Lightly Salted Lumpfish Roe
Composition, Spoilage, Safety and Preservation

by

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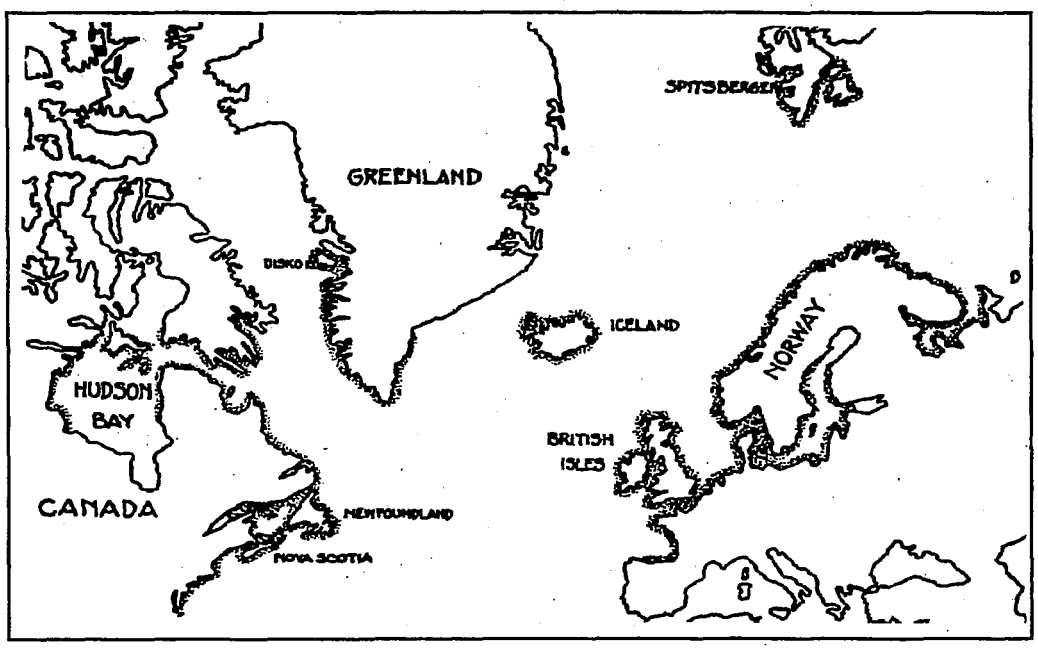

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Summary

This thesis deals with composition, spoilage, safety and preservation of lightly salted lumpfish roe. Stored heavily salted lumpfish roe is the raw material for production. The roe is desalted to obtain a salt concentration of around 4 % WPS, and the pH is adjusted to 5.4 (0.1 % w/w lactic acid). The product is vacuum packed and stored at 5°C, at which temperature a shelf life of three months should be obtained. Industrial production of salted lumpfish roe products is briefly described at the beginning of the thesis.

Proximate composition, fatty acid composition, lipid hydrolysis and oxidation and composition of total and free amino acids of desalted lumpfish roe has been examined and compared to fresh roe. Lipid hydrolysis and oxidation were shown to occur during storage of heavily salted roe prior to production, influencing the overall quality of the final products. The analysis of free amino acids indicated, that autolytical liberation of amino acids also take place during storage of heavily salted roe, increasing the total concentration and changing the composition of free amino acids. It was noted that the resulting concentrations of individual free amino acids vary among newly produced products.

Spoilage of the final roe products at 5°C was caused by bacterial activity. Spoilage as judged by odour, varied greatly among roe batches. Three situations regarding odour characteristics of roe (3.5 - 4.8 % WPS) occurred after storage: Non-spoiled roe, borderline roe (sour, marinated, rotten) and spoiled roe (sulphidy, cabbage-like, sour, rotten, green/hay).
Characterisation of the microflora (108 isolates) of five roe batches after three months of storage showed, that the microflora (> 5 log cfu/g) consisted of LAB or LAB and Enterobacteriaceae. In some roe batches *Vibrio* spp. composed a minor part of the flora. The composition of the LAB flora of lightly salted lumpfish roe covered a wide range of different LAB. The Enterobacteriaceae flora was less variable, and the tentatively identified strains belonged to/were closely related to three species: *Serratia liquefaciens, Serratia plymuthica* and *Morganella morganii.* Other Enterobacteriaceae were rarely detected. Growth of Enterobacteriaceae occurred less frequently in products with salt concentration above 4 % WPS.
Several potential chemical spoilage indicators were analysed in storage experiments. Volatile sulphur compounds were produced during storage of lightly salted lumpfish roe. Different profiles of the compounds (semi-quantitative) were obtained among stored roe batches, and seemed related to the degree of spoilage. The following compounds/groups of compounds were concluded to be without value as indicators of spoilage of the roe products: TMA, TVB, nucleotides and lactic, acetic, formic and propanoic acids.
Spoilage potential of pure cultures of LAB, Enterobacteriaceae and *Vibrio* sp. (16 strains) was assessed in pasteurised roe products, and of three Enterobacteriaceae also in lightly salted roe made from sterile fresh roe. Effect on spoilage of inoculation of low numbers of *Morganella*

morganii was examined in a normal roe product. It was concluded that *Morganella morganii* act as a spoilage organism by production of volatile sulphur compounds and probably also other volatile compounds. *Serratia liquefaciens* may also contribute in spoilage, but the compounds responsible for odours are yet to be identified. LAB is judged as less important in spoilage reactions. Yet their role need further investigation, including their possible interaction with the Enterobacteriaceae.

Potential health hazards of lightly salted lumpfish roe have been evaluated on the basis of data from the literature: *Clostridium botulinum*, *Listeria monocytogenes*, *Vibrio* spp., *Aeromonas* spp., *E. coli*, *Salmonella* spp., *Yersinia enterocolitica*, *Staphylococcus aureus*, viruses, parasites and biogene amines. It was concluded that during storage at 5°C, additional hurdles is not required to control these hazards, except for *Listeria monocytogenes*. It was shown in inoculation experiments, that *Listeria monocytogenes* (< 1 log cfu/g) grew to high numbers (> 7 log cfu/g) in lightly salted lumpfish roe within the first months of storage.

Thus in preservation Enterobacteriaceae and *Listeria monocytogenes* were the primary targets of inhibition. As the first option, the prospects was studied of biopreservation of lightly salted lumpfish roe using live cultures of LAB isolated from roe products, as this was a central wish in the industry at initiation of this project. For this purpose attempts have been made to isolate LAB from stored roe products. The screenings were carried out in roe agar at product relevant conditions. Among 43 LAB none produced inhibition zones against the Enterobacteriaceae. Yet 21 LAB produced zones against *Vibrio* spp. The nature of *Vibrio* inhibition was not further examined, as these organisms were not considered important in spoilage. A LAB strain V6 origination from sugar salted herring, previously reported to have antagonistic activity also against Gram-negative bacteria, was inoculated in lightly salted lumpfish roe. Only very weak influence on Enterobacteriaceae growth could be noted. It was concluded that prevention of spoilage by Enterobacteriaceae using LAB cultures did not offer good prospects. As *Listeria monocytogenes* failed to grow in the roe agar, a laboratory medium was used instead. Among 31 LAB 8 produced inhibition zones. Three selected LAB were coinoculated with *Listeria monocytogenes* in lightly salted roe. Significant inhibition of growth of two *Listeria monocytogenes* only occurred by a LAB strain, not producing inhibition zones in laboratory medium. The perspectives in biopreservation using LAB cultures in lumpfish roe products in general is discussed.
Preservation using sodium lactate was tested as an alternative to biopreservation. During storage for 116 days growth of Enterobacteriaceae in the product was inhibited (≤ 3.2 log cfu/g) by 2.8 % (w/w) sodium lactate. Growth of *Morganella morganii* and spoilage was prevented in a roe product inoculated with this organism. Growth of *Listeria monocytogenes* was totally inhibited by 2.8 % sodium lactate (< 1 log cfu/g). It is recommended to examine, whether other sufficiently effective combinations of salt concentration, pH and a lower sodium lactate concentration can be found, which are preferred in terms of sensory quality.

Sammendrag

Denne afhandling omhandler sammensætning, fordærv, sikkerhed og konservering af letsaltet stenbiderrogn. Den anvendte råvare er lagret højsaltet rogn, som udvandes til ca. 4 % salt i vandfasen, og pH sænkes til 5.4 (0.1 % (v/v) mælkesyre). Produktet er vakuumpakket og opbevares ved 5°C. Under disse betingelser ønskes en holdbarhed på minimum 3 måneder. Industriel produktion af saltede stenbiderrognprodukter beskrives kort i begyndelsen af afhandlingen.

Udvandet stenbiderrogn er analyseret med hensyn til overordnet sammensætning, fedtsyresammensætning, lipidhydrolyse og -oxidation og sammensætning af totale og frie aminosyrer. Lipidhydrolyse og -oxidation fandt sted under lagring af højsaltet rogn, og påvirkede produktets kvalitet. Analyse af frie aminosyrer viste, at autolytisk frigørelse af aminosyrer også fandt sted under lagringen. Resulterende absolutte koncentrationer af frie aminosyrer i rognen varierede derfor mellem forskellige nyfremstillede produkter.

Fordærv af letsaltet stenbiderrogn ved 5°C var forårsaget af bakteriel aktivitet. Graden af fordærv varierede stærkt mellem forskellige rognbatch. Der blev efter lagring af rogn (3.5 - 4.8 % salt i vandfasen) observeret 3 karakteristiske situationer: Rogn stort set uden bilugte, rogn nær kassation (sur, marineret, rådden lugt) og fordærvet rogn (svovlet, kålagtig, sur, rådden, grøn/høagtig lugt). Karakterisering af mikrofloraen (108 isolater) fra 5 rognbatch efter 3 måneders lagring viste, at mikrofloraen (> 5 log cfu/g) bestod af mælkesyrebakterier eller mælkesyrebakterier og Enterobacteriaceae. I nogle tilfælde udgjorde *Vibrio* spp. en mindre del af floraen. Mælkesyrebakteriefloraen fra rognprodukter dækkede et bredt spektrum af forskellige mælkesyrebakterier. Enterobacteriaceae-floraen var mindre variabel, og de tentativt identificerede isolater tilhørte/var nært beslægtede med 3 species: *Serratia liquefaciens, Serratia plymuthica* og *Morganella morganii.* Andre Enterobacteriaceae blev sjældent påvist. Vækst af Enterobacteriaceae forekom mindre ofte i rognprodukter med et saltindhold på over 4 % i vandfasen.
I lagringsforsøg blev potentialet af flere kemiske fordærvelsesindikatorer undersøgt. Flygtige svovlforbindelser blev produceret i rognen under lagring. Forskellige profiler af svovlforbindelser blev påvist i forskellige lagrede rognbatch, og var tilsyneladende relateret til graden af fordærv. De følgende kemiske indikatorer fandtes at være uanvendelige som mål for fordærv af letsaltet stenbiderrogn: Trimethylamin, total flygtig base, nukleotider, mælke-, eddike-, propan- og myresyre.
Fordærvelsespotentialet af rene kulturer af mælkesyrebakterier, Enterobacteriaceae og *Vibrio* spp. (16 isolater) blev undersøgt i pasteuriseret letsaltet rogn, 3 Enterobacteriaceae desuden i letsaltet rogn fremstillet af steril fersk rogn. Indflydelsen på fordærv af *Morganella morganii* blev også undersøgt ved podning i et normalt rognprodukt. Det blev konkluderet, at *Morganella morganii* optræder som fordærvelsesorganisme i letsaltet stenbiderrogn og producerer flygtige

svovlforbindelser samt sandsynligvis andre flygtige forbindelser. Det er sandsynligt, at *Serratia liquefaciens* også medvirker til fordærv af produktet, men de ansvarlige flygtige forbindelser er ikke identificeret. Mælkesyrebakterierne vurderes af mindre betydning for fordærvet, men deres mulige indflydelse bør undersøges yderligere, herunder deres interaktion med Enterobacteriaceae-floraen.

Følgende potentielle sikkerhedsrisici ved letsaltet stenbiderrogn er vurderet på basis af litteraturdata: *Clostridium botulinum*, *Listeria monocytogenes*, *Vibrio* spp., *Aeromonas* spp., *E. coli*, *Salmonella* spp., *Yersinia enterocolitica*, *Staphylococcus aureus*, virus, parasitter og biogene aminer. Det blev konkluderet at under lagring ved 5°C, er yderligere hurdles ikke påkrævede for at opnå kontrol med disse risici, med undtagelse af *Listeria monocytogenes*. I podningsforsøg blev det påvist, at *Listeria monocytogenes* (< 1 log cfu/g) kunne vokse til høje tal (> 7 log cfu/g) indenfor den første måneds lagring.

Ved konservering af letsaltet stenbiderrogn skulle vækst af Enterobacteriaceae og *Listeria monocytogenes* derfor primært forhindres. Som første prioritet blev mulighederne for biokonservering af rognen med levende mælkesyrebakterier undersøgt, fordi dette var et centralt ønske fra industrien ved den oprindelige igangsættelse af projektet. Til dette formål er det forsøgt at isolere egnede mælkesyrebakterier fra produkterne selv. Screeninger er udført i rognagar under produktrelevante betingelser. Blandt 43 mælkesyrebakterier fandtes ingen at producere hæmningszoner overfor Enterobacteriaceae. Derimod blev *Vibrio* spp. hæmmet af 21 af disse mælkesyrebakterier. Denne hæmning blev ikke nærmere undersøgt, fordi V*ibrio* spp. ikke ansås for at være væsentlige fordærvelsesorganismer i produktet. En mælkesyrebakterie V6 blev i stedet afprøvet i letsaltet stenbiderrogn. Stammen var oprindeligt isoleret fra sukkersaltet hakket sild og vist at besidde antagonisme overfor også gramnegative bakterier. I letsaltet stenbiderrogn kunne kun en ringe indflydelse på vækst af Enterobacteriaceae påvises. Det blev konkluderet, at mulighederne ikke er gode for med mælkesyrebakteriekulturer at opnå hindring af fordærv forårsaget af Enterobacteriaceae. På grund af manglende vækst i rognagar blev antagonisme overfor *Listeria monocytogenes* undersøgt i et laboratoriesubstrat. Blandt 31 mælkesyrebakterier fandtes 8 at producere hæmningszoner. Efter podning af 3 udvalgte stammer i rognproduktet sammen med *Listeria monocytogenes*, blev tydeligt reduceret vækst af *Listeria monocytogenes* blev kun opnået med en mælkesyrebakterie, der ikke havde vist hæmningszoner i laboratoriesubstratet. Perspektiverne ved biokonservering med mælkesyrebakterier i saltede stenbiderrognprodukter generelt diskuteredes.
Som alternativ blev konservering med natriumlaktat undersøgt. I en lagringsperiode på 116 dage blev vækst af Enterobacteriaceae hæmmet (≤ 3.2 log cfu/g) af 2.8 % (v/v) natriumlaktat i rognen. Vækst af *Morganella morganii* og fordærv blev forhindret i et rognprodukt podet med denne bakterie. *Listeria monocytogenes* voksede ikke (< 1 log cfu/g) i letsaltet stenbiderrogn med 2.8 % natriumlaktat. Det anbefales at undersøge hvorvidt andre ligeså effektive kombinationer af salt, pH og en lavere natriumlaktatkoncentration er at foretrække ud fra et sensorisk synspunkt.

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When first applying for working on this project, the motive was a wish of learning. After finishing the project, I am fortunately able to conclude, that this wish has been fulfilled. It has been a healthy experience trying to learn other ways of thinking, and for that I particularly wish to thank Hans Henrik Huss.

Chapter 1. Introduction

1.1 Background

There is a long tradition in Denmark for the production of salted lumpfish roe products (caviar), the main part of which is exported to both European and overseas countries. Since lumpfish with roe can be caught for only a short period of time in the spawning season, the roe is heavily salted and stored as long as necessary for maintaining a continuous production of caviar over the year. During production the roe is partly desalted by water addition and subsequent drainage. The products are preserved by the use of chemical preservatives (potassium sorbate, sodium benzoate) and/or pasteurisation. A wide range of products containing different combinations of salt concentration, preservation, colorants and flavour components were produced, when this project was initiated, and the product formulations had fundamentally been unchanged for many years. This wide range was created by different traditions and legislation of the various importing countries, or wishes by specific customers. Depending on the salt concentration and preservation method, shelf lives of up to one year is obtained at chill storage of the products produced today.
The lumpfish roe products are used mainly as decoration in different courses rather than consumed in larger amounts as a foodstuff of its own right.

An increasing interest among consumers in more "natural" foods with less salt and less chemical preservation, has created a need to develop new types of lumpfish roe products. The industry wishes to produce a lightly salted lumpfish roe product using a more "natural" type of preservation and low salt concentration. Compared to the existing products, the new product should be closer to the fresh roe regarding sensory quality, which should make it possible to market it for consumption in larger amounts.

