)
Canada	32	-	31.2	Farber (1991)
Iceland	13	23	0	Hartemink & Georgsson (1991)
Norway	33	-	9	Rørvik & Yndestad (1991)
Italy	63	0-100 ^c	0-29 ^c	Valenti <i>et al.</i> (1991)
New Zealand	12	-	75	Hudson <i>et al.</i> (1992)
Switzerland	388	-	10.0	Jemmi (1993)
Canada	39	3	0	Dillon <i>et al.</i> (1994)
UK	22	13.6	-	Fuchs & Nicolaidis (1994)
USA	61 ^d	-	79	Eklund <i>et al.</i> (1995)
Norway	65 ^e	11	11	Rørvik <i>et al.</i> (1995)

a) All *Listeria* spp.; b) Not determined; c) Varies with origin (country); d) From 6 different processing plants reporting problems with *L. monocytogenes*; e) From one smokehouse over 8 months (6 samplings).

4.2.2 Sources of *Listeria monocytogenes*.

The origin of *L. monocytogenes* in cold-smoked salmon has been the topic of two recent publications. Eklund and coworkers (1995) found through extensive surveys of several smokehouses that the bacterium came into the plants on surface areas of frozen and fresh fish. They did however, not apply any typing techniques to their survey. In Norway, Rørvik *et al.* (1995) analyzed the occurrence and presence of the bacterium in a slaughterhouse and in the adjacent smokehouse and they included typing of *L. monocytogenes* isolates with the Multilocus Enzyme Electrophoresis (MEE) technique for the subsequent epidemiological study. No *L. monocytogenes* were found on 50 freshly slaughtered fish and the prevalence of the bacterium in the slaughterhouse environment was only 7-9%. In the smokehouse, however, 29% of the environmental and 23% of the fish samples were positive, which resulted in 11% of the end products being contaminated. MEE typing showed that the type (ET-6) found on the end products had colonized the smokehouse. This type was not predominant in the slaughterhouse environment.

Information about the occurrence of *L. monocytogenes* in live salmon and in their natural environment is scarce, but the bacterium is found in coastal waters including sediments (Colburn *et al.*, 1990; Motes, 1991). In our survey of the occurrence of *Listeria* spp. in a Norwegian salmon farm and adjacent slaughterhouse, we found no *Listeria* spp. in 10 salmon sampled directly from the net cages, 8 samples of seawater from the salmon farm and in 57 samples from the slaughterhouse including freshly slaughtered fish (skin, gills, guts, stomach swaps) and environmental samples (water, ice, equipment, conveyor belts and drains) (Note F). The contamination levels of fresh fish are most likely related to pollution of waters from human and animal sources and to the hygiene during the subsequent slaughter. Jemmi & Keusch (1994) found a much higher incidence in fresh water rainbow trout reared in waters polluted from farm run off.

Thus the raw material may or may not be contaminated with *L. monocytogenes* depending on the source, but if the bacterium is introduced to the production environment, colonization of the smokehouse is likely. Other sources of *Listeria* spp. may be the personnel and the surrounding environment. With the ubiquitous nature of *L. monocytogenes* it is probably not possible to prevent introduction of the bacterium into the production environment and it is therefore important to further discuss the survival of the bacterium in the cold-smoking process and potential growth in the finished vacuum packed product before consumption.

4.2.3 Growth and survival during and after cold-smoking.

Several model experiments have been carried out to analyze the effect the cold-smoking process has on the growth and survival of *L. monocytogenes*.

Salmon fillets with surface inoculated *L. monocytogenes* were shown to survive the brining (5 h, 18-20% NaCl (w/v)) and cold-smoking processes (6 h, 25-30°C) (Macrae & Gibson, 1990; Guyer & Jemmi, 1991). The same result was obtained in cod which was cold-smoked for 3-4 h at 25-30°C (Dillon & Patel, 1993). Eklund *et al.* (1995) analyzed salmon fillets, which had been brined or injection brined with salt solutions containing *L. monocytogenes*. During the subsequent cold-smoking process, a slight reduction in the numbers on the surface inoculated fillets was seen, whereas numbers in salmon internally contaminated through the injection brining process either remained constant or increased during smoking at 17-21°C or 22-31°C, respectively, for 16 h. Rørvik *et al.* (1995), on the other hand, found that the incidence of salmon fillets containing natural levels of *Listeria* spp. dropped from 30-60% to 0% immediately after smoking, indicating the process may have

some effect on natural levels (< 100 cfu/g). However the number of post smoking samples were very small (8). In a similar survey Guyer & Jemmi (1991) found a reduction in numbers of samples positive for *L. innocua* (from 46% to 6.3%) when raw salmon was smoked. It thus seems that cold-smoking have some effect on the survival of *L. monocytogenes*, but that it is highly dependent on the size and site of the contamination. The process can, however, not be described as listeriocidal.

Table 4.2 Growth of inoculated *Listeria monocytogenes* on cold-smoked salmon.

<i>Listeria monocytogenes</i>	Inoculum (units/g)	Temperature (°C)	Time (days)	Growth (units/g)	Source
Scott A	10 ⁵	4	28/91	0/0	Macrae & Gibson (1990)
Scott A	ca. 100	4	15	2.5 x 10 ²	Farber (1991)
Shrimp isolate	ca. 100	4	15	1.0 x 10 ²	Farber (1991)
SLCC 2755	23	4 & 10	30	0	Guyer & Jemmi (1991)
SLCC 2755	2300	4 & 10	30	3.2 x 10 ²	Guyer & Jemmi (1991)
Salmon isolate	650	4 & 10	30	3.2 x 10 ⁴	Guyer & Jemmi (1991)
Mix of NCTC 7973 & 2 salmon isolates	6 (low) ^a	4	28	6.3 x 10 ⁴	Rørvik <i>et al.</i> (1991)
	600	4	28	2.0 x 10 ⁶	Rørvik <i>et al.</i> (1991)
	6 (high) ^a	4	28	2.0 x 10 ²	Rørvik <i>et al.</i> (1991)
Mix of NCTC 7973, Scott A, 3391 (human) and 3 environmental isolates	4.9 x 10 ⁴	5	25	0	Ben Embarek & Huss (1992)
	4.9 x 10 ⁴	10	25	6.5 x 10 ⁴	Ben Embarek & Huss (1992)
Mix of NCTC 7379 & smoked mussels isolate L70	1.5 x 10 ⁴	5	26	7.9 x 10 ³	Hudson & Mott (1993)
	1.5 x 10 ⁴	10	5	4.0 x 10 ³	Hudson & Mott (1993)

a) High and low refers to size of the indigenous flora (primarily lactic acid bacteria).

Model studies of the growth of *L. monocytogenes* have almost unequivocally shown that the bacteria grow and thrive on the product (Table 4.2), but that this depends on the size of the inoculum, the origin of the inoculated isolate, the size of the indigenous flora and the storage temperature. In general, salmon isolates grew better than the human Scott A strain and culture collection isolates, indicating adaptation to the environment. Larger inoculates grew to higher final levels within the shelf life of the product as shown by Guyer & Jemmi (1991) and Rørvik *et al.* (1991), indicating the importance of the levels of contamination. Also the size of the indigenous microflora affected the growth of *L. monocytogenes*, with the "inferior" quality salmon containing a larger microflora, which apparently decreased the

growth potential of the pathogen (Rørvik *et al.*, 1991). Ben Embarek & Huss (1992) found no growth at 5°C but did observe growth at 10°C. Hudson & Mott (1993) found the same final levels in the 5°C as in the 10°C samples but 21 days later. On the other hand, Guyer & Jemmi (1991) found no differences between samples inoculated with high levels of strain 2755 and the salmon isolate, indicating that refrigeration at 4°C is no guarantee against proliferation of the bacterium.

No growth was observed in one experiment only in which salmon were inoculated with 10⁵ cfu/g and stored at 4°C for up to 91 days (Macrae & Gibson, 1990). The bacteria did however, not decrease in numbers. Thus it can be concluded that the composition of cold-smoked salmon and storage conditions in no way inhibit growth of *L. monocytogenes*.

There are, however, indications that model experiments may overestimate the growth potential under normal levels of contamination (*ca.* 1-50 cfu/g, see above). The indigenous flora may contain large numbers of lactic acid bacteria (Chapter 2), of which some may exhibit antagonistic properties toward low numbers of *Listeria monocytogenes*. Antagonistic activity towards *L. monocytogenes* is common but highly variable among LAB (Holzapfel *et al.*, 1995).

In conclusion, it must be emphasized that the cold-smoking process contains no critical control points (CCP1), which eliminate the hazard of contamination with *L. monocytogenes* and the risk of subsequent growth to hazardous levels.

4.3 Other pathogenic bacteria.

4.3.1 *Aeromonas* spp.

Among other indigenous pathogens possibly occurring in cold-smoked salmon are motile *Aeromonas* spp. including *Aeromonas hydrophila*. These bacteria may readily be found on a number of different foods and marine environments as reviewed by Knøchel (1989). In one survey of foods in Switzerland an incidence of 11% *Aeromonas* spp. (*A. hydrophila* and *A. sobria*) was found in cold-smoked fish (Gobat & Jemmi, 1993). Hudson *et al.* (1992) found a much higher incidence of 44% motile *Aeromonas* spp. of which 28% were positive for *A. hydrophila* in ready-to-eat fin-fish products. Unfortunately, it was not possible to extract exact information on the incidence on cold-smoked salmon from these reports, but low levels probably occur frequently. Hudson & Mott (1993) moved on to perform inoculation experiments on vacuum packed cold-smoked salmon with *ca.* 3% SWP, where they found no growth at 5°C and 3 log cycles growth at 10°C during incubation for

16-19 days. Growth was enhanced by aerobic incubation. According to Huss (1994), *A. hydrophila*'s role in foodborne diarrhoeal diseases has not been fully clarified and a high infective dose is probably required for the bacteria to pose a risk. It, therefore, seems in case of cold-smoked salmon, that the established critical control points for *C. botulinum* should give ample protection against *A. hydrophila*, however it would appear that vacuum packaging retards the growth of *A. hydrophila* and presents an additional hurdle for the proliferation of this organism.

4.3.2 Pathogenic *Vibrionaceae*.

Vibrio spp., of which several are pathogenic, may occur on fish products. These bacteria are, however, predominantly mesophilic and adapted to tropical climates (Huss, 1994) and not likely to occur on cold-smoked salmon prepared in Denmark or other temperate climate countries. There seems to be one case in which cholera may have been caused by cold-smoked salmon (Sutton, 1974), indicating the risk, but this case probably involved gross temperature abuse. Low levels of *V. parahaemolyticus* have been shown to survive cold-smoking for 24 h at 30°C in mullet (*Mugil cephalus*) suggesting a potential hazard in other climates, especially if the fish is not chill stored (Karunasagar *et al.*, 1986).

4.3.3 *Yersinia enterocolitica*.

The incidence and impact of the psychrotrophic pathogen *Yersinia enterocolitica* in cold-smoked salmon is not well understood. The bacterium was in a Scottish survey isolated from 48% of the analyzed cold-smoked salmon, 50% of fresh salmon and 56% of rainbow trout (Bruce & Drysdale, 1989), while Hudson *et al.* (1992) found none in 25 samples of ready-to-eat fin-fish products. Parisi *et al.* (1992) also found no sign of *Y. enterocolitica* in their analysis of raw and smoked Pacific and Atlantic salmon. During inoculation studies, the organism was shown to grow slowly at 5°C on vacuum packed cold-smoked salmon with ca. 3% SWP, but not on aerobically stored smoked salmon (Hudson & Mott, 1993) presumably because *Yersinia* was out-competed by the indigenous spoilage flora. It appears that the incidence of *Y. enterocolitica* is variable, but more data are required to evaluate whether this pathogen poses any risk in relation to chill stored vacuum packed cold-smoked salmon. Insofar as cold-smoked salmon has not been involved in any case of food borne yersinosis, the risk is probably low.

4.3.4 Non-indigenous bacteria.

A number of pathogenic bacteria may occur on cold-smoked salmon as a result of excessive contamination in the production environment. These include *Salmonella* spp., *Shigella* spp., *Escherichia coli* and *Staphylococcus aureus*. Since these organisms are mainly mesophilic and not naturally occurring, their growth potential is probably limited by competition with the indigenous microflora, provided the products are not temperature abused. Control of these organisms can be obtained by enforcing adequate hygiene.

4.4 Parasites, viruses, biotoxins and chemicals.

Parasites in salmon are not killed by the cold-smoking process where the temperatures do not reach the required 55°C for 1 min (Huss, 1994). Gardiner (1990) showed in a limited survey that *Anisakis* spp. naturally occurring in wild Canadian Pacific salmon survived brining for 15-16 h followed by cold-smoking for 12 h at 26°C. He also concluded that the cold-smoking process was not likely to have any effect on cestodes (*Diphyllobothrium* spp.). According to Ahmed (1992), the presence of these two parasites poses a serious risk to the consumer. However, it is well recognized that freezing at -20°C for a minimum of 24 h will eliminate this risk and this critical control point (CCP1) has been incorporated into the EU and Danish regulations as an requirement for cold-smoke processing of wild Atlantic and Pacific salmon (EEC, 1991a; FM, 1993a, see Chapter 2.1).

Salmon may be infected with viruses of human or animal origin through pollution of the environment with sewage. Ahmed (1992) suggests that a large number of incidents of food related illnesses may have involved Norwalk or Norwalk-like viruses. This is especially true for filter feeders. The occurrence of such viruses in salmon from unpolluted water is probably very low to non-existent. Control of viruses in the production of cold-smoked salmon is therefore achieved through monitoring of the environment in which the salmon are reared. This monitoring will minimize the risk and is a critical control point (CCP2).

The risk assessment for the occurrence and prevention of biotoxins and chemical contaminants (i.e. antibiotics from aquaculture) in the raw material for production of cold-smoked salmon is parallel to that for viruses. The risk of chemically contaminating the salmon during smoking is in fact the reason for the prohibition of the use of chemically-treated wood chips for smoke generation (FM, 1993a, see section 2.1).

4.5 Biogenic amines.

Biogenic amines are non-volatile bases, which can be formed by decarboxylation of free amino acids by a number of different microorganisms including bacteria frequently found on cold-smoked salmon. The biogenic amines include histamine, tyramine, putrescine, cadaverine, tryptamine, phenylethylamine produced from histidine, tyrosine, glutamine and arginine via ornithine, lysine, tryptophan and phenylalanine, respectively (Karmas & Mietz, 1978; ten Brink *et al.*, 1990). Agmatine is another amine produced from arginine, and spermidine and spermine are further degradation products from putrescine (Karmas & Mietz, 1978). Histamine and to some extent, other biogenic amines can exhibit toxic effects after excessive oral intake with symptoms like nausea, hot flush, sweating, head ache, red rash and respiratory distress (ten Brink *et al.*, 1990).

Histamine is probably the most potent biogenic amine causing food poisoning world wide and this syndrome has especially involved fish from the *Scrombridae* family (tuna, mackerel and sardines) (Huss, 1994). Histamine can be produced by some *Lactobacillus* spp., *Vibrio* spp., *Photobacterium phosphoreum* and *Enterobacteriaceae* spp. including *Morganella morganii*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Proteus mirabilis* (Yoshinaga & Frank, 1982; Morii *et al.*, 1986; Klausen & Huss, 1987; van Spreekens, 1987; Morii *et al.*, 1988; ten Brink *et al.*, 1990; Ababouch *et al.*, 1991; Maijala, 1993, Leisner *et al.*, 1994). Most of these bacteria will not produce histamine at low temperatures (< 5°C) with the possible exception of *Photobacterium* spp. (van Spreekens, 1987). Histamine production by *M. morganii* at 5°C has, however, been reported following incubation at ≥ 10°C for 24 h mimicking temperature abuse (Klausen & Huss, 1987). This was probably caused by histidine decarboxylase enzyme released in the substrate as no growth of the bacterium was observed.

Histamine has been found in spoiled cold-smoked salmon at levels ranging from 32 to 195 mg/kg (Cantoni *et al.*, 1993). This is just under the maximum allowable safety limit of 200 mg/kg recommended by the EU regulation for fish from the *Scrombridae* and *Clupeidaceae* families (EEC, 1991a). Other confidential reports confirmed this range.

Other biogenic amines found in cold-smoked salmon include tyramine, which is produced by LAB (Edwards *et al.*, 1987; Leisner *et al.*, 1994) and the malodorous cadaverine and putrescine produced by a number of *Enterobacteriaceae* including *H. alvei* and *S. liquefaciens* (Dainty *et al.*, 1986).

Biogenic amines were suggested by Cantoni *et al.* (1993) as possible quality indicators for the product. Again the specific relation between occurrence of different bacteria and

production of specific amines makes it an impossible task to standardize quality levels, unless it is shown that a certain level of an amine is invariably connected with spoilage off-odours or -flavours (see also discussion in 2.4.3).

Cold-smoked salmon has not been identified as the causative agent in histamine or biogenic amine poisoning, and it may be that the product is spoiled before hazardous levels are obtained. It seems, however, justified to consider the hazard in relation to production and distribution of cold-smoked salmon. Control is obtained through control of the quality of the raw material and temperature control (CCP1) through the production and the subsequent distribution before consumption. Histamine production by psychrophilic *Photobacterium* spp. may, however, not be controlled by this temperature regime, but more knowledge is required about the occurrence and growth potential in cold-smoked salmon to give a qualified evaluation of this situation.

4.6 Conclusion.

In this chapter, potential hazards in relation to cold-smoked salmon have been identified and discussed. It can be concluded that all hazards except *Listeria monocytogenes* can be controlled within the process by monitoring of the quality of the raw material, ensuring a salt content of at least 3.5% SWP, compulsory freezing at -20°C for at least 24 h for wild salmon and appropriate time and temperature control throughout the process.

Control of *L. monocytogenes* remains a potential problem and it may be that an additional hurdle has to be incorporated in the production of cold-smoked salmon in order to inhibit growth of the bacterium during chill-storage. Possible solutions to this problem will be discussed in Chapter 5.

5. Critical control points in the production of cold-smoked salmon.

In Chapters 2, 3 and 4 different factors of significance for achieving a high quality and safe cold-smoked salmon product have been discussed. It can be concluded that a number of factors affecting the quality in terms of shelf life and a series of factors with possible impact on the sensory quality and safety of the product have to be controlled. These factors (or hazards) can be summarised as follows:

- 1) Quality of raw material (colour, texture, oxidative stability)
- 2) Growth and metabolic activity of psychrotrophic spoilage bacteria
- 3) Growth and toxin production of *C. botulinum*
- 4) Growth of *Listeria monocytogenes* and possibly other psychrotrophic pathogenic bacteria
- 5) Contamination with biotoxins, viruses and parasites
- 6) Growth and formation of biogenic amines by decarboxylating bacteria

Critical control points for the control of the above mentioned hazards have been identified based on the flow diagram over the production of cold-smoked salmon (Figure 2.1) and the NACMCF (1992) decision tree. Two types of CCPs are identified: **CCP1** - which will ensure full control of a hazard and **CCP2** - which will minimise but not ensure full control of the hazard. The identified critical control points are presented in Table 5.1.

The individual processing plants have to adapt these CCP's to their own facilities in order to design procedures to monitor that the CCP's are under control. This may for instance include tables of suitable weight gains during the salting process to ensure that a salt content of minimum 3.5% SWP is obtained in the final product. It is important that all control measures are defined and described in standard protocols, together with descriptions of appropriate ways of monitoring whether the CCP is under control. Also, a system for registration, data collection and record keeping has to be established.

Table 5.1 Critical control points in the production of cold-smoked salmon^a.

Process steps	Hazards	Control measures	CCP type	Monitoring
Reception of raw material	Substandard quality or unacceptable contamination with pathogenic and spoilage bacteria, parasites, viruses, chemicals and biotoxins	Ensure reliable source, preferably with quality programme	CCP2	Inspection of arriving material (temperature, sensory evaluation, documentation from supplier)
Storage and pretreatment of raw material (thawing and filleting)	Contamination and growth of bacteria of concern	(T x t) ^b control, sanitation and hygiene, water quality, separate storage facilities for raw fish	CCP1/2	Time and temperature recording, efficiency of sanitation
Salting (including equilibration)	Unacceptable salt content: a) control of <i>C. botulinum</i> , safety level $\geq 3.5\%$ SWP, b) acceptable taste, less than 6% SWP	Visual observations of salting procedures and equipment, calibration by weighing (brining & injection brining)	CCP1 (in combination with temp < 5°C)	Measurement of salt content of brine and product. Time and temperature recording.
Smoking	Chemical contaminants	Reliable source of woodchips	CCP1	Inspection of arriving material
Chilling, freezing, skinning and slicing	Contamination and growth of bacteria of concern	(T x t) control, sanitation and hygiene, separate storage facilities for processed fish	CCP1/2	Time and temperature recording, efficiency of sanitation, frequent sanitation of equipment with direct salmon contact
Packaging (vacuum)	Contamination with spoilage and pathogenic organisms (post-process & distribution), oxidation	Vacuum sealing of packages, quality of plastic	CCP1	Continuous visual and mechanical control of the vacuum sealing, documentation of plastic quality
Storage of end-products and distribution	Growth or activity of bacteria of concern	(T x t) control, separate storage facilities for packed fish, storage management system (first in, first out)	CCP1 (pathogens) CCP2 (spoilage)	Time and temperature recording (in plant), maintenance of the chill-chain in distribution, retail and homes.

a) If wild Pacific or Atlantic salmon are processed, an additional CCP1 (-20°C for ≥ 24 h) for control of live parasites is necessary; b) Time x temperature control

For a detailed description of the application of the concept of Hazard Analysis of Critical Control Points (HACCP) in quality assurance in the production of cold-smoked salmon and other fish products, the reader is referred to Huss (1994).

As outlined in Chapter 4, the hazard of unacceptable growth of *L. monocytogenes* cannot be controlled in the traditional cold-smoked salmon process. It is probably impossible to produce *Listeria* free cold-smoked salmon as this ubiquitous bacterium may enter the plant through the raw material as well as from the surrounding environment. Good manufacturing practices can however, limit the (cross) contamination to the lowest possible level. Along that line it may be necessary to accept low "natural" levels of the bacterium in salmon, but through incorporation of additional hurdles the risk of proliferation of the bacterium to hazardous levels during subsequent chill storage may be eliminated. Potential hurdles will be discussed briefly below.

5.1 Additional hurdles.

In Table 5.2 a number of chemical, biochemical and microbiological hurdles and their presumed mode of action are presented, of which some may be valuable in controlling growth of *L. monocytogenes* in chilled stored cold-smoked salmon. Only few of these compounds have been tested in salmon for their inhibitory effects, but their listericidal or inhibitory effect toward growth of *Listeria* spp. has been demonstrated in meat products or in microbiological assays.

There are numerous reports in the literature of the antilisterial effect of bacteriocins produced by lactic acid bacteria, and the application of bacteriocins in pure form seems to be a possibly way of inhibiting *L. monocytogenes* as reviewed by Hastings *et al.* (1993), Holzapfel *et al.* (1995) and Huss *et al.* (1995).

Use of lactate has shown some promising results in model systems resembling cold-smoked salmon, and Pelroy *et al.* (1994) reported total inhibition of *L. monocytogenes* inoculated in "natural" levels of 10 cells/g in comminuted salmon containing 2% (w/w) Na-lactate and 3% SWP and incubated at 5°C for up to 50 days. Lactate has also been shown to have an inhibitive effect toward *C. botulinum* (Meng & Genigeorgis, 1993) and could therefore act as an extra hurdle to control this bacteria in case of temperature abuse.

Table 5.2 Potential additional hurdles to control *Listeria monocytogenes* in the production of cold-smoked salmon.

Preservative system	Mode of action	Effect	Potential disadvantages
Bacteriocins Nisin Lactococcin Sakacin Carnosin Pediocin	Disruption of cell membranes, PMF and [H ⁺] gradient	Bacteriostatic/ bactericidal	Development of resistance. Cell repair mechanisms. Has to be approved for food. Inactivation by proteases
Organic acid Lactate Acetate	Disruption of PMF and [H ⁺] gradient	Bacteriostatic	Off-flavours in active concentrations (metallic, acidic)
Protamine	Disruption of membranes, PMF and [H ⁺] gradient. Associates with bacterial DNA.	Bactericidal/ bacteriostatic	? Has to be approved for food.
Protective cultures (Lactic acid bacteria)	Competition for nutrients. Secretion of bacteriocins, H ₂ O ₂ , organic acids, diacetyl, reuterin	Bacteriostatic & bactericidal	Has to be approved for food. Sensory characteristics.
Glucono- δ -lactone	pH?	Bacteriostatic	Sensory characteristics

Protamine is a cationic peptide extracted from fish milt, which has been shown to inhibit *Listeria monocytogenes* when grown in broth (Johansen *et al.*, 1995). It has yet to be shown that protamine exhibits the same inhibitory effect in food systems and in combination with the salt levels relevant to cold-smoked salmon.

Addition of live protective cultures would ensure a continuous antagonistic effect based on one or more inhibitory principles as indicated in Table 5.2 and reviewed by Huss *et al.* (1995). For a lightly preserved fish product such as smoked salmon where lactic acid bacteria already occur abundantly, there seems to be a potential for selecting suitable strains able to grow and compete with the indigenous (spoilage) flora and inhibit *L. monocytogenes*.

Glucono- δ -lactone is just an example of numerous other compounds, which have been found to inhibit of *L. monocytogenes* in combination with other hurdles. Qvist *et al.* (1994) used 0.25% (w/w) glucono- δ -lactone in combination with 2% (w/w) lactate to inhibit growth of *L. monocytogenes* in bologna-type sausages stored at 5°C.

6. Conclusion.

Cold-smoked salmon spoils as a result of microbiological activity, although autolysis has significant impact on the textural deterioration. The microflora on cold-smoked salmon is highly variable among production batches from the same plant and among different processing plants. A complex microflora consisting of lactic acid bacteria, *Enterobacteriaceae* and/or bacteria belonging to the group of *Photobacterium* spp. and marine vibrios develops on the vacuum packed cold-smoked salmon during storage at chill temperatures. The occurrence of bacteria from the latter group has not previously been reported in cold-smoked salmon, and further work is required to elucidate the importance of these bacteria with respect to quality.

The use of chemical quality indicators such as hypoxanthine, total volatile bases, trimethylamine, ethanol and acetic acid, is confounded by the fact that spoilage patterns are not uniform nor are the mixtures of organisms, which produce off-flavours and off-odours. The production of the tested compounds was not related to the occurrence of the typical spoilage flavours and odours but related to the metabolism of the prevalent bacteria, which produced these compounds at different rates. It was shown that *P. phosphoreum* was relatively more metabolically active than any of the other isolated bacteria. This may warrant that this bacterium possesses a higher spoilage potential at a lower cell concentration.

Preliminary model experiments indicated the existence of spoilage potentials among lactic acid bacteria, *Enterobacteriaceae* and *Photobacterium phosphoreum* strains isolated from spoiled cold-smoked salmon. Further work is required to characterise the chemistry in microbiological spoilage of cold-smoked salmon and to analyze the microbial interactions which seem to take place in the product.

Hazard analysis of factors (microbiological, biological and chemical), which may affect the safety of the product, showed that it is possible under proper conditions to control all potential hazards except the risk of growth of *Listeria monocytogenes* to unacceptable levels during chill storage of the product. It is therefore recommended that incorporation of additional hurdles into the products is considered.

Papers, manuscripts and notes.

- A. Effects of salt and storage temperature on chemical, microbiological and sensory changes in cold-smoked salmon. (*Paper, published*)**
- B. Importance of autolytic and microbiological activities on quality of cold-smoked salmon. (*Paper, accepted*)**
- C. Characteristics of the microflora isolated from spoiled cold-smoked salmon. (*Manuscript*)**
- D. Microbiological quality and shelf life of cold-smoked salmon from 3 different processing plants. (*Manuscript*)**
- E. Activity of potential spoilage bacteria from cold-smoked salmon. (*Manuscript*)**

- F. Occurrence of *Listeria* spp. in farmed salmon and during subsequent slaughter: Comparison of Listertest™ Lift and the USDA method. (*Research note, collaborative work*)**

Effects of salt and storage temperature on chemical, microbiological and sensory changes in cold-smoked salmon.

by

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Abstract.

Chemical, microbiological and sensory changes during storage of vacuum packed cold-smoked salmon were studied using a factorial experimental design with two storage temperatures (5 and 10°C) and two salt levels (2.2 and 4.6%).

The spoilage characteristics were typical of microbiological activity in all treatments, but there was no relation between sensory changes and any of the microbiological numbers (total viable counts, total psychrotrophes, lactic acid bacteria or *Enterobacteriaceae*). Total viable counts typically reached 10⁸ cfu/g weeks before sensory rejection.

Acetic acid, hypoxanthine, trimethylamine and ethanol concentrations increased with storage time in all treatments. The increase in ethanol depended on salt concentration but not storage temperature. Absolute values of trimethylamine ranged from 2-8 mg TMA-N/100 g initially, to 10 mg TMA-N/100 g at sensory rejection. Acetic acid levels increased with temperature and decreased with salt concentration, but varied between 12-23 µmol/g at rejection. Initial concentrations of hypoxanthine increased from 2-3 µmol/g to maximum 8-9 µmol/g with values of 5-7 µmol/g indicating the limit of sensory acceptability. Hypoxanthine was considered to be the best objective indicator for sensory quality of cold-smoked salmon.

Keywords: Cold-smoked salmon, vacuum packed, quality indicators, fish spoilage, hypoxanthine

Introduction.

Cold-smoked salmon is a lightly preserved fish product of considerable economic importance world-wide. Although a traditional product, microbiological or chemical criteria for objective quality evaluation of cold-smoked salmon are not well established and are not based on systematic studies of spoilage of the product.

Several surveys of the quality of cold-smoked salmon on the German market have revealed serious problems with sensory acceptability at the end of the declared minimal shelf life (Rakow, 1977; Schulze & Zimmerman, 1983; Nieper, 1986; Hildebrandt & Erol, 1988). The reasons for these serious quality problems have not been examined but could be due to consumer demands for lower salt levels.

During storage of cold-smoked and vacuum packaged salmon at chill temperatures, a complex microflora develops which is dominated by lactic acid bacteria along with fewer numbers of other groups like *Brochothrix thermosphacta*, *Vibrionaceae*, other Gram negative bacteria and yeasts (Cann *et al.*, 1984; Schneider & Hildebrandt, 1984; Jakobsen *et al.*, 1988), but no specific spoilage organisms have yet been identified. No relation between shelf life of cold-smoked salmon and total numbers of bacteria has been found (Gibson & Ogden, 1987; Hildebrandt & Erol 1988; Jakobsen *et al.*, 1988), which makes the use of any criteria based on total viable counts irrelevant as a quality parameter.

Among potential chemical quality indicators for measuring the quality of cold-smoked salmon, the development of total volatile bases (TVB-N) (Declerck & Vyncke, 1972; Declerck, 1976; Cann *et al.*, 1984; Schneider & Hildebrandt, 1984; Jakobsen *et al.*, 1988), the production of NH₃ and the increase in volatile acids (Declerck, 1976), the degradation of nucleotides expressed as an increase in hypoxanthine (Cann *et al.*, 1984) and the production of ethanol (Rehbein, 1993) have been considered. However, the origins of these chemical changes and their relation to product quality are not well established which make their use as objective quality indicators, uncertain.

Without objective criteria for quality evaluation, the producers of smoked salmon have difficulties in monitoring quality changes, determining shelf life and may be confronted with irrelevant and unreasonable demands from their customers, which primarily deal with the microbiological status of the product. Apart from the typical demand for the total viable count (TVC) to be less than 10⁵ cfu/g in the sliced, vacuum packed product, other frequent unreasonable demands deal with maximum limits on the numbers of specific types (genera) of organisms (coliforms, *Enterobacteriaceae*, *Enterococci*, lactic acid bacteria etc.) regardless of there being any scientific evidence relating levels of such organisms and the shelf life of

the product.

The objective of this study was to find suitable objective quality indicator(s) through a systematic study of chemical, microbiological and sensory changes during storage of vacuum packed cold-smoked salmon with two salt levels (*ca.* 2 and 4.5% salt in the water phase) stored at two storage temperatures (5 and 10°C).

Materials and Methods.

Salmon.

Fresh, premium quality, farmed salmon (*Salmo salar*) was slaughtered and filleted at a salmon farm in St. George, New Brunswick, Canada. Within 1-2 days after slaughter the fillets were packed in Styrofoam shipping boxes with ice (12.5 kg fillets to approximately 6-7.5 kg of ice in bags) and shipped (12 h) on a refrigerated truck to the smokehouse (Ocean Organic Ltd., Tusket, Nova Scotia, Canada). On arrival, the temperature of the fillets was 0.5-1.3°C and they were stored at 2°C for 1 day before processing. Fillets had an average weight of 1 kg. To obtain cold-smoked salmon with high and low salt levels, the salmon fillets were brined in a 23% (w/w) salt brine for 6 and 3 h, respectively, with a fish/brine ratio of 1:1 (w/w) and brine temperature of 8°C. The fish were dried for 6 h at 26°C with an initial relative humidity (rh) of 80% and final rh of 50%. They were smoked at 26°C for 6 h at a rh of 63%. Fish were sliced and vacuum packed in 50 g portions in a barrier material consisting of Nylon/polyethylene with an oxygen permeability of 0.29 mL/(100 cm² · 24 h) at 0% rh, 20°C and water vapour permeability of 0.06 mL/(100 cm² · 24 h). After packing, the fish were frozen for 8 days at -26°C and then transported to the laboratory (4 h) in sealed Styrofoam boxes. Upon arrival, the temperature did not exceed 0°C.

High and low salt packages were divided at random into treatments (see Table 1) and stored for up to 61 days at either 5 or 10°C. Six packages from each treatment were drawn at random every 8 to 12 days during the storage period and used for the chemical, microbiological and sensory analyses. All chemical and microbiological analyses were performed in triplicate (e.g. 3 different packages were analysed). Salt and moisture determinations were done separately on 6 packages (50 g each) from each treatment at the beginning of the storage experiment.

Sensory evaluation.