On this background the present industrial Ph.D. project was initiated in collaboration among a producer of lumpfish roe products (ABBA Seafood A/S), the Danish Institute of Fisheries Research, Department of Seafood Research of the Danish Ministry of Food, Agriculture and Fisheries[1)] and The Royal Veterinary and Agricultural University of Denmark. In addition the Danish Academy of Technical Sciences gave financial support.

The strategy was to study spoilage, shelf life and safety of a product without preservation except for salt (app. 4 % waterphase salt (WPS)) and lowered pH (5.4 with lactic acid). If additional preservation was necessary to ensure a shelf life of three months, a preservation method should be developed, preferably by the use of live cultures of Lactic Acid Bacteria

[1)] At that time: Technological Laboratory, Ministry of Fisheries

isolated from roe products. The organoleptic characteristics of the roe was not to be radically changed by the biopreserving culture like in a fermented product; instead the sensory effect of the culture were to be neutral or at least slight and positive, like marinated, if such could be discovered.
In addition the project should increase the knowledge on factors important to quality and deterioration, which could be of general use in the production of salted lumpfish roe products.

1.2 Definition of the lumpfish roe product

The type of product to be studied during the project was defined. This model product without preservation is throughout the thesis referred to as **lightly salted lumpfish roe** or just lightly salted roe. The product definition was: A non-pasteurised product without colorants, stabilisers and flavourings, that was to be produced from the normal raw material (i.e. **heavily salted roe**) using the existing processing equipment. The salt concentration should be around 4 % WPS, and the pH adjusted to 5.4 with lactic acid (0.1 % w/w). The lightly salted roe should be packed under partial vacuum (65 - 75 %) in glass jars closed with metal twist off caps, similar to the existing products. A relatively short shelf life of three months (5°C) was estimated by the industry to be necessary for practical production and marketing. According to the Danish legislation such a product belongs to the group of "lightly preserved fish products" defined by containing less than 6 % WPS and having a pH value higher than 5 (Fiskeriministeriet 1993).

1.3 Objectives and structure of the thesis and experimental work

The first objective was to provide basic knowledge on chemical composition of the roe, and identify factors of importance to product quality and deterioration. This included the study of changes taking place during the long storage period of the roe raw material at low temperature and high salt concentrations, and the changes occurring within the shelf life period of the final product. If spoilage of the product was concluded to be caused by bacterial activity, the spoilage bacteria responsible were to be identified. Lastly the potential safety hazards of lightly salted lumpfish roe also were to be identified.
Based on this information, the second objective was to evaluate the possibilities of using Lactic Acid Bacteria, isolated from roe products, as preservative means directed against spoilage and pathogenic bacteria. Other types of preservation were to be evaluated if necessary. Finally the effectiveness of the chosen preservation method, against spoilage and pathogenic bacteria was to be verified during a storage period of three months.

This thesis is divided into two parts. The first part (chapter 2 - 8) discusses own experimental results in relation to relevant results published by others. The second part named "Experimental section" is a detailed presentation of the experiments carried out during the project, either in the form of manuscripts for publication (papers 1 - 3) or descriptions of experiments and detailed results (notes 1 - 7).

The experiments performed during the project are listed below:

Composition, deterioration and safety

- Fresh roe, heavily salted roe and desalted roe ready for production, were examined regarding chemical composition (Paper 1, Note 1).
- Two storage experiments on lightly salted lumpfish roe with different salt levels were performed. Microbial and odour changes were studied, and flora composition was examined. Chemical changes were studied to search for spoilage indices. Sterile roe product and roe product with antimicrobial substances were included in order to reveal the significance of autolytical changes in off-odour development (Paper 2 and 3, Note 3).
- To study their spoilage potential, selected bacterial isolates were inoculated into pasteurised lightly salted roe. The ability of the isolates to produce off-odours and volatile sulphur compounds was examined. In a second experiment a few isolates were likewise studied after inoculation into lightly salted roe made from fresh sterile roe (Paper 3).
- The influence of pH and salt concentration on growth of selected Enterobacteriaceae isolates was examined by absorbance measurements in roe extract at 5°C (Note 2).
- The ability of *Listeria monocytogenes* to grow in lightly salted roe was examined in an experiment, where different strains originating from fish products were inoculated in low numbers into lightly salted lumpfish roe stored at 5° and 10°C (Note 4).

Evaluation of preservation methods

- Lactic Acid Bacteria were isolated in a storage experiment using replica plate technique and well diffusion assays, both in roe agar. The targets of inhibition were potential spoilage organisms and a pathogenic bacterium (Note 5).
- The effect of 2.8 % sodium lactate on growth of selected Enterobacteriaceae isolates was studied by absorbance measurements during growth in roe extract at 5°C (Note 2).
- The effect of selected roe lactic acid bacteria on growth of *Listeria monocytogenes* in lightly salted lumpfish roe was studied in a storage experiment at 5°C (Note 6)
- The effect of lactic acid bacteria originating from roe products or salted herring and/or 2.8 % sodium lactate on growth of *Listeria monocytogenes* or LAB and Enterobacteriaceae were studied in two storage experiments at 5°C. (Note 6).
- The effect on off-odour development and growth of lactic acid bacteria, Enterobacteria and *Listeria monocytogenes* in lightly salted lumpfish roe preserved by the use of 2.8 % sodium lactate was studied during storage at 5°C (Note 6).

- The perspectives in developing a fermented product was examined in a storage experiment with lightly salted lumpfish roe added glucose and a Lactic Acid Bacterium originating from salted herring. The effect on spoilage was examined (Note 6).
- The effect on quality of lightly salted lumpfish roe by sodium lactate or low pH (lactic acid addition) was examined by sensory evaluation (Note 7).

Chapter 2. Industrial production of salted lumpfish roe products

2.1 Introduction

In this chapter the process of converting fresh lumpfish roe into salted lumpfish roe products is described. The initial stage of processing fresh roe is based on old traditions, and is largely dependent on practical conditions including the seasonal supply and long distances from the catching locations to production facilities. With this chapter it is intended to provide background information, before factors influencing composition, deterioration and microflora of the roe products are examined in the following chapters.

2.2 The lumpfish and its distribution

The fish has been given the name lumpfish due to the short, thick and blunt body (figure 2.1), but the name "lumpsucker" is also used, due to the "sucker" under the side of the chest formed by a modification of the ventral fins. By means of this, the lumpfish can attach itself firmly to for example bottom stones. Weight proportions of individual body parts of the lumpfish are characterised by a low content of meat (25 %) and a large quantity of thick skin (18.5 %). The head accounts for 14 - 21 %. The roe reaches 35 % of the weight of whole fish. Large females will produce around 140000 eggs, but in one case 279620 eggs have been reported (Cox 1920, Bykov 1983, Leim and Scott 1966).

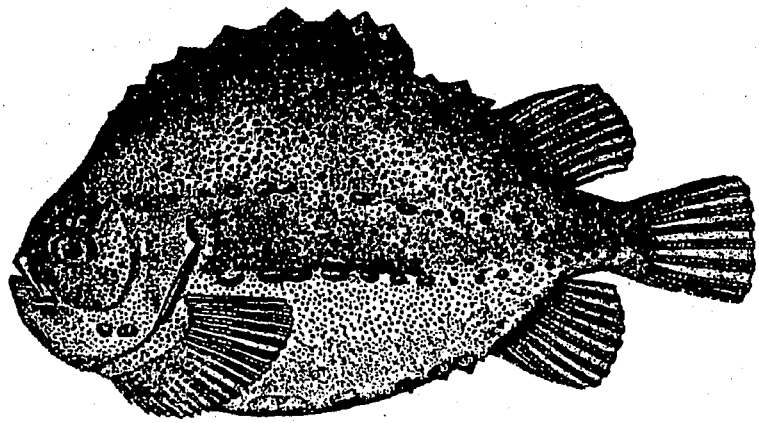


Figure 2.1 Lumpfish (*Cyclopterus lumpus*) (Cox 1920).

In the spawning season the fish approaches the coasts all around the North Atlantic, the North Sea and the bays of eastern Canada (the distribution is shown on the front page) to deposit their eggs in masses among rocks and seaweed (Cox 1920). On their way in, the fish are caught close to the shore during spring time. The exact season varies depending on geographic location, and may differ slightly from year to year depending on the weather conditions. The roe for production by ABBA Seafood A/S originates mainly from Norway, Iceland and Canada, but also Danish roe and small quantities of roe from Greenland is used. The Danish season begins in January/ February and the Canadian season lasts till the end of June. The Norwegian and Icelandic season falls in between these, and consequently the roe raw material for production can be harvested during a period of around five months.

2.3 Industrial production of lumpfish roe products

ABBA Seafood A/S has a large production of lumpfish roe products, and more than 1000 tons a year are produced. This equals around 35 % of the world's production of preserved lumpfish roe products. The main part of the production is exported to other European and overseas countries (ABBA Seafood AB, Göteborg, Sweden).

2.3.1. Harvesting, salting and storage prior to production

The catching of lumpfish is an occupation for a large number of local fishermen distributed at distant locations along the coasts of the countries mentioned above. On board the boats or as the fish are brought in, the fish are cut open and the roe sacs removed (see figure 2.2). The roe is screened into loose eggs by passing it through a screen, the holes of which has the size of the eggs. The roe is left on a screen for draining for 15 - 20 hours, normally until the next day. Then the roe is dry salted by addition of crystalline salt and mixing. The salt concentration prescribed by the production company is > 10 % (w/w). The roe is then filled into plastic barrels of 105 kg. Depending on the number of nets owned by a fisherman and depending on the weather conditions, it can take several days to fill a barrel. The barrels are left open, while the brine is forming. If necessary saturated NaCl-solution is added to make sure the roe is covered, and the barrel is sealed. During the first weeks the barrels must be turned over frequently to prevent clumping of eggs. After salt addition the eggs tends to stick together, and loose eggs can be obtained by this mechanical action. It has been experienced in the industry, that salting also causes a hardening of eggs, which is appreciated, as it reduces the loss due to crushing of eggs. Hardening of rainbow trout eggs has also been reported after light salting (4 %) by Stodolnik et al. (1992). The hardness increased from 46.5 g to 85.0 g, as measured by the stress needed to crush a single egg.

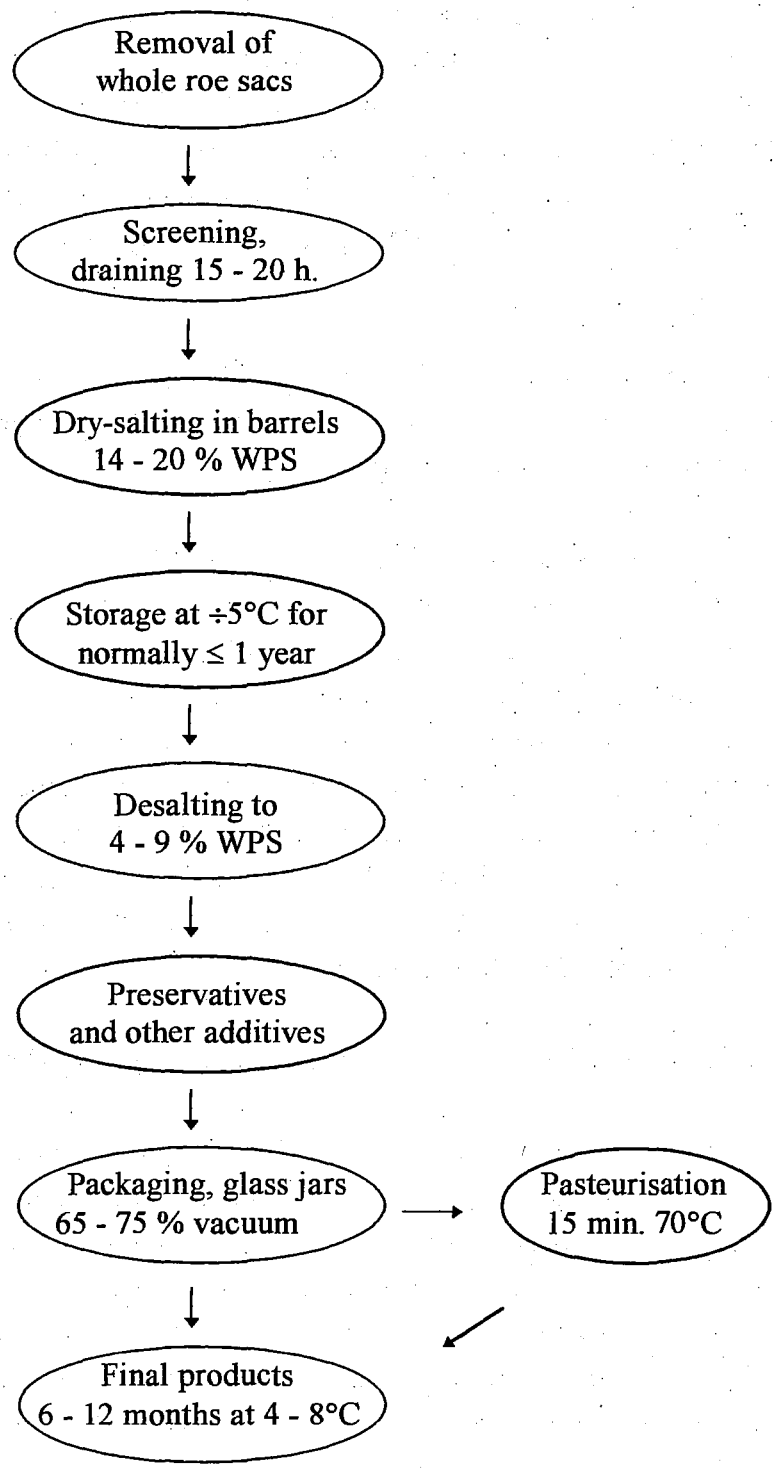


Figure 2.2 Processing of fresh lumpfish roe into salted, preserved caviar-type products.

Several export companies are involved in the collection of roe and the export by boat to Sweden (during this project, the production was moved from Denmark to Sweden). The storage time/temperature at the catching location and at the export company is dependent on the practice of each export company. The screening and salting of roe is performed at very different conditions regarding hygiene and degree of mechanisation. Thus several factors not easily controlled, influence the quality of heavily salted roe delivered to the production factory.

At the production factory the barrels are stored at ÷5°C (÷3 - ÷7°C) until needed for production. In practice the roe used for production has normally been stored for a few months up to a year, occasionally longer.

When a delivery arrives, all barrels are opened and the appearance and odour examined by laboratory personnel. In the case of off-odours, for example rancid, sour or rotten, the barrel is rejected. Saturated sodium chloride solution is added to barrels, in which the roe is not completely covered with brine. Samples are taken from 5 % of the barrels for microbiological analysis and analysis of salt concentration. If salt concentrations below 11 % (w/w) occur, additional salt is added to obtain a salt concentration in the range of 11 - 14 %. The heavily salted roe used for production has in general salt concentrations in the range of 11 - 16 % (i.e. approximately 14 - 20 % WPS) The data from the analysis is also used to decide, whether future improvements should be requested from the deliverer. All barrels are sealed and placed in the cold storage.

2.3.2. Processing of heavily salted roe into final roe products

The processing steps involved in the production are shown in figure 2.2.
During production, barrels of roe are tipped into containers and water is added. During this process the roe is desalted to the salt concentration, that is wanted in the final product. Products with a range of salt concentrations between 3.3 and 7.0 % (w/w) (i.e. approximately 4 - 9 % in the waterphase) are produced today.
After water addition the roe stands for equilibration for half an hour. The brine is sucked out and fresh water is added a number of times, depending on the salt concentration wanted. Half an hour after the last water addition the salt concentration is controlled by direct titration with $AgNO_3$ solution of a brine sample.
If additional water is needed for the salt concentration to be within the stipulated limits, brine is sucked out, and water added according to the internal standard.
In practice the actual salt concentrations of lumpfish roe products vary, when adjusted by this method. For example five barrels of heavily salted roe were in this project desalted to

obtain 3.3 % w/w salt. When analysed after desalting, the salt concentration of the five batches were 2.7, 2.8, 2.8, 2.9, 2.9 and 3.3 % w/w (3.6 - 4.2 % WPS, data are shown in paper 2, batch 1 to 5).