Sensory evaluation was carried out with 6 semi-trained judges presented with a pooled sample from each treatment for overall acceptability, with regard to taste and texture disregarding salty and smoky flavours. A hedonic smiley scale with 7 "faces" was used (Fletcher *et al.*, 1990) and later translated into numerical scores 1-7, where 1 and 7 were considered lowest and highest, respectively; 4 was considered the borderline of acceptability. The judges were also asked to comment on odour, texture, appearance and taste. On each sampling day, the effects of judges and treatment were evaluated by analysis of variance (ANOVA). Treatments receiving scores > 4 by $\geq 50\%$ of the judges were considered acceptable, treatments obtaining scores of 4 at the borderline for acceptability and scores < 4 unacceptable and rejected. The maximum shelf life for a treatment was defined as the last sampling day where the treatment received a score of 4 or above.

Microbiological analysis.

Salmon samples were homogenised in 0.9% NaCl (w/v) - 0.1% (w/v) peptone for 30 s in a Stomacher 400 Lab Blender (A. J. Seward, Bury, St. Edmunds, U. K.). **Total viable counts (TVC)** were performed by pour plating with Tryptic Soy Agar (TSA) (Difco (0369-17-6), Detroit MI, USA) and plates were incubated 3 days at 25°C. **Total psychrotrophic counts (TPC)** were done using spread plate method on TSA and plates were incubated at 10°C for 5 days. *Enterobacteriaceae* were enumerated on pour plates prepared with Violet Red Bile Glucose agar (VRBG-G) (Oxoid CM485, Unipath Ltd. Basingstoke, Hampshire, England) and TSA. All colonies with typical red violet zones after 2 days' aerobic incubation at 25°C were counted as tentative *Enterobacteriaceae* (Mossel, 1985). **Lactic acid bacteria** were counted on pour plates in Nitrite Actidion Polymyxin agar (NAP) (Davidson & Cronin, 1973) made from All Purpose Tween agar (BBL, Becton & Dickenson Microbiological Systems, Cockeysville MD, USA) with the addition of 0.6 g NaNO₂, 0.01 g Actidion (Cycloheximid, Sigma, St. Louis MO, USA) and 0.003 g polymyxin-B (Sigma) per litre agar. The pH was modified to 6.7 instead of the original 5.5 to make growth of acid sensitive lactic acid bacteria including *Carnobacterium* spp. possible (Collins *et al.*, 1987). All typical colonies were counted as tentative lactic acid bacteria after 3 days' aerobic incubation at 25°C.

Chemical analysis.

Ethanol analysis. Salmon was blended with 0.6 M perchloric acid (PCA) in a Lourdes Multi-Mixer (Model MM-1B, Lourdes Instrument Corp., Brooklyn, N.Y., USA) in a 1:2 ratio and filtered (Whatman #1). The filtrate was stored at -28°C until the analysis was performed. Samples were neutralised to pH 7 with 15% (w/v) KOH just before analysis. An enzymatic kit based on alcohol dehydrogenase from Diagnostic Chemicals Limited, Charlottetown, PEI, Canada was used.

Nucleotides. Ryder's (1985) HPLC method was used with a Waters HPLC system equipped with 2, Model 6000 A pumps, a WISP 710B autosampler, a Model 720 System Controller and Data Module and a C-18 μ Bondapak reverse phase 3.9 x 300 mm column. The mobile phase was 0.1 M potassium phosphate with pH 7.00. Samples were prepared by PCA extraction as described above, except that the neutralisation was performed immediately after the extraction. The nucleotides were identified using relative retention times and externally quantified using authentic standards obtained from Sigma, St. Louis, MO, USA. The external quantification was calibrated with fresh standards every day.

Trimethylamine (TMA). The picric acid method of Dyer & Mounsey (1945) as modified by Tozawa *et al.* (1971) was used. PCA extracts were prepared as described above for ethanol.

Organic acids. Organic acids were analysed by HPLC on a BIORAD HPX-87H Organic Acid column (300 x 7.8mm). All analyses were performed on neutralised PCA extracts. Experimental conditions were: flow rate, 0.4 ml/min; eluent, 0.008 M H₂SO₄; run time, 60 min; injection volume, 15 μ l; column temperature, 35°C and UV detection at 210 nm. Identification and quantification was done on the basis of relative retention times of authentic standards.

Salt. The AOAC (1975) method was used for salt determinations on salmon homogenates. Salt content was expressed as % NaCl in water phase of salmon.

Dry matter content was determined by oven drying 2 g salmon at 105°C for 20-24 h (until constant weight was obtained).

Statistical analyses.

The effects of the treatments and the storage time on the quality of cold-smoked salmon as measured by the chemical quality indices were analysed by a trend comparison procedure based on orthogonal polynomial contrasts with unequal intervals (Gomez &

Gomez, 1984). The linear, quadratic and cubic contrasts for each treatment and their interactions were analysed for significance by forward stepwise multiple regression using STATGRAPHICS ver. 4.0 (1989).

Results.

The salt contents and treatments of the samples and results of the sensory evaluation are shown in Table 1. High salt samples stored at 5 and 10°C were acceptable for at least 2-3 weeks more than the low salt samples stored at the same temperature. Increasing storage temperature from 5 to 10°C gave a decrease in shelf life of 1-2 weeks for high salt samples and of 2-3 weeks for low salt samples. Analysis of variance (ANOVA) showed no significant differences ($P > 0.05$) among the judges on any sampling day, indicating that the judges agreed on their evaluation (data not shown). The spoilage patterns described by the panelists were the same in all treatments and involved change from highest quality with fresh typical odours and flavours to formation of a neutral bland and slightly sour taste indicating a borderline quality. Spoiled cold-smoked salmon was characterised by bitter, faecal and rancid off-flavours regardless of storage temperature and salt level (see Table 1). The texture softened before off-odours and off-flavours were produced, and the tissue softening was often the reason for initial decrease in quality scores.

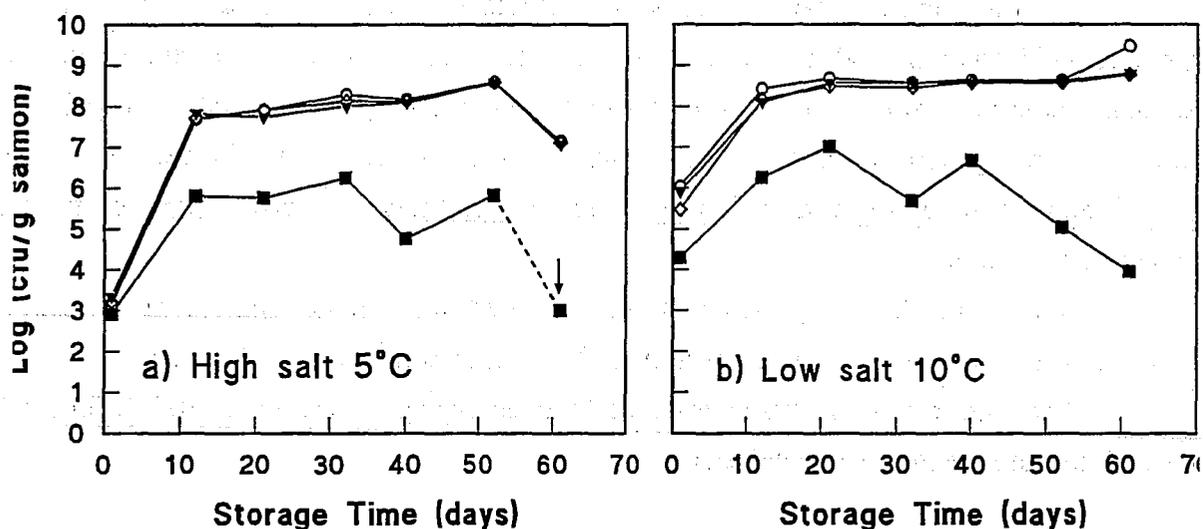


Fig. 1 The microflora during storage of cold-smoked salmon with a) 4.6% and b) 2.2% SWP. ○, TVC; ▽, TPC; ◇; LAB, ■; *Enterobacteriaceae*; ↓, below detection.

The size and development of the microflora was similar in all treatments regardless of the large differences in sensory properties.

Table 1. Sample treatment and sensory evaluation of cold-smoked salmon.

Codes	Storage temp.	Salt level (% SWP) ¹⁾	Max. acceptable shelf life (weeks) ²⁾	Borderline sensory characteristics		Rejection sensory characteristics	
				Texture	Flavour	Texture	Flavour
H10	10°C	4.6 ± 0.5	5-6	Soft	Slightly sour/bitter (32) ³⁾	Mushy, soft	Bitter, faecal, rancid, oxidised (52) ⁴⁾
L10	10°C	2.2 ± 0.6	1-2	Soft	Slightly sour (12)	Mushy, sticky	Bitter, faecal, putrid, ammonia (21)
H5	5°C	4.6 ± 0.5	7-8	Soft	Slightly sour (40)	- ⁵⁾	- ⁵⁾
L5	5°C	2.2 ± 0.6	4-5	Soft, mushy	Slightly sour (32)	Mushy	Bitter, faecal, putrid, rancid (40)

1) % SWP: Salt content in water phase ± Standard deviations (n-1); 2) Based on last acceptable sampling day; 3) Sampling day at which borderline quality is reached; 4) Sampling day at which sample is unacceptable, 5) Samples from treatment are still of borderline quality.

Figures 1a-b show the microflora of the high salt samples stored at 5°C and the low salt samples stored at 10°C, respectively, which had very different sensory properties (Table 1). The numbers of microorganisms ranged from 10^3 - 10^6 cfu/g salmon for TVC and TPC at the beginning of the storage period. After 12 days of storage, levels of 10^7 - 10^8 cfu/g salmon were reached for TVC, TPC and tentative lactic acid bacteria. Ultimately, bacterial numbers of 10^8 - 10^9 cfu/g salmon were obtained. The numbers of tentative *Enterobacteriaceae* were at least two log units lower than the TVC, TPC and tentative lactic acid bacteria counts throughout the storage period, and at the end of the period their numbers decreased to levels of $\leq 10^4$ cfu/g salmon.

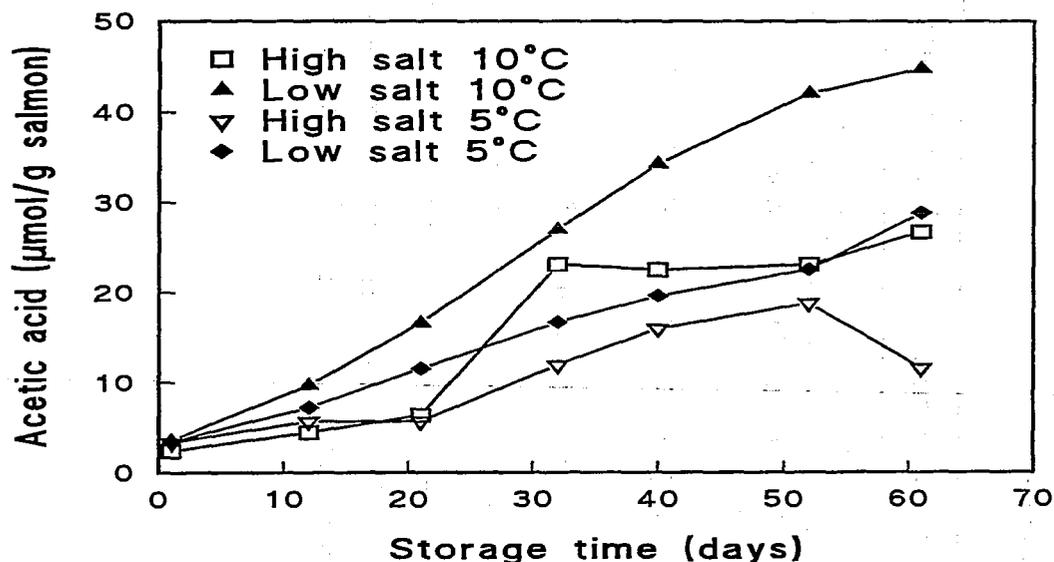


Fig. 2. Development of acetic acid during storage at 5 and 10°C of cold-smoked salmon with 2.2 and 4.6% SWP.

Figure 2 shows that the acetic acid concentration rose with storage time for all treatments, but at different rates. In most treatments the acetic acid concentrations were below 4 $\mu\text{mol/g}$ in fresh cold-smoked salmon, between 11.6-23 $\mu\text{mol/g}$ at rejection (Table 3) and rose to levels of up to 45 $\mu\text{mol/g}$ in completely spoiled salmon. The concentration of acetic acid was significantly related to the salt levels ($P < 0.001$), storage temperature ($P < 0.001$) and storage time (linear ($P < 0.001$) and cubic ($P < 0.05$)) (Table 2). However, there was a significant interaction between salt and time (linear ($P < 0.001$)).

Initial levels of lactic acid ranged from 55-105 $\mu\text{mol/g}$ and after 61 days of storage, values ranged from 81-118 $\mu\text{mol/g}$ (data not shown). The formic acid concentrations were below 1 $\mu\text{mol/g}$ salmon at the beginning of the study and reached levels of 4-11 $\mu\text{mol/g}$ salmon at day 61 (data not shown). Fumaric acid concentrations were in the range from 19-37 nmol/g salmon and 16-87 nmol/g salmon at the beginning and end of the storage period,

respectively (data not shown). None of the changes in these organic acids showed any systematic pattern related to the experimental parameters.

Initial pH values ranged from values of 5.9-6.1 to final values of 5.8-6.3, but no consistent trends with salt, storage temperatures, production batch or storage time were observed (data not shown).

At the beginning of the storage experiment, inosine monophosphate (IMP), inosine (Ino) and hypoxanthine (Hx) were found in all samples of cold-smoked salmon. Only in one treatment (H10), were traces of adenosine di- and monophosphate (ADP and AMP) found (data not shown). All IMP and Ino was converted into Hx during the storage period.

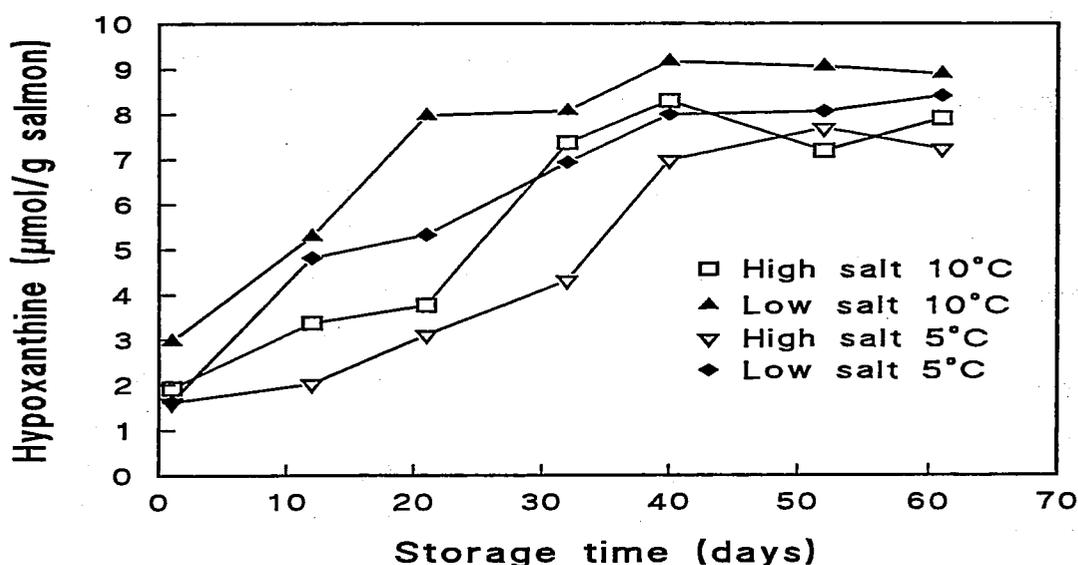


Fig. 3. Hypoxanthine content during storage at 5 and 10°C of cold-smoked salmon with 2.2 and 4.6% SWP.

Figure 3 shows the increase in Hx from initial levels of 1.6-3.0 µmol/g salmon to final levels of 8.0-9.2 µmol/g salmon, with levels of 5-7 µmol/g and 7-8 indicating borderline and rejection quality, respectively (Table 3). Trend comparison analysis of the Hx concentrations for the treatments revealed significant effects for the main factors: salt ($P < 0.001$), temperature ($P < 0.001$) and time (linear ($P < 0.001$), quadratic ($P < 0.001$) and cubic ($P < 0.05$)) (Table 2), thus decreasing salt levels and increasing storage temperature increased the rate of degradation with time in all treatments. The linear, quadratic and cubic components of the time effect and the related salt and time interaction seemed to be caused by the complete conversion of Ino to Hx. Therefore, by the end of the storage period, the rate of degradation is decreased by substrate limitations and not by salt level.

Concentrations of trimethylamine (TMA) ranged from 2-8 mg TMA-N/100 g in fresh

smoked salmon and 10-14 mg TMA-N/100 g in spoiled salmon (Table 3) and further to levels of 12-22 mg TMA-N mg/100 g in completely spoiled salmon (data not shown). The final concentration and the rate of development of TMA depended upon the salt level and storage temperature; decreasing salt levels and increasing storage temperature resulted in higher concentrations of TMA (Table 2). The interaction between salt and temperature (Table 2) meant that the low salt samples stored at 10°C had a higher TMA level than the low salt sample stored at 5°C during the storage experiment, whereas there was no significant difference ($P > 0.05$) for high salt samples.

Table 2. Trend comparison analyses of the development of TMA, ethanol, acetic acid, hypoxanthine as a function of salt concentration, storage time and storage temperature of smoked salmon (n=84).

Dependent variable	Independent variable	Coefficient	F-prob.
TMA $R^2 = 0.7588$ $SE^a) = 2.8148$ F-prob. = 0.0000	Salt	-2.8773	0.0000
	Temperature	1.0989	0.0006
	Time	0.0267	0.0000
	Salt x Temp.	-1.2889	0.0001
	Constant	10.8744	
Ethanol $R^2 = 0.7354$ $SE = 0.8917$ F-prob. = 0.0000	Salt	-0.7746	0.0000
	Time	0.0085	0.0000
	Time ² (Q)	-0.0011	0.0002
	Salt x Time	-0.0018	0.0079
	Constant	2.6821	
Hypoxanthine $R^2 = 0.8703$ $SE = 0.9290$ F-prob. = 0.0000	Salt	-0.7700	0.0000
	Temperature	0.5469	0.0000
	Time	0.0150	0.0000
	Time ² (Q)	-0.0018	0.0000
	Time ³ (C)	-0.0001	0.0430
	Salt x Time ² (Q)	0.0007	0.0208
	Salt x Time ³ (C)	-0.0001	0.0026
Acetic acid $R^2 = 0.7871$ $SE = 5.6311$ F-prob. = 0.0000	Salt	-3.7890	0.0000
	Temperature	3.6993	0.0000
	Time	0.0649	0.0000
	Time ³ (C)	-0.0003	0.0272
	Salt x Time	-0.0170	0.0002
	Constant	16.7280	

a) - Standard error of estimate

Initial ethanol concentrations in cold-smoked salmon samples were less than 1 $\mu\text{mol/g}$ salmon and rose to levels of 2.2-3.4 $\mu\text{mol/g}$ in spoiled salmon (Table 3). At final stages of storage, low salt samples reached concentrations of 4-6 $\mu\text{mol/g}$ salmon, whereas high salt samples only reached levels of 2.8-3.5 $\mu\text{mol/g}$ salmon (data not shown). Trend comparison analysis showed that the salt level and storage time (linear and quadratic) affected the concentration and development of ethanol (Table 2). However there was a significant interaction between salt and time (linear ($P < 0.05$)). The influence of storage temperature was not significant ($P > 0.05$). Samples with the high salt level developed less ethanol regardless of storage temperature.

Table 3. Changes in concentration of various chemical compounds during storage of cold-smoked salmon.

	Codes ¹⁾	H10	L10	H5	L5
	Quality ²⁾				
Hypoxanthine ($\mu\text{mol/g}$)	Fresh	1.9 ³⁾ (1) ⁴⁾	3.0 (1)	1.6 (1)	1.6 (1)
	Borderline	7.4 (32)	5.3 (12)	7.0 (40)	6.9 (32)
	Spoiled	7.2 (52)	8.0 (21)	7.2 (61) ⁵⁾	8.0 (40)
Acetic acid ($\mu\text{mol/g}$)	Fresh	2.4	3.6	3.4	3.6
	Borderline	23.1	9.7	15.9	16.7
	Spoiled	23.0	16.6	11.6 ⁵⁾	19.6
TMA (mg TMA-N/100g)	Fresh	3.6	8.9	1.9	2.0
	Borderline	7.8	16.6	12.8	11.9
	Spoiled	12.0	10.3	14.6 ⁵⁾	13.5
Ethanol ($\mu\text{mol/g}$)	Fresh	0.3	0.7	0.4	0.2
	Borderline	2.9	2.4	2.6	5.0
	Spoiled	2.2	2.5	2.8 ⁵⁾	3.4

1) Codes: See Table 1; 2) Quality as judge by sensory evaluation; 3) Values are mean of 3 vacuum packs; 4) Number in parenthesis refers to the sampling day, see Table 1); 5) Samples were not rejected at the last day (52) for sensory evaluation, values are from 61st day.

Discussion.

The shelf life of vacuum packed cold-smoked salmon varied according to salt concentration and storage temperature. The observed shelf lives were long compared to other reports in the literature, where shelf lives ranged from 21 to 27 days at 5°C and 4.3 % salt

in water phase (Cann *et al.*, 1984; Jakobsen *et al.*, 1988). However, absolute shelf lives are difficult to compare because of the lack of standardised criteria for determination and differences in production methods. The spoilage pattern and description of off-flavours were in agreement with previous descriptions in the literature by Hildebrandt & Erol (1988), Cann *et al.* (1984) and Jakobsen *et al.* (1988). This indicates the existence of a specific spoilage mechanism for the product.

The textural changes were evident before production of off-odours and off-flavours (Table 1) and may be caused by activity of muscle proteases. Herbert *et al.* (1971) found textural changes during storage of sterile blocks of cod indicating the importance of autolytic enzymes for textural changes. The tissue softening in the different treatments developed at different rates as observed by the sensory evaluation (Table 1). Part of the explanation for this may be, that increasing salt concentrations in the range of 0.5-5.0 % (w/w) decrease the activity of some proteolytic enzymes (cathepsins) as shown by Reddi *et al.* (1972).

The observed differences in shelf life were not reflected by any of the microbiological analyses (TVC, TPC, lactic acid bacteria or counts on Violet Red Bile Glucose agar). Thus, cold-smoked salmon with high numbers of microorganisms (10^8 cfu/g salmon) were not always spoiled (Fig. 1a & Table 1). Similar observations have been made in previous experiments with cold-smoked salmon (Jakobsen *et al.*, 1988) and cold-smoked herring (Magnússon & Traustadóttir, 1982). Thus microbiological counts of microorganisms not identified as specific spoilage organisms (or pathogens) are useless as quality indicators for this product.

The development of Hx, acetic acid and TMA was significantly related to salt level, storage temperature and storage time and in some cases, to interactions among parameters (Table 2). This qualified these compounds as potential quality indicators. Bitter off-flavours were characteristic for the spoiling fish (Table 1) and has previously been connected with hypoxanthine production (Fletcher *et al.*, 1990). The direct relationship to sensory properties enhances the suitability of Hx as a quality indicator for cold-smoked salmon. Cann *et al.* (1984) reported comparable levels of Hx, but because of variation in the experimental material they did not find the compound suitable as quality indicator in cold-smoked salmon nor in vacuum packed fresh salmonids. Others have found the nucleotide pattern to be appropriate for quality assessment of fresh salmonids (Blokhus, 1986; Lakshmann *et al.*, 1993). The acetic acid content in cold-smoked salmon was well explained by the trend comparison analysis (Table 2), but unlike Hx, no set concentrations could be related to the sensory evaluation (Table 3). The occurrence of sour off-flavours in aged samples (Table 1) can thus not be explained solely on the basis of the production of acetic acid. The presence

and development of acetic acid in cold-smoked salmon has not been reported previously, but development of total volatile acids with storage time in vacuum packed cold-smoked salmon has been reported by Declerck (1976). Production of ethanol did not reflect the observed sensory differences between cold-smoked salmon stored at 5 and 10°C (Table 2 and Table 3), which disqualified this compound as quality indicator. Rehbein (1993) found ethanol useful as indicator of temperature abuse (above 15°C) during storage or handling of cold-smoked salmon but not as an indicator of quality during storage at chill temperatures (5-8°C).

The TMA values obtained in this study were comparable to values earlier obtained in storage experiments with cold-smoked salmon, where practically all trimethylamine oxide (TMAO) was degraded to TMA during storage at 5°C for 35 days (Personal communication, V. F. Jeppesen, 1994). However the suitability of TMA as quality indicator is questionable because of the small and inconsequential differences between concentrations in borderline and spoiled samples (Table 3). This may be related to the fact that the TMAO content in *Salmo salar* is variable and relatively low with a reported range of 24 mg TMAO-N/100 g \pm 10 mg TMAO-N/100 g (Hebard *et al.*, 1982).

The chemical parameters including the organic acids (lactic, formic, fumaric and acetic acid), TMA and ethanol were all chosen as potential chemical indicators on the basis of their characteristics as end products of various microbiological metabolisms e.g. mixed acid fermentation (organic acids and ethanol) and anaerobic respiration (TMA) (Gottschalk, 1986). The breakdown of ATP is thought to be autolytic (Ehira & Uchiyama, 1986) apart from the degradation of Ino to Hx, which has been proven also to be related to growth of microorganisms (Ravn Jørgensen *et al.*, 1988; Surette *et al.*, 1988; Dalgaard *et al.*, 1993).

It has been tempting to assume that the tested potential quality indicators are of bacterial origin. However, the differences in sensory acceptability cannot be explained by the numbers of microorganisms. This could be an indication that the spoilage is due to autolytic changes or, more likely, that changes in the composition and metabolism of the microflora were not reflected by the microbiological methods used in this experiment. This is supported by studies in fresh fish showing that autolysis rarely causes true spoilage off-flavours and off-odours (Herbert *et al.*, 1971; Dalgaard *et al.*, 1993).

Lactic acid bacteria dominated the microflora throughout the storage period (Figs. 1a-b), and they appeared to be the only potential spoilage organisms on the product. Certain groups of lactic acid bacteria have been associated with spoilage of lightly preserved proteinaceous foods (von Holy *et al.*, 1991; Mäkelä *et al.*, 1992; Borch & Agerhem, 1992)

The lactic acid bacteria group is known to produce organic acids and ethanol as typical fermentation end products (Gottschalk, 1986). There are no reported observations in

the literature on the lactic acid bacteria's ability to produce hypoxanthine or TMA, but our unpublished preliminary model experiments have shown that lactic acid bacteria isolated from spoiled cold-smoked salmon are capable of producing Hx but not TMA in sterile fish extracts. A possible explanation for the similarity in development of lactic acid bacteria among all treatments, may be a shift in the composition of the lactic acid bacteria group during storage from harmless to spoiling species as has been reported by von Holy *et al.* (1991).

The low numbers of tentative *Enterobacteriaceae* appeared unrelated to treatments and quality of cold-smoked salmon, and typically decreased with storage time (Figs. 1a-b). The reason for this decrease in tentative *Enterobacteriaceae* is unknown, but may be caused by microbial interactions. Though this group of Gram negatives are known to produce TMA from TMAO, their numbers hardly explain the production (Strøm & Larsen, 1979, Dalgaard *et al.*, 1993). This suggests the occurrence of organisms not detected by the microbiological methods used in this experiment.

In conclusion, spoilage and shelf life of cold-smoked salmon were found to be related to salt levels and storage temperatures and this relationship was best described by the development of the chemical compounds: Hx, acetic acid and TMA. Hx was found to be superior as an indicator of quality, because a clear relation to the sensory evaluation could be established. The role of the observed microflora in spoilage of cold-smoked salmon was unclear, but apart from textural changes the off-flavours and off-odours were typical of microbiological activity. Future work will concentrate on the role of the microflora in spoilage of the product and identification of the specific spoilage organism(s).

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Importance of autolysis and microbiological activity on quality of cold-smoked salmon.

by

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Abstract.

The relative importance of autolysis and microbiological activity on spoilage of cold-smoked salmon and the origin of the chemical compounds hypoxanthine, acetic acid, trimethylamine and total volatile bases was studied in a storage experiment of dry salted and injection brined, vacuum packed salmon with normal and reduced loads of microorganisms.

Comparative studies of cold-smoked salmon with a reduced and normal load of microorganisms showed that microbiological activity caused production of the characteristic spoilage odours and flavours, while the autolytic enzymes from the fish tissue had major impact on the textural deterioration.

Total volatile bases and hypoxanthine were produced in significantly higher levels in salmon with a normal bacterial load. Sixty-eight % of the hypoxanthine found in stored samples originated from microbiological conversion of inosine to hypoxanthine. The concentration of acetic acid only increased in samples with a normal load. At the onset of spoilage the microflora in dry salted salmon was dominated by marine vibrio in contrast to the injection brined product where a mixture of *Enterobacteriaceae* and lactic acid bacteria were prevalent. The different ratios of hypoxanthine produced to trimethylamine also suggested that spoilage of salmon salted by the two methods was caused by different microorganisms

Keywords: Cold-smoked salmon, spoilage, microbiology, autolysis, sterile.

Introduction.

Storage of vacuum packed cold-smoked salmon at chill temperatures results in development of a large microflora often reaching levels of 10^7 - 10^8 cfu/g. This microbiological development does not necessarily coincide with onset of spoilage, and no relation has thus been found between the total number of microorganisms, sensory quality and shelf life (Gibson & Ogden, 1987; Hildebrandt & Erol, 1988; Jakobsen *et al.*, 1988; Truelstrup Hansen *et al.*, 1995a). Although the characteristic spoilage off-flavours and off-odours are typical of microbiological activity, the role of the microflora in spoilage of the product remains to be determined.

The importance of autolytic enzymes in quality changes of cold-smoked salmon is unknown. The temperature never exceeds 28°C during the cold-smoking process and there is therefore no heat-inactivation of native enzymes in the salmon tissue. Among several quality indicators tested including ethanol, trimethylamine, organic acids and adenosine nucleotides, hypoxanthine and to some extent, acetic acid have been found suitable for cold-smoked salmon (Truelstrup Hansen *et al.*, 1995a). Although the mode of quality deterioration is not well established, it is assumed to be microbiological.

The objectives of this work were to evaluate the relative importance of autolytic and microbiological processes during chill storage of vacuum packed cold-smoked salmon and to establish the origin of the following chemical quality indicators: hypoxanthine, acetic acid, trimethylamine and total volatile bases. In addition, two different methods of salt application were tested (dry salting and injection brining) for their effect on spoilage characteristics of the product.

Materials and methods.

Salmon.

Freshly slaughtered and gutted salmon (*Salmo salar*) were obtained from a salmon farm in Ålesund, Norway. The salmon were shipped (16 h) in Styrofoam shipping boxes packed with ice on a refrigerated truck (temperatures < 2°C) to the smokehouse in Skagen, Denmark. The fish arrived to the smokehouse 3 days after slaughter and were stored on ice at 0 to 2°C for 2 days before processing.

The salmon was randomly separated into two groups, and one group was filleted by machine according to smokehouse's standard procedure. The other group was filleted

manually with knives and cutting boards sanitised in ethanol (70% v/v) to decrease the initial microbial load on the fillets. One half of the salmon from the filleting procedures were dry salted by applying crystalline salt on the meat side of fillets. The salt was left on the fish for 3 h to equilibrate and then removed with tap water. The other half of the salmon fillets were salted by either automatic injection brining (23% salt w/v) for machine filleted salmon or by manual injection by applying heat-sterilised brine (23% w/v) with sterile syringes (manually filleted salmon). Salmon from both salting methods were left for 14-18 h at 2°C before being dried (2 h at 24°C, rh 50%) and cold-smoked (4 h, 18°C, rh 60%) according to the smokehouse's normal procedure. The smoked fillets were chilled immediately after smoking to 5°C. Salmon produced with a reduced load of microorganisms were aseptically cut into muscle blocks without skin of approximately 150 g each. Muscle blocks of the same specifications were also cut from salmon with a normal load of microorganisms but under normal good manufacturing practices. Each muscle block was divided into two halves aseptically and the pair was then vacuum packed separately in a high barrier material (Riloten/x 40/50 Conovac from Otto Nielsen Emballage A/S, Lyngby, Denmark. Permeabilities for O₂ - 1.0 cm³/m² and CO₂ - 4.0 cm³/m² in 24 h, 1 atm. at 23°C, 95% rh) and stored at -30°C for 3 days.

Storage and sampling.

At the beginning of the storage experiment the vacuum packs from the four treatments (see Table 1) were thawed and then stored at 5°C. Three paired vacuum packs from the treatments with normal loads of microorganisms and 5 pairs from treatments with reduced loads of microorganisms were sampled randomly every 7th day during the storage period and used for the chemical, microbiological and sensory analyses. One vacuum pack from each pair was used for microbiological analysis, while the other was used for chemical and sensory analysis. Salt and moisture determinations were performed on 9 packs and 15 packs from the treatment with normal and reduced loads of microorganisms, respectively.

Sensory evaluation.

Sensory evaluation was carried out with 5-6 semi-trained judges presented with a pooled sample from each code for the overall acceptability with regard to taste and texture and disregarding salty and smoky flavours. The judges were also presented with freshly thawed samples (from storage at -30°C) of injection brined and dry salted cold-smoked

salmon from the same production batch. These samples served as references. A hedonic smiley scale with 5 "faces" was used (modified after Fletcher *et al.*, 1990) and later translated into numerical scores 1-5, where 1 and 5 were considered lowest and highest, respectively; 3 was considered the borderline of acceptability. The judges were also asked to comment on odour, texture, appearance and taste. On each sampling day, the effects of judges and treatment were evaluated by analysis of variance (ANOVA). Sample treatments receiving scores > 3 by $\geq 50\%$ of the judges were considered acceptable, sample codes obtaining scores of 3 at the borderline for acceptability and scores < 3 unacceptable and rejected. The minimum shelf life for a treatment was defined as the last sampling day where the sample code received a score of 3 or above.

Microbiological analysis.