If a coloured product (black or red colours) is produced, the colouring is added when the correct salt concentration is obtained, and the roe is allowed to stand again. The brine is drained from the roe through a screen lid (centrifugation using specially designed equipment).
The last step before packaging is addition of a mixture in water of stabilisers, preservatives, acids and flavour components. A number of different mixtures are used depending on the specific product, the legislation of the country for which it is produced and the wishes of the customer regarding water content and flavour. After thorough mixing the roe product is ready for packaging and is kept at 2°C, if a packaging line is not immediately free. The roe is filled into glass jars (usually 100 g for retail and larger glass jars for catering). Before mounting the lid (metal twist off caps), the 100 g glass jars pass under a steam jet, and when the jars are closed, a vacuum of 65 - 75 % has been obtained.
Some products are then pasteurised by passing through showering hot water (76 °C). A temperature of 70°C for 15 minutes is obtained in the centre. All glasses are labelled and packed and placed in a 5°C storage room until delivery shortly after.
The prescribed storage temperature for the final products after delivery are in the range of 4 - 8°C, and depending on the type of preservation (chemical preservatives and/or pasteurisation) and the salt concentration, commercial shelf lives in the range of 6 - 12 months are guaranteed.

Chapter 3. Chemical characteristics of lumpfish roe - composition and changes during processing

3.1 Introduction

Chemical characteristics of the eggs/roe of the lumpfish have not been much studied. Data on proximate composition and composition of total amino acids of commercial caviar-type products, and fatty acid composition of fresh lumpfish roe have however been reported (Cantoni et al.1975, Rehbein 1985). Data on lipid content of final products and heavily salted roe, as well as certain changes occurring during storage of both, are available from earlier investigations in this laboratory (Huss et al. 1984). In this chapter characteristics of the lumpfish egg are briefly described (section 3.2), and own data on proximate composition of fresh, heavily salted and lightly salted roe are presented (section 3.3). Also composition of fatty acids, total and free amino acids and measures of lipid hydrolysis and oxidation have been examined in a range of commercial barrels of roe after desalting (sections 3.4 and 3.5). By comparison to fresh roe examined the same way, indications on changes taking place during processing of the roe are obtained. The fresh roe examined included screened roe of five lumpfish analysed separately. The fish were landed on the same day in Copenhagen, and killed right before sampling. The lightly salted roe included ten barrels originating from Canada, that had been stored at the production factory for 8 months at ÷5°C. The barrels were desalted to a level of 3 - 6 % w/w salt, by the normal desalting procedure described in section 2.3.1 (salt concentrations are shown in table 3 of note 1). Thus apart from pH adjustment the roe equalled newly produced products, with the chemical constituents that characterises the roe, as the shelf life begins.

3.2 The fish egg

3.2.1 Structure and colour

In the two ovaries of each fish, the eggs are protected by an envelope of epithelial membrane, within which the eggs are embedded in connective tissue. When the eggs are separated from the ovaries by passing through a screen (see chapter 2), the epithelium and connective tissue is broken (Zaitsev 1969).

The lumpfish egg, that was first described and figured by Cunningham (1887), is surrounded by a soft shell (double layer egg membrane named chorion or cortex, Ø 45 - 63 µm). The membrane is permeated by fine pore canals and slightly thickened at one spot to form the

micropyle, that come into use during fertilisation. Inside the shell the yolk forms the greater part of the egg, and is surrounded by a thin layer of cytoplasm (periblast). The egg contains a large number of oil globules. The yolk completely fills the available space, and contains the nutrients for larval development. The eggs are spherical, and the size has been reported in the range of 2.2 - 2.6 mm in diameter (Cunningham 1887, Devillers 1961, Ehrenbaum 1904, Russell 1976a and 1976b).

The eggs have by different authors been described as bluish-red, red, pale red, pinkish, yellow, pale green or nearly white (Cox 1920, Cunningham 1887, Leim and Scott 1966, Russell 1976b). Mikulin et al. (1978) examined the pigmentation of lumpfish eggs, and reported that the colour of eggs obtained from different females ranged from practically colourless over yellow-orange to purple violet; the depth of coloration also varied considerably. The authors concluded, that the colour of lumpfish eggs, in all its diversity, was governed by different combinations of a whole series of pigments both of a carotenoid and non-carotenoid nature. Carotenoids were present in free form in the plasma part and in the oil drops, while in the yolk carotenoids had formed complexes with protein. The nature of the non-carotenoid colouring matter was not established.

3.2.2 Antimicrobial activity

Some studies have been published on possible mechanisms of self-protection by fish eggs against microorganisms. Kudo and Inoue (1986) examined the effect on growth of bacteria (*Aeromonas hydrophila, Vibrio anguillarum,* and *Escherichia coli*) by extracts from eggs of rainbow trout (*Salmo gairdneri*). The bacteria were incubated with extracts of the outermost layer of fertilisation envelope (FE) from fertilised eggs and the outermost layer of vitelline envelope (VE) from unfertilised eggs. FE extracts were shown to have a bactericidal effect on *Aeromonas hydrophila* (decreasing survival rate with increasing incubation time) but no effect on the other two organisms. The VE extracts had no effect on growth of the bacteria. Structural changes were shown to occur during the formation process of the fertilisation envelope after fertilisation of the egg, and the outermost layers of fertilised and unfertilised eggs differed from one another. It was suggested that the demonstrated effect contributed to protection of the egg in the period of time from fertilisation till hatching, but apparently not in the (shorter) time from ovulation till fertilisation. Similarly the same authors reported a strong bactericidal effect on *Vibrio anguillarum* (not on *Aeromonas hydrophila* and *E. coli*) of FE-estracts from the fish *Cyprinus carpio* and *Plecoglossus altivelis*; no effect was observed by the VE-extracts of the same fish (Kudo and Inoue 1989). In a later study Kudo (1992) reported that FE extracts from rainbow trout had an antifungal or fungicidal effect on *Saprolegnia parasitica* (no effect of VE) and that several enzymes may contribute to the effect; those included a protease and (low activity) lysozyme.

In another study of eggs from the fish *Tribolodon hakonensis* Kudo et al. (1988) demonstrated peroxidase activity in both VE's and FE's. Both had a bactericidal effect on the three bacterial species mentioned above, when combined with NaI and H_2O_2.
The authors suggested, that the peroxidase may contribute to the defence against microbial infection, and discussed how endogenous H_2O_2 is supplied to exert a bactericidal effect. Thus the exact mechanism and the significance of such a defence is yet to be clarified.
It is not known whether any antimicrobial activities are present in lumpfish eggs, and it has not been studied in the present project, whether such activities can be retained during processing and storage.

3.3 Proximate composition

Fresh roe

Proximate composition has in the present project been examined in portions of screened fresh roe landed at two locations in Canada (5 kg portions of a mixture of roe from several fish landed on the same day) and one location in Denmark (roes from five fish landed on the same day). Moisture and especially protein content varied among the roes of five individual fish (table 2 of paper 1, detailed data in table 1 of note 1). The following proximate composition of fresh roe expressed as mean values of the three catches mentioned above, was obtained:

Moisture: 79.2 % w/w
Crude protein: (N x 6.25): 15.5 % w/w
Lipid: 5.8 % w/w
Ash: 1.1 % w/w

The moisture content is rather high compared to roe of both sturgeons (51.5 - 55 %, Zaitsev et al. 1969), rainbow trout (63.1 %, Vuorela et al. 1979) and chum salmon (57.6 %, Iwasaki and Harada 1985), also traditionally used for production of salted caviar-type products.
Vuorela et al. (1979) examined the proximate composition of roe from 10 rainbow trout (*Salmo gairdneri*) landed during a four months season, and reported a pronounced variation among individual fish, including fish landed on the same day. Variation was observed in both crude protein (26.1 - 32.6 %), lipid (7.1 - 9.7 %) and moisture (57.8 - 63.7 %) content. Kaitaranta (1980) reported, that no systematic influence by the year of catch occurred on the lipid content of whitefish (*Coregonus albula*) roe (9.1 - 11.4 %). Thus proximate composition of fresh roe varies among individual fish, but not as the result of any systematic influence by catching year or time in the season. The variation is to some extend equalised

during processing, as roe from a varying number of fish is mixed during screening, salting and desalting.

Heavily and lightly salted roe

Proximate composition of six barrels of heavily salted roe from two catching seasons (three barrels stored for two months and three barrels stored for one year at ÷5°C) was at a similar level as in the fresh roe. Moisture content was in the range of 75.8 - 77.8 %, crude protein in the range of 14.4 - 16.8 % and lipid in the range of 4.7 - 5.7 %, when calculated as % of roe with no extra salt (0.9 % salt) (based on data shown in note 1). During desalting of roe stored for two months, moisture content increased slightly (80.2 - 82.3 %) (table 2 of paper 1, detailed data in table 1 of note 1).

3.4 Lipids

3.4.1 Fatty acid composition

Fresh roe

Fatty acid composition of fresh roe of five lumpfish was determined (paper 1). The roe lipids were highly unsaturated with a calculated iodine value of 248 ± 2.1. An iodine value of 210 has been calculated for whitefish (*Coregonus albula*) roe lipids, which was higher than for the flesh (194) of the same fish (Kaitaranta 1980).

In the fresh lumpfish roe 27 fatty acids were identified. The composition of fatty acids is shown in figure 3.1. Around half the amount of fatty acids were n-3 fatty acids. Four fatty acids completely dominated, composing around three quarters of total fatty acids: Palmitic acid (16:0) 14.4 ± 0.46 %, oleic acid (18:1(n-9)) 15.9 ± 0.86 %, eicosapentaenoic acid (20:5(n-3)) 17.4 ± 0.75 % and docosahexaenoic acid (22:6(n-3)) 25.8 ± 0.64 %. Three fatty acids were present at concentrations around 3 % of total fatty acids (18:0, 18:1(n-7) and 20:1(n-9)), while concentrations of all the remaining 20 fatty acids were below 2 %, for the majority even below 1 % of fatty acids. Among 5 % of fatty acids, that remained unidentified, no single acid was present at concentrations above 1 % of total fatty acids (paper 1, detailed data are shown in note 1).

Cantoni et al. (1975) examined the fatty acid composition of fresh lumpfish roe. Direct comparison to the data obtained in this project is difficult, as the range of identified fatty acids present at low concentrations is not identical, and the grouping of acids differs. However dominance by the same four fatty acids as listed above, was reported by Cantoni et al. (68.6 %). The concentration of 18:1 was slightly higher and of 20:5 and 22:6 slightly lower than found in this project.

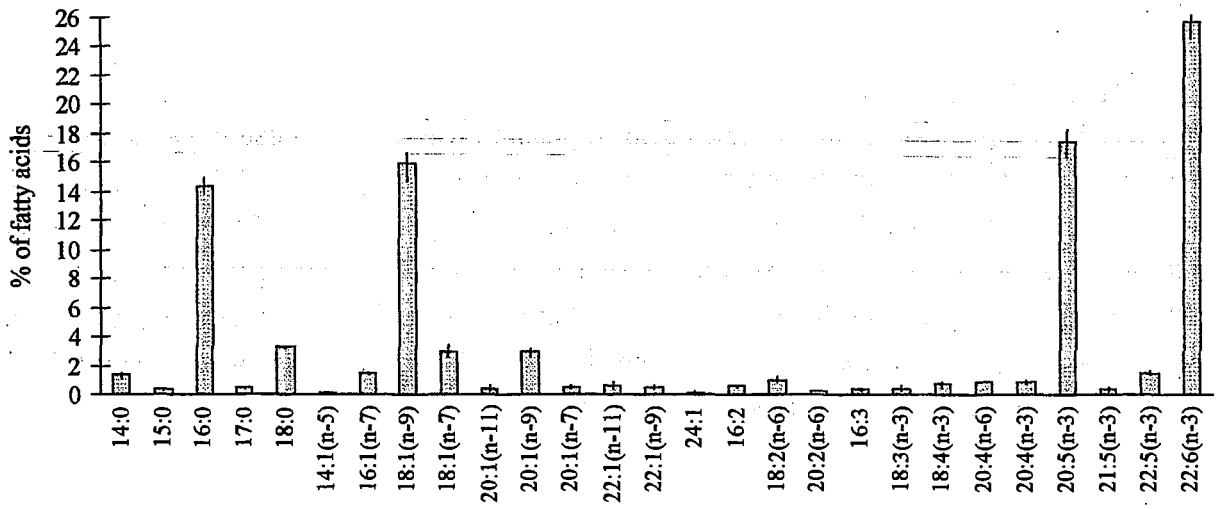


Figure 3.1 Fatty acid composition (mean values and range) of fresh roe of five lumpfish (data from table 3 of paper 1, detailed data in table 2 of note 1).

In cod (*Gadus morhua*) roe lipids, domination of the same fatty acids as in lumpfish roe lipids, has been reported, only two other acids exceeded 2 % (Ackman and Burgher 1964). In whitefish roe the four acids were also shown to compose a major part. The domination was however less extreme (around half the total fatty acids), and five other acids were present at concentrations around 5 % (Kaitaranta 1980). Tocher and Sargent (1984) examined the roe lipids of seven Northwest European marine fish (cod (*Gadus morhua*), herring (*Clupea harengus*), haddock (*Melanogrammus aeglefinus*), saithe (*Pollachius virens*), whiting (*Merlangus merlangus*), sand eel (*Ammodytes lancea*) and capelin (*Mallotus villosus*)), and found the four fatty acids to compose in the range of 62 - 77 % of total fatty acids.

Lightly salted roe

Fatty acid composition of ten barrels of heavily salted roe was similar to the fatty acid composition of fresh roe reported in the previous section. Only slight differences were observed, and the iodine value was similar (248 ± 2.6) to that of fresh roe (table 3 paper 1, detailed data in table 2 of note 1).

3.4.2 Lipid hydrolysis and oxidation

Though not revealed from the analysis of fatty acid composition, changes in the lipid fraction had occurred in the lightly salted roe compared to the fresh roe. As measures of lipid hydrolysis and oxidation, free fatty acids (FFA), peroxide value (PV) and thiobarbituric acid

value (TBA-value) were analysed of the ten stored, desalted barrels of roe. The results are shown in figure 3.2.

The concentration of FFA in fresh lumpfish roe were in the range of 2.5 - 4.3 % of lipid (paper 1). FFA concentrations of several other fish roes have all been reported in the range of 2 - 7 % of lipid: Rainbow trout (*Salmo gairdneri*) and whitefish (*Coregonus albula*) (Kaitaranta 1982), perch (*Perca fluviatilis*) (Linko et al. 1980) and Baltic herring (*Clupea harengus*) (Linko et al. 1979).
In lightly salted lumpfish roe an increased level of FFA in the range of 4.8 - 10.8 % was detected. Thus lipid hydrolysis had occurred during storage of heavily salted roe (paper 1). In all the above mentioned reports FFA in heavily salted (14 - 16 % w/w) roe of the different fish species listed, increased significantly during chill (2 - 5°C) storage for one year. Thus it is well established, that lipid hydrolysis occurs during storage of fish roe at conditions close to those applied in lumpfish roe processing. In a study in this laboratory Huss et al. (1984) reported a FFA level of 1.8 - 2.2 % in heavily salted lumpfish roe, which increased to 5.1 % in roe with 20 % salt and to 8.2 % in roe with 15 % salt after 9 months at 5°C. Linko et al. (1979) concluded, that lipid hydrolysis in herring roe occurred enzymatically and could be prevented by pasteurisation and freezing (÷20°C for one year), but not by heavy salting (16 % salt). By comparison to whitefish roe stored at similar conditions, the authors also concluded, that the rate of FFA liberation was dependent on the fish species. Similarly Kaitaranta (1982) found the increasing FFA concentration during storage of rainbow trout and whitefish roe to be resulting from enzymatical reactions. That author suggested, that different sensitivities of the lipolytic enzyme systems of the roes could be responsible for different effects of salt concentration on lipid hydrolysis in roes of different fish species.

Increased levels of both primary and secondary oxidation products were detected in the roe, as measured by PV and TBA-values respectively (figure 3.2). For both parameters however pronounced variation was noted. In some of the ten lightly salted roes examined, double determinations also differed considerably (table 3 of note 1). During desalting the roe from top and deeper parts of a barrel is mixed to a certain extent, and the original location in the barrels of roe samples analysed, is no longer known. In a student's project performed in connection to the present project it was indicated, that the location in the barrels during storage is important in lipid oxidation. Moradjow-Namin (1995) examined six barrels of normal heavily salted roe stored for 1 - 13 months. Samples were taken from top and lowest half of barrels. The PV's of deep samples ranged from 3.1 to 8.3 (one at 17.3 meqv./kg lipid), and of top samples from 9.5 to 14.7 (one at 30.0 meqv./kg lipid). For deep samples TBA-values ranged from 15.0 to 19.5 (one at 27.5 µmoles/kg roe), and for top samples from 21.6 to 31.1 (one at 17.9 µmoles/kg roe). FFA concentration was evenly distributed in the barrels. In all barrels higher PV's and TBA-values were noted for top than deep samples.