Salmon samples were homogenised in Peptone Saline (PS, 0.9% (w/v) NaCl and 0.1% (w/v) Bacto Peptone, Difco) for 30 s in a Stomacher 400 Lab Blender (A. J. Seward, Bury, St. Edmunds, U. K.). Total viable counts (TVC) were performed in pour plates with Tryptic Soy Agar (TSA, Merck, Darmstadt, Germany). Total psychrotrophic counts (TPC) were done using the spread plate method on TSA. *Enterobacteriaceae* were enumerated on pour plates prepared with Violet Red Bile Glucose agar (VRBG, Oxoid CM485, Unipath Ltd., Basingstoke, Hampshire, England) and bottom layer of TSA. Lactic acid bacteria were counted on pour plates in Nitrite Actidion Polymyxin agar (NAP) modified after the original recipe by Davidson & Cronin (1973) with pH 6.7 instead of 5.5 (Truelstrup Hansen *et al.*, 1995a). For specific counts of *Brochothrix thermosphacta*, spread plates were prepared with Streptomycin Thallous Acetate agar (STAA, Oxoid CM881 & SR151) as described by Gardner (1966). Pour plates in Oxytetracycline Gentamycine Yeast Extract agar (OGYE, Oxoid CM545 & SR121) were performed for counts of yeasts and moulds. All agar plates were incubated aerobically at 21°C except for TPC, which was incubated at 10°C. VRBG plates were incubated for 2 days. TVC, NAP & STAA plates were incubated for 3 days. TPC & OGYE plates were incubated for days, after which either all colonies (TVC & TPC) or typical colonies (VRBG, NAP, STAA & OGYE) were counted. Tentative identity of colonies was confirmed by phase contrast microscopy and colonies from STAA and NAP were also checked for their catalase reaction.

Tentative identity of isolated microorganisms.

During storage, representative colonies from all media were selected and grown in Brain Heart Infusion broth (BHI) at 25°C, streaked out on TSA and identified tentatively using the following criteria: Gram-reaction by the KOH method (Gregersen, 1978), cytochrome oxidase by Bactident® Oxidase strips (Merck) and catalase by the 3% H₂O₂ method. Shape, size and motility of strains grown in Brain Heart Infusion broth (BHI) for 24 h at 25°C or in BHI for 24 h at 15°C (large cells) were examined by phase contrast microscopy. Glucose metabolism was tested using the Oxidative/Fermentative test (O/F-test) of Hugh & Leifson (1953) incubated at 25°C. Large (3-5 µm) cells often appearing as pairs of oval to kidney shaped cells, were tested in O/F media containing 3.5% NaCl incubated at 15°C, as they would not grow in the standard O/F media containing 0.5% NaCl. Eighteen of these large cells isolated from throughout the storage period were further tested for their sensitivity to Vibriostaticum (150 µg O/129 discs, Rosco A/S, Taastrup, Denmark) on TSA and their ability to bioluminesce (Bauman & Bauman, 1984; Dalgaard, 1995). Tentative identification of strains was based on their phenotypical characteristics (Dainty *et al.*, 1979; Lee *et al.*, 1979; Dalgaard, 1995).

Chemical analysis.

Nucleotides.

Perchloric acid (PCA) extracts of salmon were prepared as described by Truelstrup Hansen *et al.* (1995a) and the neutralised extract was stored at -30°C until the analysis was performed. The HPLC method of Murray *et al.* (1985) was used to analyze the nucleotide catabolites using external standards from Sigma (St. Louis MO, USA) to quantify and identify unknowns.

Total volatile bases (TVN), trimethylamine (TMA) and trimethylamine oxide (TMAO).

Conway and Byrne's (1933) microdiffusion method was performed on PCA extracts neutralised just before analysis.

Organic acids and glucose.

Organic acids and glucose were analyzed by HPLC on a BioRad HPX-87H Organic Acid column (300 x 7.8mm) (BioRad, Hercules, CA, USA). All analyses were performed on neutralised, filtered PCA extracts. Experimental conditions were: flow rate, 0.6 ml/min; eluent, 0.008 M H₂SO₄; run time, 30 min; injection volume, 40 µl; column temperature, 50°C; UV detection at 210 nm. Identification and external quantification was done on the basis of relative retention times of authentic standards. For lactate and acetate, the results from the HPLC method were verified by analysis of selected samples using the respective enzyme kits from Boehringer Mannheim (Germany).

Salt and moisture content.

Salt content was analyzed in homogenates using the modified method of Schroeder-Hellich (Nordlab, 1994). The method is based on a AgNO₃ titration of Cl⁻ and results are expressed as % NaCl in water phase of salmon. Moisture content was determined by oven drying 2 g salmon at 105°C for 20-24 h (until constant weight was obtained).

Statistical analyses.

The effects of the treatments and storage time on quality of cold-smoked salmon as measured by the chemical quality indices were analyzed by a trend comparison procedure based on orthogonal polynomial contrasts with equal intervals and missing observations (Gomez & Gomez, 1984). The linear contrast for each treatment and for the effect of storage time also the quadratic and cubic contrasts and their interactions were analyzed for significance by forward stepwise multiple regression using STATGRAPHICS ver. 4.0 (1989). Linear regression equations were analyzed by testing for the homogeneity of the slopes (Gomez & Gomez, 1984).

Results.

Salt levels obtained in the salmon from the four treatments ranged from 3.6 to 4.4% in the water phase (Table 1). The standard deviations were larger in the dry salted samples. The dry matter content ranged between 34.8 and 40.3% and injection brined salmon with a normal load of microorganisms had the lowest level of dry matter (Table 1).

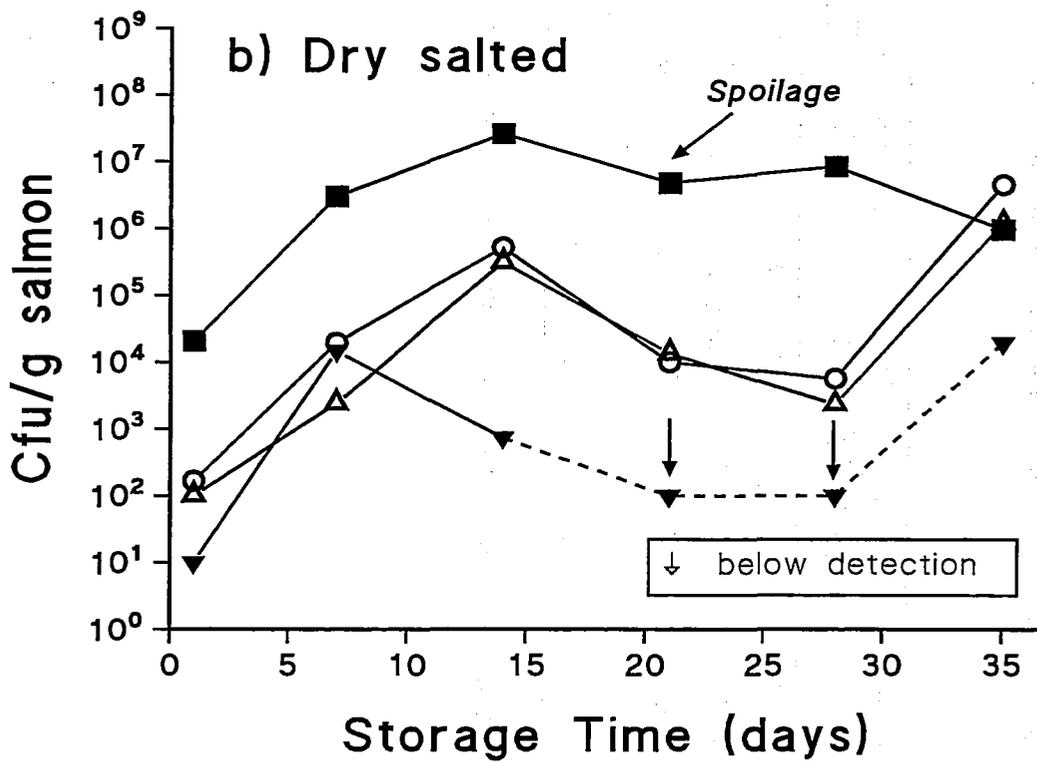
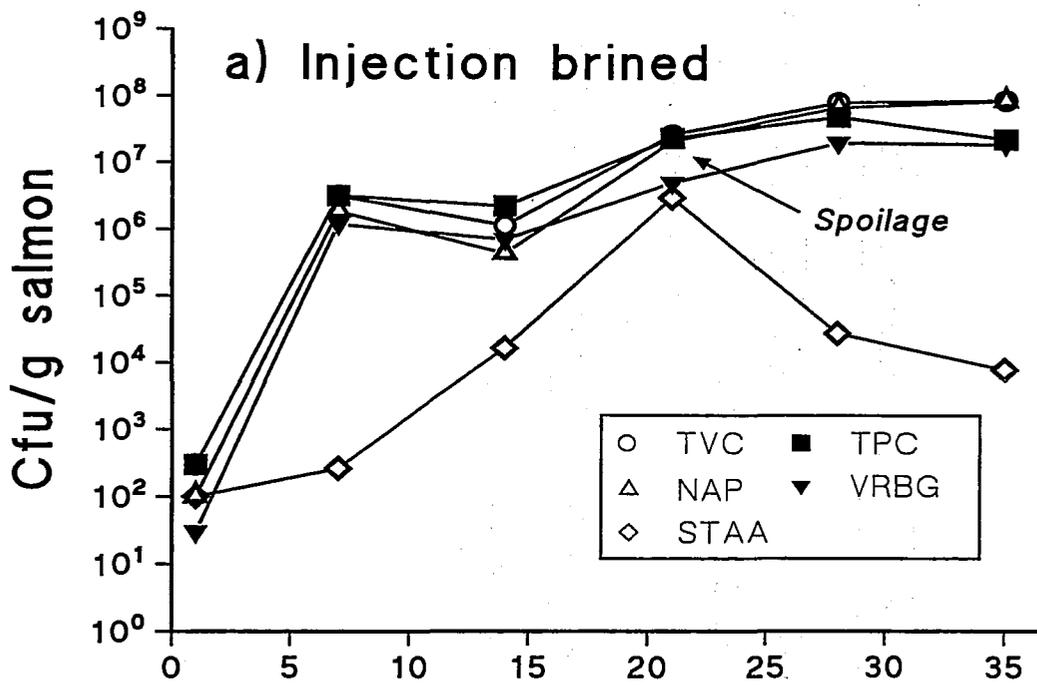


Table 1. Sample treatment and sensory evaluation for cold-smoked salmon.

Treatments (salting method & load of microorganisms)	Salt level (% SWP) ¹⁾	Dry matter (%)	Day of rejection	Comparison of sensory characteristics at day 28	
				Texture	Flavour & Odour
Dry salted & normal load (DN)	3.9 ± 0.9	39.4 ± 2.9	21	Soft, sticky	Putrid, astringent, sour, faecal
Dry salted & reduced load (DR)	4.0 ± 0.9	40.3 ± 2.4	>28 ²⁾	Soft, mushy, oily	Slightly bitter, neutral, bland
Injection brined & normal load (IN)	4.4 ± 0.4	34.8 ± 2.6	21	Soft, mushy, creamy	Bitter, sour, ammonia fruity, astringent, sharp
Injection brined & reduced load (IR)	3.6 ± 0.5	38.7 ± 3.6	>28 ²⁾	Soft, mushy, crumbling	Slightly sour, bitter, neutral

1) % SWP: Salt content in water phase ± Standard deviations (n-1); 2) Sample day is still of borderline quality at the last day (28) for sensory evaluation.

The efforts to produce cold-smoked salmon with a reduced load of microorganisms using both salting methods gave at least 2 out of 5 vacuum packs at each sampling day throughout the experiment with less than 10^3 cfu/g in all microbiological analyses (data not shown). Only these vacuum packs were used to analyze the effect of the reduced load treatments.

Shelf lives of both dry salted and injection brined cold-smoked salmon with normal loads were judged to be approximately 2.5 weeks by the sensory panel (Table 1). Analysis of variance showed that the judges agreed on their evaluations. Initial sensory properties were characteristic of the fresh product. The texture was, however, firmer in the dry salted products, which may be related to the higher dry matter content. At rejection, the salmon from both treatments had a soft and mushy texture and bitter, sour (astringent) and putrid off-flavours. Fruity and ammonia-like off-odours were also observed in injection brined salmon.

Not all vacuum packs from the reduced load treatments contained less than 10^3 cfu/g. Therefore reduced load samples were pre-screened for the presence of microorganisms by direct microscopy of a 10^{-1} dilution and only used for the last taste panel session after 28 days of storage if they appeared to contain low levels of microorganisms. This modified sensory evaluation scheme showed that salmon containing reduced loads were not spoiled but of borderline quality, whereas the normal samples were completely spoiled (Table 1). The judges characterised samples with reduced loads as having soft and mushy texture and slightly bitter and sour off-flavours (Table 1).

The microflora in dry salted and injection brined salmon with normal loads developed very differently as shown in Figures 1a-b. The total number of microorganisms in the dry salted product reached final levels of 10^7 cfu/g, whereas counts on the injection brined samples reached final levels of 10^8 cfu/g. TVC, TPC and NAP (lactic acid bacteria) rose to levels of 5×10^7 cfu/g on injection brined salmon at time of spoilage, while VRBG (*Enterobacteriaceae*) counts were slightly lower (Figure 1a). Examination of colonies from agar plates revealed that the microflora consisted mainly of a mixture of Gram-negatives (predominantly *Enterobacteriaceae*) and lactic acid bacteria throughout the storage, with fewer marine vibrio. TPC counts on dry salted salmon were 2-3 log(cfu/g) higher than all other counts until day 28 (Figure 1b). Colonies isolated from these TPC agar plates were tentatively identified as marine vibrio or *Photobacterium* spp., whereas lactic acid bacteria and *Enterobacteriaceae* predominated on the last sampling day (35). The Gram-negative marine vibrio or *Photobacterium* spp. could in general be described as large (3-5 μ m) coccobacilli with a negative or very slow oxidase reaction. Strains matching this description

were not isolated from pour plates (TVC), which indicated that these large coccibacilli were heat labile. Also the strains from TPC were only motile in BHI incubated at 15°C and would only ferment glucose in H&L medium with 3.5% (w/v) NaCl. Eighteen of these isolates were tested and found to be sensitive to Vibriostaticum (O/129) and 4 strains amongst these showed bioluminescence. All strains were tentatively identified as marine vibrio, possibly *Photobacterium phosphoreum*. P. Dalgaard has later confirmed the identification of 5 of these strains (both bioluminous and non-bioluminous) as *Photobacterium phosphoreum* (Personal communication, 1994). *Brochothrix thermosphacta* were found in levels 1-4 log(cfu/g) lower than the other microbiological counts in injection brined salmon but not in the dry salted salmon. Yeasts and moulds were not isolated from any of the treatments. *B. thermosphacta*, yeasts and moulds were not considered to be of any importance for spoilage in this study.

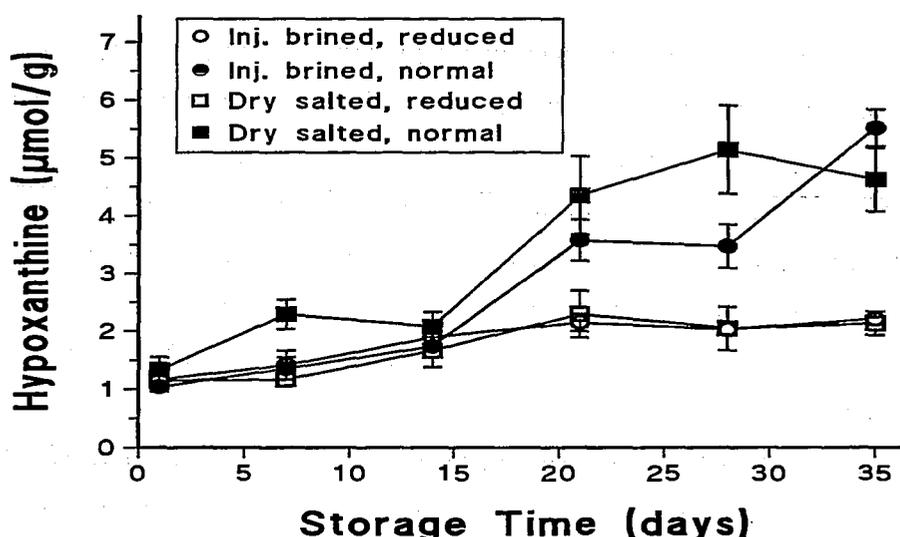


Figure 2. Development of hypoxanthine in injection brined and dry salted cold-smoked salmon with reduced or normal loads of microorganisms during storage at 5°C. Error bars are standard deviations (n-1).

The amount of hypoxanthine (Hx) increased with storage time in samples with normal loads from initial levels of 1-1.4 µmol/g to 4.5 µmol/g (Figure 2). In samples with reduced loads, the Hx content also increased with time but at a slower rate with initial values ranging between 1.0-1.3 µmol/g and final values of 2.2 µmol/g (Figure 2). Trend comparison analysis of the Hx production revealed significant effects of the main factors: reduced/normal loads, salting method and storage time (linear) and some two-factor interactions (Table 2). Production of Hx was significantly slower in samples with reduced loads and in normal samples production

Table 2. Trend comparison analysis of the development of hypoxanthine, acetic acid, TVB and TMA related to microbiological load, salting method and storage time (n = 72).

Dependent variable	Independent variable	Coefficient	F-probability
Hypoxanthine $R^2 = 0.776$ $SE^1) = 0.596$ F-prob. = 0.0000	Reduced/Normal	0.557	0.0000
	Salt	0.188	0.0093
	Time(Linear)	0.237	0.0000
	Reduced/Normal x Salt	0.222	0.0024
	Reduced/Normal x Time(Linear)	0.127	0.0000
Acetic acid $R^2 = 0.909$ $SE = 1.912$ F-prob. = 0.0000	Constant	2.333	
	Reduced/Normal	3.195	0.0000
	Salt	1.146	0.0000
	Time(Linear)	1.037	0.0000
	Time(Quadratic)	0.274	0.0000
	Reduced/Normal x Time(Linear)	0.911	0.0000
	Reduced/Normal x Time(Quadratic)	0.248	0.0000
	Salt x Time(Quadratic)	-0.244	0.0000
TVB $R^2 = 0.814$ $SE = 4.448$ F-prob. = 0.0000	Constant	6.073	
	Reduced/Normal	4.252	0.0000
	Salt	1.254	0.0198
	Time(Linear)	1.933	0.0000
	Time(Cubic)	0.218	0.0263
	Reduced/Normal x Salt	2.108	0.0002
	Reduced/Normal x Time(Linear)	0.936	0.0000
	Salt x Time(Linear)	0.750	0.0000
	Salt x Time(Quadratic)	-0.332	0.0210
	Constant	24.763	

1) Standard error of estimate.

of Hx started to increase earlier in the dry salted salmon, but the interactions precluded any further interpretation (Table 2).

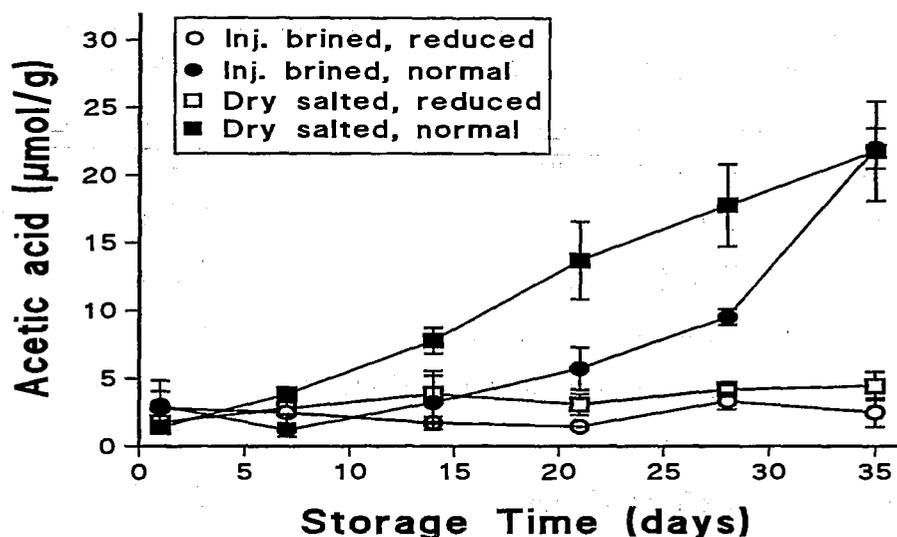


Figure 3. Acetic acid content in injection brined and dry salted cold-smoked salmon with a reduced or normal load of microorganisms during storage at 5°C. Error bars are standard deviations (n-1).

Acetic acid was only produced in normal samples, where concentrations increased from initial levels of 2-3 $\mu\text{mol/g}$ to final levels of 23 $\mu\text{mol/g}$ after 35 days' of storage (Figure 3). In samples with reduced load, no significant increases were found (Figure 3). Trend comparison analysis supported the fact that acetic acid levels started to increase first in dry salted salmon, and the relationship between acetic acid and storage time was quadratic (Figure 3 & Table 2) for injection brined samples with normal bacterial loads. However, after 35 days the levels of acetic acid were the same for both salting methods.

None of the other organic acids (lactic, formic or propionic) were found to increase systematically with storage time (data not shown).

Initial levels of trimethylamine-oxide nitrogen 15-25 mg TMAO-N/100 g were completely converted into trimethylamine (TMA) by the end of the storage period in normal samples, whereas there were no increases in the TMA levels in samples with reduced loads (data not shown). The total volatile bases (TVB) increased from initial values of 9 mg TVB-N/100 g to final values of 50 mg TVB-N/100 g in normal dry salted salmon, whereas in normal injection brined TVB values increased from 18 mg TVB-N/100 g to 40 mg TVB-N/100 g (Figure 4). The TVB values in samples with reduced loads from both salt treatments only reached final levels of 25 mg TVB-N/100 g (Figure 4). Trend comparison analysis showed that 81% of the variation in TVB content could be explained by the experimental factors: microbiological load, salting method, time (linear and cubic) and some

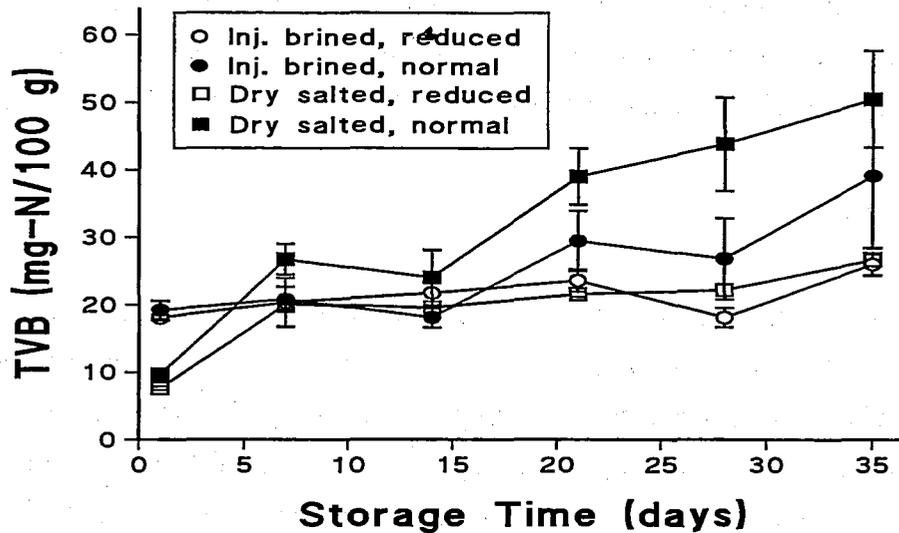


Figure 4. Development of total volatile bases (TVB) during storage at 5°C in cold-smoked salmon with normal and reduced loads of microorganisms. Error bars are standard deviations (n-1).

two factor interactions (Table 2). Only 70 % of the variation in the TMA content was explained by the main experimental factors (data not shown).

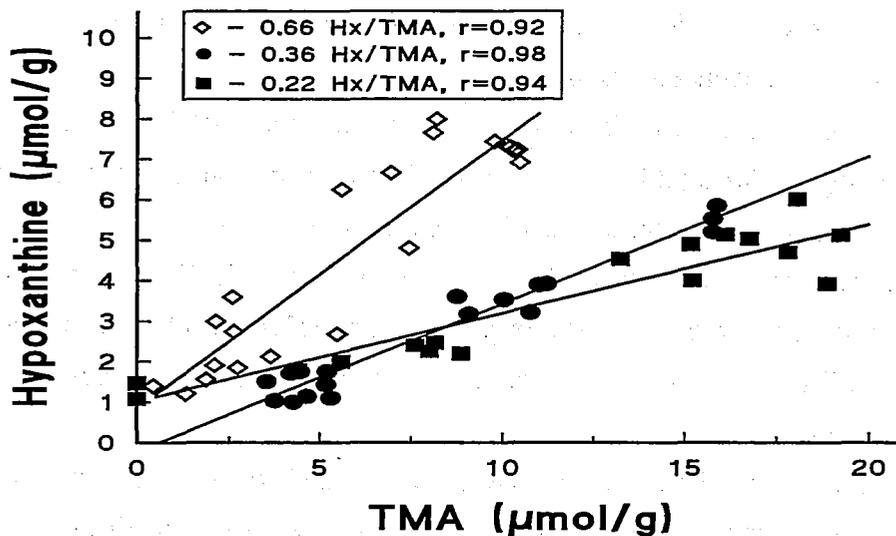


Figure 5. Relation between hypoxanthine and trimethylamine in cold-smoked salmon. \diamond , Brined and sliced (4.6% NaCl); \bullet , Dry salted (3.9% NaCl); \blacksquare , Injection brined (4.4% NaCl); r, Correlation coefficient.

Production of Hx was correlated with the production of TMA in normal samples ($r > 0.9$). However, the ratio of Hx/TMA varied from 0.36 $\mu\text{mol Hx/TMA}$ for dry salted to 0.22 $\mu\text{mol Hx/TMA}$ for injection brined (Figure 5). In a previous study a ratio of 0.66 $\mu\text{mol Hx/TMA}$ was obtained in brined and sliced cold-smoked salmon with 4.6% salt stored at 5°C

(Truelstrup Hansen *et al.*, 1995a, Fig. 5). The significantly ($P < 0.001$) different chemical patterns also suggested presence of different microflora on the products, and in fact, this difference may explain the long shelf-life of 7-8 weeks found in the latter study.

Discussion.

Salmon with normal bacterial loads developed offensive off-flavours and odours, which limited the shelf life to approximately 2.5 weeks (Table 1). In contrast, removal or reduction of the microflora resulted in only neutral to slightly sour and bitter flavours being produced after 28 days' storage, suggesting that production of the characteristic spoilage off-odours and off-flavours observed in cold-smoked salmon (Cann *et al.*, 1984; Hildebrandt & Erol, 1988; Jakobsen *et al.*, 1988; Truelstrup Hansen *et al.*, 1995a) were caused by microbiological activity. Absence of off-odours were also observed during prolonged storage (up to 24 weeks) of sterile muscle blocks of cod under aerobic and anaerobic conditions at 0-2°C (Herbert *et al.*, 1971; Dalgaard *et al.*, 1993). On the other hand Fletcher and Statham (1988) found the spoilage characteristics to be identical regardless of the microbiological load during storage of yellow-eyed mullet, when they compared sterile and normal fish. The relative importance of microbiological and autolytic activities on development of spoilage must therefore be dependent upon the fish species.

In contrast, samples with reduced and normal bacterial loads were judged to soften to the same degree during 28 days of storage (Table 1), indicating that textural changes were mainly due to autolysis. Herbert *et al.* (1971) also suggested that textural deterioration of sterile cod was due to autolysis. Muscle tissue softening in fish has been connected with activity of different cathepsins and though increasing salt concentrations reduce the catheptic activity (Reddi *et al.*, 1972), they are still active at salt levels relevant to smoked salmon (Makinodan *et al.*, 1991).

Marine vibrio dominated at the onset of spoilage (day 21) in normal dry salted salmon, while injection brined salmon was found to support growth of a mixture of lactic acid bacteria and *Enterobacteriaceae*. Marine vibrio including *Photobacterium phosphoreum* have been found on vacuum packed and lightly salted matjes herring by van Spreekens (1987), and *Photobacterium phosphoreum* has been held responsible for sour, TMA and ammonia-like spoilage odours in fresh cod packed in vacuum and modified atmospheres (Dalgaard *et al.*, 1993; Dalgaard, 1995). It is likely that these potential spoilage organisms have been overlooked in the past due to their heat labile nature and lack of specific detection methods. Injection brined salmon tasted and smelled different than the dry salted product,

suggesting a different bacterial flora causing spoilage. Previous studies of vacuum packed, sliced cold-smoked salmon suggested the importance of lactic acid bacteria in spoilage (Schneider & Hildebrandt, 1984; Jakobsen *et al.*, 1988; Truelstrup Hansen *et al.*, 1995a). The present study shows not only the importance of *Enterobacteriaceae* and marine vibrio but also the variability of the spoilage pattern as being dependant on the production method. Recent work in our laboratory (unpublished work) shows that there is considerable variation in the cold-smoked salmon microflora among different smokehouses and production batches from the same smokehouses.

Levels of Hx, acetic acid and TVB increased in normal samples between 14 days and 21 days of storage and this increase coincided with rejection by the sensory panel and thereby suggested a possible connection to the growth of spoilage organisms (Figures 2, 3 & 4). After storage for 35 days, the molar ratio of Hx found in normal and reduced load samples were about 2.3:1, which suggested that about 68% of Hx found in this product originated from microbiological activity. Fletcher & Statham (1988) and Surette *et al.* (1988) observed a delayed production of Hx in chill stored sterile fish samples and concluded microorganisms to be responsible for part of the Hx production. Several bacteria including *Shewanella putrefaciens*, *Proteus vulgaris* and other *Enterobacteriaceae*, *Photobacterium phosphoreum* and some lactic acid bacteria have been found to convert inosine into Hx (Gram, 1989; Surette *et al.*, 1990; own unpublished work) and this property seems to be widespread amongst bacteria found on fish products.

Acetic acid was only produced in normal samples (Figure 3) and autolytic enzymes therefore produced no acetic acid. Acetic acid is a common metabolite for a number of bacteria, such as lactic acid bacteria (Axelsson, 1993), *Enterobacteriaceae* (Gottschalk, 1986) and *Photobacterium phosphoreum* (Baumann & Baumann, 1984). The slight increase in TVB in samples with reduced loads may origin from autolytic deamination of free amino acids produced by proteolysis and degradation of nucleotides, while the larger increase in TVB in normal samples was most likely caused by a combination of microbiological and autolytic deamination of amino acids and the complete reduction of TMAO to TMA.

By using the same reasoning as Dalgaard *et al.* (1993), it could be shown how spoilage of normal salmon from the two salting methods must have been caused by different organisms, as the ratios of Hx produced to TMA were significantly different (Figure 5). Also, the ratios of Hx and TMA obtained in this study were significantly lower than the ratio found for sliced cold-smoked salmon in a previous experiment. Lactic acid bacteria seemed to dominate the microflora completely in the latter experiment (Truelstrup Hansen *et al.*, 1995) but preliminary experiments in our laboratory have shown that lactics produce Hx but

not TMA. The variability found in the microflora as affected by the production method, initial microflora and processing environment, makes it difficult to use set levels of chemical compounds like Hx, acetic acid and TVB as reliable indicators of quality, because different organisms produce such indicators at different rates.

In conclusion, spoilage off-flavours and off-odours characteristic for cold-smoked salmon was found only in samples with high bacterial loads indicating the relative importance of spoilage organisms in product shelf-life. The development of Hx, acetic acid, TMA and TVB was also related to growth and activity of microorganisms. However, activity of autolytic enzymes were found to have major impact on the textural quality of cold-smoked salmon during the early stages of deterioration but did not produce the characteristic spoilage off-odours and off-flavours. Autolytic degradation of the muscle tissue can therefore ultimately limit the shelf life and may lead to the early downgrading of product quality when the numbers of spoilage organisms are relatively low. Future work will concentrate on characterization of the microflora on spoiled cold-smoked salmon and the influence of the production environment.

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Characteristics of the microflora isolated from spoiled cold-smoked salmon.

by

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Abstract.

The microflora on spoiled, sliced, vacuum packed, cold-smoked salmon from 3 smokehouses were quantified and characterised in two independent experiments. Tentative identification of the isolated bacteria indicated large variations in the microflora both within (i. e. among vacuum packs from the same batch) and among the smokehouses. Lactic acid bacteria dominated the microflora, which reached 10^7 cfu/g. In one experiment high numbers of *Enterobacteriaceae* were also present. The numbers of microorganisms were not related to quality, though spoilage characteristics were typical for microbiological spoilage. Amongst the lactic acid bacteria, *Lactobacillus curvatus* (ca. 55%) was the most common species in both experiments with *Lactobacillus sake/bavaricus*, *Lactobacillus plantarum*, *Carnobacterium* spp. and *Leuconostoc* spp. present in smaller numbers. Tentatively identified species among the *Enterobacteriaceae* were *Serratia liquefaciens*, *Enterobacter agglomerans* and *Hafnia alvei*. The microflora on cold-smoked salmon seemed to be related to the source of contamination i.e. the smokehouse or the raw material.

Keywords: Cold-smoked salmon, microbiology, lactic acid bacteria, *Enterobacteriaceae*.

Introduction.

Cold-smoked salmon is a lightly preserved fish product, which undergoes a mild salt cure followed by cold-smoking at temperatures below 28°C. In Denmark, Germany and other European countries the vast majority of this product is sold as a sliced, vacuum packed, ready-to-eat product to be stored at temperatures between 3-8°C. Freshly packed smoked salmon is not sterile and will ultimately spoil due to microbiological activity when stored at chill temperatures (Truelstrup Hansen *et al.*, 1995b). Basic knowledge about the product's microflora can help cold-smoked salmon producers in their optimisation of daily production routines and in relation to demands from customers and public authorities.

A number of different bacteria including lactics, *Brochothrix thermosphacta*, *Enterobacteriaceae* (Cann *et al.*, 1984; Schneider & Hildebrandt, 1984; Truelstrup Hansen *et al.*, 1995a & 1995b) and marine vibrios including *Photobacterium phosphoreum* (Truelstrup Hansen *et al.*, 1995b) and occasionally yeast have been found to develop during storage. Lactic acid bacteria appear to be the predominant group of microorganisms, but their role on the quality deterioration of cold-smoked salmon is unknown. Also, the microflora on cold-smoked salmon have been shown to vary among different smokehouses (Truelstrup Hansen *et al.*, 1995d). Variability in the composition of the microflora at time of spoilage makes it difficult to identify which organisms are responsible for spoilage.