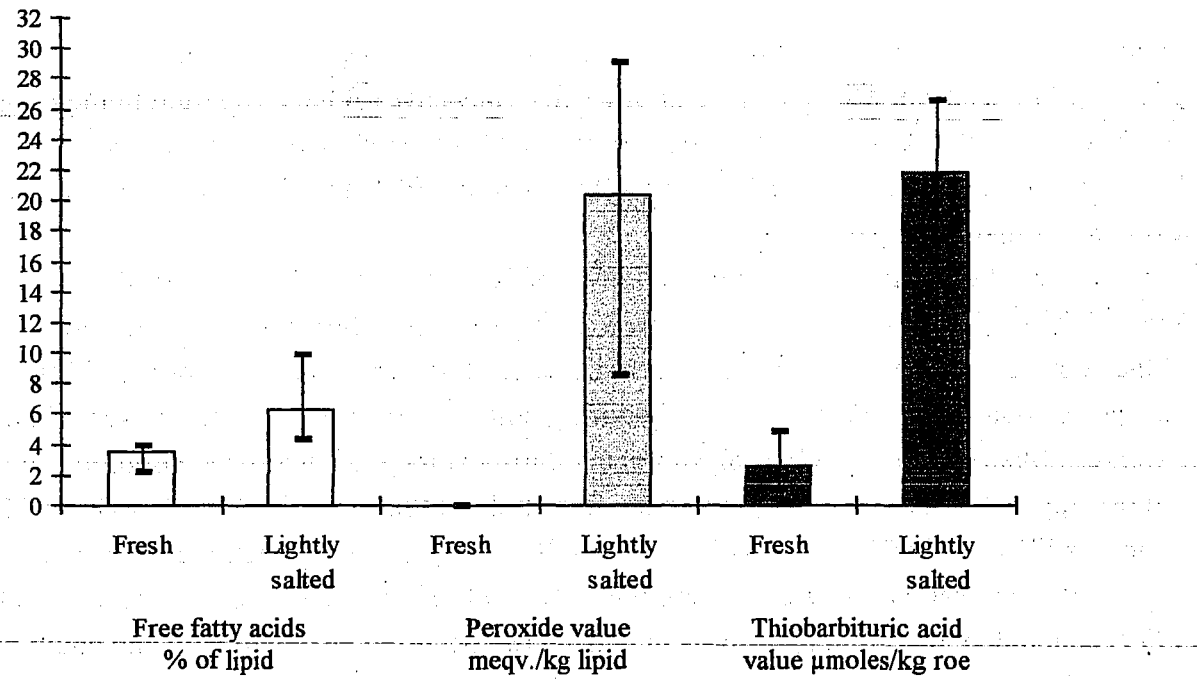


Figure 3.2. Free fatty acids, peroxide value and thiobarbituric acid value (mean values and range) of fresh roe of five lumpfish and of lightly salted roe, made by desalting of ten barrels of heavily salted roe stored for 8 months at ÷5°C (data from table 4 of paper 1, detailed data in table 3 of note 1).

Linko et al. (1979) examined the effect of different storage conditions on peroxide values in herring roe. Lower PV's during one year of storage of non-salted roe could be obtained by reducing the temperature from 5°C to ÷20°C. Further reduction in PV's were obtained by limiting the availability of oxygen through air-proof vacuum packaging (glass jars). During frozen storage in glass jars, no influence of 5 % w/w salt was observed on lipid oxidation. During storage of dry-salted roe with 6 and 16 % salt (no vacuum) at 5°C for one year, the authors reported a faster onset of lipid oxidation in heavily salted roe, but at the end of storage similar PV's were detected in both salted roes. Linko et al. (1983) studied lipid oxidation during storage of roe from Baltic herring, perch and rainbow trout, and concluded, that lipid oxidation is not generally accelerated by salt addition, but the effects of the salt may depend of the lipid and fatty acid composition of a specific roe product.
Shaban et al. (1984) measured TBA-values of chum salmon (*Oncorhynchus keta*) roe with two salt levels (not analysed) during storage at various temperatures. They showed that to prevent an increase of TBA-values during storage for 11 months without vacuum packaging, a temperature as low as ÷40°C was necessary. Even at ÷30°C slight increase in TBA-values occurred at both salt levels.

Changes in the lipid fraction of lumpfish roe reported in the present section, had occurred before the final roe products were even produced. The degree of lipid oxidation varied among samples originating from different barrels of roe, and occasionally among roe samples originating from the same barrel. As extracted from data reported by other workers, a low storage temperature is preferred in order to reduce the rate of lipid hydrolysis and oxidation, while limiting the availability of oxygen is an important factor in lipid oxidation only. In today's production of lumpfish roe, the temperature in storage of heavily salted roe has been lowered to ÷5°C compared to the 5°C, that was used some years back. Frozen storage of roe, which is one alternative to heavy salting, can have other drawbacks, apart form being expensive. Stodolnik et al. (1992) reported, that membrane hardness (resistance to crushing) of rainbow trout eggs stored at ÷25°C decreased markedly as early as after 4 months, which became more pronounced through storage for one year.
Though quality improvement of the heavily salted roe as raw material for production is beyond the scope of this thesis, it can within the existing procedures be suggested to give higher priority to the complete covering of roe with brine, before the storage is initiated. It could even prove useful in reduction of lipid oxidation, to avoid filling the barrels completely, and leave the upper part of barrels for filling with brine, as suggested by the differences in PV and TBA-values in top and deeper layers of barrels reported in this section. Processing means alternative to those used today are not easy to find, as such means should be applicable to a large number of local fishermen, working at very distant geographic locations along the coasts of Norway, Iceland and Canada.

3.5 Amino acids

3.5.1 Total amino acids

Fresh roe

The total amount of amino acids detected in fresh roe of five freshly killed lumpfish varied (87.3 - 108.7 mg/g roe), but the composition was rather uniform. 17 amino acids were identified, and the dominating amino acids (% w/w of identified amino acids) were glutamic acid (12.9 ± 0.10 %), leucine (9.9 ± 0.10 %) and aspartic acid (9.6 ± 0.09 %). Methionine and cyst(e)ine were present at the lowest concentrations, 2.6 ± 0.03 % and 1.5 ± 0.26 % respectively. The data on methionine may however be too low, as a certain amount of methionine is easily lost during the analysis; a peak (non-quantified) of oxidised methionine was also present in the chromatograms (paper 1). Apart from a lower concentration of glutamic acid detected in this study, the composition was similar to that reported by Rehbein (1985), who examined a commercial lumpfish roe product. The sulphur containing compound taurine (2-aminoethanesulphonate) was in the present study detected at a

concentration of 0.5 - 0.6 mg/g roe. (Total amino acids and taurine in fresh roe are shown in table 5 of paper 1, detailed data in table 4 of note 1).

Lightly salted roe

The total amount of amino acids varied among ten roe barrels after desalting (84.5 - 102.1 mg/g roe or 88.8 - 104.3 mg/g when corrected for the extra salt), and the composition was similar to that of fresh roe. Taurine was detected at a concentration of 0.1 - 0.2 mg/g roe. (Total amino acids and taurine in lightly salted roe is shown in table 5 of paper 1, detailed data in table 4 of note 1).

3.5.2 Free amino acids

Fresh roe

Free amino acids of fresh roe composed 0.28 ± 0.03 % of total amino acids, and the concentration ranged from 21.5 - 28.4 mg/100g (table 3.2). The composition differed from that of total amino acids, and a higher proportion of glutamic acid (29.1 - 36.7 % of free amino acids) and slightly higher of glycine were detected among free amino acids. Methionine could not be detected, and cyst(e)ine only in three of the five fresh roes. Tryptophane, not detected among total amino acids (lost during the acid hydrolysis), was detected at a concentration of 0.3 - 1.5 % of free amino acids. The composition of free amino acids (% of amino acids) is illustrated in figure 3.3. The actual concentrations (mg/100g roe) of individual free amino acids detected in fresh roe is illustrated in figure 3.4A.

The composition of free amino acids was less uniform among different fish than the composition of total amino acids. This may be explained by the fact, that composition of free amino acids changes considerably during the last few weeks before spawning (Gjessing 1963), and fish with ripe and less ripe roes can be caught throughout the season (Vuorela et al. 1979).

Taurine concentration ranged from 44.4 - 63.1 mg/100 g in fresh lumpfish roe. As reported in the previous section, taurine concentration detected in the analysis of total amino acids (0.5 - 0.6 mg/g) was at a similar level. Thus taurine, that is not a regular amino acid, is not incorporated into protein and is only present as the free compound.

Lightly salted roe

Free amino acid concentration in lightly salted roe varied in the range of 14.8 - 51.9 mg/100g roe or 15.2 - 54.4 mg/100 g, when corrected for the extra salt (table 3.2). The composition of free amino acids differed from that of fresh roe, which is illustrated in figure 3.3. Glutamic acid, glycine and alanine were present at a lower concentration, while isoleucine, leucine,

tyrosine, phenylalanine, tryptophane and arginine composed a greater part of free amino acids than in the fresh roe.

Taurine concentration was much lower than in fresh roe, and in the range of 6.6 - 22.0 mg/100 g (table 3.1). It seems reasonable, that taurine is washed out during desalting. This is a similar situation to what was reported by Herbert and Shewan (1975), who observed leaching (melt-water) of taurine from cod muscle kept in ice. The difference in taurine concentration between fresh and desalted roe, roughly equals the reduction in salt concentration during desalting of the ten barrels to 3.0 - 5.8 % (heavily salted roe 11 - 16 %, chapter 2) . In spite of the desalting, eight of the ten desalted roes had a higher total concentration of free amino acids than any of the fresh roes. Free amino acids composed 0.16 - 0.61 % of total amino acids compared to 0.24 - 0.30 % in the fresh roe (table 3.1), the lowest concentrations were detected in roe with low salt concentrations.

When taurine concentration is calculated relative to the total concentration of free amino acids, it is observed, that taurine concentration is around double the concentration of free amino acids in the fresh roe, while in lightly salted roe taurine concentration is only around one third the concentration of free amino acids (table 3.1).

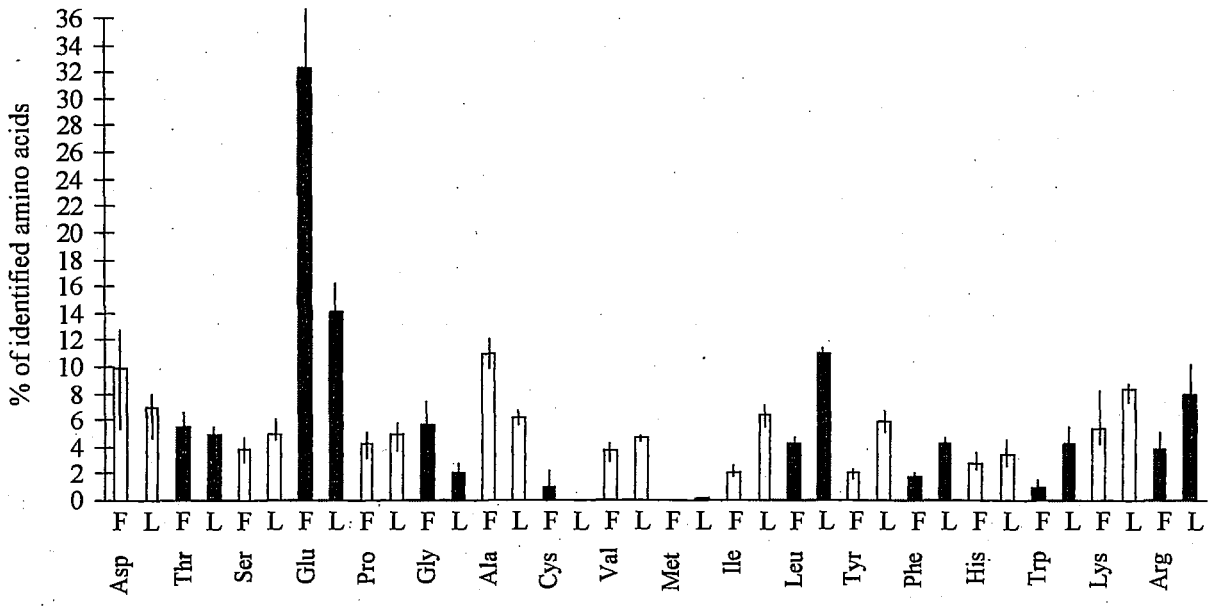


Figure 3.3 Composition of free amino acids (% of amino acids, mean values and range) of (F) fresh roe of five lumpfish and (L) lightly salted lumpfish roe made by desalting of ten barrels of heavily salted roe stored for 8 months at ÷5°C (Data from table 5 of paper 1, detailed data in table 5 of note 1).

Table 3.1. Free amino acids and taurine of desalted lumpfish roe (3.6 - 5.8 % w/w) made from ten barrels of heavily salted roe stored for 8 months at ÷5°C, and of fresh roe of five lumpfish. (Data from table 5 of paper 1, detailed data in table 5 of note 1).

	Fresh roe [1] Mean (range)	**Lightly salted roe** [2] Mean (range)
Free amino acids, mg/100g [3]	25.8 (21.5 - 28.4)	39.8 (15.2 - 54.4)
Free amino acids as % of total amino acids	0.28 (0.24 - 0.30)	0.41 (0.16 - 0.61)
Taurine mg/100g [3]	53.0 (44.4 - 63.1)	14.0 (6.6 - 22.0)
Taurine/free amino acids	2.1 (1.8 - 2.3)	0.35 (0.3 - 0.4)

1) Fresh roe of five lumpfish. 2) Lightly salted roe (3.0 - 5.8 % w/w) made from ten barrels of heavily salted roe, stored for 8 months at ÷5°C. 3) Concentrations in lightly salted roe are calculated as mg/100g of roe with 0.9 % salt.

The results indicate that, as free amino acids must be expected to be washed out during desalting together with other soluble compounds, the pool of free amino acids in lightly salted roe was supplemented during the long storage by breakdown of protein/peptides. The altered composition of free amino acids in lightly salted roe (figure 3.3) indicates, that some amino acids are liberated to a greater extent than others. This could be resulting from the specific amino acid compositions of various proteins/peptides attacked by autolytical enzymes at the actual storage conditions (11 - 16 % salt, ÷5°C).

The results presented in this section also showed, that pronounced variation occurred in the concentration of free amino acids in lightly salted roe (the actual concentrations of individual free amino acids detected in the ten batches of roe are illustrated in figure 3.4.B). This is probably due to the combined effects of variation in the extent of proteolysis and variations in the extent of washing out during desalting. Salt concentration of both heavily and lightly salted roe varies, and subsequently the total amount of water added to 105 kg roe during desalting also varies, even when products of similar salt concentration are produced. Thus in lightly salted lumpfish roe products, produced by standard procedures (see chapter 2), amino acids readily available for bacterial growth is not uniform among production batches.

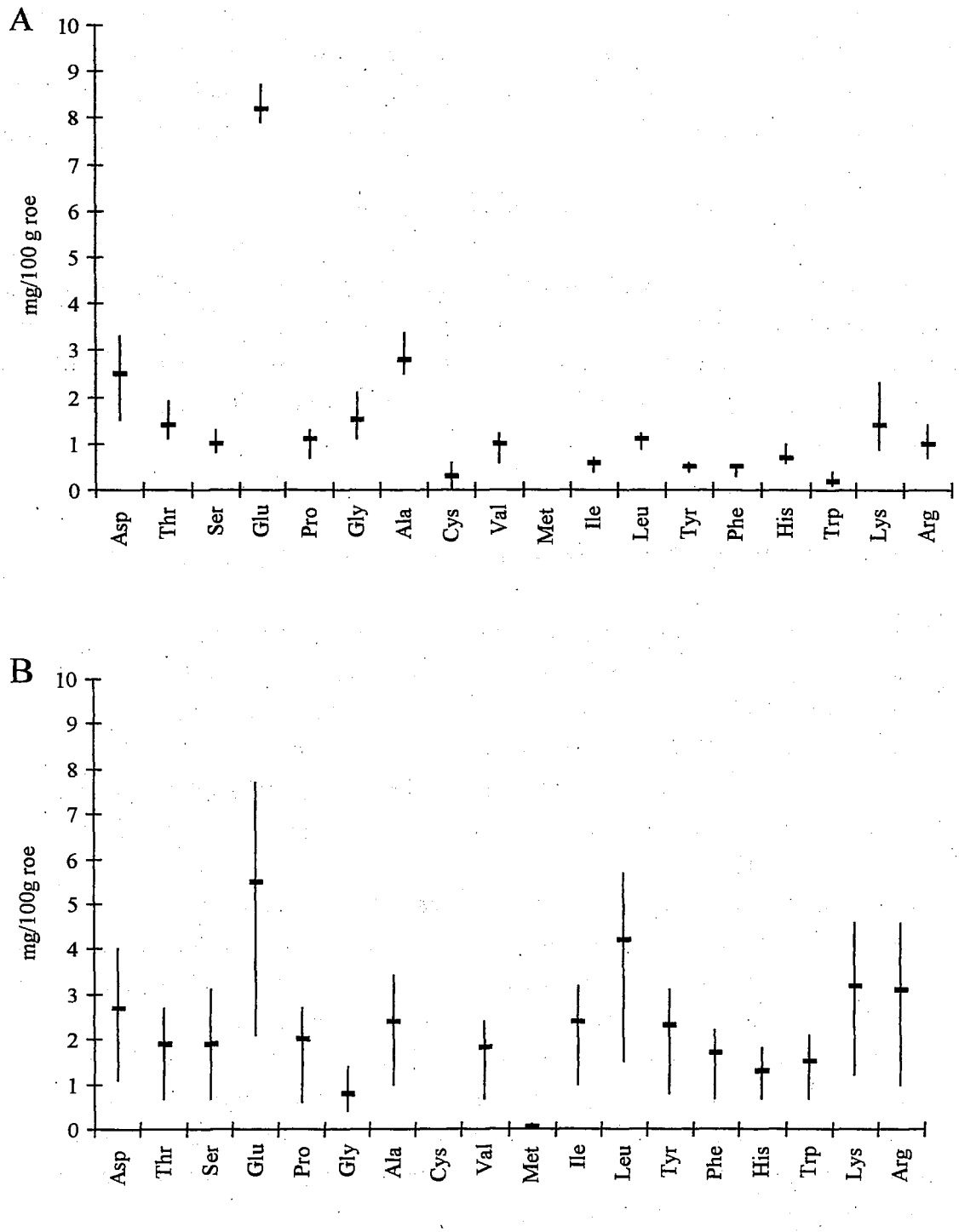


Figure 3.4. Actual concentration of free amino acids (mg/100g roe, mean values and range) of (A) fresh roe of five lumpfish (B) lightly salted lumpfish roe (salt 3.0 - 5.8 % w/w) after desalting of ten barrels of heavily salted roe stored for 8 months at ÷5°C (based on data from table 5 of note 1).

A large increase in free amino acids was observed by Chiou and Konosu (1988) during processing (salting, drying and sun-drying) of mullet (*Mugil cephalus*) roe. In the final product, the concentration of free amino acids was more than three times the concentration in fresh roe. During processing (soaking in 15 % saline and draining) of Alaska pollack (*Theragra chalcogramma*) roe into the salted roe product "Tarako", an increase of 14 % in free amino acids was reported by Chiou et al. (1989a). During the process 30 % of initial taurine was lost.
Chiou al. (1989b) found that caseinolytic, acid protease and aminopeptidase activities were present in the roes of mullet and Alaska pollack. The authors suggested that the enzymes responsible for the activities may be cathepsin D and/or a cathepsin D-like enzyme and at least two different aminopeptidases. The study supported the assumption, that the observed increases in free amino acids in the two types of roe may be caused by the combined actions of proteolytic enzymes. The enzymes were shown to be active to a different extent at the individual steps of processing.

3.6 Total volatile bases, trimethylamine and trimethylamineoxide

Concentrations of trimethylamineoxide and trimethylamine were determined in fresh, heavily salted and lightly salted roe. Both were low in all roes: ≤ 4.4 mgN/100 g and ≤ 1.3 mgN/100 g respectively (paper 1). In several roes the compounds were not detectable. Equally low levels of TMAO as detected in this study, were reported in herring roe by Huss and Larsen (1979) and Linko et al. (1979). Chiou and Konosu (1988) detected only traces of TMAO in fresh mullet roe, while 2 - 3 mgN/100 g has been reported for roe of Alaska pollack (Chiou et al. 1989a).
The concentration of total volatile bases, thus consisting of ammonia, of both heavily salted roe in barrels and fresh roe was in the range of 24 - 33 mgN/100 g, and in ten barrels after desalting TVB concentration was in the range of 4 - 12 mgN/100 g, generally being lowest in roe with the lowest salt concentrations (paper 1). Huss and Larsen (1979) and Linko et al. (1979) reported a TVB level in herring roe of 15 mgN/100 g and 10 - 20 mgN/100 g respectively. In a study in this laboratory on preservation of caviar-type lumpfish roe products (5.7 - 9.5 % salt), Huss et al. (1984) reported TVB concentrations in newly produced products (processing procedures as described in chapter 2) to be in the range of 5.0 - 16.7 mgN/100 g, based on analysis of 20 products.
(Detailed data on TVB, TMA and TMAO are shown in table 6 of note 1).

3.7 Summary and conclusions

Data on proximate composition of fresh lumpfish roe has been presented, and the composition did not change during processing except for a slight increase of moisture content during desalting.
Proximate composition varied among roes of individual fish. According to the literature the variation in proximate composition of fish roe is not the result of any systematic influence by catching season or time in the season.

Fatty acid composition of fresh and lightly salted lumpfish roe was similar and dominated by palmitic, oleic acid, eicosapentaenoic and docosahexaenoic acid.
During storage of heavily salted roe in barrels at ÷5°C lipid hydrolysis and oxidation occurred, as measured by increased levels of free fatty acids, peroxide values and thiobarbituric acid values in lightly salted roe. The most important factors in controlling these reactions were concluded from the literature to be low storage temperature and limitation of oxygen availability.

Composition of total amino acids of fresh and lightly salted lumpfish roe were similar. 17 amino acids were identified, among which the major acids were glutamic acid, leucine and aspartic acid. Composition of free amino acids in fresh roe was less uniform than that of total amino acids, and greater domination of glutamic acid was observed.
In spite of desalting, higher concentrations of free amino acids were detected in most lightly salted roes compared to fresh roe. The composition of free amino acids also differed from that of fresh roe.

The results indicated, that free amino acids were supplemented by autolytical liberation from peptides and/or proteins during storage of heavily salted roe. Some amino acids were released to a greater extent than others, probably depending on the nature of peptides and proteins attacked by autolytical enzymes. As a combined effect of proteolysis and washing out of soluble compounds during desalting, the actual concentrations of individual free amino acids in lightly salted roe varies among different production batches.

Lumpfish roe contained no or low levels of trimethylamineoxide (≤ 4.4 mgN/100 g). Total volatile bases in fresh and heavily salted roe was in the range of 25 - 30 mgN/100 g, which decreased during desalting to 4 - 12 mgN/100 g.

Chapter 4. Spoilage and microflora of lightly salted lumpfish roe

4.1 Introduction

This chapter will deal with sensory, microbial and chemical changes, that are taking place in lightly salted lumpfish roe during storage at 5°C and eventually lead to spoilage of the product. Spoilage is understood as changes resulting in the presence of unacceptable sensory characteristics.
The objective has been to point out potential spoilage organisms against which preservation of the product should be directed, and to contribute to an understanding of the spoilage mechanism of lightly salted lumpfish roe products.

4.2 Off-odour development

Off-odour development and thereby the shelf life of lightly salted lumpfish roe is highly variable. This was observed in storage experiments including eleven batches of lightly salted lumpfish roe with salt concentrations in the range of 3.5 - 4.8 % WPS. The roe originated from normal barrels of heavily salted roe, and the products were stored at 5°C for two and a half or three months.
Differences in off-odour development among batches were reported as different degrees of spoilage after a certain storage time rather than different shelf lives. This strategy was chosen to allow comparison of flora composition and chemical parameters among roe batches exhibiting different odour characteristics. At the end of storage three different situations regarding odour had occurred among batches (paper 2):

1) Practically no off-odour. Roe acceptable.
2) Off-odours described as: Sour, marinated, fruity and rotten. Roe close to borderline.
3) Strong off-odours described as: Sulphidy or cabbage-like as the dominating impression, in different combinations with rotten, sour, green/hay, fruity and ammonia-like. Roe rejected.

Thus within a range of salt concentrations on both sides of 4 % WPS, these different spoilage situations are likely to occur in a three months period, corresponding to the shelf life wanted by the industry (see chapter 1). Differences in flora composition were observed among batches with different odours, as it will be discussed in section 4.6.

4.3 Significance of microbial and non-microbial changes in quality and deterioration

4.3.1 Significance in development of spoilage off-odours

As spoilage can be the result of both microbial and non-microbial quality changes, sterile model systems of various kinds have been used in order to separate these and identify the main causes of spoilage. The ideal system used in studies of fish spoilage, is muscle blocks cut from the fish at sterile conditions (Herbert et al. 1971). Sterilisation after dilution as extracts have also been widely used, either by heat treatment (Gram et al. 1987, Dalgaard 1995) or filter sterilisation (Jørgensen and Huss 1989, Lerke et al. 1963).

In this project attempts have been made to produce filter sterilised roe extract from heavily salted roe after desalting (i.e. the normal raw material for production) by modification of the method of Jørgensen and Huss (1989). The method involves mincing, water addition, filtration, centrifugation (43.500 x g) and pressure filtration through 8 µ, 0.45 µ and 0.22 µ filters. The roe mince/water mixture could however not readily be filtered, therefore a mechanical press was used to squeeze the liquid from the solid material. Even after enhanced centrifugation (49.500 x g) only very small amounts of liquid could be pressure filtered through a series of filters. The risk of filter bursting was high and the yield per kg of roe was very low. Due to these obstacles, production of filter sterilised roe extract in sufficient amounts was not considered possible in practice.

Two different methods have been used instead. Lightly salted lumpfish roe have been added antimicrobial substances (paper 2) and roe product have been produced from sterile roe obtained from just killed lumpfish (paper 3). None of these methods are entirely satisfactory. Addition of chemical substances creates a risk of irrelevant chemical reactions, and just removed sterile roe is, though close, not identical to the heavily salted stored roe used for production, where changes in the lipid and free amino acid fractions have been shown to take place (chapter 3). The ideal system for imitating normal product conditions would be roe products made from sterile heavily salted roe stored at conditions used in the industry. Still both the above experiments showed that off-odours characteristic to spoilage described the previous section, did not develop when no microbial growth occurred.
In addition naturally contaminated lightly salted lumpfish roe products have, during this project, failed to develop off-odours though stored for three months (paper 2). This was not likely to occur, if non-microbial changes were significant in the formation of spoilage off-odours.
Adding these results it can be concluded, that lightly salted lumpfish roe at 5°C spoils due to bacterial activity.

4.3.2 Non-microbial changes influencing overall product quality

Though spoilage of the final roe products is caused by bacterial activity, the overall product quality is influenced by the non-microbial changes taking place, before the products are produced.
Changes in the lipid fraction of heavily salted roe described in section 3.4.2, have a direct influence on quality due to rancidity and complicates the odour assessments of the lumpfish roe products during storage. A constant level of rejections of 10 - 15 % due to rancidity, which did not increase as storage of the products proceeded, was noted in the off-odour assessments of lightly salted roe (paper 2).
In order to identify off-odours of bacterial origin, it has throughout the project been necessary to separate rancid off-odours already present in the newly produced product, from off-odours developing during storage of the lightly salted roe. Despite this separation, compounds e.g. aldehydes and ketones resulting from lipid breakdown might still have an unknown impact on off-odours of the product when combined with volatiles of bacterial origin. This theoretical impact has not been possible to eliminate in the performed experiments.

Liberation of free amino acids occurs during storage of heavily salted roe (section 3.5.2), and may also occur during storage of the product.
Some amino acids are bitter tasting including tyrosine, leucine, isoleucine, valine, phenylalanine and tryptophane (Wieser and Belitz 1975). Those amino acids except for valine, were present at higher concentrations in the lightly salted roe compared to fresh roe. In addition bitter tasting peptides could also have been formed due to proteolysis, as reported in cheddar cheese (Lee and Warthesen 1996). An increase of oligopeptides has been reported during processing of Alaska Pollack roe (Chiou et al. 1989a), while in Mullet roe fluctuations occurred during processing, but the changes were rather small (Chiou and Konosu (1988).
Quality improvement of the raw material (i.e. heavily salted roe) has not been an objective of this project. Therefore it has not been examined, whether the increased concentration of free amino acids has any direct influence on the taste of lightly salted roe.

4.4 Microflora of lightly salted lumpfish roe

4.4.1 Microflora developing during storage

The flora composition of five batches of lightly salted lumpfish roe after three months of storage at 5°C, are shown in table 4.1. The roe was produced from five barrels of heavily salted roe originally landed at different locations in Canada, that reflected a variety of normal roe raw material (paper 2).

Table 4.1. Flora composition [1] (counts > 5 log cfu/g) in five batches of lightly salted lumpfish roe after three months of storage (log cfu/g, blood agar with 3 % of salt at 10°C). The roe was vacuum-packed in glass jars and stored at 5°C. (Data from paper 2 and 3).

Batch	**1**	**2**	**3**	**4**	**5**
Salt concentration, % WPS [2]	4.2	3.7	3.8	3.6	3.8
Total viable count	**7.0**	**7.3**	**7.3**	**7.1**	**7.3**
Lactic Acid Bacteria (total)	**6.9**	**7.0**	**7.2**	**6.7**	**7.1**
Lactococcus sp. (A1)	6.7	-	-	-	-
Lactococcus sp. (A2)	-	6.0	6.5	6.3 [3]	7.0
Carnobacterium sp. (B1)	6.5	[4]	7.1	6.5	6.3
Carnobacterium sp. (B2)	-	7.0	-	-	-
Carnobacterium sp. (C)	-	-	-	5.7	-
Enterobacteriaceae (total)	**≤ 5**	**6.9**	**6.5**	**6.9**	**6.6**
Serratia liquefaciens (1)	-	6.8	5.7	6.9	-
Serratia plymuthica (2)	-	-	6.4	-	6.6
Morganella morganii (3) [5]	-	6.4	-	-	-
***Vibrio* spp.**	**6.1**	**≤ 5**	**6.1**	**≤ 5**	**5.5**

1) Based on differential counting, isolation (108 isolates) and basic testing. LAB (70) and Enterobacteriaceae (30) were further biochemically tested, grouped by numerical taxonomy and presumptively identified. Details are reported in paper 2 and 3. 2) Calculated from mean values of total salt and dry matter content. Standard deviations in these analysis were ≤ 3 % and ≤ 2 % respectively. 3) Group A2 was not recovered from Blood-agar, the count originates from APT-agar. 4) Low numbers of group B1 is also present, one single isolate was obtained from the ÷5 dilution. 5) At Statens Seruminstitut, Copenhagen by further testing identified as *Morganella morganii* subsp. *sibonii*

The total viable counts of the newly produced products were < 2 - 3 log cfu/g and increased to around 7 log cfu/g in three to four weeks (paper 2). This level was maintained throughout storage. The microflora at the end of storage consisted of Lactic Acid Bacteria (LAB), Enterobacteriaceae and *Vibrio* spp. Except for batch 1, LAB and Enterobacteriaceae in turn yielded the highest counts while *Vibrio* spp. generally composed a smaller part of the flora. In batch 1 Enterobacteriaceae failed to grow (< 5 log cfu/g), which may be due to the higher salt concentration of this batch (see section 4.4.2).

LAB and Enterobacteriaceae repeatedly developed as major parts of the microflora in experiments reported elsewhere in this thesis (paper 2, chapter 6 and 7). Total viable counts after storage of around 7 log cfu/g, have also continuously been observed, while levels of 8 log cfu/g only occasionally were noted (paper 2).

Indications on the variability of composition within the groups of LAB and Enterobacteriaceae have been obtained:

- In a storage experiment 28 LAB isolates were randomly chosen from six batches of roe stored for two and a half months at 5°C. Results from biochemical testing of the isolates (table 1 of note 5) showed, that all LAB isolated in that experiment were different from the LAB flora shown in table 4.1. Thus a wide range of LAB are able to grow in lightly salted lumpfish roe, and the genera showed in table 4.1 only reflects the LAB flora of those particular five batches.

- Enterobacteriaceae isolates were occasionally picked up from stored (5°C) lightly salted lumpfish roe products included in other experiments of this project (counts 5 log cfu/g or higher). Results from biochemical testing showed, that among 18 isolates obtained, 6 isolates were identical to or closely related to strains of group 1 (*Serratia liquefaciens*), 10 isolates were identical to or closely related to strains of group 2 (*Serratia plymuthica*) and one isolate was identified as *Morganella morganii* (subsp. *morganii*). The remaining isolate differed more, and was by the API 20E identification system suggested to be *Enterobacter agglomerans* (note 3).

The number of isolates are limited, and they cannot be claimed to reflect the total Enterobacteriaceae flora of the roe products from which they were isolated. The results show however, that when bacteria present in significant numbers are randomly picked up from a variety of chill stored lumpfish roe products, the majority of isolates are either identical or closely related to strains of the three groups shown in table 4.1, and other genera are rarely found. Thus the Enterobacteriaceae able to grow during chill storage of lightly salted lumpfish roe constitutes a much narrower range of organisms than does the LAB.