The purpose of the present study was to characterise and tentatively identify the microflora on spoiled cold-smoked salmon from 3 different smokehouses using a combination of several agar substrates, and biochemical tests on the subsequently isolated microorganisms. Two independent experiments were performed and the microorganisms were separated into subgroups for the purpose of analyzing the variations in the microflora within and among the smokehouses. In the second experiment the subgrouping was based on numerical taxonomical methods.

Materials and methods.

Two sets of storage experiments were conducted, where the microflora on spoiled cold-smoked salmon (*Salmo salar*) was quantified and microorganisms isolated. In the first experiment cold-smoked salmon were stored until spoiled and then analyzed. In the second experiment, the microflora were continuously monitored throughout storage and spoilage

1) Screening of microorganisms on spoiled cold-smoked salmon.

Salmon and storage.

Cold-smoked salmon from plant No. 1 was produced from fresh Norwegian farmed salmon, never frozen and stored at 5°C immediately after slicing and vacuum packing in a barrier material (Walkiter x 60E: PET 12/PVDC/PE 60, permeability for O₂ 8 cm³/m² x 24 h and for CO₂ 40 cm³/m² x 24 h at 23°C, rh 50%, Walki-Pak, Finland). Sliced, vacuum packed salmon produced from Norwegian farmed salmon by plant No. 2 was frozen immediately after production and stored at -28°C for 4 to 5 months prior to thawing and chill storage, a practice commonly used in the industry. The fish were packed in a barrier material (S Vac PETX/PE 15/70, permeability for O₂ 10 cm³/m² x 24 h and for CO₂ 30 cm³/m² x 24 h at 23°C, rh 60%, Scandiflex AB, Landskrona, Sweden). Cold-smoked salmon from plants No. 1 and No. 2 were stored for 38 days at 5°C until spoilage off-odours were obvious. Two vacuum packs (200 g each) from each plant were sampled and analyzed microbiologically and chemically (salt and dry matter). The two vacuum packs from plant No. 1 represented the same production batch, where those from plant No. 2 represented two different production batches produced within a month of one-another using the same quality raw material, recipe and equipment.

Microbiological analysis.

Microbiological analyses were performed by mixing 10 g salmon sampled from all parts of vacuum packs to obtain a representative sample with 90 ml Peptone Saline (PS, 0.1% (w/v) Bacto Peptone, Difco, 0.9% NaCl (w/v)) for 30 s in a Stomacher 400 Lab Blender (A. J. Seward, Bury, St. Edmunds, UK). Appropriate dilutions were plated in pour plates with Tryptic Soy agar (TSA, Merck, Darmstadt, Germany) for total viable counts (TVC), Nitrite Actidion Polymyxin agar (NAP) for lactic acid bacteria (Davidson & Cronin, 1973, pH 6.7 see Truelstrup Hansen *et al.*, 1995a) and in Violet Red Bile Glucose agar (VRBG, Oxoid CM485, Unipath Ltd., Basingstoke, Hampshire, England) with bottom layer of TSA for *Enterobacteriaceae*. For specific counts of *B. thermosphacta*, spread plates with Streptomycin Thallium Actidion agar (STAA, Oxoid CM881 & SR151) (Gardner, 1966) were performed. Pour plates in Oxytetracycline Gentamycin Yeast Extract agar (OGYE, Oxoid CM545 & SR121) were prepared for enumeration of yeast and mould. All agar plates were incubated aerobically at 25°C for 3 days except VRBG (2 days) and OGYE (5 days), after

which either all colonies (TSA) or typical colonies only (NAP, VRBG, STAA & OGYE) were counted. Dilutions of samples (10^{-1}) and colonies on OGYE were also examined by phase contrast microscopy.

Isolation of microorganisms.

Based on colony morphology and occurrence, representative colonies were randomly selected from the highest countable dilutions (10-300 colonies) from all substrates. Approximately 20 colonies from TSA and NAP and 10 from STAA, VRBG and OGYE (or as many as possible) were isolated from each vacuum pack. Lactic acid bacteria and *B. thermosphacta* were grown in APT broth (Difco) and on APT agar aerobically at 25°C. Isolates from VRBG and OGYE were grown in Brain Heart Infusion broth (BHI, Difco) and TSA under the same conditions. When necessary strains would be stored at -80°C in the freezing media of Gibson & Khoury (1986).

Characterisation and tentative identification.

Gram-negative and -positive isolates were initially characterised according to the schemes of Dainty *et al.* (1979). Lactic acid bacteria (LAB) were further divided into groups based on criteria recommended by From (1991). Methods used for the basic criteria were: Gram reaction - 5% KOH-method (Gregersen, 1978), catalase - 3% H_2O_2 , cytochrome oxidase - dry slides (Difco), form and motility - phase contrast microscopy of broth cultures (24 h, 25°C), glucose metabolism - Hugh & Leifson's medium (1953), trimethylamine-oxide (TMAO) reduction - media by Gram *et al.* (1987), H_2S -production - stab inoculation in iron agar (Gram *et al.*, 1987). *Enterobacteriaceae* were further characterised using the API 20E system (BioMérieux SA, MEDA, Glostrup, Denmark), respectively. Selected LAB were further characterised using the following biochemical tests: gas production from glucose (all LAB isolates) in modified MRS broth with Durham tubes (Schillinger & Lücke, 1987a), NH_3 production from arginine in modified MRS broth with 0.05% (w/v) glucose (Schillinger & Lücke, 1987a), growth on acetate agar (pH 5.6, Leisner *et al.*, 1994), reduction of 0.01% (w/v) tetrazolium and 0.05% (w/v) potassium tellurite (Wilkinson & Jones, 1977) and production of acid from the following sugars and alcohol: cellubiose, galactose, lactose, maltose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sucrose, trehalose and xylose, using the method described by Jeppesen & Huss (1993). All tests were incubated aerobically at 25°C and repeated on 2 strains. Selected strains among all LAB

groups (21 strains), *B. thermosphacta* (2), *Enterobacteriaceae* (4) and yeast (2) were inoculated in salmon juice with 3 % salt and screened for production of off-odours after anaerobic growth at 15°C for 7 days. The salmon juice was prepared as described by Dalgaard (1995). One trained judge scored presence or absence of off-odours in comparison with sterile salmon juice incubated under the same conditions. The judge subsequently described the off-odours.

Chemical analysis.

Salt analyses were performed using the AOAC (1975) method on salmon homogenates. Salt content was expressed as % NaCl in water phase. Dry matter content were determined by drying 2 g salmon (± 0.0001 g) at 105°C for 20-24 h (until constant weight was obtained).

2) Development and characteristics of microflora during storage of cold-smoked salmon.

Salmon.

Sliced, vacuum packed cold-smoked salmon were obtained in portions of 200 g from 3 smokehouses in Denmark and transported to the laboratory. The details of production and storage can briefly be described as follows:

a) Plant No. 1. Fresh, farmed salmon (3-4 kg) from Norway was filleted, salt cured by a combination of injection brining and dry salting and cold-smoked for 5 h at 18-24°C. The salmon was sliced at -1.5°C and vacuum packed in a barrier material (Walkiter x 60E: PET 12/PVDC/PE 60, permeability for O₂ 8 cm³/m² x 24 h and for CO₂ 40 cm³/m² x 24 h at 23°C, rh 50% from Walki-Pak, Finland). After production, the fish was stored at -2 to 0°C for 7 days prior to start of the storage experiment.

b) Plant No. 2. Frozen, farmed salmon was obtained from Chile. The temperature never exceeded -20°C during transport and storage. After thawing the salmon was filleted, injection brined, cold-smoked for 5 h at 20-28°C and sliced partially frozen (-12 to -7°C). The fish was vacuum packed in a barrier material (PETPX 15 μ /PE 80 μ SPEC., permeability for O₂ 10 cm³/m² x 24 h and for CO₂ 40 cm³/m² x 24 h at 23°C, 0% rh from Raackman, Horsens, Denmark). The fish were stored for 19 days at -24°C between smoking and the storage experiment.

c) Plant No. 3. Fresh, farmed salmon from the Faroe Islands was filleted, injection

brined and cold-smoked for 7 h at 20-25°C. After slicing the fish at a temperature of -14°C, it was packed in a barrier material (CCP 30/PA 30/PE 50 E, permeability for O₂ 36 cm³/m² x 24 h and for CO₂ 160 cm³/m² x 24 h at 23°C, 50% rh from DTI, Copenhagen, Denmark). The fish was stored frozen at -24°C for 13 days between smoking and the storage experiment.

Storage and sampling.

Before the beginning of the storage experiment, salmon from plants No. 2 and 3 were thawed overnight at 10°C with the temperature in fish never exceeding 5°C. All samples were transferred to 5.0-5.5°C, and this time was considered to be day 0 in the storage experiment. Sets of 3 separate vacuum packs from each plant were regularly withdrawn throughout the storage period of 35 days (plant No. 2) and 42 days (plants No. 1 and No. 3) and analyzed by sensory panel and microbiologically.

Chemical analyses.

Salt and dry matter content were determined in 9 vacuum packs randomly sampled from each plant by using the same methods as described above.

Sensory analysis.

Between 8 and 10 trained judges evaluated a pooled sample from each plant, compiled by the 3 vacuum packs used for microbiological analyses. Panelists checked for overall acceptability with regard to odour, flavour and texture and disregarding salty and smoky flavours. The judges were presented with a freshly thawed sample from the same production batch but stored at -30°C. This sample served as a standard. A hedonic smiley scale with 5 "faces" was used as described by Truelstrup Hansen *et al.* (1995b), and a maximum shelf life for each product was determined. The judges were also asked to comment on odour, texture and flavour.

Microbiological analyses.

For quantification of the size and composition of the microflora throughout the storage experiment, the same sampling technique as just described was used. Total viable counts

(TVC) were performed by pour plate in Iron Agar (IA, Oxoid) with an overlayer of IA and plates were incubated aerobically for 3 days at 25°C. Total psychrotrophic counts (TPC) were done using the spread plate method on TSA and plates were incubated at 10°C for 5 days. *Enterobacteriaceae* counts on VRBG and lactic acid bacteria counts on NAP agar were performed as described above. In the case of LAB, NAP agar with both the original pH 5.5 (Davidson & Cronin, 1973) and the modified pH 6.7 was used to obtain counts of aciduric and non-aciduric LAB. The microflora was also evaluated by phase contrast microscopy of the 10⁻¹ dilution.

Isolation of microorganisms.

On the day the taste panellists rejected the salmon from the respective plants, 20 colonies were selected with regard to colony morphology and occurrence, from TPC plates with 20 to 100 colonies from each of 3 vacuum packs, giving a total of 60 isolates per plant. Culture in APT broth and APT agar for LAB and BHI and TSA for Gram-negative isolates as well as maintenance of the isolates was performed as described above.

Characterisation and tentative identification.

The 180 isolates were tentatively identified and characterised as described above except, the following tests were not performed on the LAB isolates: reduction of tetrazolium, potassium tellurite and TMAO, production of H₂S (in Iron agar) and off-odours. The ability to degrade inosine to hypoxanthine and ribose with subsequent acid production from ribose was tested by adding 2% (w/v) inosine (Sigma) to the same basic medium used for carbohydrates and then following the procedure described by Jeppesen & Huss (1993). API 20E identification was performed on all Gram-negative isolates. For 3 Gram-negative *Enterobacteriaceae* (one from each of the clusters I, IIA and IIB, Table 5) the API 20E identification was confirmed using the VITEK Gram-negative identification card (GNI) with 31 tests according to the instructions of the manufacturer in the Vitek Senior System (bioMérieux Vitek Inc., Hazelwood, Missouri, USA) fitted with the Industrial Information System as identification data base.

Cluster analysis.

Results from the biochemical tests were scored 0 and 1 for negative and positive

results, respectively. LAB and *Enterobacteriaceae* were subsequently analyzed separately for similarities between strains using the Simple Matching coefficient S_{SM} and clustered on the basis of the dendrograms constructed with the Unweighed Pair Group Method with Averages (UPGMA) following the method described by Sneath (1978). The similarity matrix and dendrograms were computed and constructed using the NT-SYS package (version 1.8, Exeter Software, Setauket, NY, USA). Five LAB strains and 3 *Enterobacteriaceae* strains were tested for all characteristics in duplicate or triplicate to calculate the average probability of erroneous test results (p_i) (Sneath and Johnson, 1972).

The following reference strains donated by V. F. Jeppesen (Dept. of Seafood Research) were included in the analysis of the LAB: *Pediococcus pentaceus* (Pedio-start 40 from Chr. Hansen's Laboratorium (CHL), Hørsholm, Denmark), *Carnobacterium divergens* (MR373 from AFRC Institute of Food Research (AFRC), Bristol Laboratory, UK), 2 *Lactobacillus plantarum* (1-PM from Prof. Cherl-Ho-Lee, Korea and L73 from CHL) and *Leuconostoc mesenteroides* (MR327 from AFRC). Also, a strain from the screening experiment (from group A, 1T18, see Table 2) was included.

Results.

Screening of microorganisms on spoiled cold-smoked salmon.

The microflora on cold-smoked salmon stored for 38 days at 5°C was dominated by lactic acid bacteria (LAB) in all vacuum packs from both plants (Table 1). Phase contrast microscopy of 10⁻¹ dilutions showed presence of small non-motile rods, which supported results from the plate counts. The total numbers of bacteria on TSA and NAP reached 1.2 - 5.1 x 10⁷ cfu/g and yeast were present at levels of 2.1 - 4.3 x 10³ cfu/g in all vacuum packs. *B. thermosphacta* and *Enterobacteriaceae* were found on 3 of 4 vacuum packs in levels of 1.6 - 4.8 x 10⁴ cfu/g and of 3.9 x 10³ to 10⁵ cfu/g, respectively. Vacuum packs from the same plant were similar with regard to TSA, NAP and OGYE counts, but not with regard to STAA and VRBG counts (Table 1).

A total number of 238 microorganisms were isolated from spoiled cold-smoked salmon and tentatively characterised (Table 1). Amongst those were 154 LAB, of which 149 strains were homofermentative and only 5 heterofermentative. Twenty-nine out of 30 isolates from STAA were tentatively identified as catalase-positive, non-motile *B. thermosphacta*. One isolate from STAA was identified as a homofermentative LAB.

Table 1. Composition of the microflora on spoiled vacuum packed sliced cold-smoked salmon from 2 smokehouses stored for 38 days at 5°C. (A total number of 238 microorganisms were characterised)

Source	Salt in water phase (%)	Dry matter (%)	TSA Total viable count (TVC)		NAP Lactic acid bacteria (LAB)		STAA <i>B. thermosphacta</i>		VRBG <i>Enterobacteriaceae</i>		OGYE Yeast & mould	
			Log ^c	Isolates	Log	Isolates	Log	Isolates	Log	Isolates ^f	Log	Isolates
Plant 1, 1 ^a	4.4	33.4	7.31	19 homof. LAB ^d , 1 heterof. LAB ^e	7.30	20 homof. LAB	4.20	9 <i>B. thermosphacta</i> , 1 homof. LAB	<3	None	3.38	10 yeasts
Plant 1, 2 ^a	5.0	33.5	7.60	20 homof. LAB	7.71	20 homof. LAB	4.33	10 <i>B. thermosphacta</i>	4.90	8 <i>Serratia</i> spp.	3.32	10 yeasts
Plant 2, 3 ^b	5.9	35.7	7.61	20 homof. LAB	7.08	12 homof. LAB	4.68	10 <i>B. thermosphacta</i>	3.00	4 <i>Serratia</i> spp.	3.66	10 yeasts
Plant 2, 4 ^b	5.1	31.0	7.57	14 homof. LAB, 4 heterof. LAB	7.41	23 homof. LAB	<2	None	5.00	3 <i>Serratia</i> spp.	3.63	10 yeasts

a) Vacuum packs from the same production batch; **b)** Vacuum packs from two different production batches; **c)** Log₁₀(cfu/g); **d)** homof. LAB - homofermentative lactic acid bacteria; **e)** heterof. LAB - heterofermentative lactic acid bacteria; **f)** Results from API 20E identifications.

Table 2. Characteristics and tentative identity of 36 out of 154 lactic acid bacteria (23% of total) isolated from spoiled sliced, vacuum packed cold-smoked salmon after storage for 38 days at 5°C.

Characteristics ^a	Numbers of positive strains							
	Group A (10) <i>Lb. saké/bavaricus</i> ^b	Group B (12) <i>Lb. curvatus</i>	Group C (3) <i>Lb. curvatus</i>	Group D (4) <i>Leucó. carnosum</i>	Group E (3) <i>Carnobacterium</i> spp.	Group F (3) <i>Lb. curvatus</i>	Group G (1)	
Gas from glucose	0	0	0	4	0	0	0	
Arginine (0.05% glucose)	5	0	1	0	3	0	0	
Acetate agar (growth)	10	12	3	3 (weak)	0	3	1	
Tetrazolium (reduction)	0	0	0	0	1	0	0	
Tellurite (reduction)	10	11	3	3	3	3	0	
H ₂ S	3	0	0	0	2	0	0	
Cellulbiose	9	11	3	4	1	0	0	
Galactose	10	11	3	0	3	3	0	
Lactose	0	1	3	0	2	3	0	
Maltose	1	12	3	1	3	3	1	
Mannitol	0	1	0	0	0	0	0	
Melibiose	10	0	0	0	0	0	0	
Ribose	10	12	3	1	3	3	1	
Salicin	9	11	3	0	3	0	0	
Sucrose	10	3	2	4	3	0	0	
Trehalose	10	12	3	4	3	3	0	
Xylose	0	0	3	0	0	0	0	
Off-odours in fish juice	1/5 ^c	1/5	0/3	2/3	3/3	0/2	-	
Percentage of isolates ^d	30.2	36.3	9.1	3.3	9.1	9.1	3.0	
Origin ^e	Plant No. 1	1 (6), 2 (1)	1 (3), 2 (2)	2 (1)	1 (1)	2 (2)	1 (1)	2 (1)
	Plant No. 2	4 (3)	3 (5), 4 (2)	3 (2)	4 (3)	3 (1)	4 (2)	-

a) All strains were Gram-positive rods, non-motile, catalase negative, fermented glucose and produced no acid from melezitose, raffinose and rhamnose; b) Phenotypic group, number of strains and tentative identity; c) One positive strain of 5 tested; d) Calculated group percentage of 154 isolates; e) Origin of isolates: 1 - vacuum pack (see Table 2), (6) - number of strains from vacuum pack identified to phenotypic group.

All 15 isolates from VRBG were tentatively identified as *Serratia* spp., more specifically as *Serratia marcescens* or *Serratia liquefaciens*. However, VRBG is not strictly selective for *Enterobacteriaceae* as also reported by Cyzeska *et al.* (1981) and the results from VRBG should probably be expressed as Gram-negative count instead of *Enterobacteriaceae* count. Only yeast and no mould was found on spoiled cold-smoked salmon. None of the Gram-negative, *B. thermosphacta* or yeast strains gave positive reactions for TMA or H₂S-production, but all fermented glucose (data not shown). *B. thermosphacta* strains produced burnt and malty off-odours when grown in salmon juice, where all the *Serratia* spp. produced faecal, acidic, pungent and nauseous off-odours resembling those found on spoiled cold-smoked salmon (data not shown). One of the yeast strains produced faecal and nauseous off-odours, whereas the other only produced a slightly aromatic odour.

Thirty-six strains selected among the initially screened 154 LAB were further characterised using 20 additional biochemical tests. All isolates could in general be described as small to coccoid rods occurring in pairs or short chains or occasionally in small irregular bundles of 6-8 cells. On the basis of their biochemical characteristics the strains could be divided into 7 groups (Table 2). Strains from 6 groups were tentatively identified according to keys of Schillinger & Lücke (1987), Collins *et al.* (1987 & 1993), Döring *et al.* (1988), Shaw & Harding (1989) and From (1991). Only one homofermentative strain (group G) did not resemble any known group or species of LAB (Table 2). The vast majority of isolates belonged to *Lactobacillus saké/bavaricus* subgroups (30.2%) in addition to 3 different subgroups of *Lb. curvatus* (54.5%). Minor groups were *Carnobacterium* spp. (9.1%) found in group E, which were identified on the basis of their fermentation patterns, arginine metabolism and inability to grow on acetate agar. Only 3.3% of the LAB were obligate heterofermenters. These strains were identified as *Leuconostoc carnosum* on the basis of their weak to no growth on acetate agar, lack of NH₃ production from arginine and fermentation pattern (Table 2). These strains were described as small coccoid rods as examined by phase contrast microscopy, thus supporting the *Leuconostoc* identity.

Off-odour production among the LAB was not evenly distributed. Among the tentative *Leuc. carnosum* sp. (group D) and the *Carnobacterium* spp. (group E), 5 out of 6 strains produced off-odours, which were described as fruity, malty, burnt and sweet (nauseous). On the other hand, only 2 out of 15 tested strains in the *Lb. saké/bavaricus/curvatus* groups (A, B, C & F) produced off-odours, which were similar to the off-odours just described.

Analysis of the distribution of different lactic acid bacteria indicated that the composition of the lactic acid bacteria flora varied between vacuum packs from same

production batch (plant No. 1) as well as between production batches (plant No. 2, Table 2).

Development and characteristics of the microflora during storage of cold-smoked salmon.

Cold-smoked salmon from the 3 smokehouses contained salt in the range of 4.2 - 5.7% in the water phase and dry matter in the range of 30.4 - 36.0% (Table 3).

The taste panellists rejected the salmon after 24, 28 and 42 days of storage from plant No. 3, No. 2 and No. 1, respectively (Table 3). Spoiled salmon from all plants were characterised by soft texture, sour and pungent off-odours and off-flavours. Salmon from plant No. 1 was dominated by fruity off-odours and sweet, nauseous spoilage off-flavours and salmon from plant No. 2 by faecal, sour and cabbage-like off-odours and pungent, sour, bitter and rancid off-flavours. Salmon from plant No. 3 differed somewhat by being rejected partially because of a very penetrating plastic off-flavour and off-odour, which cannot be considered to be of microbiological origin, in addition to bitter, ammonia, rancid and sweet off-flavours more typical of microbial spoilage (Table 3).

The size and development of the microflora during the storage period varied among plants (Figure 1a-c). The initial number of organisms (TVC & TPC) was lower on salmon from plant No. 1 (10^2 cfu/g) than on salmon from the other plants (10^3 - 10^4 cfu/g, Figure 1a-c). A short lag phase of approximately 7 days was only seen in salmon from plants No. 2 and 3 before growth proceeded towards the ultimate levels of 10^7 - 10^8 cfu/g for TVC, TPC and NAP 6.7. Lactic acid bacteria counts on NAP 6.7 were similar to TVC and TPC except for at the beginning of the

Table 3. Characteristics of chill stored (5°C) sliced and vacuum packed cold-smoked salmon at the time of rejection by sensory panellists.

Plant	Salt in water (%) ^a	Dry matter (%) ^a	Spoilage (days) ^b	Spoilage characteristics ^c			Microbiology (Log ₁₀ (cfu/g) ± standard deviation (n-1))				
				Off-odours	Off-flavours	Texture	TVC ^d	TPC ^e	NAP 6.7 ^f	NAP 5.5 ^g	VRBG ^h
1	4.8 (0.3)	35.3 (2.0)	35-42	Fruity, sweet, pungent	Neutral, sweet, nauseous	Soft, mushy, oily	7.08 (0.8)	7.21 (0.6)	6.95 (0.4)	6.71 (0.4)	6.78 (1.0)
2	4.2 (0.5)	30.4 (1.0)	24-28	Faecal, sour, cabbage	Pungent, sour, bitter, rancid	Soft, crumbling	7.11 (0.5)	7.07 (0.3)	7.01 (0.3)	6.05 (0.1)	6.32 (1.0)
3	5.7 (0.3)	36.0 (1.3)	21-24	Pungent, nauseous, rancid	Pungent, bitter, ammonia, sweet, plastic	Soft	6.94 (0.5)	6.60 (0.3)	6.74 (0.5)	6.35 (0.2)	5.52 (1.6)

a) Average of 9 individual vacuum packs and the standard deviation (n-1); b) Last acceptable sampling day and day of rejection; c) Sensory characteristics at day of rejection; d) TVC - TVC on Iron agar; e) TPC - total psychrotrophic count, TSA surface, 10°C; f) NAP 6.7 - LAB on NAP with pH 6.7; g) NAP 5.5 - LAB on NAP with pH 5.5; h) VRBG - tentative *Enterobacteriaceae* counts.

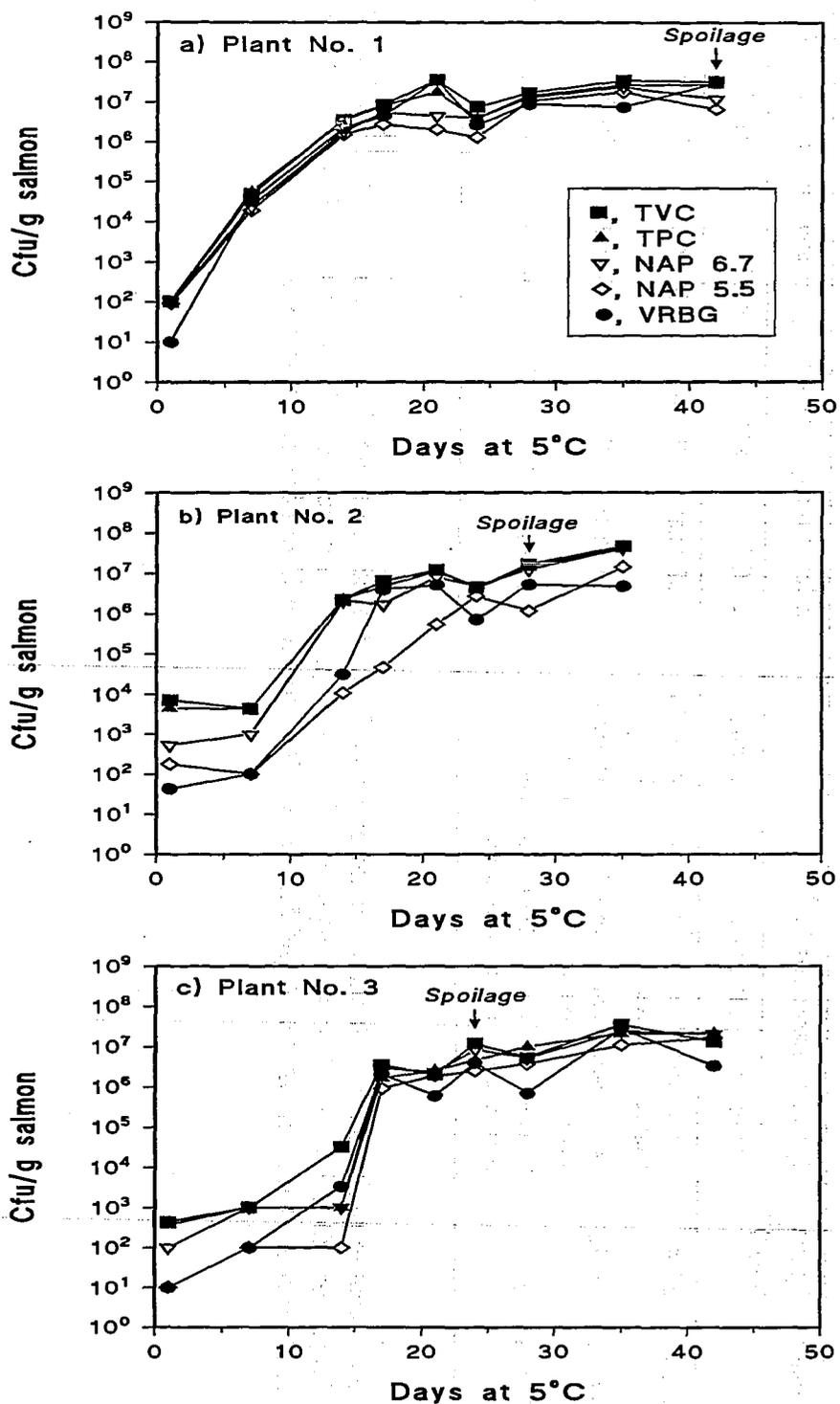


Figure 1a-c. Development in microflora during storage of sliced, vacuum packed cold-smoked salmon (averages of 3 vacuum packs) produced by 3 smokehouses.

storage period (Figure 1a-c and Table 3). NAP agar with pH lowered to 5.5 supported growth of fewer (up to 10^3 cfu/g) LAB than the same agar with pH 6.7, indicating the presence of non-aciduric LAB. Especially salmon from plant No. 2 contained a substantial number of such acid sensitive LAB (Figure 1b and Table 3). As can be seen from Figure 1a-c the number of organisms counted on VRBG varied somewhat irregularly. Lactic acid bacteria dominated the microflora on salmon from all plants at the time of spoilage (Table 3). At the same time, the average number of Gram-negatives tended to be only 0.3 -1.0 $\text{Log}_{10}(\text{cfu/g})$ below the LAB but with standard deviations in the range of 1.0 - 1.6 $\text{Log}_{10}(\text{cfu/g})$, and variations among individual vacuum packs were thus relatively large (Table 3). Detection of H_2S -producing bacteria (black colonies) in the Iron agar (IA) was scattered and non-systematic, in that their occurrence was not related to storage time, sensory quality or specific plants. Also the numbers never reached more than 0.1-1 % of the total microflora counted on the IA plates (data not shown). Phase contrast microscopy of the 10^{-1} dilutions of aged samples showed presence of small to coccoid rods and fewer motile rods.

Nineteen to 20 colonies from each vacuum pack and 59 - 60 organisms from each plant were tentatively identified. All organisms belonged either to the lactic acid bacteria (74%) or the *Enterobacteriaceae* (26%) families (Table 5). Further phenotypic characterisation of the lactic acid bacteria isolates resulted in the formation of 5 clusters using the simple matching coefficients (S_{SM}). The clusters were separated at 80% similarity level or lower. Cluster I consisted of 2 sub-clusters (IA and IB) separated at 85% similarity level (Table 4). The average probability of erroneous results (p_i) was calculated to be 1.1%.

LAB-cluster IA was apparently identical to group B from the screening experiment (Table 2) and therefore tentatively identified as *Lb. curvatus*. The *Pedioc. pentaceus*, reference strain was phenotypically similar to the isolates in LAB-cluster IA, but the strains from salmon were morphologically characteristic by being small to coccoid rods in pairs and short chains and not resembling the cocci in the tetrads typical for the reference strain and *Pediococcus* spp. in general (Garvie, 1986).

The strain (1T18) included from the screening experiment's group A (Table 2) grouped with the storage experiment's LAB-cluster IB (Table 4), and this cluster was tentatively identified as *Lb. saké/bavaricus*. LAB-cluster IB and group A resembled one another apart from variable acid production from maltose and production of NH_3 from arginine.

LAB-cluster II was characterised by 100% of strains being positive for acid production from rhamnase and mannitol and only 4% of strains being able to grow on acetate agar (Table 4). The cluster consisted of coccoid to slender rods often found in short chains.

This homofermentative *Lactobacillus* cluster did not resemble any groups from the screening experiment nor any of the reference strains and could not be identified further.

Table 4. Clusters and characteristics of 6 reference strains and 132 lactic acid bacteria isolated from spoiled, sliced, vacuum packed cold-smoked salmon after storage at 5°C until rejection by sensory panel. The salmon was produced by 3 different smokehouses.

Characteristics	Percentage of positive strains in clusters					
	Cluster I (80) ^a		Cluster II (24)	Cluster III (12)	Cluster IV (16)	Cluster V (6)
	IA (72)	IB (8)				
Gas	0 (—) ^b	0 (—) ^c	0	17 (+) ^d	0 (—/—) ^e	100 (+) ^f
Arginine (0.05% glucose)	3 (+)	25 (+)	25	58 (+)	38 (—/—)	0 (—)
Acetate	100 (+)	100 (+)	4	0 (—)	13 (+/+)	0 (—)
Cellulbiose	97 (+)	100 (+)	75	83 (+)	100 (+/+)	100 (+)
Galactose	99 (+)	100 (+)	17	0 (—)	100 (+/+)	17 (+)
Lactose	0 (—)	13 (—)	8	8 (—)	94 (+/+)	67 (+)
Maltose	100 (+)	75 (—)	88	100 (+)	100 (+/+)	100 (+)
Mannitol	0 (—)	0 (—)	100	83 (—)	100 (+/+)	17 (+)
Melezitose	0 (—)	0 (—)	0	75 (+)	88 (—/+)	0 (—)
Melibiose	0 (—)	100 (+)	0	0 (—)	81 (—/+)	100 (+)
Raffinose	0 (—)	0 (—)	0	0 (—)	25 (—/+)	100 (+)
Rhamnose	0 (—)	13 (—)	100	0 (—)	6 (—/—)	0 (—)
Ribose	100 (+)	100 (+)	100	100 (+)	100 (+/+)	100 (+)
Salicin	100 (+)	100 (+)	100	100 (+)	100 (+/+)	50 (—)
Sucrose	22 (—)	100 (+)	4	100 (+)	100 (+/+)	100 (+)
Trehalose	99 (+)	100 (+)	100	100 (+)	100 (+/+)	100 (+)
Xylose	3 (—)	0 (—)	0	0 (—)	0 (—/—)	100 (+)
Inosine (+ ribose)	13 (—)	0 (—)	46	92 (+)	94 (—/—)	100 (+)
Percentage of all (132) isolates	54.1	6.0	18.1	9.0	12.0	4.5

a) Number of strains in cluster inclusive reference strains; b) Reactions by *Pedioc. pentaceus* reference strain; c) Reactions by strain 1T18 (Group A, Table 2); d) Reactions by *Cb. divergens* (MR373); e) Reactions by *Lb. plantarum* L73 and 1-PM; f) Reactions by *Leuco. mesenteroides* (MR327).

Cb. divergens (MR373) grouped with the lactic acid bacteria belonging to LAB-cluster III (Table 4). This cluster had a unique inability to grow on acetate agar or to produce acid from galactose. Almost 60% of the strains produced NH₃ from arginine, 75% acid from melezitose and 83% from mannitol. This cluster resembled group E (Table 2).

Both *Lb. plantarum* strains (1-PH, Korea & CHL) grouped with LAB-cluster IV. This cluster was characterised by the wide range of carbohydrates fermented. This cluster did not resemble any groups from the screening experiment and within the cluster, salmon

strains differed from the reference strains by their inability to grow on acetate agar. In spite of this, the cluster was tentatively assigned to the variable *Lb. plantarum* group.