4.4.2 Factors affecting flora composition

The vacuum-packed lightly salted lumpfish roe product has, as defined in chapter 1, a salt concentration of around 4 % WPS and a pH of 5.4 (0.1 % w/w lactic acid); the product is stored at 5°C. It was shown in the previous section, that these conditions allows growth of LAB, Enterobacteriaceae and *Vibrio* spp..
Truelstrup Hansen (1995) reported that during a number of storage experiments, three different situations occurred regarding flora composition on cold smoked salmon stored at 5°C (3.9 - 4.8 % WPS): 1) Domination by LAB (7 - 9 log cfu/g), 2) Domination by LAB and Enterobacteriaceae (7 - 8 log cfu/g) or 3) Domination by *Photobacterium*/marine vibrios (6 - 7 log cfu/g). The typical microflora of spoiling vacuum packed normal pH meat (5.4 - 5.8) is dominated by LAB, while in vacuum packed high pH meat (> 6.0) other bacteria are also able to grow to higher numbers: *Shewanella putrefaciens*, Enterobacteriaceae and *Brochotrix thermosphacta* (Dainty and Mackey 1992, Borch et al. 1996). In "gravad" fish (lightly preserved fish product with 4 % of salt, no acid but sugar added) From and Huss (1988) found the flora of the spoiled product to consist of LAB, Enterobacteriaceae, Vibrionaceae, *Brochotrix thermosphacta* and *Shewanella putrefaciens.*

When comparing flora composition of lightly salted lumpfish roe to the above products, the differences maybe explained as the effect of a few intrinsic parameters characteristic to the products.
In spite of the lower pH and a higher salt concentration compared to meat, Enterobacteriaceae counts of 6 - 7 log cfu/g occurred in stored, lightly salted lumpfish roe (table 4.1). A higher concentration of lactic acid is however characteristic of normal pH meat (0.9 % w/v, Dainty and Mackey 1992) . Since lactic acid is inhibitory to psycrotrophic Enterobacteriaceae at anaerobic conditions (*Serratia liquefaciens* and *Enterobacter cloacae*, Grau 1981), these bacteria are inhibited in normal pH meat.
The pH of lightly salted lumpfish roe is comparable to that of normal pH meat, but lower than that of cold smoked salmon and "gravad" fish (5.8 - 6.2). *Vibrio* spp. does not compete well at low pH (<5.5) and lack of oxygen (Walker 1992). Growth of *Brochotrix thermosphacta* is at low oxygen tension inhibited in meat at pH 5.8 or lower (Campbell et al. 1979), while in the absence of lactic acid it has been reported to grow anaerobically down to pH 5.5 (Grau 1980). *Shewanella putrefaciens* does not grow on meat of normal pH, and is unable to grow below pH 6.0 at refrigeration temperature (Gill and Newton 1979).
Thus the lower pH of lightly salted lumpfish roe may explain, why the flora composition is less variable than observed for the other lightly preserved fish products mentioned above.

As discussed in section 4.4.1 the range of different Enterobacteriaceae able to grow is rather narrow and dominated by *Serratia* spp. (*Ser. liquefaciens* and *plymuthica*). *Serratia* spp.

occur in the natural environment (soil, water, plant surfaces), *Serratia liquefaciens* being the most prevalent species. The principal habitat of *Serratia plymuthica* is probably water, the same species being isolated from marine and fresh water (Grimont and Grimont 1984, Grimont and Grimont 1992). Therefore *Serratia* spp. can easily be introduced into foods. An additional factor in lumpfish roe production is the large amounts of tap water added directly into the roe during the desalting process. The apparently random occurrence of *Morganella morganii* (table 4.1 and note 3) may be determined by the presence or absence in the initial flora. *Morganella morganii* is not part of the flora typical to newly caught cold water fish, and are likely to be added to fish/fish products during handling (Liston 1992). The roe originates from different geographic locations, and is handled by a large number of small producers at very different hygienic conditions.

Serratia spp. is known among Enterobacteriaceae to tolerate relatively high salt concentrations, several species does at certain conditions grow at 7 % salt, a few even higher (Grimont and Grimont 1984). When inoculated in lumpfish roe extract, most of the isolated strains did not grow at such high levels of salt, which is illustrated in figure 4.1. The combination of salt (4 % WPS) and pH (5.4) in the defined roe product is close to the limit of growth of most of the strains. Lowering the pH of the product by half a unit or increasing the salt concentration to 4.5 % has a considerable inhibitory effect. Still growth of one of the tested strains (F 79) was only slightly inhibited by the combination pH 4.9 and 5 % of salt. Growth in roe extract may be less advantageous due to the heat treatment and dilution included in the processing (note 2). The inoculated *Morganella morganii* strains F39-1 and E5 failed to grow in the roe extract, but they were originally isolated from stored roe products (pH 5.4) with salt concentrations of 3.7 % (section 4.4.1) and 4.5 % WPS (note 3) respectively.

The results shown in figure 4.1 indicate that a balance between pH, varying salt concentration and initial flora of *Serratia* spp. could determine which species, if any, will grow in a specific lightly salted lumpfish roe product, with salt concentrations between 4 and 5 %. This suggestion was to some extend supported by the data obtained on stored products. In six batches of lightly salted lumpfish roe (two and a half months at 5°C) LAB and Enterobacteriaceae counts were determined using selective agars. When these results are combined with those shown in table 4.1, eleven roe batches with salt concentrations ranging from 3.5 % WPS to 4.8 % WPS are obtained (table 1 of paper 2). Among four batches (1, 9 - 11) with salt concentrations above 4 % WPS, growth of Enterobacteriaceae (> 5 log cfu/g) only occurred in one (10). In all the remaining seven batches (3.5 - 3.8 % WPS) growth occurred, in two batches (6 and 8) however only to a level of 5.3 and 5.5 log cfu/g (see also table 4.3). These low counts may be influenced by the use of selective agar, which in general yielded lower counts than those obtained by isolation and characterisation from non-selective agar.

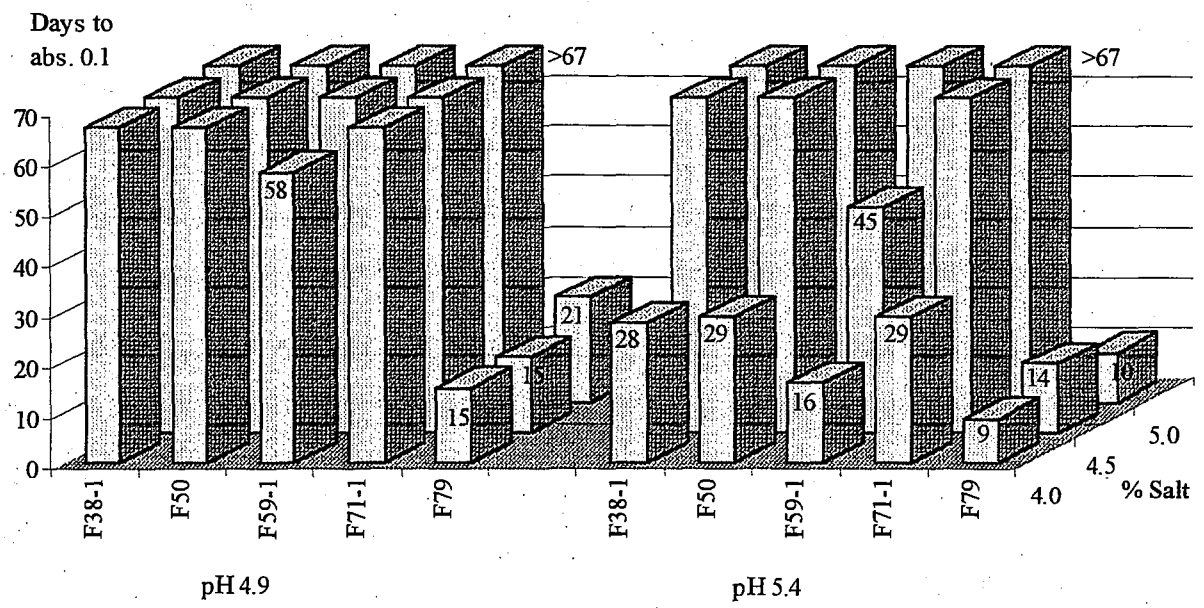


Figure 4.1. Growth of different *Serratia* isolates in roe extract at 5°C as affected by pH and salt concentration (days until absorbance (600 nm) of 0.1 was reached). Initial inoculum ≈ 5 log cfu/g. F38-1, F50 and F59-1: Group 1 (*Serratia liquefaciens*), F71-1 and F79: Group 2 (*Serratia plymuthica*) (data from note 2).

4.5 Chemical spoilage indicators

When the concentrations of particular compounds/groups of compounds, resulting from bacterial activity, during storage increases above their thresholds, off-odours and thereby spoilage begin to appear. Several compounds have been suggested as chemical indicators of spoilage of fish and/or meat. The potential of some of these as indicators of lightly salted lumpfish roe spoilage are evaluated in the following sections.

4.5.1 Trimethylamine

Trimethylamineoxide (TMAO) plays an important role in fish spoilage by its microbial reduction to trimethylamine (TMA) contributing to off-odours and off-flavours. TMAO serves as electron acceptor during anaerobic respiration by well known fish spoilage bacteria as *Shewanella putrefaciens* and *Photobacterium phosphoreum* (Gram et al. 1987, Dalgaard et al. 1993).

Fresh, heavily salted and desalted lumpfish roe analysed during this project, contained no or very small amounts of TMAO (section 3.6). This is in accordance with Huss and Larsen

(1979), who found both initial TMAO and resulting TMA concentrations in herring roe at 10°C to be very low (< 4 mgN/100g). During storage of Baltic herring roe (6 % salt) at 5°C Linko et al. (1979) found TMA concentration to be < 1 mgN/100g and to remain at that level for several months, but then an increase of TMA concentration was observed. However no values were given on the magnitude of the increase, and no suggestions on the mechanism involved. Jessen (1986) found cod roe to contain 6 - 7 mgN/100g of TMAO, and the TMA concentration to increase to more than 15 mgN/100g during storage in ice. It was therefore suggested that endogenous synthesis of TMA occurred in cod roe.

Due to these reports on TMA of roes increasing to higher levels than initial TMAO, TMAO and TMA analyses were included in a storage experiment of this project. During storage of six roe batches at 5°C for two and a half months no increase in TMA concentration was observed, neither in spoiled nor non-spoiled roe (paper 2).

4.5.2 Total volatile bases

Analysis of the concentration of total volatile bases (TVB) determines the production of ammonia by bacterial deamination of amino acids during storage (since TMA, part of this fraction, is negligible). This indicator may be useful in products without TMA production, providing the formed ammonia is important in spoilage.

TVB concentration in six batches of lightly salted lumpfish roe stored at 5°C, showed a uniform increase (final value 19.5 ± 1.7 mgN/100g after three months) despite a very different off-odour development (figure 1 of paper 2, detailed data in table 6 of note 1). Thus TVB concentration showed no correlation to sensory characteristics of stored lumpfish roe products, and was rather reflecting bacterial growth in general. This makes the TVB concentration an equally unsuitable spoilage indicator in lightly salted lumpfish roe, as are the total viable counts of the product.

4.5.3 Nucleotides

The K-value based on nucleotide degradation in fish muscle was originally proposed by Saito et al. (1959) as an indicator of fish freshness. The K-value is defined as the percentage of total nucleotide concentration that is composed by inosine and hypoxantine, resulting from a series of degradation reactions:
Adenosine triphosphate (ATP) → Adenosine diphosphate (ADP) → Adenosine mono-phosphate (AMP) → Inosine monophosphate (IMP) → Inosine (Ino) → Hypoxantine (Hx)

In the two last steps of degradation bacteria have been shown to contribute to the process (Jørgensen and Huss 1989, Ravn Jørgensen et al. 1988, Surette et al. 1988).
Truelstrup Hansen et al. (1995) reported a clear relation between hypoxanthine and sensory quality of cold smoked salmon in the manner, that values of 5 - 7 µmol/g corresponded to the limit of overall sensory acceptability, as assessed by a panel. Although the long storage period of lumpfish roe before production might create a completely different situation regarding nucleotide degradation, a few preliminary analyses were performed in this project using the analytical procedure described by Truelstrup Hansen et al. (1995).
Nucleotide concentrations of five batches of lightly salted roe stored for three months at 5°C (paper 2, batch 1 to 5) were low and uniform among batches. Hypoxanthine concentration of the five roe batches were 0.19 ± 0.03 µmol/g. Lower levels of IMP were detected in four of the roes (0.13 ± 0.02 µmol/g) and Ino in two (0.05 and 0.06 µmol/g). Due to the low concentrations detected in both spoiled and non-spoiled roe, the changes in nucleotide concentrations during storage of lightly salted lumpfish roe have not been examined.

4.5.4 Formation of acids

Acids are produced during the fermentation of carbohydrates, examples of which are the production of lactic acid from glucose by homofermentation, and the production of formic, acetic and lactic acid in butanediol fermentation in some Enterobacteriaceae (Gottschalk 1986, Axelsson 1993, Brock and Madigan 1991). Acids are also produced in amino acid degradation, and acetic acid can further be produced from pyruvate formed during deamination of various amino acids (Eskin et al. 1971).
Accumulation of short chain fatty acids occurred during chill storage of vacuum packed beef exhibiting sour/acid/cheesy off-odours (Dainty et al. 1979), and elevated levels of acetic and butyric acid were judged as likely contributors to the odour. Sutherland et al. (1976) attributed the acidic odour of chill stored vacuum packed beef to the presence of short chain volatile fatty acids, mainly acetic acid. Truelstrup Hansen et al. (1995) also reported microbial production of acetic acid during chill storage of cold smoked salmon, but the concentration at sensory rejection varied considerably (12 - 23 µmol/g).
Own investigations showed, that lactic acid concentration of lightly salted lumpish roe did not exceed the added amount (0.1 % w/w), and only slight changes occurred during storage, while small amounts of acetic acid was formed. Formic and propionic acid could not be detected (paper 2, detailed data in table 7 of note 1). Final concentrations of acetic acid in five batches ranged between 3.2 ± 0.05 and 6.3 ± 0.23 µmol/g, and a higher concentration was detected in a borderline batch compared to a heavily spoiled batch.

4.5.5 Volatile sulphur compounds

Volatile sulphur compounds have been shown to contribute largely to the development of off-odours during storage of several fish and meat products including chill stored cod (Herbert et al. 1971, Herbert et al. 1975, Herbert and Shewan 1976), high pH (pH 6.1 - 6.3) vacuum packed pork (Edwards and Dainty 1987), high pH (pH 6.2 - 6.6) vacuum packed beef (Dainty et al. 1979), ground beef (Stutz et al. 1991) and in some cases vacuum-packed sliced bacon (Gardner and Patterson 1975). Through several of these reports it is well established, that volatile sulphur compounds detected in significant amounts at spoilage, are the result of bacterial activity and do not accumulate in products without bacterial growth. Low odour thresholds are characteristic to several volatile sulphur compounds (Whitfield and Tindale 1984, Belitz and Grosh) and off-odours at rejection are by the above authors described as sulphidy, putrid, sour-sink and cabbage-like.

Precursors of some of the volatile sulphur compounds are known. The precursor for hydrogensulphide production by fish spoilage bacteria was shown to be the sulphur containing amino acid cyst(e)ine, while methionine was the precursor of both methanethiol and dimethylsulphide (Herbert and Shewan 1976).

The following reaction leading to hydrogensulphide production was suggested by Chatagner and Sauret-Ignazi (1956) (c.f. Herbert and Shewan 1975):
3 **cysteine** $\leftrightarrow$ NH_3 + pyruvic acid + **H_2S** + cystine
Methionine is decomposed through a two step reaction (Segal and Starkey 1969):
Methionine $\rightarrow$ NH_3 + ketomethyl mercapto butyric acid $\rightarrow$ **methanethiol** + keto butyric acid
(By further oxidation half a mole **dimethylsulphide** (and carbondioxide and water) can be formed from methanethiol).
Precursors and mechanisms of production of several volatile sulphur compounds are not clear, and chemical reactions transforming microbially produced sulphur compounds into other sulphur compounds could also be involved. Lee and Simard (1984) detected methanethiol after growth of LAB in different agar media, and suggested a reaction between produced hydrogensulphide and free methionine to be responsible for the detected methanethiol.

The bacteria examined by Herbert and Shewan (1976) were spoilage bacteria of aerobically stored cod and belonged to the genera *Pseudomonas* and *Alteromonas* including strains now classified as *Shewanella putrefaciens*. These genera do not develop during storage of lightly salted lumpfish roe (see section 4.4.1). The ability to produce hydrogensulphide from cystein is however distributed among many other bacteria, including some of concern in lightly

salted lumpfish roe. The production of volatile sulphur compounds by potential spoilage organisms of lightly salted lumpfish roe is discussed in more detail in section 4.6.
In this project several different volatile sulphur compounds have been detected in the headspace of naturally spoiled lightly salted lumpfish roe. Opposed to the other spoilage indicators examined, volatile sulphur compounds in the roe headspace does seem related to the odour characteristics of the spoiled roe products (paper 2).