All strains in LAB-cluster V produced gas from glucose, acid from melibiose, raffinose and xylose, while none grew on acetate agar or produced NH₃ from arginine (Table 4). The *Leuc. mesenteroides* (MR327) reference strain grouped with this cluster, which then was tentatively identified as such. This was supported by phase contrast microscopy by which the LAB-cluster V strains were described as being very coccoid rods.

Conversion of inosine to hypoxanthine and ribose would only be detected if the LAB was able to produce acid from ribose. All of the isolated LAB were able to ferment ribose and a total of 52 strains were able to break inosine down and subsequently produce acid from ribose (Table 4). The majority of the isolates (92-100%) from LAB-clusters III, IV and V possessed this ability, while 46% from cluster II and only 13% from cluster IA were positive. No strains from cluster IB were able to convert inosine (Table 4).

Among the 47 isolated *Enterobacteriaceae* strains, 3 *Enterobacteriaceae*-clusters could be formed and the distribution of strains and tentative identities based on the API 20E and VITEK identification is shown in Table 5. The clusters were separated at 72% level of similarity for cluster III, 84% for cluster I and 90% level for sub-clusters IIA & IIB. The average probability of erroneous results (p_i) were calculated to be 2.9%. Forty percent of the 60 isolates from plant No. 1 belonged to the *Enterobacteriaceae*, where only 18% and 20% of the isolates from plants No. 2 & 3, respectively, were identified as *Enterobacteriaceae*.

The 20 strains found in cluster I were tentatively identified as *Enterobacter agglomerans* by API 20E, which was confirmed by VITEK on one strain (data not shown). The 13 strains from clusters IIA and IIB were identified as *Hafnia alvei* and *Serratia liquefaciens*, respectively. These were also confirmed by VITEK identification of two representative strains from the clusters (data not shown). Cluster III only contained one strain, which could not be further identified with the API 20E method.

In Table 5 the results from the clustering and tentative identification of all 159 strains are presented with emphasis on the origin of strains. All LAB from clusters II, III, IV originated from plant No. 2. Also, LAB from clusters IA & IB were only found on cold-smoked salmon from plants No. 1 & 3. The 5 strains in the LAB cluster V all came from one vacuum pack from plant No. 1. A similar distinct picture was obtained when the distribution of strains in the *Enterobacteriaceae* clusters were evaluated.

Table 5. Composition of the microflora at point of sensory rejection on individual vacuum packs of sliced, cold-smoked salmon produced by 3 smokehouses and stored at 5°C.

Bacteria groups ^a	Plant No. 1			Plant No. 2			Plant No. 3		
	Vacuum packs			1	2	3	1	2	3
No. of lactic acid bacteria (LAB) isolates	12	10	14	14	20	15	9	20	18
LAB cluster IA <i>Lb. curvatus</i> ^b	1	10	14	-	-	-	8	20	18
IB <i>Lb. saké/bavaricus</i>	6	-	-	-	-	-	1	-	-
II <i>Lactobacillus</i> spp.	-	-	-	10	9	5	-	-	-
III <i>Carnobacterium</i> spp.	-	-	-	3	7	1	-	-	-
IV <i>Lb. plantarum</i>	-	-	-	1	4	9	-	-	-
V <i>Leuc. mesenteroides</i>	5	-	-	-	-	-	-	-	-
No. of <i>Enterobacteriaceae</i> isolates	8	10	6	6	0	5	10	0	2
Ent. cluster I <i>Enterobacter agglomerans</i>	8	-	2	5	-	5	-	-	-
IIA <i>Hafnia alvei</i>	-	9	4	-	-	-	-	-	-
IIB <i>Serratia liquefaciens</i>	-	-	-	1	-	-	10	-	2
III <i>Enterobacteriaceae</i> spp.	-	1	-	-	-	-	-	-	-

a) Bacteria groups and clusters according to results from biochemical characterization (18 tests for lactic acid bacteria and 20 for *Enterobacteriaceae*) and numerical cluster analysis using S_{SM} ; b) Tentative identity.

Thus, all *Hafnia alvei* in cluster IIA came from plant No. 1, and 12 out of 13 *Serratia liquefaciens* strains in cluster IIB were found on salmon from plant No. 3. *Enterobacter agglomerans* from cluster I were isolated on salmon from plants No. 1 & 2.

The intraplant variations (e.g. variations among vacuum packs from the same production batch and smokehouse) in the composition of the isolates were as striking as the differences among the plants. The numbers of *Enterobacteriaceae* isolated from vacuum packs from plants No. 2 & 3 varied for example between 0 and 10, which also was reflected in the standard deviation on the VRBG counts on the vacuum packs (Table 3). Also, one vacuum pack from plant No. 1 contained LAB from clusters IB and 5, which were not found on the other vacuum packs from the same plant.

Discussion.

Composition of microflora at time of spoilage.

At the time of spoilage in the screening experiment LAB seemed to dominate with the occurrence of *B. thermosphacta*, *Enterobacteriaceae* and yeast at significantly lower levels (Table 1). LAB were also found to occur in high numbers at spoilage in the storage experiment, but here the levels of *Enterobacteriaceae* were similar to those of LAB but highly variable among vacuum packs (Table 3). This rather variable picture of the microflora at the time of spoilage with the LAB representing the vast majority of the isolates recovered from TVC/TPC procedures and with *Enterobacteriaceae* varying from insignificant levels to comparable levels to those of LAB, has previously been reported (Cann *et al.*, 1984; Schneider & Hildebrandt, 1984; Truelstrup Hansen *et al.*, 1995a, 1995b, 1995d). The size of the microflora was not related to spoilage and quality and is therefore not recommended as an indicator of spoilage or quality (Figures 1a-c).

Selective agars made it possible to detect the presence of *B. thermosphacta* and yeast at levels 10^3 - 10^4 cfu/g lower than the TVC level. It is unlikely that such low numbers would have any effect on the quality of cold-smoked salmon in spite of their potential for production of off-odours when inoculated in salmon juice. The spoilage bacteria *Photobacterium phosphoreum* and/or other marine vibrios have previously been found at high levels of 10^6 - 10^7 cfu/g on spoiled cold-smoked salmon (Truelstrup Hansen *et al.*, 1995b, 1995d). In this study phase contrast microscopy of samples failed to reveal the presence of large coccobacilli typical for *P. phosphoreum* (Dalgaard, 1995) and these organisms were likely not present in any significant numbers. In the storage experiment this was supported by TVC and TPC being identical and the initial levels of 10-14 mgN/100 g TMAO not being converted to TMA during storage (data not shown).

Sensory characteristics of spoilage.

Spoilage of cold-smoked salmon is primarily caused by microbiological activity (Truelstrup Hansen *et al.*, 1995b) and the spoilage characteristics in the storage experiment presented in Table 3 resembled those previously described (Cann *et al.*, 1984; Truelstrup Hansen *et al.*, 1995a). The plastic off-flavour found in salmon from plant No. 3 was, however, more likely related to diffusion of contaminants from the packaging material. *Enterobacteriaceae* were capable of producing the typical spoiled salmon odours when

inoculated into sterile salmon juice. Only 7 out of 21 LAB were able to produce such odours under the same conditions, which indicated a variable spoilage potential within the group.

Occurrence and origin of lactic acid bacteria.

LAB dominated the isolates in both the screening and storage experiment, where 100% and 74% of organisms isolated from TVC and TPC were LAB, respectively. Dominance of LAB in chill stored lightly preserved fish products has been reported by Magnússon & Traustadóttir (1982), Schneider & Hildebrandt (1984), Jeppesen & Huss (1993) and Leisner *et al.* (1994) covering a number of different products such as vacuum packed cold-smoked herring, cold-smoked salmon, sugar-salted (gravad) fish (salmon, Greenlandic halibut, mackerel), brined prawns, shell fish salads, fish paté and vacuum packed minced herring with 5% sucrose and 4.6% NaCl.

The origin of LAB could be the raw material as LAB is found on fresh fish in smaller numbers (Schröder *et al.*, 1980; Knöchel, 1981; Mauguin & Novel, 1994). Some LAB are linked with diseased salmonid fish such as *Carnobacterium piscicola* and *Vagococcus salmoninarum* (Hiu *et al.*, 1984; Collins *et al.*, 1987; Wallbanks *et al.*, 1990). Contamination from the in-house microflora could also contribute and in fact, a previous study showed that different microflora would develop on smoked salmon from the same batch, which had been vacuum packed either immediately after smoking or after slicing (Truelstrup Hansen *et al.*, 1995d). Similarly, variations in the microflora on vacuum packed emulsion type sausages from 3 subsequent production days were shown to involve contamination with LAB from the production environment (Borch *et al.*, 1988).

Biochemical characterization of the isolates.

Biochemical characterization of the LAB and Gram-negative isolates was primarily performed for the purpose of distinguishing between the different sub-groups within the bacterial families to allow comparison of the microflora between smokehouses, and thus it was chosen to limit the number of characteristics tested. However, it was possible to identify the microorganisms tentatively on the basis of these biochemical tests, and to our knowledge this is the only reported characterization of the microflora on spoiled, cold-smoked salmon.

Characteristics of lactic acid bacteria.

The properties of Group A and LAB-cluster IB LAB were in good agreement to profiles previously reported for *Lb. saké* or *Lb. bavaricus* isolated from numerous meat products (Kandler & Weiss, 1986; Schillinger & Lücke, 1987a; Borch & Molin, 1988; Döring *et al.*, 1988; Grant & Patterson, 1991; Dykes *et al.*, 1994; Samelis *et al.*, 1994). Most probably 35% of the *Lb. saké/bavaricus* strains were *Lb. saké* on the basis of their ability to produce NH₃ from arginine.

Eighty-nine strains from groups B, C, F and LAB-cluster IA (Tables 2 & 4) were tentatively identified as *Lb. curvatus*. *L. curvatus* was the most frequently occurring LAB in both the screening experiment (55%) and in the storage experiment (54%). The *Lb. curvatus* profiles agreed well with strains described from red meat products by Grant & Patterson (1991), Hugas *et al.* (1993) and Samelis *et al.* (1994) but not by Dykes *et al.* (1994), where 33% of the strains were unable to grow on acetate agar and 50-83% unable to produce acid from cellubiose, salicin and trehalose.

Jeppesen & Huss (1993) found a high number of *Lb. saké* but not *Lb. curvatus* among the LAB from a variety of seafoods, whereas Mauguin & Novel (1994) found neither *Lb. saké/bavaricus* nor *Lb. curvatus* in their screening of seafoods.

Occurrence of *Leuconostoc* spp. in seafood was reported by Jeppesen & Huss (1993) and Mauguin & Novel (1994). *Leuconostoc* spp. did not dominate the LAB flora on cold-smoked salmon, where the tentative *Leuc. carnosum* from the screening experiment and *Leuc. mesenteroides* from the storage experiment only accounted for 3.3% and 4.5% of the LAB-flora, respectively (Tables 2 & 4).

Group E and LAB-cluster III were tentatively identified as *Carnobacterium* spp., but none of the salmon strains produced gas from glucose. The production of gas by *Carnobacterium* spp. can be difficult to detect as pointed out by Collins *et al.* (1987) and one report indicates that *Carnobacterium* in fact is homofermentative (de Bruyn *et al.*, 1987). Only 9% of the LAB were found to be *Carnobacterium* spp. in both experiments, which was in contrast to domination by *Carnobacterium* spp. found in a lightly preserved fish product (gravad salmon) by Leisner *et al.* (1994). In other screenings of LAB from seafood, Mauguin & Novel (1994) found that 16 out of 86 strains belonged to *Carnobacterium* spp., and Jeppesen & Huss (1993) reported 2-7 of 61 strains to be *Carnobacterium* spp.

Group G from the screening experiment (Table 2) contained one homofermentative *Lactobacillus* sp. with a very narrow fermentation pattern (glucose, maltose and ribose). LAB-cluster II strains (24) resembled *Lb. casei* subsp. *rhamnosus* (or *Lb. rhamnosus*) but

contrary to LAB-cluster II this species produces acid from lactose, melezitose and sucrose (Döring *et al.*, 1988; Collins *et al.*, 1989). Strains in LAB-cluster II and group G did not resemble any groups found in seafood (Jeppesen & Huss, 1993; Mauguin & Novel, 1994) or any groups found in meat products (Shaw & Harding, 1984; Morishita & Shiromizo, 1986; Borch & Molin, 1988; Grant & Patterson, 1991; Hugas *et al.*, 1993; Dykes *et al.*, 1994; Samelis *et al.*, 1994).

Both *Lb. plantarum* reference strains were found in LAB-cluster IV in spite of their variable fermentation patterns and ability to grow on acetate agar (Table 4). There are numerous reports of the presence of *Lb. plantarum* in seafood (Schröder *et al.*, 1980; Jeppesen & Huss, 1993; Mauguin & Novel, 1994) and meat products (Morishita & Shiromizo, 1986; Hugas *et al.*, 1993; Samelis *et al.*, 1994), but the description of biochemical properties varies considerably.

Characteristics of Gram-negative isolates.

The psychrotrophic Gram-negative species on spoiled cold-smoked salmon characterised in this study consisted of *Serratia* spp. (*liquefaciens* and *marcescens*), *Enterobacter agglomerans*, *Hafnia alvei* and an unidentified *Enterobacteriaceae* sp. (Tables 1 & 5). Similar species have previously been found on cold-smoked salmon (Nieper, 1986; From & Huss, 1991; Cantoni *et al.*, 1993). From & Huss (1991) also found *Vibrionaceae* spp., which were not encountered in this study. *Enterobacteriaceae* and *Vibrionaceae* are ubiquitous microorganisms (Krieg & Holt, 1984), and the above mentioned species have often been isolated from vacuum packed red meat products and related to spoilage (Hanna *et al.*, 1979; Dainty & Mackey, 1992).

Inosine metabolism of lactic acid bacteria.

The ability to convert inosine into hypoxanthine and ribose was widespread among the LAB with 92-100% of the tentative *Carnobacterium* spp., *Lb. plantarum* and *Leuconostoc mesenteroides* spp. being positive. Borch & Molin (1988) assayed LAB's ability to assimilate inosine and also found that *Carnobacterium* spp. (Cluster 1 & 2) possessed that ability. This property is interesting in relation to the use of hypoxanthine as a quality parameter.

Intra- and interplant variations and existence of one specific microflora for cold-smoked salmon.

The intra- and interplant variations in the microflora indicated by the screening experiment were much more pronounced in the storage experiment (Table 5), where a larger number of isolates and vacuum packs were analyzed. On cold-smoked salmon from plant No. 2 both LAB and Gram-negative isolates were significantly different between batches (Table 1, 2 & 5). However, the considerable vacuum pack to vacuum pack variation could disturb this picture. Borch *et al.* (1988), Samelis *et al.* (1994) and Hitchener *et al.* (1982) all found batch to batch variations in the LAB flora on vacuum packed emulsion sausages, naturally fermented salami and vacuum packed beef, respectively, from the same source. On the variation in the LAB microflora on the same type of products from different sources Morishita & Shiromizu (1986) and Hugas *et al.* (1993) found similar variations to those observed on cold-smoked salmon in this study. Based on those results it is not likely that there exists one specific microflora on cold-smoked salmon. The product rather represents a frame where a number of different organisms can grow and thrive depending on whether they are successfully introduced either through the raw material or from the production environment. Spoilage occurs as a result of microbiological activity and will therefore be influenced by the spoilage potential among the microorganisms present.

Conclusion.

The microflora on cold-smoked salmon appeared to be very similar, when agar methods alone were used. Characterization of the microflora proved that there were considerably variations between smokehouses. Also, when vacuum packs from the same production batch were compared there were distinct differences. Apart from the unidentified LAB-cluster II and LAB group G, it was possible to tentatively identify the isolated lactic acid bacteria on the basis of the biochemical characteristics and the literature on LAB in seafood and vacuum packed red meat. The significance of the bacteria isolates and the variable microflora found on spoiled cold-smoked salmon in relation to spoilage remains to be analyzed.

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Microbiological quality and shelf life of cold-smoked salmon from 3 different processing plants.

by

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Abstract.

Vacuum packed whole fillets and sliced cold-smoked salmon from the same production batch were stored at 5°C for up to 51 days, sampled regularly and analyzed for sensory and microbiological changes using taste panel, traditional agar plating methods (total viable counts, psychrotrophic counts, lactic acid bacteria and *Enterobacteriaceae*) and Malthus conductance methods for total viable counts and *Enterobacteriaceae*. The experiment was performed twice with salmon from 3 different smokehouses. Sliced salmon was rejected after 21-36 days and fillets after 32-49 days of storage with no apparent relation to the microflora or salt content (4.1-6.1% in the water phase). Whole fillets developed the same spoilage characteristics as sliced salmon but had a softer texture at the time of rejection. Plate counts and Malthus detection times revealed a highly variable microflora among the 3 smokehouses. The microflora differed between whole fillets and the corresponding sliced salmon from 2 smokehouses, indicating microbiological contamination from the production environment may have influenced the composition of the microflora. Lactic acid bacteria, *Enterobacteriaceae* and psychrotrophic marine vibrio and *Photobacterium* spp. were found on spoiled salmon. Use of Malthus methods calibrated to traditional agar methods was problematic, because the microflora on cold-smoked salmon was so variable.

Keywords: Cold-smoked salmon, microbiology, spoilage, conductance assays

Introduction.

Cold-smoked salmon is a lightly preserved fish product with a typical salt content of 3.5-5% in the water phase, water content between 65-70% and pH between 5.8-6.2. This product spoils due to microbiological activity when vacuum packed and stored at chill temperatures (Truelstrup Hansen *et al.*, 1995b). It has been proposed that there exists a specific spoilage flora for cold-smoked salmon in parallel to what has been found for cod stored on ice (Gram *et al.*, 1987) and in modified atmosphere (Dalgaard *et al.*, 1993). However, reports on development of the microflora on cold-smoked salmon are not consistent (Cann *et al.*, 1984; Schneider & Hildebrandt, 1984; Truelstrup Hansen *et al.*, 1995a,b,c), and the microflora therefore seem to be rather variable.

Lightly preserved fish products like cold-smoked salmon undergo more processing steps than chilled groundfish like cod, and these processes i.e. injection brining (addition of salt and penetration of tissue), drying and smoking will alter the physical and chemical properties of the raw material and possibly the size and composition of the indigenous microflora. Also, each processing step will increase the exposure of the fish to the in-house microflora. The importance of contamination with the in-house flora for shelf life and spoilage is not known.

Rapid microbiological methods based on changes in electrical properties (capitance, conductance and impedance) caused by microbiological activity have been developed for foods (Silverman & Monoz, 1979; Firstenberg-Eden & Tricarico, 1983; Gibson & Hobbs, 1987; Ravn Jørgensen *et al.*, 1988). The detection time (DT) will under the given assay conditions be related to the numbers and kinds of organisms present. DT can be converted into colony forming units (cfu) through calibration with traditional agar plating methods. However, as a result of differences in metabolism, end products or morphological characteristics, identical plate counts of various microorganisms do not necessarily correspond to the same DTs (Firstenberg-Eden & Tricarico, 1983; Gibson *et al.*, 1984; Dumont & Slabyj, 1994).

This study was undertaken to determine whether any consistent patterns could be found in the development of sensory and microbiological properties during storage of vacuum packed cold-smoked salmon from 2 individual production batches produced under identical conditions by each of 3 smokehouses. Influence of the exposure to the in-house microflora during slicing and packaging procedures was evaluated by comparison of salmon vacuum packed immediately after smoking with normally processed sliced, vacuum packed cold-smoked salmon from the same batch. The microbiological testing involved traditional agar plating methods and the Malthus conductance methods for measuring total viable counts

(TVC) and *Enterobacteriaceae*, which allowed an alternative way of analyzing for differences in the microflora.

Material and methods.

Production of samples of cold-smoked salmon.

Farmed Atlantic salmon (*Salmo salar*) were obtained from Norway by 3 different smoke-houses in northern Denmark, stored, filleted and salted according to their usual procedures (Figure 1). Immediately after the drying and cold-smoking processes whole unsliced fillets were put into vacuum pouches using sterile gloves and vacuum packed. The vacuum packed whole fillets followed the rest of the cold-smoked salmon during the routine procedures of chilling (freezing), skinning, slicing, laying onto gold-laminated card boards, weighing and vacuum packing. In this way all the salmon from the same production batch got the same temperature x time treatment (Figure 1). The vacuum packed whole sides (W) and the normal sliced cold-smoked salmon (S) were shipped to the laboratory (temperature < 3°C), where the storage experiment began. This procedure was repeated after 7 or 12 days at all 3 smokehouses to produce a second production batch using the same recipes, equipment and fish of comparable quality.

For each plant the following conditions were specified:

i. Plant No. 1. Norwegian farmed salmon were stored for 6 days on ice between slaughter and beginning of both production batches (1.1 & 1.2). The salmon was salted by a combination of injection brining and dry salting. The fish was sliced at -1°C. All salmon were vacuum packed in laminates of oriented polyamide (OPA) and polyethylene (PE) for the upper layer (OPA/PE 15/70) and polyamide (PA) and PE for the under layer (PA/PE 30/70) supplied by SL-emballage, Copenhagen, Denmark. Permeability for O₂ were 22-35 cm³/m² x 24 h and for CO₂ 115-140 cm³/m² x 24 h at 25°C, 0% rh.

ii. Plant No. 2. Norwegian farmed salmon stored for 7 days on ice after slaughter were used for the first production (2.1). Norwegian farmed salmon stored 2 days on ice and frozen (-35°C) for 7 days, were used for the second production batch (2.2). The salmon was injection brined and sliced at -12°C. Sliced salmon were vacuum packed in laminates consisting of PETX/PE 15/70 for the upper layer and PETM/PE 12/60 for the under layer with permeability for O₂ of 5-10 cm³/m² x 24 h and for CO₂ of 15-30 cm³/m² x 24 h at 23°C, rh 90% (Scandiflex, Landskrona, Sweden). Whole fillets were packed in Riloten 50/100 Conospeed (Otto Nielsen, Lyngby, Denmark) with the following properties: PA/LDPE

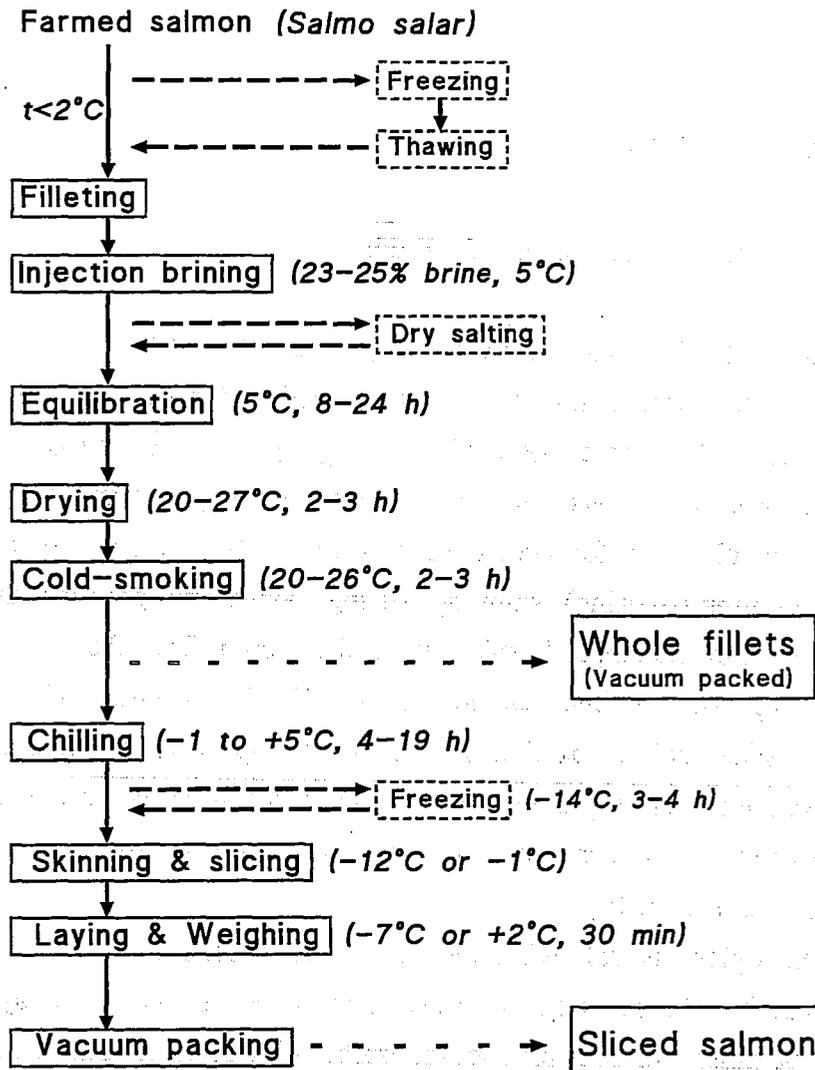


Figure 1. Flow chart of cold-smoking process and sample preparations. Range of the time and temperatures for each process is given.

55/100; O₂ 12 cm³/m² x 24 h; CO₂ 54 cm³/m² x 24 h at 23°C, 90% rh.

iii. Plant No. 3. For production of both batches (3.1 & 3.2) Norwegian farmed salmon were slaughtered and iced for 7 days and then processed. The salmon was injection brined and sliced at -14°C. Sliced salmon were packed in Nyloflex with upper layer consisting of PA/PE 15/70 and under layer of PA/PE 40/70 with permeability for O₂ of 29-44 cm³/m² x 24 h and for CO₂ of 117-175 cm³/m² x 24 h at 23°C, 90% rh (BA Emballage, Horsens, Denmark). Whole fillets were packed in PA/PE 50/100 with permeability for O₂ of 18 cm³/m² x 24 h and for CO₂ of 55 cm³/m² x 24 h at 23°C, 90% rh (Innopac, Tåstrup, Denmark).

Storage and sampling.

Whole fillets and sliced salmon were stored at $5 \pm 1^\circ\text{C}$ in the dark for up to 51 days, sampled regularly and analyzed by microbiological methods (2 vacuum packs) and by taste panel (1 vacuum pack). The microbiological and sensory analyses on each set of samples continued at least until the taste panel rejected the salmon. Salt and dry matter content were determined in 5 whole fillets from each production batch from the 3 smokehouses.

Sensory analysis.

Samples from all treatments and smokehouses were evaluated for overall acceptability with regard to odour, flavour and texture and disregard to salty and smoky flavours. The 8-10 trained panellists were also presented with a freshly thawed sliced sample from the same production batch stored at -30°C throughout the experiment, and this sample served as a standard. All samples were served sliced, so the judges would have no preference. The hedonic Smiley scale with 5 "faces" was used as described by Truelstrup Hansen *et al.* (1995b), and the samples were considered unacceptable when 50% or more of the judges discarded the salmon. The judges were also asked to comment on odour, texture and flavour.

Chemical analysis.

Approximately 100 g samples were homogenised and the AOAC (1975) method was used to determine the salt content expressed as % NaCl in water phase in homogenates. Dry matter content was determined by oven drying 2 g salmon at 105°C for 20-24 h (until constant weight was obtained).

Microbiological analysis.

Traditional plating methods.

Ten g salmon sampled from representative parts of the vacuum packs were homogenised in 90 ml Peptone Saline (PS, 0.9% (w/w) NaCl and 0.1% (w/w) Bacto Peptone, Difco) for 30 s in a Stomacher 400 Lab Blender (A. J. Seward, Bury, St. Edmunds, U. K.). Total viable counts (TVC) and counts of H_2S -producers (black colonies) were performed by using appropriate dilutions in pour plates (double layer) with Iron Agar (IA) (Gram *et al.*,

1987). Counts of total psychrotrophs (TPC), total viable counts in Tryptic Soy Agar (TSA), tentative *Enterobacteriaceae* in Violet Red Bile Glucose agar (VRBG) with bottom layer of TSA and lactic acid bacteria in Nitrite Actidion Polymyxin agar (NAP) with pH adjusted to 6.7 were performed as described by Truelstrup Hansen *et al.* (1995a). All colonies (IA and TSA) or typical colonies (NAP) were counted after 3 days' aerobic incubation at 21°C. On TPC plates, all colonies were counted after 5 days' aerobic incubation at 10°C, whereas typical colonies on VRBG plates were counted after 2 days at 21°C.

Malthus methods.

From the same 10⁻¹ dilution (10 g sample + 90 ml Saline Peptone), which was made for the traditional analyses, 1 ml was pipetted into Malthus cells with 2 ml Special Peptone Yeast Extract broth (SPYE, No. 490-001, Malthus, Crawley, West Sussex, England) for determination of total viable counts (TVC-Malthus). Numbers of *Enterobacteriaceae* (*Enterobacteriaceae*-Malthus) were determined by pipetting 1 ml sample into Malthus cells with 5 ml *Enterobacteriaceae* medium (No. 490-004, Malthus) with addition of 0.05 g/l Monensin (Sigma). The cells were incubated in a Malthus 2000 (Malthus Instruments Ltd., Crawley, West Sussex, England) at 21°C (TVC) and 36°C (*Enterobacteriaceae*) for up to 50 h. Linear regression was used to analyze the relationship between detection times (DT, time until changes in conductance) and plate counts. The linear regression analyses were performed using Fig.P version 6.0 (Fig.P Software Corporation, Durham, NC, USA).

Statistical methods.

Differences between regression equations were tested either by modelling the results in a first order equation ($\text{Log}_{10}(\text{cfu/g}) = \alpha_i + \beta_i \cdot \text{DT}$) with indicator variables and interactions to allow analyses for both differences among slopes (β_i) and intercepts (α_i) or by first testing for homogeneity of the regression coefficients (slopes β_i) and then, if the regression coefficients were homogeneous, a covariance analysis for differences among the intercepts (α_i) was carried out. These tests were performed as described in Gomez & Gomez (1984) and carried out using SAS version 6.09 (SAS Institute Inc., Cary, NC, USA). Differences among the regression correlation coefficients (r) were tested as described in Gomez & Gomez (1984). When appropriate, analyses of variance (ANOVA) were performed.

Results.

Sensory changes and maximum shelf-lives of whole and sliced cold-smoked salmon.

The salt and moisture content in cold-smoked salmon ranged between 4.1 and 6.1% in the water phase and 62.5 to 67.1%, respectively (Table 1). The salt contents were significantly ($P < 0.05$) different between the two production batches from plant No. 3. The shelf life of cold-smoked salmon was not related to the salt content alone as is obvious from Table 1 and Figure 2, but strongly related to whether the salmon was vacuum packed as a whole fillet or sliced. In Table 1, a set of codes are presented to symbolise the individual treatments, where 1.1W represents whole sides from plant No. 1's first production and 1.1S the sliced salmon from the same plant and batch.

Table 1. Shelf life, salt and moisture content in whole and sliced cold-smoked salmon from 2 production batches from each of 3 smokehouses.

Plant	Production batch	Salt % (in water phase)	Water %	Shelf life (days) at 5°C	
				Whole fillets	Slices
1	1	5.2 ± 0.3	67.1	42-47 ^a (1.1W) ^b	21-26 (1.1S)
	2	4.8 ± 0.3	66.9	32-35 (1.2W)	24-28 (1.2S)
2	1	4.1 ± 0.4	64.3	38-43 (2.1W)	30-36 (2.1S)
	2	4.4 ± 0.3	66.3	34-36 (2.2W)	25-29 (2.2S)
3	1	5.2 ± 0.3	63.1	45-49 (3.1W)	22-29 (3.1S)
	2	6.1 ± 0.3	62.5	38-42 (3.2W)	25-28 (3.2S)

a) Day in bold represents day at which the sensory panel rejected the sample; b) Sample coding to used in the presentation of results.

Cold-smoked salmon gradually lost the sensory quality during storage (Figure 2). The estimated shelf life at 5°C of sliced salmon ranged between 21 to 36 days and of whole sides between 32 to 49 days (Table 1 & Figure 2). Shelf lives of whole sides were always longer than shelf lives of the corresponding sliced product.

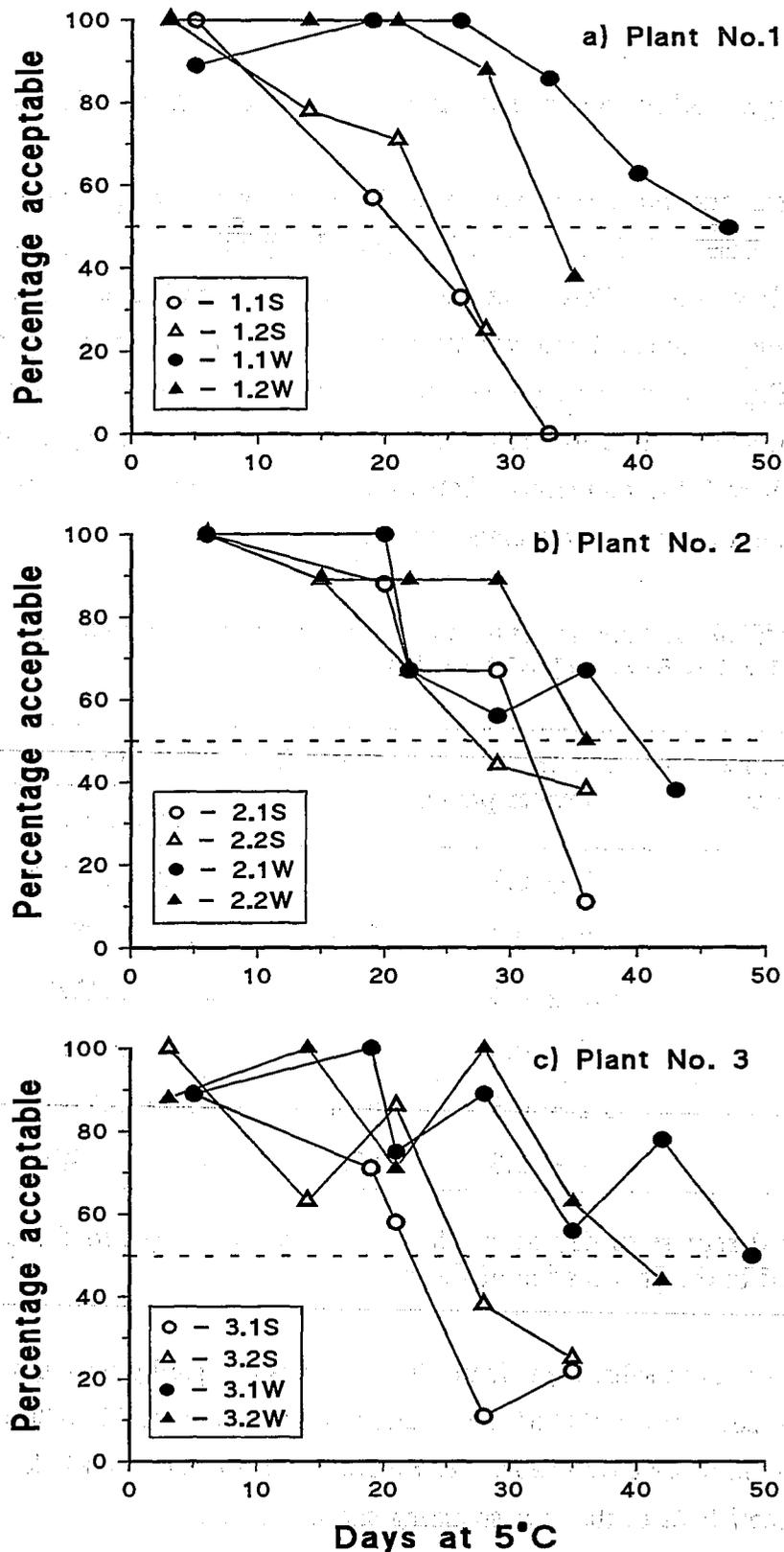


Figure 2. Sensory acceptability of whole sides (W) and sliced (S) cold-smoked salmon during storage. Dashed lines are borderline of acceptability (50%).

The odour of sliced cold-smoked salmon changed during storage from fresh and typical to neutral and slightly sour, sweet and putrid. Strong sweet, nauseous, sour, putrid and in some samples (1.2S & 2.2S) ammonia-, amine- and cabbage-like off-odours were reported at time of rejection. The characteristic flavour was initially mild and sweet, but also slightly rancid in 2.2S & 3.2S. The flavour of borderline salmon was slightly sweet and could in addition be slightly rancid, bitter, oily and astringent. The scores and descriptions of salmon of borderline quality revealed great variation among samples from the same vacuum pack (Figure 2). Off-flavours in spoiled salmon matched the characteristics of borderline salmon but had become more intense. The texture of spoiled salmon was described as soft, pasta-like, mushy and butter-like. Samples, that initially were slightly rancid, became very rancid at the time of rejection (2.2S & 3.2S), but also 3.1S became rancid during storage.

The sensory changes, which developed during storage of whole fillets, resembled those observed and described above for sliced salmon. In general, the textural deterioration was more pronounced in rejected whole fillets, where several judges noted that they rejected the salmon on the basis of the texture rather than any other characteristics.

Microflora during storage (traditional plate count methods).

The traditional plate count methods yielded considerable variations in both the size and the composition of the microflora on sliced and whole fillets. The microflora also varied between production batches. Large standard deviations (0.5 to 1.0 $\text{Log}_{10}(\text{cfu/g})$) were often seen between the two independent samples analyzed on each sampling day. The initial numbers of microorganisms were in general low ($10^2 - 10^3$ cfu/g) and this indicated that appropriate good manufacturing practices had been used by the smokehouses.

Figures 3a-d show the batch variation on sliced cold-smoked salmon from the same smokehouse (No. 1). Sliced salmon from the first production batch was dominated by psychrotrophs (10^6 cfu/g) at the time of time of spoilage, while sliced salmon from the second batch was dominated by lactic acid bacteria (LAB, 5×10^7 cfu/g) (Figures 3a & c). Lower numbers of microorganisms were found on whole fillets from both productions than on the corresponding sliced salmon (Figures 3b & d). Whole fillets from the first production (1.1W) and second batch (1.2W) contained at spoilage approximately 10^5 cfu/g and 10^6 cfu/g, respectively, and the flora was in both cases dominated by psychrotrophs. *Enterobacteriaceae* counts were lower than any other agar counts in all salmon from plant No. 1 (Figure 3).

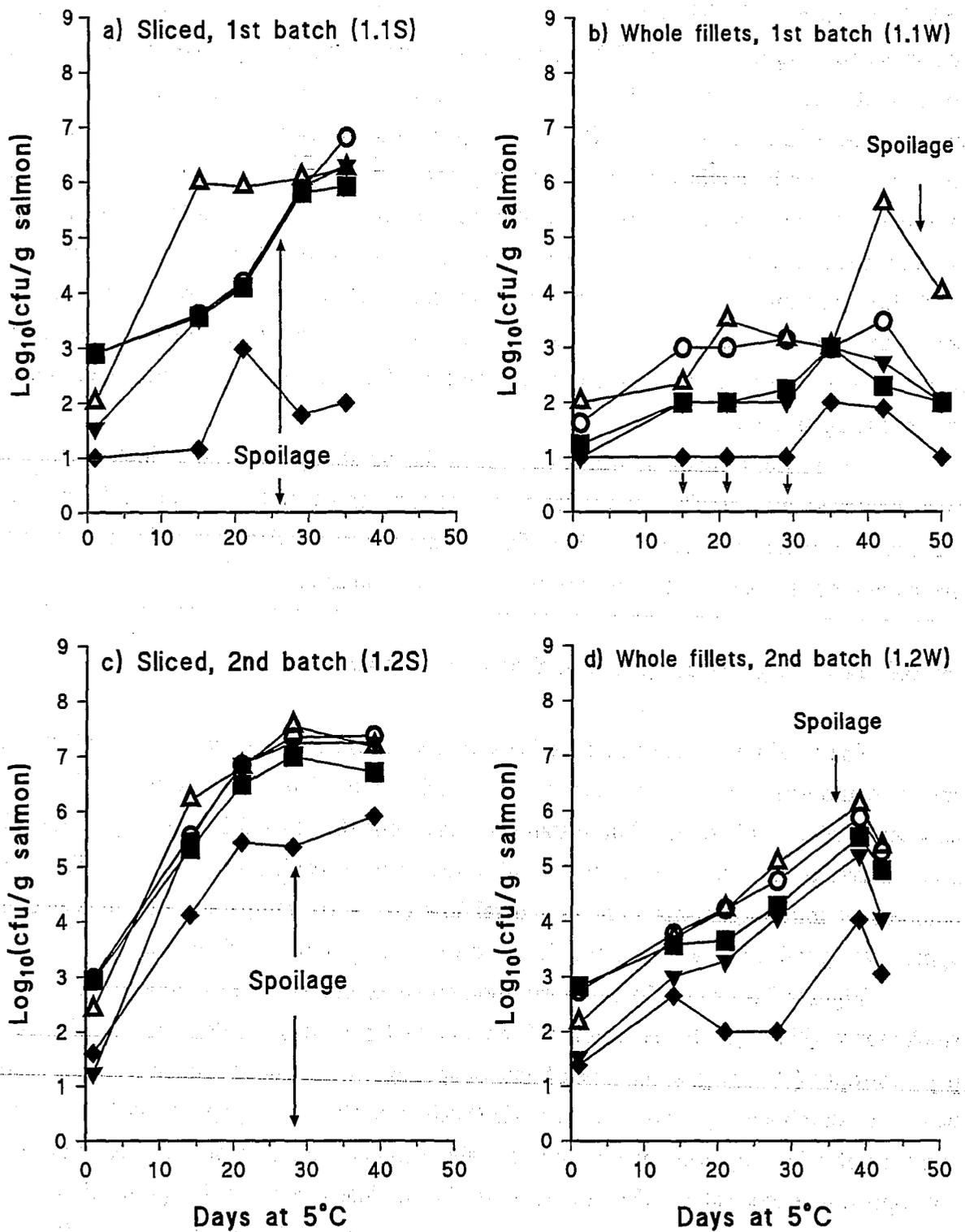


Figure 3a-d. Development of the microflora on 2 different batches of cold-smoked salmon made by plant No. 1. ■ - TVC; △ - TPC; ▼ - NAP; ◆ - VRBG and ○ - TSA.

Sliced salmon from plant No. 2's first batch reached final numbers of $10^7 - 10^8$ cfu/g and was dominated by LAB (Figure 4a). The microflora on whole fillets from the same batch (2.1W, Figure 4b) was of the same size as the sliced but contained very few LAB ($10^3 - 10^4$ cfu/g). The second batch from the same smokehouse differed from the first batch in that sliced salmon (2.2S) spoiled with 10^8 cfu/g LAB and whole fillets (2.2W) with a mixture of bioluminescent bacteria and LAB totalling 10^6 cfu/g (data not shown).

The microflora on whole fillets from plant No. 3's second batch reached higher final levels (10^8 cfu/g) on 3.2W (Figure 4d) with LAB, than the flora on sliced salmon did (10^7 cfu/g, Figure 4c). Sliced salmon from the first batch (3.1S) was similar to the second, whereas the microflora on whole fillets (3.1W) contained lower numbers (10^6 cfu/g) and large variations between double determinations (data not shown).

The variation in size and composition of the microflora within a vacuum pouch was further analyzed by sampling the same pack 3 times at different spots with subsequent separate analyses. This local variation in microbiological load was largest for whole fillets with standard deviations of up to $2 \text{ Log}_{10}(\text{cfu/g})$ on all agar counts and only $0.1-0.5 \text{ Log}_{10}(\text{cfu/g})$ for sliced salmon. Also composition of the flora determined with the 5 agar substrates varied considerably on whole sides but not on sliced salmon.

Bioluminescent strains of *Photobacterium phosphoreum* and some marine vibrio are known to be heat sensitive and to occur in fish products (Baumann & Baumann, 1984; van Spreckens, 1987; Dalgaard *et al.*, 1993). Presence of these bacteria were indicated in samples, where counts on TPC were much higher than on TVC/TSA (Figures 3a & 4c). Screening of selected TPC plates showed presence of bioluminescent bacteria on salmon from plant No. 2's second batch in levels of *ca.* 5×10^6 cfu/g and *ca.* 3×10^5 cfu/g on sliced and whole salmon after 38 days, respectively. Bioluminescent isolates from plant No. 1 (1.1S, Figure 3a) were characterised as large Gram-negative and oxidase-negative coccobacilli. No bioluminescent bacteria were found on TPC from other production batches, but this may be due to the limited screening. Non-bioluminescent strains of these organisms occur frequently in fish (Dalgaard, 1995) and may not have been detected in this study.

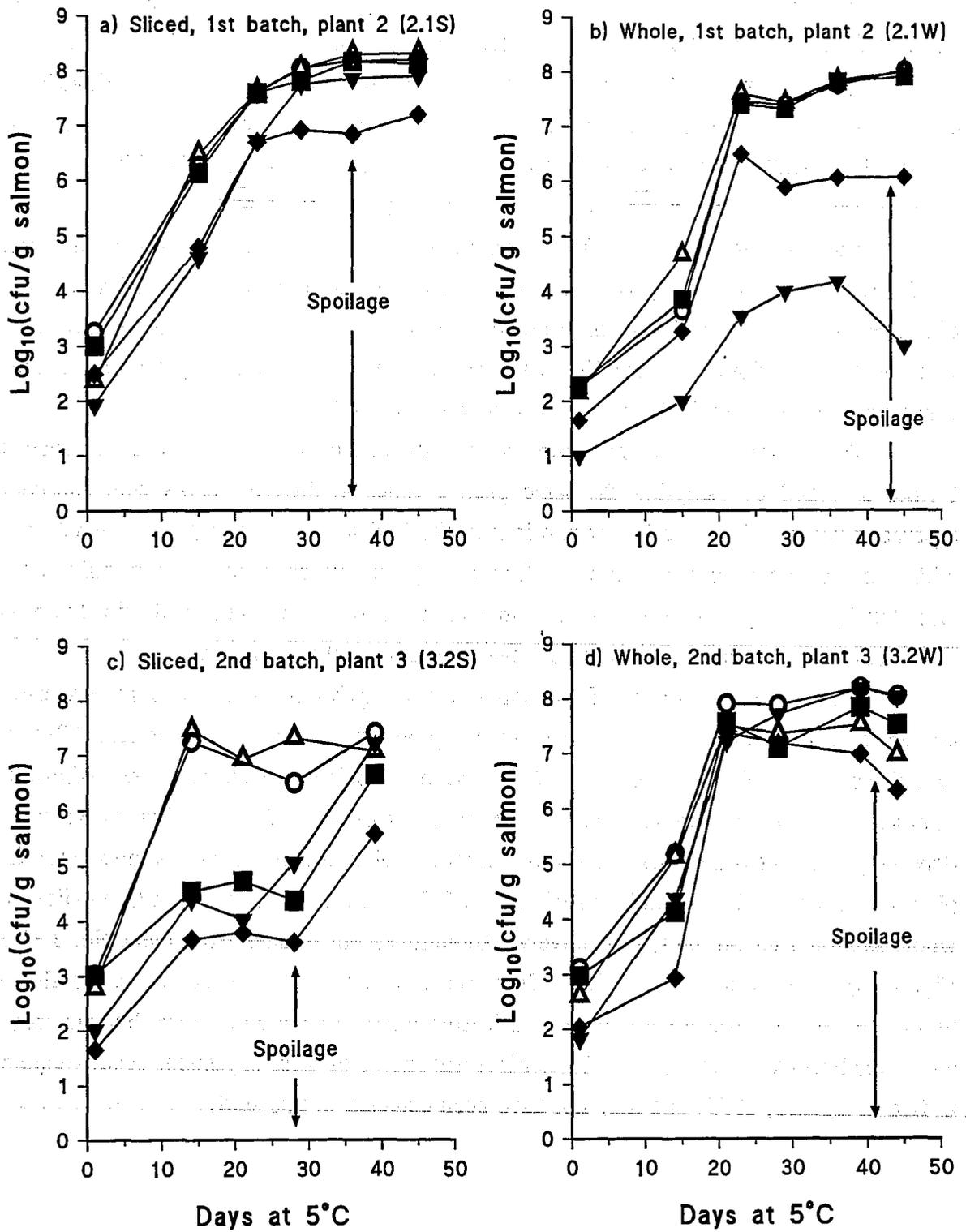


Figure 4a-d. Development of the microflora during storage of cold-smoked salmon from plant No. 2 and plant No. 3. ■ - TVC; △ - TPC; ▼ - NAP; ◆ - VRBG and ○ - TSA.

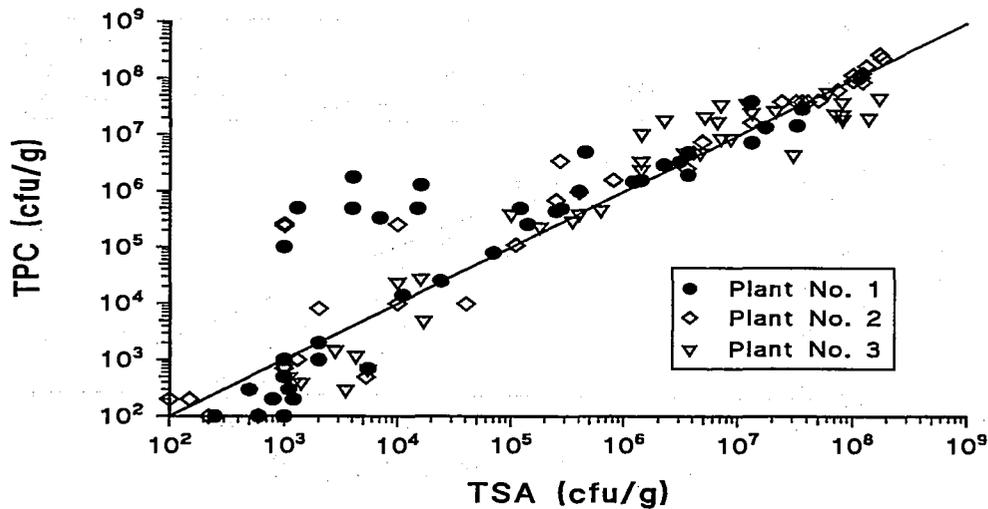


Figure 5. Relationship between TPC and TSA plate counts from the 3 smokehouses.

Comparison of the microflora from the smokehouses using the Malthus results.

It is clear from Figure 5 showing the relationship between total psychrotrophic counts (TPC) and total viable counts on TSA, that TPC allowed for enumeration of heat sensitive bacteria. At counts below 10^3 cfu/g TPC tended to underestimate the numbers of bacteria. However, TPC was preferred in the analysis of the relationship between total bacterial numbers as measured by traditional plate counts and TVC-Malthus, because TPC gave a more accurate estimate of the numbers of bacteria in the majority of the samples.

Figure 6a shows the linear relationships between $\text{Log}_{10}(\text{TPC})$ and the detection time (DT) for the TVC-Malthus method from all 3 smokehouses with Pearson correlation coefficients (r) between -0.88 and -0.95. For the relationship between TPC and TVC-Malthus for all samples from each plant pooled, it was found that the linear regression equation describing the microflora on smoked salmon from plant No. 1, was significantly different ($P < 0.001$ for slope and $P < 0.05$ for intercept) from the equations describing the flora on salmon from plants No. 2 and No. 3, indicating a difference in the microflora between plant No. 1 on one side and plants No. 2 & 3 on the other side. Similarly the regression equations for DT *Enterobacteriaceae*-Malthus and VRBG counts showed that the Gram-negative (*Enterobacteriaceae*) flora from plant No. 2 was significantly ($P < 0.05$) different from the Gram-negative microflora on plants No. 1 & 3 (Figure 6b). Combined, these results strongly suggested that the microflora on cold-smoked salmon from the 3 smokehouses were different from one-another.

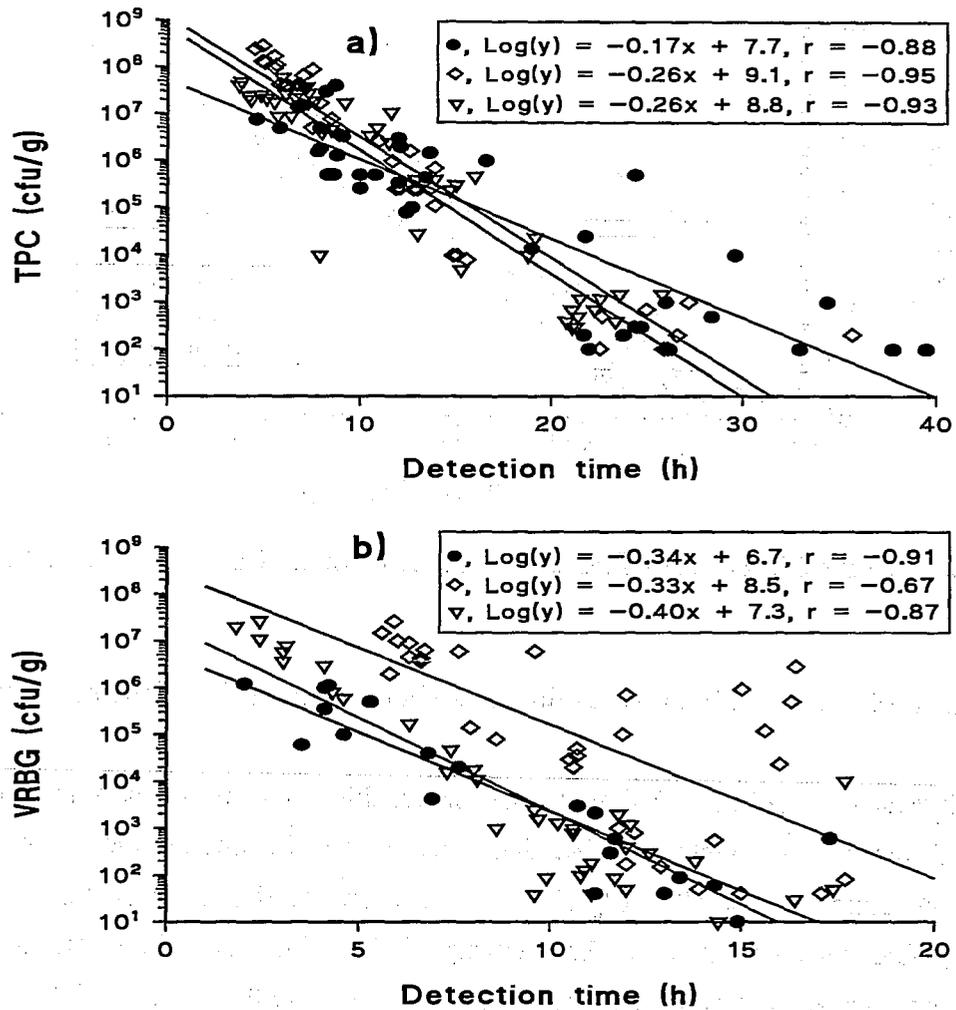


Figure 6. Calibration curves between a) Malthus detection time (DT) and TPC or b) DT and VRBG in cold-smoked salmon (all treatments). ●, Plant No. 1; ◇, Plant No. 2; ▽, Plant No. 3.

Further data analysis revealed that the regression lines for TPC and DT (TVC-Malthus) for whole and sliced salmon were significantly different from one-another on salmon from plants No. 1 and 3 but not from No. 2 (Table 2). This suggested that the microflora on sliced salmon from plants No. 1 & 3 was different from the flora found on the corresponding whole fillets. The data from whole fillets contained more variation, which could not be described by the simple linear model. The linear regression correlation coefficients from sliced salmon were therefore always higher ($r_{\text{sliced}} = [| 0.921; 0.980 |]$) than those for unsliced salmon ($r_{\text{whole}} = [| 0.807; 0.906 |]$, Table 2). Larger variation among duplicate samples from wholes sides were also seen in the results from the plating methods (data not shown).

Table 2. Linear regression between Malthus detection time for total psychrotrophic counts (TPC) and DT for whole fillets and sliced cold-smoked salmon from 3 smokehouses. (Model: $\text{Log}_{10}(\text{TPC}) = \beta_i \times \text{DT} + \alpha_i + \varepsilon$)

Plant	Sliced/ Whole	Slope (β)	Intercept (α)	r^a	T_β^b	DF ^c
1	S	-0.2022	8.1633	-0.9210	2.4667*	36
	W	-0.1282	6.9527	-0.8068		
2	S	-0.2626	9.1839	-0.9634	1.0332 ^{NS}	38
	W	-0.2373	8.5758	-0.9043		
3	S	-0.3156	9.5582	-0.9795	3.2528**	42
	W	-0.2231	8.2461	-0.9059		

a) r - Correlation coefficient; b) T_β - calculated t-value using the test for homogeneity of slopes; c) DF - degrees of freedom; * - significant at 5% level; ** - significant at 1% level; NS - not significant.

Discussion.

Smoked salmon spoil as a result of microbiological activity (Truelstrup Hansen *et al.*, 1995b), and the shelf life extension found for whole fillets in comparison with the sliced salmon will be related to factors affecting growth of microorganisms. In general, lower bacterial counts were found on whole fillets. The surface of sliced salmon is contaminated with the in-house flora through processes like skinning, slicing etc. The relatively large surface area of sliced product provides more growth space per g than in the intact salmon tissue. Also, more nutrients will be readily available from the cut surfaces with ruptured cells than from the smoked surface on fillets.

The sensory characteristics at the time of rejection for whole fillets and sliced salmon were similar with regard to odour and flavour. The judges' description of the gradual quality loss resembled descriptions previously reported (Cann *et al.*, 1984; Truelstrup Hansen *et al.*, 1995a). However, whole fillets with low microbial numbers were frequently rejected on the basis of textural deterioration and shelf life of cold-smoked salmon with low numbers of microorganisms may therefore be determined by the degree of autolytic texture degradation as suggested by Truelstrup Hansen *et al.* (1995b).

The shelf life of sliced cold-smoked salmon was at least 21 days and independent of

salt levels ranging from 4.1 to 6.1% in the water phase (SWP) (Table 1). It may however, be difficult to compare salmon produced by different smokehouses because a number of important factors such as packaging material, production method, size and composition of initial flora, numbers of freezing and thawing cycles and quality of raw material, varied and may thus explain why salmon with 4.1 and 6.1% SWP had comparable shelf lives. Shelf life has previously been found to increase when salt levels was increased from 2.2% to 4.6% SWP in smoked salmon otherwise produced under identical conditions (Truelstrup Hansen *et al.*, 1995a).

The rancid off-flavours seemed to be related to initial presence of slight rancidity in freshly produced salmon, gas permeability of the packaging material and the relative size of meat surface, which may explain differences between rancidity in whole fillets and sliced salmon from the same batch, and these factors of importance for development of rancidity in cold-smoked salmon have not been studied systematically to our knowledge, but a preliminary study indicated that significant lipid hydrolysis occurs during chill storage of cold-smoked salmon (unpublished data).

The microbiological patterns found in this study covered the range of different microflora reported to develop on cold-smoked salmon (Cann *et al.*, 1984; Schneider & Hildebrandt, 1984; Truelstrup Hansen *et al.*, 1995a,b,c). The microflora varied among products from the 3 smokehouses, but large variations were also seen in salmon from the same smokehouse (Figure 3). Characterization of representative isolates from spoiled cold-smoked salmon from 3 different smokehouses revealed similar differences (Truelstrup Hansen *et al.*, 1995c), and similar batch to batch variations have been found in vacuum packed red meat products (Hitchener *et al.*, 1982; Borch *et al.*, 1988, Samelis *et al.*, 1994).

LAB, psychrotrophs (marine vibrio and *Photobacterium* spp.) and *Enterobacteriaceae* all appeared in numbers, where they could be considered to be of importance, at the time of spoilage. Plate counts on Iron agar (TVC) tended to be lower than on TSA (Figure 3b; Figures 4c & d). There is no obvious explanation since typical bacteria including LAB from smoked salmon normally thrive in both Iron agar and TSA (unpublished data). H₂S-producing bacteria were only detected occasionally in Iron Agar (data not shown), indicating such bacteria played no part in the spoilage. Heat sensitive marine vibrio and *P. phosphoreum* have previously been found on cold-smoked salmon (Truelstrup Hansen *et al.*, 1995b), and it appears that *Photobacterium* spp. and/or marine vibrio occur regularly on vacuum packed smoked salmon. *P. phosphoreum* has been associated with spoilage of packed cod fillets (Dalgaard *et al.*, 1993) and histamine production in vacuum packed lightly salted mackerel (van Spreekens, 1987), which could be of potential concern.

Calibration curves for TPC and DT (TVC-Malthus) and for VRBG and DT (*Enterobacteriaceae*-Malthus) revealed that the microflora on salmon from the 3 different smokehouses were different from one-another, and best described by 3 separate sets of calibration curves. Gibson & Ogden (1987) found different regression lines in repeated experiments with cod and smoked trout under different packaging conditions (vacuum and modified atmosphere) and concluded, this was due to prevalence of different microorganisms. Several researchers have suggested the use of different electrometric methods for the rapid estimation of bacteriological quality of fish products (Gibson *et al.*, 1984; Gibson, 1985; Ogden, 1986; van Spreckens & Stekelenburg, 1986; Huss *et al.*, 1987; Ravn Jørgensen *et al.*, 1988). In the present study it was found that the variability in the microflora and thus the Malthus responds rendered the use of standard Malthus methods difficult.

Several of the processing steps following cold-smoking may cause intensive exposure of the salmon to the in-house flora (Figure 1) and influence the composition and development of the microflora on the finished product. This may in part explain the differences found between the microflora on whole and sliced salmon from plants No. 1 & 3 (Figures 3 & 4a-b, Table 2). Borch *et al.* (1988) found the successful introduction of in-house originating spoilage bacteria through contamination on a processing line for emulsion sausages to be highly dependant upon the competitiveness of the surviving indigenous microflora after the heat process. Such differences in competitiveness between the indigenous flora on the raw material and the in-house microflora may explain part of the reason for the highly variable microflora found on cold-smoked salmon.

Conclusion.

The observed differences among the microflora isolated from products from three different smokehouses and production batches indicated that no typical microflora for cold-smoked salmon exists. The microflora is highly variable and partially influenced by the production environment in the smokehouse. The Malthus' standard methods could not be used to determine the total viable count and *Enterobacteriaceae* on cold-smoked salmon, when calibrated to the traditional agar methods, without specific and frequent calibrations for each smokehouse.

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Activity of potential spoilage bacteria from cold-smoked salmon.

by

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Abstract.

The growth and metabolism of 6 lactic acid bacteria (LAB), 4 *Enterobacteriaceae* and one strain of *Photobacterium phosphoreum* isolated from spoiled cold-smoked salmon were studied during growth in sterile salmon juice incubated in 100% N₂ or on vacuum packed salmon block incubated at 5°C for 15 and 20-21 days, respectively. *Lactobacillus sake* 1T18, *Lactobacillus curvatus* 3N6, *Carnobacterium* sp. 2T2, *Serratia liquefaciens* 2R4 and *Photobacterium phosphoreum* P66 all grew and produced hypoxanthine (Hx) and acetic acid in salmon juice but at different rates and cell concentrations. No trimethylamine (TMA) and only small amounts of acetic acid and Hx were produced on muscle blocks with cell counts of less than 10³ cfu/g. Forty-eight % of all salmon blocks were contaminated with indigenous bacteria mainly of the *Photobacterium*/marine vibrio type. *P. phosphoreum* grew to final levels of 10⁶ cfu/g but were up to 100 times more metabolically active per cell than any other test organisms, indicating a high spoilage potential at lower cell concentrations. The lactics grew to final levels of 10⁸ cfu/g and produced off-odours resembling those found on smoked salmon. Two LAB strains produced cabbagey off-odours and *Lb. sake* 1T18 was shown to produce H₂S. Growth of the *Enterobacteriaceae* on contaminated salmon blocks were affected by indigenous bacteria, *Hafnia alvei*, however, colonized the salmon with the production of stale, nauseous and fruity off-odours.

Introduction.

Cold-smoked salmon is a lightly preserved fish product and will ultimately spoil due to bacteriological activity during chill storage (Truelstrup Hansen *et al.*, 1995b). Among the microorganisms developing on the product during storage are: Lactic acid bacteria, *Brochothrix thermosphacta*, *Enterobacteriaceae* (Cann *et al.*, 1984; Schneider & Hildebrandt, 1984; Jakobsen *et al.*, 1988, Truelstrup Hansen *et al.*, 1995a, b & d), marine vibrio and *Photobacterium phosphoreum* (Truelstrup Hansen *et al.*, 1995b). Considerable variations have been found among the microflora on products from different smokehouses (Truelstrup

Hansen *et al.*, 1995c & d). It has therefore not been possible to establish one general microbiological mechanism for the spoilage of cold-smoked salmon.

The spoilage potential amongst bacteria isolated from spoiled foods have been evaluated in several model systems under conditions resembling the authentic products as much as possible. Off-odour production has been screened in sterilized juice made from the various food products in question (Castell & Anderson, 1948; Lerke *et al.*, 1963; Gram *et al.*, 1987; Jeppesen & Huss, 1993), while other researchers have used inoculation of bacteria onto products with no or reduced loads of indigenous microorganisms and subsequently monitored production of metabolites and off-odours (Herbert *et al.*, 1971; Dainty *et al.*, 1989; Borch & Agerhem, 1992; Dalgaard *et al.*, 1993). Microbiological spoilage potential can also be evaluated by quantitative measurement of the organisms' metabolic activity, and this approach in the form of yield factors was used by Dalgaard (1995) to show that *P. phosphoreum* spoiled vacuum and modified atmosphere packed cod fillets.

In this study, selected strains previously isolated from spoiled cold-smoked salmon and characterised (Truelstrup Hansen *et al.*, 1995b & c) were evaluated with regard to their spoilage potentials during growth in sterile salmon juice incubated anaerobically or on vacuum packed cold-smoked salmon. Microbiological, chemical and sensory changes were monitored during storage at 5°C.

Material and methods.

A number of model experiments with isolates from previously described storage experiments (Truelstrup Hansen *et al.*, 1995b & c) were carried out in salmon juice or on muscle blocks made from cold-smoked salmon prepared with reduced loads of microorganisms.

Preparation of model systems.

Preparation of salmon juice.

Salmon juice was made from salmon fillets (*Salmo salar*) and tap water in the ratio 2:1. Briefly described, the process involved following steps: salmon was boiled with water for 10 min, the resulting juice filtered through a standard coffee filter and the remaining tissue fluid expressed manually in a filter press and then filtered. Most of the lipid was discarded with the coffee filters, resulting in less than 3% lipid in the juice. The juice was

buffered with 0.1 M phosphate buffer (44 mmol/l KH_2PO_4 and 56 mmol/l K_2HPO_4 (Merck, Darmstadt, Germany)), and pH adjusted to 6.2 with 0.1 M HCl and salt (4.3 % w/v) was added to match levels found in cold-smoked salmon. After heat treatment for 30 min at 100°C, the juice was added 5 mmol/l inosine (Sigma, MO, USA) and 7 mmol/l TMAO (Sigma, 10 mg-N/100 ml) from filter sterilised stock solutions to compensate for dilution and heat decomposition.

Preparation of sterile salmon blocks.

Whole gutted salmon (*Salmo salar*) were obtained fresh from Norway and filleted with knives and cutting boards sanitised in ethanol (70% v/v) to decrease the initial microbial load on the fillets. The unskinned fish were dry salted with crystalline salt on the flesh sides and left to equilibrate for 4 h, after which the salt was removed with tap water. The salmon were left for 14-18 h at 2°C before being dried (2 h at 24°C, 54% rh) and cold-smoked (4 h at 22°C, rh 60%). After smoking, the fillets were aseptically cut into muscle blocks without skin of approximately 50-75 g each. The muscle blocks were vacuum packed in a high barrier material (Riloten/x 40/50 Conovac) from Otto Nielsen Emballage A/S, Lyngby, Denmark with the permeability for O_2 of 1.0 cm^3/m^2 and for CO_2 of 4.0 cm^3/m^2 in 24 h, 1 atm at 23°C, 95% rh. The samples were stored at -30°C until beginning of the model experiments.

Bacterial cultures.

The test organisms were isolated from spoiled vacuum packed cold-smoked salmon and tentatively identified as previously described (Truelstrup Hansen *et al.*, 1995b & c). Table 1 lists the strains, their origin and performed model experiments.

Preparation of inoculum.

Isolates were stored at -80°C in the freezing media of Gibson & Khoury (1986) and subcultured twice in salmon juice at 10°C for 48 h before inoculation. Numbers of cells in the second subculture were estimated by phase contrast microscopy under the assumption that one cell per field equals 5×10^5 cfu/ml. Appropriate dilutions were made in Peptone Saline (PS, 0.1% (w/v), Bacto Peptone [Difco, Detroit, MI, USA] and 0.9% NaCl (w/v)). Identity and purity of strains were confirmed by streaking strains out on either Tryptone Soy Agar

Table 1. Overview of performed model experiments.