Due to the cabbage-like, sulphidy nature of off-odours detected in heavily spoiled roe products (see section 4.2), volatile sulphur compound analysis was performed on roe products exhibiting different degrees of spoilage. The results are shown in table 4.2. A relation seem to exists between volatile sulphur compounds and the degree of spoilage. In spoiled batches (6, 7 and 10) four compounds were detected, while in the non-spoiled and borderline batches two or more of the compounds were detected in smaller amount or not detected at all.

Table 4.2 Odour characteristics and volatile sulphur compounds of six batches of lumpfish roe (pH 5.4 and 3.5 - 4.8 % WPS) after vacuum packed storage for two and a half months at 5°C (data from paper 2).

Batch	**6**	**7**	**8**	**9**	**10**	**11**
%-rejection	100	100	50	25	75	25
Odour	sulphidy, green/hay, sour, rotten	sulphidy, sour, rotten	sour (borderline)	(rotten, acceptable)	sulphidy, rotten	(sour, green, rotten, acceptable)
Volatile sulphur compounds:						
H_2S	+[1)]	+	(+)	-	+	(+)
CS_2 [2)]	+	-[3)]	+	-	+	(+)
CH_3SH	+	+	(+)	-	+	+
CH_3CH_2SH/ CH_3SCH_3 [4)]	+	+	(+)	-	+	+

1) +: The compound detected, (+): Peak area max. 25 % of the largest peak area of the same compound in the other roe batches, - : Peak area below detection limit. 2) Likely identity. Retention time 0.64 - 0.71 min. 3) Overflow of the coloumn by H_2S might have hidden the peak. 4) Retention times of the two standards very close, identity inconclusive.

The method does not allow direct quantification and comparison to odour thresholds, but the peak area of a compound can be compared to the peak area of the same compound in the headspace of other batches of roe. Quantitative comparison cannot be made between different compounds of the same batch. Thus the results represent a rough profile of volatile sulphur compounds in the headspace, and the main obstacle is that odour thresholds of the compounds (or combination of compounds) relevant in off-odour of this particular product is not known. Odour threshold above which a compound has a significant impact on spoilage odours may for example lie within the range of concentrations represented as "+" for one compound, and within the range of "(+)" for another compound.

Still it can be concluded that several volatile sulphur compounds are produced during storage of lightly salted lumpfish roe. When the sulphidy nature of odours of some spoiled batches, and the low odour threshold of several of the compounds are considered, volatile sulphur compounds are likely to be relevant in spoilage of lightly salted lumpfish roe, and worth further investigation.

4.5.6 Indole

Indole concentration have not been much studied as a spoilage indicator. Some reports however suggest, that indole formation can play a role in spoilage of certain foods. Giaccone et al. (1994) concluded that indole is a reliable indicator of sensory and hygienic quality of bovine tripe, and attributed the increase of indole concentration to growth of Entero-bacteriaceae. Dainty (1996a) mentioned some unpublished results showing, that a case of unusual spoilage in vacuum-packed ham was caused by a *Morganella morganii*, which produced both indole and hydrogensulphide. Off-odours due to indole are by Whitfield and Tindale (1984) described as faecal and moth ball-like (threshold 300 µg/kg).
As one mole of indole is produced from one mole of tryptophane (Whitfield and Tindale 1984), it can be calculated, that 0.05 mg/100 g tryptophane is needed for 300 µg/kg indole to be formed. Tryptophane concentration is in desalted roe ready for production: 0.7 - 2.1 mg/100g, (based on data shown in table 5 of note 1). Through the further reactions skatole can be formed exhibiting faecal odours (threshold 10µg/kg) (Whitfield and Tindale 1984). During storage of lightly salted lumpfish roe inoculated with *Morganella morganii* faecal odours developed among others at an early stage, before volatile sulphur compounds could be detected (see section 4.6.1). Indole and skatole was not determined in this project, but can be suggested included in future studies of spoilage reactions due to growth of *Morganella morganii*.

4.6 Potential spoilage organisms

By comparison of flora composition of stored roe products exhibiting different odour characteristics, indications can be obtained on which organisms may be important in spoilage.
In table 4.3 eleven batches of stored roe products are grouped according to their odour characteristics. The odour groups corresponds to those described in section 4.2.

Table 4.3 Off-odours and counts of LAB and Enterobacteriaceae of eleven batches of lightly salted lumpfish roe (pH 5.4, 3.5 - 4.8 % WPS, vacuum packed and stored at 5°C). Batch 1 - 5 had been stored for three months, batch 6 - 11 for two and a half month. For definition of groups see table 4.1 and paper 3. (Data from paper 2 and 3).

Odours	**%-rejection**	**Batch**	**Enterobacteriaceae** log cfu/g	**LAB** log cfu/g
Very weak	10-25	1 [1)]	≤ 5	6.9 (group A1, B1)
		9,11 [2)]	≤ 5	7.3 - 7.6
Sour, marinated, rotten	37-53	3 [3)]	6.5 (group 1, 2)	7.2 (group A2, B1)
		4	6.9 (group 1)	6.7 (group A2, B1, C)
		5 [4)]	6.6 (group 2)	7.1 (group A2, B1)
	50	8 [2)]	5.5	7.7
Sulphidy or cabbage-like, sour, rotten green/hay	73	2	6.9 (group 1, 3)	7.1 (group A2, B2)
	100	6, 7 [2)]	5.3 - 5.8	8.0 - 8.1
	75	10 [2)]	5.8	7.3

1) And 6.1 log cfu/g *Vibrio* spp. 2) Counts of batch 6 - 11 were obtained on selective agars. 3) And 6.1 log cfu/g *Vibrio* spp. 4) And 5.5 log cfu/g *Vibrio* spp.

In general LAB counts were at a level of around 7 log cfu/g regardless of the odour characteristics of the stored roe. In the heavily spoiled batches 6 and 7, however higher counts of around 8 log cfu/g were noted. As Enterobacteriaceae counts were low in those batches, spoilage by LAB were indicated. The results of those particular batches should however be interpreted with precaution, as this roe may have been spoiled for some time (%-rejection 100) when analysed. As earlier discussed, the counts of Enterobacteriaceae on selective agar tended to be lower than on non-selective agar. Among LAB, the group B2 also draws attention, as it was only detected in the heavily spoiled batch 2.

In none of the batches with very weak odours, growth of Enterobacteriaceae had occurred (≤ 5 log cfu/g). When roe batches of which the composition of Enterobacteriaceae were known, was compared, the results indicated, that group 3, *Morganella morganii* (spoiled batch 2) may be able to produce more offensive odours at similar counts, than the group 1 and 2, *Serratia* spp. (borderline batches 3 - 5).
The highest count of *Vibrio* spp. was detected in the non-spoiled batch 1, and *Vibrio* spp. were not detected in borderline batch 4 and spoiled batch 2, indicating that those organisms were unimportant in spoilage of the roe.

Spoilage potential of the three groups of bacteria was further examined, and is discussed in the following sections.
The spoilage potential of pure cultures of bacterial isolates from stored roe products was examined in two inoculation experiments by the determination of produced off-odours and volatile sulphur compounds. Pasteurised lightly salted roe made from normal desalted roe was used as a model system. This system was preferred to heat treated juice, due to the absence of dilution, the milder heat treatment and the structural similarity to normal roe products. 16 strains were inoculated into the roe (4 - 5 log cfu/g) representing all the groups and subgroups of table 4.1. Two *Morganella morganii* produced strong off-odours (see section 4.6.1) and two *Carnobacterium* spp. produced weak off-odours (see section 4.6.2). For the remaining 12 strains very weak off-odours were occasionally detected during storage (103 days in total) without any systematic relation to neither storage time nor viable counts. The counts of all 12 strains were between 5.8 and 7.3 log cfu/g at the end of storage (paper 3).

As a majority of strains failed to produce off-odours, lightly salted roe products made from sterile roe obtained from newly killed lumpfish was inoculated in a second experiment (paper 3). This was done in the lack of a better model system, in order to avoid the heat treatment, despite the fact that fresh roe is not used in the normal production. The removal of roe form the fish is complicated by the location of the roe sacs right next to the intestines, and by their "non-solid" nature, which is difficult to handle. Consequently a low level of contamination (< 1 log cfu/g) occurred in several roes, developing into high levels during storage. Therefore only a few results on roe inoculated with Enterobacteriaceae are available from that experiment (see section 4.6.1).

4.6.1 Enterobacteriaceae

Enterobacteriaceae are generally considered organisms of a high spoilage potential. When carbohydrates are exhausted and amino acids attacked, sulphur compounds can be produced

and in addition acids and amines from decarboxylation of amino acids as reviewed by Gill (1986).
Hydrogensulphide production from cysteine is common among Enterobacteriaceae (Cowan 1974). Production of volatile sulphur compounds by different species of Enterobacteriaceae are frequently reported, and members of the family are therefore believed to contribute to spoilage of vacuum packed meat at certain conditions favouring their growth (Edwards and Dainty 1987, Gill 1986, Dainty and Mackey 1992).

Morganella morganii
Unpublished results showing hydrogensulphide production in vacuum packed ham by *Morganella morganii* has been mentioned by Dainty (1996a). The organism also produced methanthiol (Dainty 1996b). Production of volatile sulphur compounds by related organisms of the genus *Proteus,* which at that time included *Proteus morganii* (now *Morganella morganii*, Brenner 1984) has also been reported. Segal and Starkey (1969) showed production of methanethiol from methionine by *Proteus vulgaris,* and *Proteus inconstans* have been reported to cause an unusual kind of spoilage of vacuum packed sliced bacon due to production of methanethiol and "cabbage odour" (Gardner and Patterson 1975).

In the present study, pasteurised roe inoculated with two different *Morganella morganii* (isolates F39-1 supsp. *sibonii* (section 4.4.1) and E5 subsp. *morganii* (note 3)) was rejected after one week, due to off-odours described as sulphidy, faecal and green/decaying grass (counts of F39-1 were 5.8 log cfu/g and of E5 5.4 log cfu/g), but no volatile sulphur compounds were detected (paper 3).
After three weeks as the roe was heavily spoiled (rejected due to strong sulphidy, cabbage-like, faecal, sewer-like off-odours by all panellists) counts had reached levels of 7.4 (F39-1) and 6.9 (E5) log cfu/g. Several volatile sulphur compounds were detected and identical profiles were obtained for the two strains (figure 4.2 A, see also table 4.4). As perfectly detectable amounts of four volatile sulphur compounds were earlier detected in a non-spoiled roe product (batch 11, table 4.2), the absence of at least those compounds at first off-odour detection in pasteurised roe with *Morganella morganii,* was not likely to be due to inadequacy of the sulphur compound analysis.
Morganella morganii (F39-1) caused rejection of lightly salted lumpfish roe made from fresh roe at counts of 6.3 log cfu/g, but odours were transient, and the storage was continued. When counts had reached 7.3 log cfu/g odours were described as cabbage-like, sulphidy and sewer-like, and several volatile sulphides were detected (figure 4.2 B, table 4.4).

From these experiments it can be concluded that *Morganella morganii* produced sulphidy, cabbage-like, faecal off-odours and several volatile sulphur compounds in both pasteurised and fresh roe at counts of 6 - 7 log cfu/g. In the naturally spoiled batch 2 (cabbage-like,

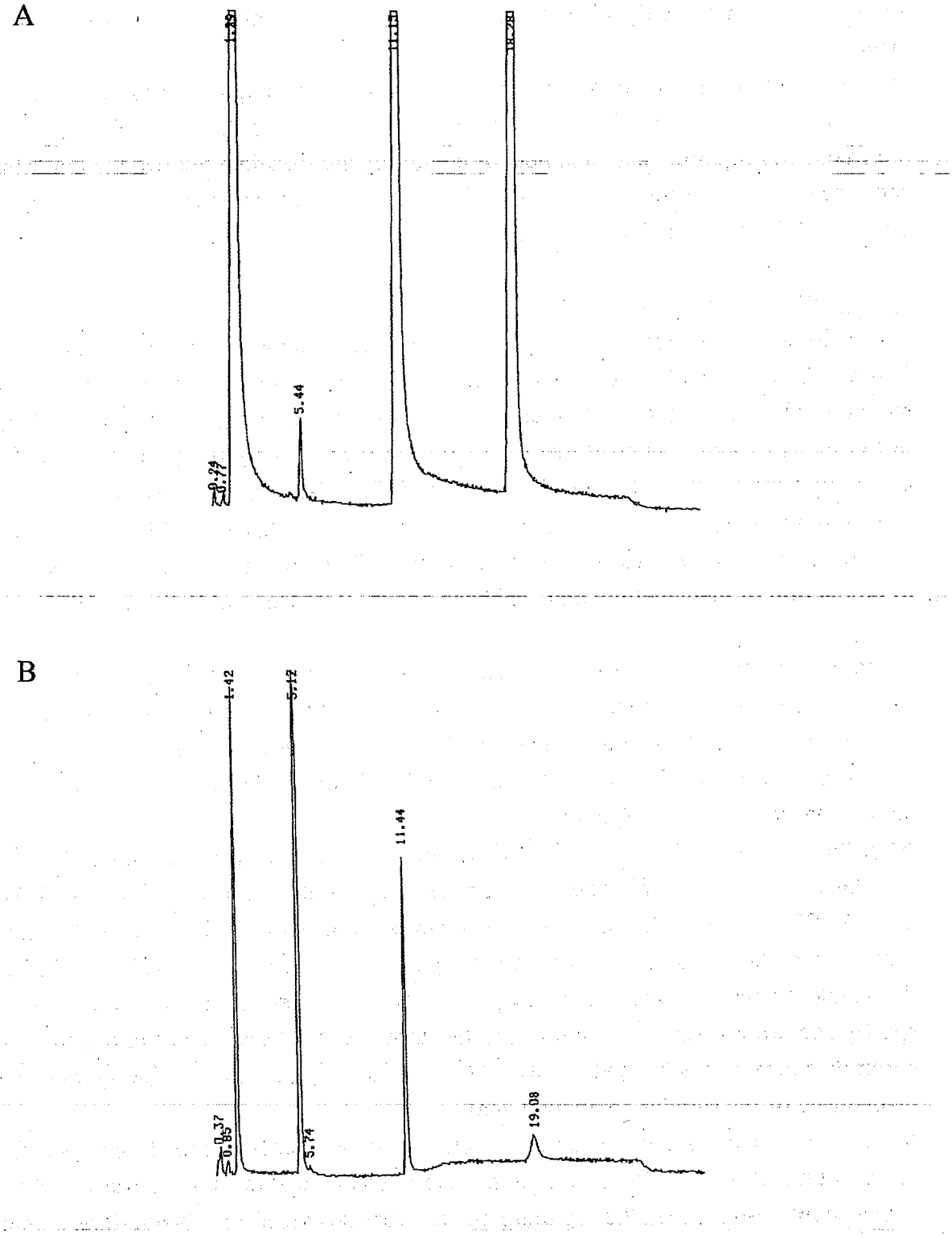


Figure 4.2.. Chromatograms of volatile sulphur compounds in headspace gas (3 ml gas injected) from spoiled lightly salted roe inoculated with *Morganella morganii* (F39-1). In headspace gas from corresponding sterile controls only traces were detected. **A**: Pasteurised roe product (3.6 % WPS). **B**: Roe product of fresh sterile roe (3.0 % WPS). The products were vacuum packed and stored at 5°C, pH was 5.4.

ammonia-like, fruity, rotten (table 4.3), from which F39-1 was originally isolated, counts of *Morganella morganii* were 6.4 log cfu/g (table 4.1), thus at a similar level.
The profiles of sulphur compounds produced in the two types of inoculated roe were not identical, and may be influenced by several factors including variation in salt concentration and storage times among experiments, and by unknown effects of the difference in roe substrate. The relation to profiles of sulphur compounds in naturally spoiled roe, and the significance of available precursors for the production is discussed in section 4.8. Off-odours were in both types of roe detected before the volatile sulphur compounds, and other volatile compounds are likely to contribute to the spoilage odours. One suggestion to explain the faecal odours is the formation of indole from tryptophane, as discussed in section 4.5.6.

Serratia spp.
Serratia liquefaciens readily produced off-odours, and appeared to be the major cause of spoilage in DFD meat (Gill and Newton 1979). The end products responsible for the off-odour were however not examined in that study. Production of spoilage odours by *Serratia liquefaciens* was likewise reported by Patterson and Gibbs (1977), who also detected hydrogensulphide production by the organism, when inoculated into vacuum-packed high pH meat at 4°C.