Strain	Origin ^a	Group/Cluster ^b	Model experiments
<i>Lactobacillus saké</i> 1T18	Screening experiment (plant No. 1)	Group A (LAB cluster IB)	Salmon juice, salmon blocks, production of H ₂ S.
<i>Lactobacillus curvatus</i> 3N6	Screening experiment (plant No. 2)	Group B	Salmon juice
<i>Carnobacterium</i> spp. 2T2	Screening experiment (plant No. 1)	Group E	Salmon juice
<i>Lactobacillus saké/bavaricus</i> S1.11	Storage experiment (plant No. 1)	LAB Cluster IB	Salmon blocks
<i>Lactobacillus</i> sp. (homofermentative) R1.12	Storage experiment (plant No. 2)	LAB Cluster II	Salmon blocks
<i>Lactobacillus curvatus</i> N1.4	Storage experiment (plant No. 3)	LAB Cluster IA	Salmon blocks
<i>Serratia liquefaciens</i> 2R4	Screening experiment (plant No. 1)	Ent. Cluster IIB	Salmon juice, salmon blocks
<i>Hafnia alvei</i> S2.4	Storage experiment (plant No. 1)	Ent. Cluster IIA	Salmon blocks
<i>Enterobacter agglomerans</i> R3.3	Storage experiment (plant No. 2)	Ent. Cluster I	Salmon blocks
<i>Serratia liquefaciens</i> N1.6	Storage experiment (plant No. 3)	Ent. Cluster IIB	Salmon blocks
<i>Photobacterium phosphoreum</i> P66	Autolysis experiment (dry salted, plant No. 1)	-	Salmon juice, salmon blocks

a) Details on isolation, characteristics and tentative identity of strains in Truelstrup Hansen *et al.* (1995b & c); b) Groups and clusters refer to study by Truelstrup Hansen *et al.* (1995c).

(TSA, Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) for Gram-negatives or All Purpose Tween agar (APT, Difco) for Gram-positives, phase contrast microscopy and appropriate biochemical tests (Gram reaction, morphology, catalase, ability to bioluminesce, fermentation patterns etc.) using the techniques described by Truelstrup Hansen *et al.* (1995b & c).

Inoculation, incubation and sampling.

Growth in salmon juice.

Each of the test organisms (Table 1) were inoculated in two screw cap flasks with 225 ml salmon juice at levels of approximately 10⁴ cfu/ml. The flasks were incubated in

anaerobic jars (Oxoid) in 100% N₂ at 5°C for 15 days. Samples were withdrawn regularly from the stationary cultures and analyzed microbiologically and chemically (nucleotides and acetic acid). The anaerobic conditions were restored immediately following the sampling with 100% N₂ and incubation at 5°C continued. Two uninoculated flasks receiving identical treatment served as control samples. The production of off-odour was assessed after 15 days by 3 judges.

Growth on salmon muscle blocks.

The test organisms (Table 1) were diluted in PS, and 2 portions of 0.05 - 0.1 ml of an appropriate dilution for achieving an inoculum of approximately 10³ cfu/g salmon were injected onto the surface of vacuum packed salmon muscle blocks through two membranes. The vacuum pack was massaged to obtain an even distribution of the inoculum. This procedure was found to give a satisfactory distribution in a preliminary experiment, where 2 portions of 0.05 ml of an indicator colour (0.5% (w/v) Victoria Blue B, Sigma) were injected onto 75 g vacuum packed salmon blocks to visualize the distribution. The salmon was incubated for 20 to 21 days at 5°C, and two vacuum packs per test organism were withdrawn regularly and analyzed with microbiological and chemical methods (nucleotides, organic acids and volatile bases) and for production of off-odours. At each sampling day, 3 non-inoculated vacuum packs were withdrawn, analyzed and served as controls. Approximately 50% of these control samples were, however, found to support growth of more than 10³ cfu/g and the samples were therefore discarded and the analytical results not used. This experiment was performed twice for *P. phosphoreum* P66.

Microbiological analysis.

Salmon juice.

Microbiological counts were performed by mixing 1 ml salmon juice aseptically sampled from the thoroughly mixed screw cap flask with 9 ml PS. Appropriate dilutions were plated onto spread plates with TSA, which were incubated aerobically at 25°C for 3 days or at 10°C for 5 days (*P. phosphoreum* P66) and then counted. Purity of strains were checked throughout the incubation period on TSA plates and with direct phase contrast microscopy.

Salmon muscle blocks.

Microbiological analyses were performed by mixing 10 g salmon sampled from all parts of vacuum packs to obtain a representative sample with 90 ml PS for 30 s in a Stomacher 400 Lab Blender (A. J. Seward, Bury, St. Edmunds, UK). For all bacteria and uninoculated samples, spread plates were made from TSA and counted after 5 days at 10°C for total psychrotrophic counts (TPC). Lactic acid bacteria strains were enumerated on the selective agar Nitrite Actidion Polymyxin agar (NAP) with pH 6.7 (Davidson & Cronin, 1973; Truelstrup Hansen *et al.*, 1995a) incubated for 3 days at 25°C, after which typical colonies were counted. Violet Red Bile Glucose agar (VRBG, Oxoid) with bottom layer of TSA was used to enumerate *Enterobacteriaceae* strains and after 2 days at 25°C, typical colonies with a violet precipitation zone were counted. An estimate of the heat sensitive *P. phosphoreum* was obtained by subtracting pour plate counts in TSA (3 days at 25°C) from TPC. All samples were also examined by phase contrast microscopy on 10⁻¹ dilutions. The tentative identity of the bacteria growing on the salmon blocks was monitored on isolates from TPC plates using the criteria mentioned above.

Sensory analysis.

Production of off-odours by the inoculated bacteria was evaluated in relation to uninoculated samples by 2-3 judges. The uninoculated samples served as references. Approximately 10 g salmon or 5 ml salmon juice was incubated in petri dishes at room temperature (21-23°C) for 30 min. The judges described the presence, characteristics and intensity of off-odours in the samples as compared to the reference samples with their own words.

Chemical analysis.

Salt and dry matter.

Salt analysis were performed using the AOAC (1975) method on salmon homogenates or salmon juice. Salt content was expressed as % NaCl in water phase of salmon. For determination of dry matter content, approximately 2 g salmon (± 0.0001 g) was weighed and dried at 105°C for 20-24 h (until constant weight was obtained).

Nucleotides.

The HPLC method of Murray *et al.* (1985) was used on neutralised and filtered perchloric acid (PCA) extracts prepared from 20 g salmon and 40 ml 0.6 M PCA or 10 ml salmon juice and 15 ml 0.6 M PCA. Unknown peaks were identified and quantified externally with freshly prepared standards from Sigma.

Organic acids.

Acetic acid in salmon juice was determined enzymatically with the kit from Boehringer Mannheim (Biochemica, Mannheim, Germany) on the neutralised and filtered PCA extract following the manufacturer's instruction. Organic acids in salmon muscle blocks were determined on PCA extracts with the HPLC method described by Truelstrup Hansen *et al.* (1995b) and unknown peaks were identified and quantified externally with appropriate standards.

Total volatile bases (TVN), trimethylamine (TMA) and trimethylamine oxide (TMAO).

Conway and Byrne's (1933) microdiffusion method was performed on PCA extracts prepared from salmon muscle blocks.

Calculation of yield factors.

The maximum cell concentration (N_{\max}) in the salmon juice experiment was calculated with the 3 parameter logistic model using the curve fitting programme FIGP ver. 6.0. (Fig.P Software Corporation, Durham, NC, USA):

$$\text{Log}(N(t)) = \text{Log}\left(\frac{N_{\max}}{1 + \text{EXP}(-\mu_{\max}(t - t_i))}\right)$$

- where:
- $N(t)$ - cell concentration (at time t), cfu/ml
 - N_{\max} - maximum cell concentration (stationary phase)
 - μ_{\max} - maximum specific growth rate, hours⁻¹ (exponential phase)
 - t_i - time at inflection point, hours

The yield factor is calculated from the equation:

$$Y_{\text{Metabolite}} = \frac{([\text{Metabolite}]_{\text{Final}} - [\text{Metabolite}]_{\text{Initial}})}{(\text{Cfu}_{\text{Final}} - \text{Cfu}_{\text{Initial}})}$$

where: $[\text{Metabolite}]_{\text{Final or Initial}}$ - Concentration of metabolite at N_{max} and N_{min}
 $\text{Cfu}_{\text{Final or Initial}}$ - N_{max} and N_{min}

(After Dalgaard, 1993 & 1995)

Production of volatile sulphur compounds by *Lactobacillus sake* (1T18).

The strain was qualitatively examined for production of volatile sulphur compounds during growth on cold-smoked salmon using the GC method of Dalgaard *et al.* (1993). The strain was inoculated on vacuum packed cold-smoked salmon with reduced loads of microorganisms as described above, at levels of approximately 10^6 cfu/g and stored at 15°C for 18 h and at then 10°C for 4 days. Ten g salmon sample was weighed into a 50 ml Pyrex flask with a membrane stopper and equilibrated at 25°C for 30 min, before 1 ml of the headspace was injected onto the GC column. Uninoculated salmon from the same production batch were incubated and analyzed under the same conditions and served as control samples. Production of off-odours was assessed by two experienced judges.

Results.

Growth and metabolism in salmon juice.

The inoculated bacteria reached final levels of 10^8 cfu/ml for the lactics (*Lb. sake* 1T18, *Lb. curvatus* 3N6 and *Carnobacterium sp.* 2T2), 10^9 cfu/ml for *S. liquefaciens* 2R4 and only 10^6 cfu/ml for *P. phosphoreum* P66 after growth in salmon juice at 5°C and 100% N_2 for 15 days (Figure 1a).

P. phosphoreum P66 had the highest production of both acid acid and hypoxanthine (Hx), reaching maximum levels of $7.7 \mu\text{mol/ml}$ acetic acid and $6.3 \mu\text{mol/ml}$ Hx (Figure 1b & c). Among the lactic acid bacteria (LAB), 1T18 produced the highest maximum levels of $5.5 \mu\text{mol/ml}$ acetic acid and $4.2 \mu\text{mol/ml}$ Hx, while the other LAB produced much lower levels. *S. liquefaciens* 2R4 produced maximum levels of $7.3 \mu\text{mol/ml}$ acetic acid and

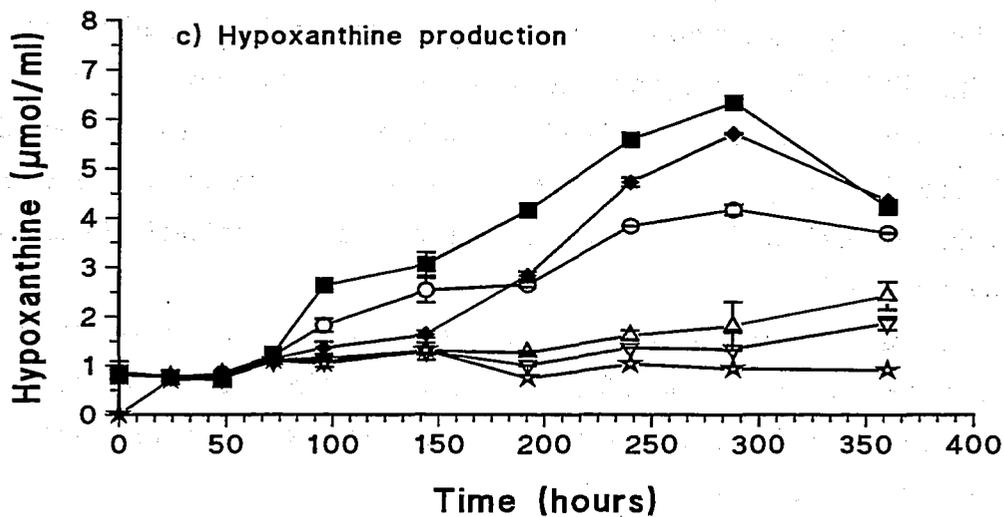
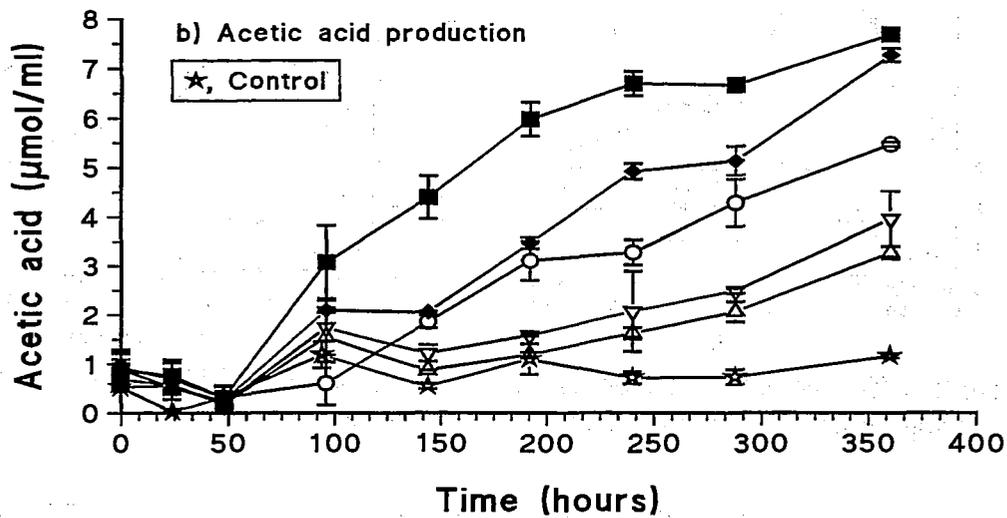
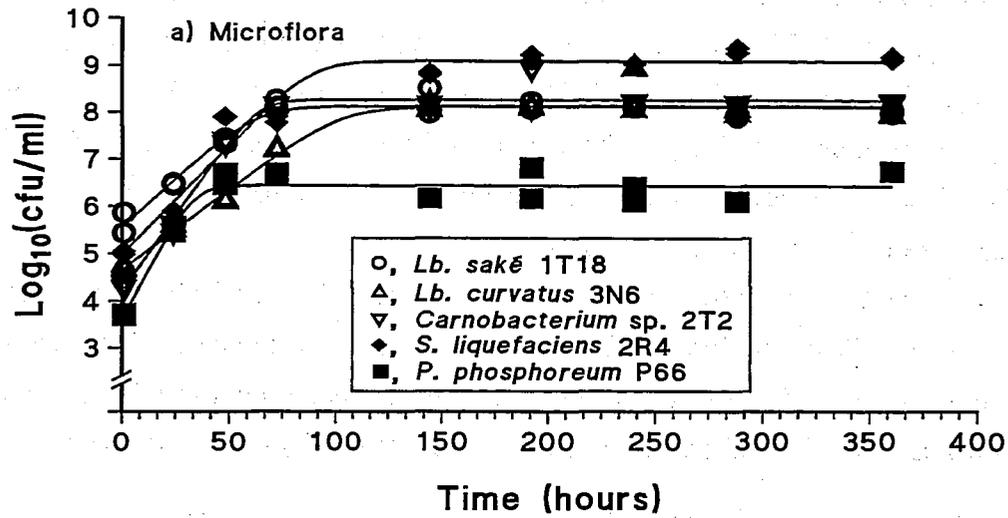


Figure 1. Growth and metabolism of 5 bacteria in salmon juice at 5°C for 15 days. a) Development of microflora; b) Production of acetic acid and c) hypoxanthine. Error bars are SD (n-1).

5.7 $\mu\text{mol/ml}$ Hx. Levels of acetic acid increased with time in all samples except the sterile control sample, while Hx levels increased in all samples except the control sample until day 12 (288 h), after which the Hx level in P66 and 2R4 fell (Figure 1b & c). This decrease in Hx may be explained by further degradation of Hx into xanthine and uric acid by the bacteria. The stable levels of Hx and acetic acid in control samples showed autolytic enzymes were denatured during the preparation of juice.

S. liquefaciens 2R4 and *P. phosphoreum* P66 actively converted inosine monophosphate (IMP) into inosine (Ino) as well as Ino into Hx (Figure 2), whereas the LAB strains only attacked Ino (data not shown). There were only trace levels of adenosine monophosphate (AMP) and no adenosine diphosphate (ADP) present in the salmon juice

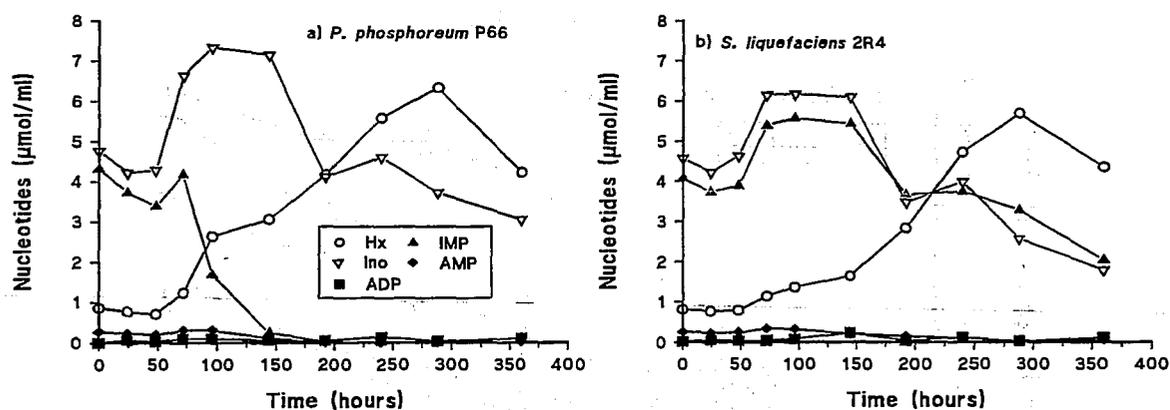


Figure 2. ATP-nucleotide metabolism in a) *P. phosphoreum* P66 and b) *S. liquefaciens* 2R4 during anaerobic growth in salmon juice at 5°C.

In Table 2, the logarithms of yield factors for acetic acid ($\text{Log}(Y_{\text{Ace}})$) and Hx production ($\text{Log}(Y_{\text{Hx}})$) are shown. For LAB, values for $\text{Log}(Y_{\text{Ace}})$ ranged between -7.49 and -7.76 with 1T18 having the highest value and thus the highest activity per cell. The same trend was found for $\text{Log}(Y_{\text{Hx}})$. *S. liquefaciens* 2R4 had yield factors for acetic acid and Hx of -8.31 and -8.42, respectively. The yield factors for *P. phosphoreum* P66 were -5.61 for acetic acid and -5.71 for Hx, and each P66 cell was thus at least 75-500 times more active than any other strain.

All strains caused production of off-odours after the 15 days of incubation. The Gram-negative *P. phosphoreum* P66 and *S. liquefaciens* 2R4 produced ammonia and amine-like odours, whereas the LAB produced sour, astringent and sweet/burnt odours. *Lb. sake* 1T18 also produced fruity off-odours.

Table 2. Apparent yield factors for acetic acid and hypoxanthine and N_{max} estimated from the 3-parameter logistic model for growth.

Strains	Acetic acid $\text{Log}(Y_{Ace})^a$	Hypoxanthine $\text{Log}(Y_{Hx})^a$	N_{max} cfu/ml	r^2 (model)
<i>Lb. saké</i> 1T18	-7.49	-7.63	1.4×10^8	0.97
<i>Lb. curvatus</i> 3N6	-7.71	-7.94	1.4×10^8	0.97
<i>Carnobacterium</i> sp. 2T2	-7.76	-8.26	1.9×10^8	0.98
<i>S. liquefaciens</i> 2R4	-8.31	-8.42	1.3×10^9	0.95
<i>P. phosphoreum</i> P66	-5.61	-5.71	2.8×10^6	0.92

a) $\text{Log}_{10}(\mu\text{mol}/\text{cfu})$

Growth and metabolism on salmon blocks.

The salmon blocks contained $64.6\% \pm 2.1\%$ (SD) water and $3.3\% \pm 0.3\%$ salt in the water phase. Production of blocks with a reduced load of microorganisms was difficult and 48% of the uninoculated control samples had to be discarded because of growth of the indigenous microflora to levels higher than 10^3 cfu/g (Table 3).

Table 3. Development of volatile bases, acetic acid and breakdown products from ATP-nucleotides during storage of vacuum packed cold-smoked salmon with reduced loads of microorganisms (control samples) at 5°C for 21 days. (Averages of samples with less than 10^3 cfu/g)

Day	No. samples	TVB ^{a)} mg-N/100 g	TMA ^{b)} mg-N/100 g	TMAO ^{c)} mg-N/100 g	Acetic acid $\mu\text{mol}/\text{g}$	Hx ^{d)} $\mu\text{mol}/\text{g}$	IMP ^{e)} $\mu\text{mol}/\text{g}$	Ino ^{f)} $\mu\text{mol}/\text{g}$
0	3 (3) ^{g)}	16.5	0.6	17.5	3.2	1.4	1.1	6.6
3	3 (3)	17.4	0.3	15.7	1.4	1.4	0.8	6.3
7	1 (3)	19.0	1.9	15.7	2.9	1.6	0.2	5.7
10	2 (3)	18.4	1.1	15.3	3.8	1.8	0.1	5.5
14	2 (3)	22.7	1.0	14.4	2.6	2.1	0.1	6.5
16	1 (3)	24.8	1.0	8.2	2.0	2.2	0.3	6.9
21	3 (6)	22.0	1.4	17.0	6.2	2.3	0.1	6.3

a) TVB - total volatile bases; b) TMA - trimethylamine; c) TMAO - trimethylamine oxide; d) Hx - hypoxanthine; e) IMP - inosine monophosphate; f) Ino - inosine; g) Total number of uninoculated vacuum packs sampled (3) of which 3 contained less than 10^3 cfu/g.

Microscopy and tentative characterization of colonies from TPC plates revealed that the naturally occurring microflora mainly consisted of marine vibrio including

Photobacterium spp. and occasionally, LAB. These organisms reached final numbers of 10^6 cfu/ml for former group and 10^8 cfu/g for the latter group on contaminated control samples (data not shown).

Reduction of microbiological numbers to levels below 10^3 cfu/g on control samples largely prevented the production of TVB and TMA and the corresponding degradation of TMAO (Table 3). Levels of acetic acid only increased slightly but inconsistently during 21 days of storage. Hx rose from initial $1.4 \mu\text{mol/g}$ to $2.3 \mu\text{mol/g}$ after 21 days, while Ino remained almost constant and IMP decreased from $1.1 \mu\text{mol/g}$ to $0.1 \mu\text{mol/g}$. These chemical changes were assumed to be autolytic, since the microbiological population remained lower than 10^3 cfu/g. Also, there were no production of off-odours on salmon blocks with reduced levels of microorganisms.

P. phosphoreum P66 inoculated on salmon blocks rapidly grew from initial levels of 10^3 - 10^4 cfu/g to final levels of 10^6 cfu/g, and growth was closely followed by degradation of TMAO and the production of TMA (Figure 3a & b). Hx increased from initial concentrations of $1.1 \mu\text{mol}$ to 4.4 - $4.8 \mu\text{mol/g}$, while concentrations of Ino (not shown) dropped correspondingly. Levels of acetic acid rose from $2 \mu\text{mol/g}$ to final levels of 30 - $35 \mu\text{mol/g}$. *P. phosphoreum* P66 caused production of characteristic amine- and ammonia-like off-odours in both experiments and in addition words like fishy, sour, astringent and old "storage room" were used. *P. phosphoreum* P66 is bioluminescent and the "purity" of microbiological growth on inoculated vacuum packs was confirmed by observing the proportion of bioluminescent colonies on TPC. Indigenous bioluminescent *Photobacterium* and marine vibrio may therefore be included in the results, but their potential presence did not change the general pattern.

The apparent yield factors for muscle blocks with *P. phosphoreum* P66 were calculated for Hx, acetic acid and TMA, and the standard deviations between the two independent experiments were in the range of ± 0.3 - $0.4 \text{ Log}_{10}(\mu\text{mol/cfu})$ (Table 4).

Inoculation with LAB at levels of 10^3 to 4.1×10^4 cfu/g resulted in high final levels of 1.5 - 4.5×10^8 cfu/g of the inoculated strains as confirmed by tentative characterization of colonies from agar plates. *L. saké* 1T18 and *L. saké/bavaricus* S1.11 presumably produced 0.4 - $2.8 \mu\text{mol/g}$ Hx, 18.9 - $31.2 \mu\text{mol/g}$ acetic acid and 11.6 - 14.9 mg-N/100 g TMA during storage for 21 days (Figure 4). However, LAB have no anaerobic respiration and therefore no use for the reduction of TMAO to TMA. LAB do therefore not reduce TMAO to TMA. This was confirmed in a preliminary experiment, where *Carnobacterium* sp. 2T2 grew to final levels of 10^8 cfu/ml in salmon juice with no production of TMA in the presence of 11 mg-N/100 ml TMAO during anaerobic incubation at 15°C for 13 days (data not shown).

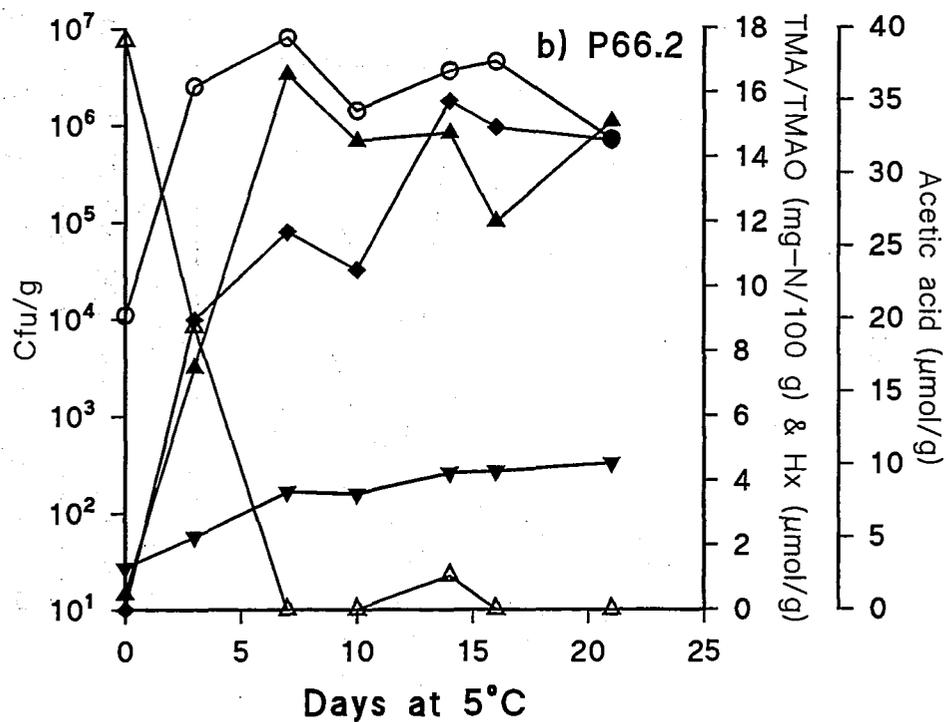
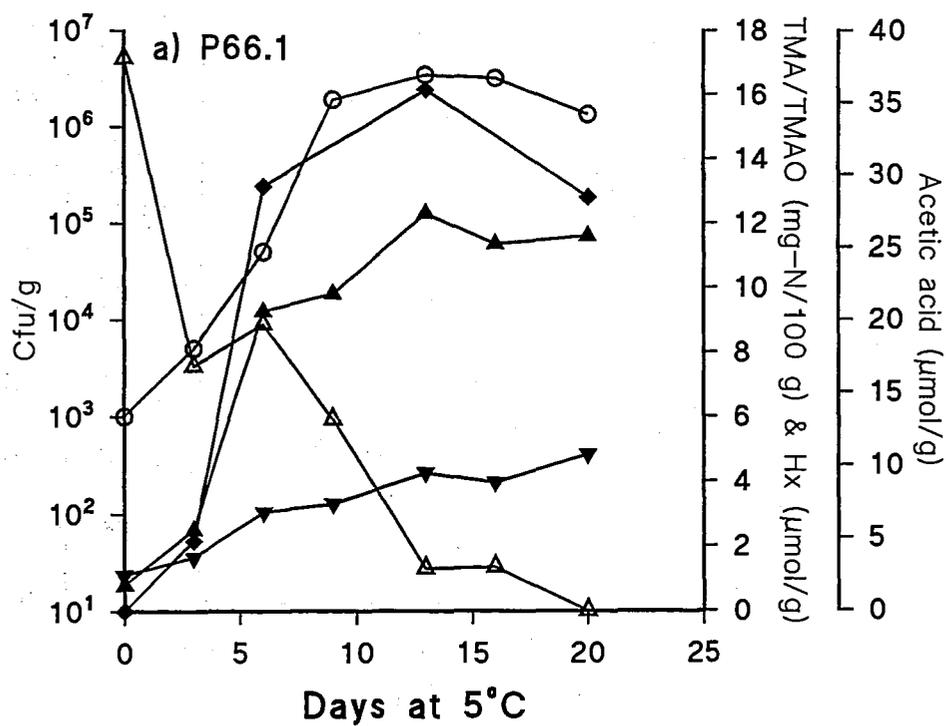


Figure 3. Growth and metabolism of *P. phosphoreum* P66 on cold-smoked salmon in a) 1st and b) 2nd experiment. ○, TPC - TSA; ▲, TMA; △, TMAO; ▼, Hx and ◆, acetic acid.

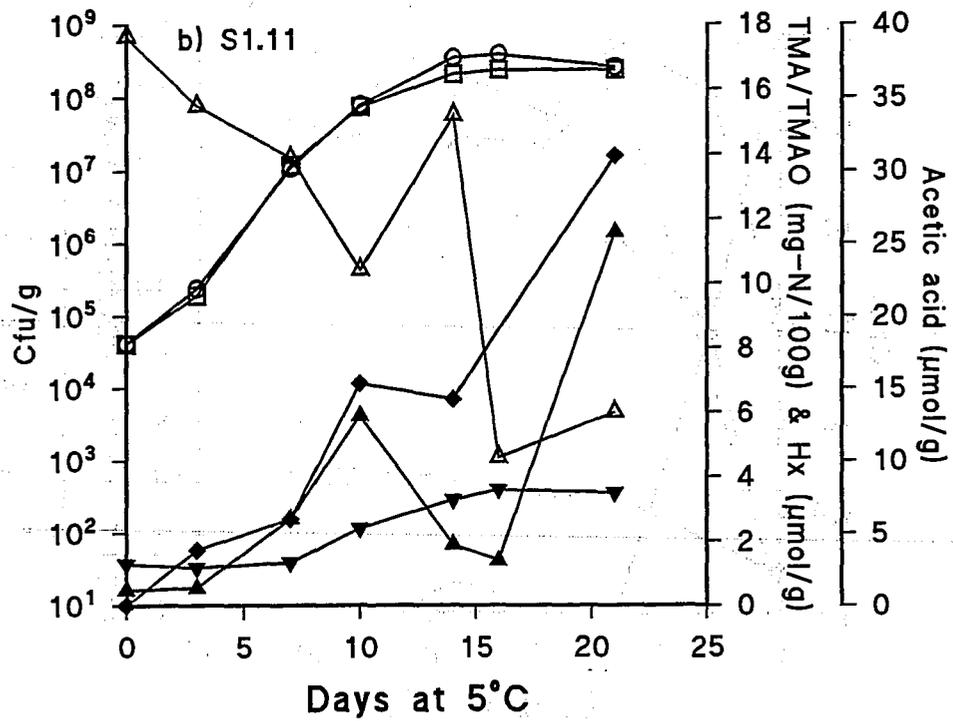
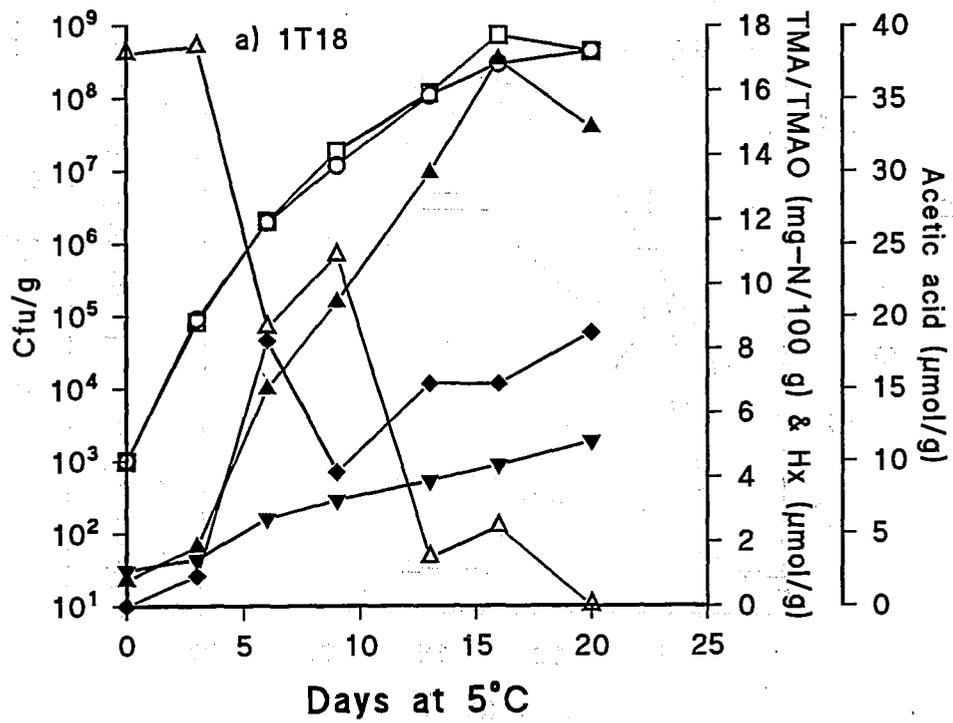


Figure 4. Growth and metabolism of a) *L. saké* 1T18 and b) *L. saké/bavaricus* S1.11 on cold-smoked salmon. ○, TPC; □, NAP; ▲, TMA; △, TMAO; ▼, Hx and ◆, acetic acid.

Table 4. Apparent microbial yield factors for hypoxanthine (Y_{Hx}), acetic acid (Y_{Ace}) and trimethylamine (Y_{TMA}) for bacteria strains inoculated on vacuum packed cold-smoked salmon blocks stored at 5°C for 20-21 days.

Strains	Cfu _{final} ^a (cfu/g)	Cfu _{initial} (cfu/g)	Hx _{final} ^b (μmol/g)	Ace _{final} (μmol/g)	TMA _{final} (mgN/100 g)	Log(Y_{Hx})	Log(Y_{Ace})	Log(Y_{TMA})
<i>Lb. saké</i> 1T18	4.5 x 10 ⁸	1.4 x 10 ³	2.8	13.9	14.9	-8.21 ^c	-7.61 ^c	-9.51 ^d
<i>Lb. saké/bavaricus</i> S1.11	2.4 x 10 ⁸	4.1 x 10 ⁴	1.2	31.0	11.6	-8.30	-6.89	-9.33
<i>Lactobacillus</i> sp. R1.12	1.5 x 10 ⁸	2.5 x 10 ⁴	0.4	12.7	0.9	-8.56	-7.07	-10.55
<i>Lb. curvatus</i> N1.4	4.1 x 10 ⁸	2.1 x 10 ⁴	1.0	8.9	2.9	-8.63	-7.66	-10.23
<i>S. liquefaciens</i> 2R.4	9.4 x 10 ⁶	3.8 x 10 ³	2.2	31.8	14.4	-6.62	-5.47	-7.83
<i>H. alvei</i> S2.4	3.8 x 10 ⁸	5.6 x 10 ⁴	1.4	35.2	11.9	-8.43	-7.03	-9.52
<i>E. agglomerans</i> R3.3	9.5 x 10 ⁶	7.0 x 10 ²	1.6	46.8	20.1	-7.49	-6.03	-8.41
<i>S. liquefaciens</i> N1.6	2.2 x 10 ⁷	4.7 x 10 ⁴	1.7	48.2	12.8	-7.12	-5.66	-8.25
<i>P. phosphoreum</i> P66, 1st experiment	1.4 x 10 ⁶	1.0 x 10 ³	2.5	28.5	11.6	-5.73	-4.72	-7.10
<i>P. phosphoreum</i> P66, 2nd experiment	4.5 x 10 ⁶	1.1 x 10 ⁴	2.2	32.4	14.4	-6.30	-5.14	-7.51

a) Maximum cfu/g levels obtained by the inoculated strains; b) Final microbial Hx with Hx produced by autolysis subtracted (see Table 3); c) Log₁₀(μmol/cfu); d) Log₁₀(mg-N/cfu)

Indigenous marine vibrio and/or *Photobacterium* spp. were found to coexist with the inoculated LAB at levels estimated to be 10⁵-10⁶ cfu/g by direct microscopy, and this may explain the slow production of TMA. Growth of *L. saké* 1T18 resulted in sour, cabbage and sulphurous off-odours, resembling those described for *L. saké/bavaricus* S1.11 except the latter strain produced weaker cabbage and slightly fruity off-odours. The logarithm to the apparent yield factors for Hx, acetic acid and TMA were -8.21, -7.61 and -9.51 for 1T18 and -8.30, -6.89 and -9.33 for S1.11 (Table 4). Similar apparent yield factors were obtained for *Lactobacillus* sp. R1.12 and *Lb. curvatus* N1.4, except for apparent yield factors for TMA, which was lower (Table 4). This suggested the contamination with *Photobacterium* sp. and marine vibrios may have been smaller, which was confirmed through direct microscopy. Off-odour production by strains R1.12 and N1.4 were initially described as slightly acidic, burnt and buttermilk after 16 days, but after 21 days of storage, astringent and faecal off-odours were also detected.

The *Enterobacteriaceae* strains were less successful in colonizing the salmon blocks containing low levels of the indigenous microflora. Only *H. alvei* S2.4 grew and reached maximum levels of 3.8 x 10⁸ cfu/g (Table 5). This strain achieved apparent yield factors of -8.43, -7.03 and -9.52 for Hx, Acetic acid and TMA, respectively (Table 4). During the storage period, *H. alvei* S2.4 produced stale, nauseous and slightly fruity off-odours.

Table 5. Bacterial counts during incubation at 5°C of vacuum packed cold-smoked salmon inoculated with *Enterobacteriaceae*.

Strain	Storage time (days)	TPC (Log ₁₀ (cfu/g))		<i>Enterobacteriaceae</i> (VRBG Log ₁₀ (cfu/g))		
		Vacuum pack	A	B	A	B
<i>S. liquefaciens</i> 2R4	0		3.58	3.62	3.51	3.65
	3		6.11	5.59	6.46	5.92
	7		6.89	7.71	5.57	5.95
	10		7.00	^a	5.43	-
	14		-	5.32	-	5.57
	16		8.20	6.90	8.20	6.30
	21		6.30	6.64	6.38	6.45
<i>H. alvei</i> S2.4	0		4.76	4.74	4.75	4.73
	3		6.00	6.61	5.68	6.59
	7		7.81	6.73	7.66	6.53
	10		8.23	6.77	8.45	6.00
	14		6.86	8.04	6.90	8.04
	16		8.38	6.28	8.57	5.70
	21		8.48	8.68	8.61	8.56
<i>E. agglomerans</i> R3.3	0		3.00	2.95	2.79	2.91
	3		4.71	3.93	4.49	4.00
	7		7.45	6.90	5.28	5.56
	10		7.11	5.94	6.79	5.71
	14		6.76	5.92	3.00	5.92
	16		7.88	7.00	7.40	6.56
	21		5.48	5.72	5.30	4.60
<i>S. liquefaciens</i> N1.6	0		4.00	4.67	4.76	4.58
	3		5.66	6.80	5.94	6.11
	7		6.85	7.08	6.60	6.11
	10		-	7.18	-	7.26
	14		6.58	8.08	6.45	8.34
	16		8.28	-	8.26	-
	21		6.77	6.88	6.84	6.08

a) Vacuum pack discarded due to loss of vacuum.

The yield factors obtained by *S. liquefaciens* 2R4 and N1.6 were comparable with those obtained for *P. phosphoreum* P66 indicating heavy contamination with *Photobacterium* spp. and marine vibrio (Table 4). Results from TPC and VRBG inclusive tentative characterization of colonies and direct microscopy confirmed this but also revealed that the inoculated strains grew to maximum levels of 10^6 - 10^7 cfu/g (Table 5). Vacuum packs inoculated with *E. agglomerans* R3.3, developed a mixed flora consisting of the inoculated strain at maximum levels of 10^7 cfu/g (day 16) in coexistence with indigenous LAB and marine vibrio (Table 5). The unsuccessful colonization of *E. agglomerans* R3.3 may have been caused by the very low inoculation level (700 cfu/g) making the strain unable to compete with the indigenous microflora on contaminated muscle blocks. The apparent yield factors for *E. agglomerans* R3.3 reflected the metabolism of the mixed microflora (Table 5). Old stale, sweet and nauseous off-odours were typical of aged salmon inoculated with the *Serratia* strains and *E. agglomerans* R3.3.

Production of volatile sulphur compounds by *Lactobacillus saké* (1T18).

Lactobacillus saké 1T18 produced large amounts of H_2S but not any other volatile sulphur compounds (data not shown). There was no production of volatile sulphur compounds in the uninoculated control samples. Inoculated samples contained astringent, sweet and sulphurous (cabbage) off-odours, while the odour of the uninoculated salmon still was typical for cold-smoked salmon.

Discussion.

Growth and metabolism in salmon juice.

Results from the salmon juice experiment revealed that *P. phosphoreum* P66 maintained metabolic activity levels 75-500 times higher than the levels obtained by the LAB strains and *S. liquefaciens* 2R4 (Figures 1a-c, Table 2). If the compounds responsible for the detected off-odours are produced at rates related to the metabolism of acetic acid and Hx, *P. phosphoreum* P66 may be responsible for spoilage at much lower cell concentrations than any of the other test strains. Acetic acid may be produced from carbohydrates including ribose from inosine and amino acids by all the inoculated strains (Axelsson, 1993; Baumann & Baumann, 1984; Gottschalk, 1986). *P. phosphoreum* P66 and *S. liquefaciens* 2R4 produced amine and ammonia-like off-odours, which may be caused by TMA production not

measured here. *P. phosphoreum* is an active producer of this compound (Figure 3; van Spreekens, 1977; Dalgaard, 1995), whereas *S. liquefaciens* was negative when inoculated in the TMAO medium by Gram *et al.* (1987) (Truelstrup Hansen & Huss, 1995c). The off-odours produced by the LAB were described as sour, astringent, sweet and burnt.

Nucleotide metabolism in salmon juice.

S. liquefaciens 2R4 and *P. phosphoreum* P66 both actively converted IMP to Ino and subsequently Ino to Hx (Figure 2), whereas the LAB only produced Hx from Ino. *P. phosphoreum* has previously been reported to degrade IMP to Ino followed by production of Hx from Ino (van Spreekens, 1977). Surette *et al.* (1988) found that *Proteus* spp. produced Hx from Ino in spoiling cod, and it is therefore not surprising that the related and biochemically very active *S. liquefaciens* 2R4 shares this ability. Degradation of IMP to Ino has previously been reported to be autolytic (Jones, 1965; Gill, 1990), but the present study confirms that bacteria may participate in the conversion. LAB from smoked salmon were tested in a previous experiment for their ability to degrade Ino to Hx with subsequent acid production from ribose, and positive strains were found among tentative *Lb. curvatus*, *Carnobacterium* spp., *Lb. plantarum* and *Leuco. mesenteroides* (Truelstrup Hansen & Huss, 1995c). *Lb. sake* 1T18 was, however, negative in this test, for which there is no obvious explanation other than the arginin-dihydrolase positive strain may have produced NH₃ from peptides in the substrate and thereby neutralised the acid from ribose fermentation. Borch & Molin (1988) found *Carnobacterium* spp. (cluster 1 & 2) and homofermentative *Lactobacilli* spp. (clusters 11, 12 & 14) to assimilate Ino, whereas other *Lactobacillus* and *Leuconostoc* clusters were negative. These results confirmed the active role of microorganisms on cold-smoked salmon in the production of Hx previously reported (Truelstrup Hansen *et al.*, 1995b).

Salmon blocks with reduced loads and natural contaminants.

It proved to be difficult to produce cold-smoked salmon blocks with a reduced load of microorganisms. A success rate of approximately 50% was obtained, which was comparable to previous results using the same technique (Truelstrup Hansen *et al.*, 1995b). The origin of acetic acid, TMA and the main proportion of Hx and TVB was again shown to be microbiological (Table 1). The occurrence of indigenous *Photobacterium* spp./marine vibrio and LAB on approximately 50% of the salmon necessitated careful data interpretation. The high metabolic activity levels of the former bacteria meant that they could disturb the

chemical data even when present at 2 log cycles under the inoculated strains. However, when these considerations were taken into account, the model experiments revealed some general patterns.

Characteristics of *P. phosphoreum* P66 on salmon blocks.

P. phosphoreum P66 grew to approximately the same maximum of cell numbers ($1-8 \times 10^6$) on salmon blocks as in salmon juice (Figure 2a & b, Table 4). Off-odours produced by *P. phosphoreum* P66 on salmon blocks closely resembled those found during growth in cod juice as well as vacuum and modified atmosphere packed (MAP) cod (Dalgaard *et al.*, 1993; Dalgaard, 1995). On the other hand, van Spreekens (1987) found that *P. phosphoreum* produced no strong off-odours in lightly salted vacuum packed matjes herring with 2-3% salt. *Photobacterium* spp. dominated the flora in levels of 6.0-6.5 Log₁₀(cfu/g) at the time of rejection by the taste panellists, but unfortunately the sensory characteristics were not described. *P. phosphoreum* rapidly converted all TMAO to TMA and produced high levels of acetic acid (29-32 $\mu\text{mol/g}$), which may have contributed to the amine and sour off-odours also detected.

Characteristics of lactic acid bacteria on salmon blocks.

It is possible that salmon blocks inoculated with LAB were influenced by the presence of naturally occurring *Photobacterium* spp., marine vibrio and LAB. The spoilage characteristics were however, clearly different from those observed for *P. phosphoreum* P66 and differences were observed among the strains. The sensory characteristics described as sour, astringent, faecal and slightly fruity found only in vacuum packs with LAB, have previously been described in spoiled cold-smoked salmon with high apparent numbers of LAB (Truelstrup Hansen *et al.*, 1995a), and it seems likely that LAB parallel to findings on vacuum packed beef and beef products can be held responsible for spoilage (Egan, 1983). Borch & Agerhem (1992) also found production of sour off-odours, acetic acid, L-lactic acid and H₂S by a homofermentative *Lactobacillus* spp. and putrid/tainted off-odours, D-lactic acid, ethanol and some H₂S by a *Leuconostoc* sp. inoculated onto MAP beef stored at 4°C.

Characteristics of *Enterobacteriaceae* on salmon blocks.

H. alvei S2.4 was the only strain to successfully colonize the salmon blocks, whereas

E. agglomerans R3.3 and *S. liquefaciens* 2R4 and N1.6 were only partially successful (Table 5). Large variations have been seen on the numbers of *Enterobacteriaceae* on vacuum packed cold-smoked salmon (Schneider & Hildebrandt, 1984; Truelstrup Hansen *et al.*, 1995d), but they have occasionally been found in numbers of relevance for spoilage. It is interesting to note, that these *Enterobacteriaceae* strains isolated from vacuum packed cold-smoked salmon with 4-5% SWP compete so poorly with the naturally occurring microflora. LAB are reknown for producing antagonistic substances, whereas this never has been elucidated for *Photobacterium* spp. and marine vibrio.

H. alvei and the other *Enterobacteriaceae* have been related to spoilage of vacuum packed meat products (Dainty *et al.*, 1992). In inoculation experiments on meat Hanna *et al.* (1979, 1983) and Dainty *et al.* (1989a) found *H. alvei* strains produced H₂S. The description of off-odours produced by this strain on cold-smoked salmon did not indicate presence of H₂S, and the strain tested negative for H₂S-production in the API 20E (data not shown), indicating the ability to produce H₂S may vary among *H. alvei* strains.

Production of H₂S by *Lb. saké* 1T18.

Growth of *Lb. saké* 1T18 on salmon blocks resulted in sour and sulphurous off-odours. This prompted the GC-analysis for production volatile sulphur compounds, where it was found to produce H₂S but not CH₃SH or CH₃-S-S-CH₃. This strain also produced H₂S in Iron agar (Truelstrup Hansen & Huss, 1995c), and *Lb. saké* has previously been shown to produce H₂S from cysteine in vacuum packed beef (Egan & Shay, 1989). Jeppesen & Huss (1993) screened 61 LAB strains from fish including 24 tentative *Lb. saké* for production of off-odours in sterile shrimp juice but detected no sulphur-like off-odours. Also, *Lb. saké* 1T18 produced no such odours when inoculated in salmon juice in the present study. It may be that LAB strains' ability to produce off-odours is restricted and therefore underestimated in the fish juice model system due to lack of natural substrates like cysteine for H₂S-production etc.

Yield factors.

Apparent yield factors were calculated for the bacterial production of acetic acid, TMA and Hx as a measurement of the inoculated bacteria's metabolic activity. Acetic acid and TMA were exclusively produced by the microflora, whereas a portion of Hx came from autolytic conversion of Ino during storage (Table 1, Truelstrup Hansen *et al.*, 1995b).

Corrected values for Hx were used for calculation of yield factors.

Total agreement between yield factors found in salmon juice and on salmon blocks could not be established. *P. phosphoreum* P66 had for example, an average yield factor for acetic acid on muscle blocks of -4.93 ± 0.30 (SD) and -5.61 in salmon juice. These differences may be caused by lower substrate concentrations in the later medium for acetic acid production such as carbohydrates, amino acids etc. The yield factors for *P. phosphoreum* P66 acid were constantly 2-3 Log(Y) higher than the yield factors for other strains not heavily contaminated with indigenous *Photobacterium* spp. and marine vibrio in both the salmon juice and muscle block experiment. *P. phosphoreum* P66 produced an average Log(Y_{TMA}) of -7.31 ± 0.29 (SD), which was comparable to the -8.0 ± 0.3 Log(mg-N/cfu) found for *P. phosphoreum* during anaerobic growth in cod juice at 0°C (Dalgaard, 1995). Further work is needed to establish whether the concept of yield factors can be used to identify the spoilage flora in cold-smoked salmon, as none of the chemical parameters (Hx, acetic acid and TMA) have been found to correlate consistently to spoilage of the product (Truelstrup Hansen *et al.*, 1995b).

Conclusion.

Model experiments with bacteria isolated from spoiled cold-smoked salmon reproduced some of the off-odours found on spoiled cold-smoked salmon. Contamination of the salmon blocks limited, however, the interpretation of the results especially for 3 out of 4 *Enterobacteriaceae*, which grew poorly in the presence of the indigenous bacteria. *P. phosphoreum* were shown to be up to approximately 100 times more biochemically active per cell than LAB and successful *Enterobacteriaceae* in salmon juice and on salmon blocks. This may indicate that *P. phosphoreum* is able to spoil at much lower cell concentrations. LAB produced off-odours in the model systems and one *Lb saké* (1T18) were also shown to produce H₂S, indicating these bacteria may contribute to spoilage. Further work is required to elucidate the bacterial spoilage of cold-smoked salmon.

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Research Note.

Occurrence of *Listeria* spp. in farmed salmon and during subsequent slaughter: Comparison of Listertest™ and the USDA-method.

by

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Abstract.

Salmon and environmental samples from a fish farm and a salmon slaughterhouse were analyzed for *Listeria* spp. using the USDA method and the rapid quantitative Listertest™. *Listeria* spp. were not detected in any of the 119 samples analyzed. However, *Corynebacterium* spp. gave false positive results with the USDA method, and with the Listertest when its plates were incubated at 20-25°C in stead of 37°C as recommended by the manufacturer. Means of distinguishing between *Corynebacterium* spp. and *Listeria* spp. are discussed.

Keywords: *Listeria* spp., salmon, fish, Listertest™, USDA method, *Corynebacterium aquaticum*, CDC group A-4.

Introduction.

Listeria monocytogenes has been recognised as a major food pathogen for the last ten years. The large majority of human listeriosis cases occur sporadically and the implicated food items are almost never identified. However, there have been at least three sporadic cases with documented seafood implications (Facinelli *et al.*, 1989; Frederiksen, 1991; Anonymous, 1993; Baker *et al.*, 1993).

L. monocytogenes is frequently isolated from frozen, cooked and lightly preserved fish products (Weagant *et al.*, 1988; Farber, 1991; Noah *et al.*, 1991; Jemmi, 1993) and its prevalence and significance in seafood have been extensively reviewed (Dillon & Patel, 1992;

Fuchs & Reilly, 1992; Jemmi, 1993; Ben Embarek, 1994).

Listeria spp. have been isolated from the marine environment in water samples and sediments (Colburn *et al.*, 1990) but data on the natural presence and level of the bacteria on seafood are limited (Colburn *et al.*, 1990; Motes, 1991).

The present work was done to determine if *L. monocytogenes* could be found naturally on aquacultured salmon and to determine the degree of contamination in a nearby salmon slaughterhouse. Due to the increasing need for quantitative data the quantitative Listertest™ was used together with the qualitative U.S. Department of Agriculture (USDA) method.

Materials and Methods.

Sample Collection

(i) **From the fish farm.** In a low density populated area, on the Norwegian west coast, samples of fish and water were collected from a salmon farm which use net-cages in the sea. Ten salmon (*Salmo salar*) weighing 4-5 kg each were picked up from a net pond, killed, put in individual plastic bags and packed in styrofoam boxes with ice. From the same site, 10 surface water samples (approx. 5 cm below the surface) were each collected in 1 l sterile bottles. Fish and water samples were transported to a nearby laboratory and analyzed within 16 h. No sediment samples were collected as the bottom was made of rocks.

At the laboratory, from each salmon, gills were aseptically removed, split into 3 pieces and placed into three stomacher bags for the *Listeria* analyses according to the USDA method and to the Listertest™, as well as for total viable count (TVC). Skin and gut samples were treated similarly.

Seawater samples were filtered on sterile nitro-cellulose filters (0.2 μm, Satorius). One filter was used for each sample.

(ii) **From the slaughterhouse.** In the slaughterhouse, the salmon were bled in a bleeding-tank, gutted, rinsed with water, heads removed and the fish were packed in boxes with ice. Samples were collected as follows: 1) gills were removed aseptically from three salmon heads into three sterile stomacher bags. 2) Three slaughtered salmon were removed before packing and skin samples were collected as for the farmed salmon. 3) Whole guts, from three salmon were collected immediately after removal into three stomacher bags. 4) Surface samples from the visceral cavity of five slaughtered and gutted salmon together with 14 surface samples from walls, tables and knives during the slaughtering and evisceration

process were collected with a sterile swab (approx. 10 cm² of plane surfaces, whole knife surface or approx. 20 cm linear swab) into screw cap tubes containing 10 ml physiological saline (PS) (0.1% peptone and 0.9% NaCl).

In addition, five water samples from the bleeding tank, two water samples from the inlet to the slaughterhouse, three water samples from the cleaning line, two from the drains in the floor, and one from the outlet of wastewater outside the slaughterhouse were collected in sterile screw cap tubes (ca. 25 ml). One sample of the ice used in the packaging of the slaughtered fish was also included. Samples were transported to the laboratory and analyzed the same day as samples from the farm.

Microbiological Analysis

Most of the liquid samples (water and swabs) were split and analyzed by all methods. Samples from fish analyzed by different methods were not the same samples but were taken from the same area on the same fish.

USDA Method. A total of 50 samples (Table 1 and Table 2) were analyzed according to the USDA method (McClain and Lee, 1988) with enrichment of 5 g sample or swabs in 45 ml UVM1 broth (Oxoid, CM863 + SR142; 24 h, 30°C). Then 0.1 ml was transferred into 10 ml UVM2 broth (Oxoid CM863 + SR143; 24 h, 30°C) and subsequently plated on Oxford agar plates (Oxoid, CM856 + SR140) which were incubated at 30°C and examined for growth after 24 h, 48 h and 72 h.

Vicam Listertest™ Method. A total of 59 samples (Table 1 and Table 2) were analyzed using the Listertest™ following the instructions from the manufacturers (Listertest™ user's guide, version 1.4).

Briefly, a 25 g sample was mixed in a stomacher bag with 15 ml buffer for 30 s and filtered. For environmental samples, swabs were mixed in 2.5 ml buffer. Two ml were transferred to capture vials. Immunobeads coated with antibodies were added. After 2 h incubation on a rotator at room temperature, the beads were captured on a magnetic rack, the fluid removed and the beads washed twice in buffer. The beads were resuspended in 0.2 ml buffer and plated onto agar plates (Brain Heart Infusion agar (52 g/l), lithium chloride (5 g/l) and ceftazidime (0.05 g/l) incubated at 37°C for at least 22 h. Membranes were then placed over the plates to create a replica of the colonies. Colony material was fixed on the membranes with ethanol, membranes washed and blocked to avoid non-specific reactivity,

and then two antibodies were added together with a colour reagent. *Listeria* colonies appeared as dark blue spots on the membrane within 5-15 min. A strain of *L. monocytogenes* (057) was used as positive control.

Master agar plates can be used for further identification. Due to practical reasons, plates in our survey were incubated at room temperature (20°C-25°C) for 72 h before the lift procedure on the membrane.

Total Viable Count. Thirty-seven samples (Table 3) were analyzed for total viable count (TVC). Five to seven g were diluted (1:10) in PS and appropriate serial dilutions were pour-plated in iron agar (Oxoid, CM867) with overlay. Plates were incubated for 3 days at 25°C.

Isolation and Identification

Black colonies on oxford agar plates and colonies matching blue to dark blue on Listertest™ membranes were considered as presumptive *Listeria* colonies, recovered in brain heart infusion broth (BHI) (Difco, 0037) and were streaked onto tryptone soya agar (TSA) (Oxoid, CM131) plates. After incubation (25°C, 24 h), they were tested for Gram reaction in 3% KOH (Gregersen, 1978), catalase activity in 5% H₂O₂ and cytochrome oxidase by Dry slide™ oxidase (Difco, 3530). Motility and morphology were determined by phase contrast microscopy of BHI cultures incubated for 24 h at 25°C.

Gram positive, catalase positive, oxidase negative, short rods motile with characteristic tumbling motility were further tested for growth on Oxford agar, haemolysis on 5% calf blood agar and acid production from D-glucose, D-mannitol, L-rhamnose, D-xylose, methyl- α -D-mannopyranoside, maltose and L-arabinose.

Results and Discussion.

Table 1 and 2 show the results of the *Listeria* analysis performed on the samples collected at the salmon farm and in the slaughterhouse.

From all the Oxford agar plates with black colonies (USDA method) one to five colonies were picked up from each plate and all colonies were collected from agar plates with spots on matching membranes (Listertest™). A total of 123 *Listeria*-presumptive isolates

Table 1. Results of *Listeria* analysis on samples from the salmon farm analyzed with the USDA-method and the>Listertest™.

Source	USDA-method			Listertest™		
	No. of samples	Black colonies on Oxford (Samples)	White colonies ^a on Oxford (Samples)	No. of samples	Spots on membrane ^b (Samples)	Growth on selective agar (Samples)
Water	7	0	4	1	0	1
Gills	10	0	10	9	2	7
Skins	10	1	10	9	4	4
Guts	6	1	5	10	3	5
Total	33	2	29	29	9	17

a) Esculin negative colonies; b) Plates were incubated at 20-25°C for 72 h.

Table 2. *Listeria* analysis results from the salmon slaughterhouse with the USDA-method and the>Listertest™.

Source	USDA-method			Listertest™		
	No. of samples	Black colonies on Oxford (Samples)	White colonies ^a on Oxford (Samples)	No. of samples	Spots on membrane ^b (Samples)	Growth on plates (Samples)
Gills	3	1	3	2	1	2
Skin	3	1	3	3	2	2
Guts	2	0	2	2	0	0
Stomach swab	5	0	5	0	-	-
Water (bleeding tank)	5	5	4	3	2	2
Water (inlet)	2	1	0	2	1	2
Water (cleaning line)	3	0	2	3	3	3
Water (drain)	2	2	2	2	2	2
Water (outlet)	1	0	0	1	1	1
Ice	1	0	0	1	0	0
Swabs	0	-	-	11	5	11
Total	27	10	21	30	17	25

a) Esculin negative colonies; b) Plates were incubated at 20-25°C for 72 h.

were recovered; 22 isolates by the USDA method (18%) and 101 isolates by the Listerest (82%). After testing for Gram reaction, catalase, oxidase, morphology, motility and growth on Oxford agar plates, 106 (80.3%) were identified as non-*Listeria* spp. and discarded.

The remaining 26 (19.7%) isolates were motile short Gram+ rods, catalase+, oxidase-, and able to grow on Oxford agar with black colonies. All but one of these 26 isolates (65% from the USDA method and 35% from Listerest) showed a similar carbohydrate fermentation pattern for glucose (+), mannitol (+), xylose (+), mannopyranoside (-), maltose (+) and arabinose (+). Fermentation of rhamnose gave negative results in 14 isolates and positive results in 11. Four of these latter isolates (two rhamnose positive and 2 negative) and the last isolate with a different carbohydrate fermentation pattern (BS3B) were sent to a reference laboratory, the Danish Serum Institute for further identification.

The 4 strains representing the 25 isolates were identified as *Corynebacterium aquaticum*. The majority (23) were isolated from different water samples in the slaughterhouse. Two were isolated from the skin of a salmon from the farm and the skin of a slaughtered salmon from the slaughterhouse. The isolate BS3B was identified as belonging to the CDC coryneform group A-4 (Krech & Hollis, 1991). It was isolated from the bleeding tank in the slaughterhouse.

As stated above, agar plates from Listerest™ were incubated at room temperature for 72 h and not at 37°C for 22 h as recommended by the manufacturer. The *Listeria* control gave positive results. In order to assess the importance of incubation parameters on the detection of *C. aquaticum* with Listerest™, the four isolates mentioned above were tested with Listerest™. The isolates were grown in BHI at 25°C and then 10-fold dilutions were made in PS and 0.1 µl of the appropriate dilution injected into sterile cod sticks (10 g) to give approx. 10⁴ cfu/g fish. The sticks were then run through Listerest™ according to the manufacturers recommendations (Listerest™ manual). Plates were incubated at 37°C for 22 and 48 h. No growth was observed on the agar plates after 22 h, while poor growth was seen after 48 h. No signals were detected on the membranes. *L. monocytogenes* Scott A was treated similarly and run in parallel as a positive control.

The levels of the TVC found on water (10³ CFU/g), gills (10⁵ CFU/g), skins (10⁴ CFU/g) and intestines (10⁵-10⁶ CFU/g) from the fish samples from the farm are in agreement with other reports on the microbial load of fish from temperate waters (Liston, 1980; Shewan, 1977). TVC results from the slaughterhouse did not show any exceptional levels (data not shown).

In the present study, *Listeria* were not isolated from any of the fish or environmental

samples tested. Rørvik *et al.* (1995) found no *Listeria* in 50 freshly slaughtered farmed salmon, while environmental samples from the slaughterhouse were positive. Incoming frozen and fresh salmon were, however, identified as the major source of contamination in smokehouses by Eklund *et al.* (1995). The occurrence of *Listeria* on salmon probably varies among farms and processing plants depending of the prevalence of the organism in the surrounding environment, and the raw material may therefore be the source of the high levels of *Listeria*, which have been found on smoked salmon products (Ben Embarek, 1994a).

Corynebacterium isolates gave disturbing false-positive results with the USDA method. *Corynebacterium* isolates were also isolated with Listertest™ but probably only because of the low incubation temperature.

On this basis, it might be safe to conclude that while some corynebacteria may produce false positives with the USDA method, they do not produce false positives when analysis is done with Listertest™. However, incubation at temperatures below the recommended 37°C or for more than 24 h, may allow growth and subsequently positive Listertest reactions by non-*Listeria* bacteria.

Recently, Rørvik & Heidenreich (1993) reported false-positive results caused by *Corynebacterium brevis* (sic) and *Actinomyces pyogenes* using a *Listeria* ELISA kit (*Listeria* Tek) on samples from a salmon slaughterhouse and a smoke house. False-positive results in analyzing fish and fishery products for *Listeria* spp. have also been reported by Dillon & Patel (1992), Paranjpye *et al.* (1992) and Spanggaard & Jørgensen (personal communication).

According to Seeliger & Höhne (1979), there seem to be overlapping antigens between *Listeria* and motile corynebacteria which could explain a possible cross-reaction with antibodies to *Listeria* antigens. Positive results from immunological test kits may therefore need confirmation by time-consuming biochemical tests. *Corynebacterium* spp. have been found on fish and in seawater (Horsley, 1973; Shewan, 1977) and the natural habitat of *C. aquaticum* is fresh water (Krech & Hollis, 1991) but no information is available on their incidence in seafood.

Corynebacterium aquaticum is not recognised as a species of the genus *Corynebacterium* by Bergey's Manual of Systematic Bacteriology (Collins & Cummins, 1986). Its motility excludes it from the genus *Corynebacterium* (von Graevenitz & Krech, 1992). It is recommended that aerobic/facultative anaerobic growth and colony pigmentation be included in the initial testing of presumptive *Listeria* colonies when analyzing fresh seafoods since these characteristics differentiate the two organisms. The BS3B isolate could be differentiated from *Listeria* spp. by positive carbohydrate reactions in both D-mannitol and xylose.

Listertest™ Lift was shown to be more sensitive and rapid (24 h, instead of one to two weeks) than the traditional enrichment based methods in isolating *Listeria* from low-level inoculated seafood and meat (Jackson *et al.*, 1993), and from spiked stainless steel surfaces (Mitchell *et al.*, 1994). Farber (1993) found Listertest™ Lift to be effective in isolating *Listeria* spp. from raw milk and pasteurised milk but reported interference with the fat of raw milk samples. We experienced similar difficulties analyzing the gills samples because of their large content of slime which agglutinate the beads and interfered with the ELISA reaction on the membrane.

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DFU-rapporter

- nr. 4 Fiskeundersøgelse i Vejle Fjord 1993-1994
Hanne Nicolajsen, Josianne Støttrup, Leif Christensen
- nr. 5 En undersøgelse af maveindholdet af Østersølaks i 1994-1995
Ole Christensen
- nr. 6 Udsætningsforsøg med Østersølaks
Heine Glüsing, Gorm Rasmussen
- nr. 7 Kampen om Limfjorden - Livsformer, miljøværdier og reguleringsformer
Kirsten Monrad Hansen
- nr. 8 Tangetrappen 1994-95
Anders Koed, Gorm Rasmussen, Gert Holdensgård, Christian Pedersen
- nr. 9 Status over bundgarnsfiskeriet i Danmark 1994
Anders Koed, Michael Ingemann Pedersen
- nr. 10 Måling af kvalitet med funktionelle analyser og protein med nærinfrarød refleksion (NIR) på frosne torskablokke
Niels Bøknæs
- nr. 11 Acoustic monitoring of herring
J. Rasmus Nielsen
- nr. 12 Blåmuslingers vækst og dødelighed i Limfjorden
Per Dolmer
- nr. 13 Mærkningsforsøg med ørred og regnbueørred i Århus Bugt og Isefjorden
Heine Glüsing, Gorm Rasmussen
- nr. 14 Jomfruhummerfiskeriet og bestandene i de danske farvande
Mette Bertelsen
- nr. 15 Bærekapacitet for havørred (*Salmo trutta* L.) i Limfjorden
Kaare Manniche Ebert
- nr. 16 Sild og brisling i Limfjorden
Jens Pedersen
- nr. 17 Produktionskæden fra frysetrawler via optøning til dobbeltfrossen torskfilet. Optøningsrapport (del 1)
Niels Bøknæs
- nr. 18 Produktionskæden fra frysetrawler via optøning til dobbeltfrossen torskfilet. Optøningsrapport (del 2)
Niels Bøknæs
- nr. 19 Automatisk inspektion og sortering af sildefileter
Stella Jónsdóttir, Magnus T. Ásmundsson, Leif Kraus
- nr. 20 Udsætning af helt, *Coregonus lavaretus* L., i Ring Sø ved Brædstrup
Thomas Plesner, Søren Berg
- nr. 21 Udsætningsforsøg med ørred, (*Salmo trutta* L.) i jyske og sjællandske vandløb
Heine Glüsing, Gorm Rasmussen
- nr. 22 Kvalitetsstyring og målemetoder i den danske fiskeindustri. Resultater fra en spørggebrevsundersøgelse
Stella Jónsdóttir
- nr. 23 Quality og chilled, vacuum packed cold-smoked salmon
Lisbeth Truelstrup Hansen (Ph.D. thesis)