When inoculated into pasteurised roe none of the *Serratia* spp. (three *Serratia liquefaciens* and three *Serratia plymuthica*) caused rejection of the roe during 103 days at 5°C (paper 3). Counts of two strains (one of each *Serratia* spp.) did not exceed 6.4 log cfu/g, while the remaining four strains reached maximum counts between 7.0 and 8.0 log cfu/g in two weeks. Two different *Serratia liquefaciens* (86 % similarity in numerical taxonomy analysis, paper 3) were successfully tested in lightly salted lumpfish roe made from fresh roe.
Serratia liquefaciens (F38-1) grew to 6.5 log cfu/g in two weeks, and did not cause rejection of the roe. *Serratia liquefaciens* (F50) caused rejection due to off-odours described as sour, sulphidy and faecal at counts of 8.0 log cfu/g. Only very small amounts of an unidentified sulphur compound not detected in naturally spoiled products, was noted (table 4.4). Thus *Serratia liquefaciens* (F50) is able to produce off-odours similar to those observed in naturally spoiled products, but at considerably higher counts. Since *Serratia liquefaciens* was inoculated in pure culture, a competitive flora of around 7 log cfu/g of LAB typical to the normal products, was not present. It is possible, that higher counts can be obtained at such conditions, before a shift in substrate utilisation from a limited amount of carbohydrate to amino acids is necessitated. Still the role in spoilage by *Serratia* spp. needs further investigation, with special emphasis on identification of volatile compounds responsible for the off-odours, and factors affecting their production. Longer chained acids, than analysed in this study, and various amines, both originating from amino acid breakdown (Eskin et al. 1971) could be examined in future experiments. Among amines production of putrescine and

cadaverine by *Serratia liquefaciens* as reported in vacuum packed meat by Dainty et al. (1986) could be considered.

4.6.2 Lactic Acid Bacteria

Some LAB are able to produce hydrogensulphide from cysteine. An often quoted example is *Lactobacillus sake,* that caused spoilage of vacuum-packed meat by hydrogensulphide production (Shay and Egan 1981 and Egan et al. 1989). Truelstrup Hansen (1995) likewise showed hydrogensulphide production by a *Lb. sake* during growth on cold smoked salmon. Other reports indicate the ability among certain Lactic Acid Bacteria to produce hydrogensulphide during extended storage of meat in anaerobic atmospheres, when glucose is depleted (Borch et al. 1996). Leisner (1992) found, that the majority of LAB strains from vacuum packed gravad fish were able to produce hydrogensulphide from cysteine at 4°C in a laboratory medium. Lee and Simard (1984) found that most of 41 *Lactobacillus* strains were able to produce hydrogensulphide in laboratory media. In one case production of volatile sulphur compounds other than hydrogensulphide has been reported. Edwards and Dainty (1987) mentioned unpublished results showing that some meat strains of Lactic Acid Bacteria could produce dimethylsulphide during growth on normal pH meat. However no details were given on the experiments, the mechanism of production or the possible significance of this ability in spoilage.

Gram and Huss (1996) mentioned some preliminary results showing that a shelf life extension could be obtained of cold smoked salmon by inhibition of the LAB flora using nisin. This indicated that the LAB contributed in spoilage reactions, either directly by production of off-odours on their own or in interaction with Enterobacteriaceae, or indirectly by consumption of carbohydrates available in limited amounts, and thereby accelerating the onset of spoilage caused by other organisms. Several workers have reported only weak off-odour production by LAB strains or inoffensive off-odours of stored fish and meat products dominated by LAB (Leisner 1992, Edwards and Dainty 1987, Truelstrup Hansen et al. 1995, Magnusson and Traustadottir 1982). A domination by LAB have therefore in general been thought to ensure a maximum shelf life compared to products, that allow growth of bacterial species of higher spoilage potential than the LAB (Gill 1983, Gill 1986, Egan et al. 1989, Dainty et al. 1979).

In the present study five LAB were inoculated into pasteurised roe (paper 3). Two presumptive *Carnobacterium* spp. (group B1 and B2, table 4.1) produced off-odours described by the words sour, sweet, malty, bread and fruity after two weeks of storage, and were both rejected by 40 % of the panellists (i.e. close to borderline). The counts of the organisms were 7.0 and 6.7 log cfu/g respectively. Through the remaining 87 days of storage

the counts varied slightly but remained between 6.0 and 7.0 log cfu/g. The odours did not develop further neither in nature or strength, and the roe was not rejected. *Carnobacterium* sp. (group C, table 4.1) reached maximum counts of 7.9 log cfu/g in two weeks, decreasing to 7.0 log cfu/g at the end of storage. Only very weak off-odours were produced. The two *Lactococcus* spp. (group A1 and A2, table 4.1) produced no off-odours, and maximum counts were 6.0 and 6.3 log cfu/g respectively.

The off-odours produced by *Carnobacterium* spp. resembled to some extend those described by Truelstrup Hansen (1995). In her study sour, astringent, sweet, burnt and fruity odours were produced by LAB including a *Carnobacterium* spp. in heat sterilised salmon juice. In salmon different *Lactobacillus* strains produced acidic, burnt, buttermilk-like odours developing into a sour and almost faecal nature, and *Lactobacillus sake* produced sour, cabbage-like, sulphurous odours in the salmon. Hydrogensulphide production by *Lactobacillus sake* in the salmon was also demonstrated. Both sour, buttermilk and sulphur-like odours produced by LAB were described by Hanna et al. (1983). The odours were detected after counts had reached 8 log cfu/cm^2 in meat, and in general odours were not strong. All the strains examined by Truelstrup Hansen grew to levels of 8 log cfu/g(ml) or higher during anaerobic incubation. Such high levels are not typical in lightly salted lumpfish roe, but have occasionally occurred (table 4.3).

The off-odours (sour, sweet, malty, bread, fruity) produced by LAB (B1 and B2) in pasteurised roe were not sufficiently strong to cause rejection. Sour odours were noted in stored naturally contaminated roe products (section 4.2), but were also produced by *Serratia liquefaciens*. Group B1 *Carnobacterium* spp. tested in pasteurised roe, was present in batch 1 at the same level of counts at the end of storage, that was observed in the inoculation experiment (table 4.1). This batch was practically without off-odour. The role in spoilage by *Carnobacterium* spp. (B1 and B2) is inconclusive. Though these organisms did not cause spoilage of the roe in pure culture, they may still have an impact on the over all odour of products in which they grow. Stutz et al. (1991) examined spoiled beef and beef inoculated with potential spoilage organisms from the product, and found that only half the number of volatiles found in spoiled beef were detected in inoculated beef, indicating that apparently less important parts of the flora might also contribute in the formation of odours.

Based on the data obtained in the present study LAB are apparently less important in spoilage of lightly salted lumpfish roe, than the Enterobacteriaceae producing the more offensive sulphidy/cabbage-like odours. A general conclusion on the role of LAB in spoilage of lightly salted lumpfish roe is however hindered by several obstacles. As concluded in section 4.4.1 a wide range of LAB are able to grow in the roe products, and LAB not tested (or yet to be detected in lightly salted roe) may have other properties than the strains

examined in the inoculation experiment described in this section. Due to this great variation, a large number of different strains needs to be included in experiments regarding spoilage potential of LAB in lightly salted lumpfish roe. Another severe obstacle is the lack of an appropriate sterile model system, with greater similarity to normal products produced by desalting of heavily salted roe after storage for various periods of time. LAB did not cause rejection of pasteurised roe, but neither did *Serratia liquefaciens.*

4.6.3 *Vibrio* spp.

Vibrionaceae isolated from fish spoiled at 20°C have been shown to produce hydrogensulphide from cysteine and produce off-odours when grown in fish juice (Gram et al. 1987).
Vibrio spp. constitutes only a minor part of the flora of lightly salted lumpfish roe. One of the highest counts was observed in the practically unchanged batch 1 (table 4.1). Three *Vibrio* isolates were included in the inoculation experiment in pasteurised roe. The strains reached counts of maximum 6.3 log cfu/g, thus comparable to those of the products, and none of the strains produced any off-odour in the storage period of 103 days. *Vibrio* spp. are not considered important in spoilage of lightly salted lumpfish roe at 5°C.

4.7 Available substrates

It is a widely accepted fact, that growth of bacteria in both fresh fish and meat, occurs by consumption of water soluble substances such as sugars and free amino acids. Proteolysis does not occur until after spoilage has been recognised (Dainty et al. 1975, Lerke 1967). Production of the necessary exoenzymes are suppressed by readily utilised carbohydrate substrate, and is usually delayed until the late logarithmic phase of growth (Glenn 1976, Gill and Newton 1980). During the prolonged shelf lives of lightly preserved fish products it is however not known, whether bacterial proteolysis or breakdown of peptides occur.
The total carbohydrate fraction of lumpfish roe was not examined in this project, but a few data are available on the concentration of glucose and ribose. In five batches of lightly salted roe glucose concentration ranged from 1.2 to 6.7 mg/100g (table 8 of note 1). After 3 months of storage, levels between 1.6 and 2.5 mg/100g was detected in the five roe batches. Changes could not be detected of the low initial concentrations of ribose (max. 2.2 mg/100g, table 8 of note 1). Compared to this the glucose concentration of meat is 10 mg/100 g, though higher levels have also been reported (Gill and Newton 1978, Gill 1986). Other carbohydrates may however be available for bacterial growth. In the roes of mullet (Chiou and Konosu 1988) and Alaska pollack (Chiou et al. 1989a) a glucose concentration of around 3 mg/100 g has been reported, while myo-inositol (21 - 22 mg/100 g), mannitol (8 - 10 mg/100 g) and mannose (around 6 mg/100 g) were present at higher concentrations. In lightly salted roe

carbohydrates are likely to be washed out to a varying extend during desalting as demonstrated for taurine (see section 3.5.2), and carbohydrate concentration must be expected to vary as does the concentration of free amino acids (see section 3.5.2).

From studies with meat it is known, that carbohydrates are utilised first, then amino acids (Newton and Gill 1978). These authors showed that a *Lactobacillus* utilised glucose and then arginine. Growing anaerobically on meat surface, a *Lactobacillus* produced concentration gradients of glucose and arginine, but not until maximum cell density is approached (Gill 1976, Gill and Newton 1978). When glucose solutions were added to meat slices in inoculation experiments, the increase in maximum cell densities were directly proportional to the amount of glucose added. The authors concluded, that under anaerobic conditions limited availability of substrates determines the maximum bacterial cell density of meat. In lightly salted lumpfish roe similar situations of substrate limitation may occur.
Gill (1983) calculated, that a glucose concentration of 10 mg/100g in meat would support growth to 7 log cfu/cm^2 by fermentation, and after this point amino acid breakdown would be initiated. A shift in substrate utilisation from carbohydrates to amino acids introduces additional end products including acids, ammonia, amines and volatile sulphur compounds, some of which are malodorous (Eskin et al. 1971, Gill 1986, Dainty 1982). There are however deviations from this general order of substrate utilisation. Gill and Newton (1979) showed, that *Serratia liquefaciens* utilised serine in the presence of glucose. Spoilage odours were however not produced until glucose was exhausted and other amino acids were attacked.

The precise range and order of substrates utilised by the bacteria growing in lightly salted lumpfish roe is yet to be examined. This knowledge is however necessary, if a full understanding of the spoilage mechanism of lightly salted lumpfish roe is to be obtained. Such an investigation could also reveal the significance of bacterial interaction in terms of competition for substrates. For example LAB could contribute to spoilage in an indirect manner by utilising carbohydrates, and forcing other bacteria into attacking amino acids at an earlier stage. LAB could also benefit from compounds released during growth of Enterobacteriaceae, and thereby contribute to spoilage, which would not be recognised for LAB grown in pure culture. An example of such interactions was reported by Borch et al. (1996). Spoilage off-odours typical for the product developed in sterile vacuum packed meat (4°C), when a LAB mixture were inoculated together with *Hafnia alvei*. Off-odours of a different nature developed, when the LAB mixture was inoculated alone.

4.8 Volatile sulphur compounds, precursors and significance in off-odour

The volatile sulphur compounds detected during this project in spoiled roe products are summarised in table 4.4. Hydrogensulphide was detected in three naturally spoiled batches of roe after two and a half months at 5°C (see also table 4.2). The concentration of cyst(e)ine, precursor of hydrogensulphide, in the roe is:
Desalted roe: not detected (n.d.). Fresh roe: min.: n.d., maximum 0.6 mg/100 g (calculated from data shown in table 5 of note 1).
The odour threshold of hydrogensulphide is by Belitz and Grosh (1987) reported to be 5 µg/l, while Shewan and Murray (1979) reported 40 µg/l to exhibit slight hydrogensulphide odour. When 3 moles of cysteine leads to 1 mole of hydrogensulphide (see section 4.5.5) this means, that 0.04 mg/100 ml cyst(e)ine is needed for 40µg/kg of hydrogensulphide to be formed. Higher concentrations than this have been detected in some fresh roes, while in others and in the desalted roe used for production, the concentration was below detection limit. Some of the cyst(e)ine consumed by bacteria is probably also directly incorporated into the growing cells, and not all converted to hydrogensulphide as discussed by Herbert and Shewan (1975). It is from the calculation not possible to conclude, whether sufficient amounts of precursor is available in the roe products (produced from desalted roe), for hydrogensulphide to reach concentrations of significance in off-odours.

Hydrogensulphide was not detected in pasteurised desalted roe and lightly salted roe made from fresh roe, inoculated with Enterobacteriaceae (table 4.4). This difference may, apart from being caused by differences among experiments (e.g. salt concentration), indicate that hydrogensulphide in naturally spoiled roe was produced by other bacteria (LAB, *Vibrio* spp.). Still the lack of hydrogensulphide production by the Enterobacteriaceae was indeed unexpected (see section 4.6.1), and another explanation can be suggested. If the reason was in fact lack of sufficient amounts of cyst(e)ine in the inoculation experiments, then the hydrogensulphide production in naturally spoiled products, was made possible by additional sources of cyst(e)ine. Autolytical (or microbial) liberation of cyst(e)ine from proteins/ peptides during storage of the products is a likely source. In the pasteurised roe autolysis did obviously not occur, and in both inoculation experiments the storage time was much shorter than in natural spoilage (table 4.4).
It has however not been shown, that the amounts of hydrogensulphide detected was in fact significant in the odours of naturally spoiled products. Off-odours similar to those of spoiled products were produced by *Morganella morganii* without production of hydrogensulphide.

Table 4.4. Overview of volatile sulphur compounds detected in the headspace of naturally spoiled and spoiled inoculated lightly salted lumpfish roe products. The roe was vacuum packed in glass jars and stored at 5°C. +: The compound detected. ÷: Compound not detected/amounts negligible.

Roe products	**Naturally spoiled** (from sec. 4.5.5) **Normal products stored 2½ months**	**Spoiled lightly salted roe inoculated with Enterobacteriaceae** (from sec. 4.4.1)			
		Pasteurised roe products stored 3 weeks		**Fresh roe products stored 2 weeks**	
	Spoiled batches 6, 7 and 10	Morganella morganii F39-1	Morganella morganii E5	Serratia liquefaciens F50	Morganella morganii F39-1
Salt, % WPS	3.5 and 4.5	3.6	3.6	3.5	3.0
Off-odour	Sulphidy, green, decaying hay, sour	Sulphidy, sewer-like, faecal, cabbage-like	Sulphidy, cabbage-like, faecal	Sour, sulphidy, faecal [5]	Sulphidy, sewer-like, faecal, cabbage-like
Hydrogensulphide H_2S	+	÷	÷	÷	÷
Carbondisulphide CS_2 [1]	+	÷	÷	÷	÷
Methanethiol CH_3SH	+	+	+	÷	+
Dimethylsulphide [2] **CH_3SCH_3 or Ethanethiol CH_3CH_2SH**	+	÷	÷	÷	+
Dimethyldisulphide [2] **CH_3SSCH_3 or 1,2 Ethanedithiol $HSCH_2CH_2SH$**	÷	+	+	÷	+
Unidentified [3]	÷	+	+	÷	÷

1) Likely identity, retention time 0.64 - 0.71 min. 2) Retention times of two standards equally close, identity inconclusive. 3) Possible identity according to retention time of standard (18.01 min.): 1,4 butanedithiol ($HS(CH_2)_4SH$), other possibilities exists in this area. 4) Overflow of the coloumn by H_2S might have hidden the peak of CS_2. 5) Very small amounts of an unidentified larger compound. Retention time 25.5 min.

Methanethiol was produced in naturally spoiled roe and in both fresh and pasteurised roe inoculated with *Morganella morganii* (table 4.4). The concentration of methionine, the precursor of methanethiol and dimethylsulphide production, in the roe is:
Desalted roe: min. n.d., max. 0.1 mg/100 g. Fresh roe: n.d. (calculated from data shown in table 5 of note 1).
The threshold value of methanethiol is reported to be 0.02 µg/l by both Belitz and Grosh (1987) and Guadagni et al. (1963), while threshold value for cabbage-like odour to be recognised is reported to be 0.2 µg/kg food (Whitfield and Tindale 1984). Shewan and Murray (1979) reports the threshold of slight sour and sulphide odour to be 0.5 µg/l in aqueous solution, while slight cabbage odour is recognised at 0.7 µg/l.
Even if the value of 0.5 µg/kg is used for calculation, methionine concentration needed (1.6×10^{-4} mg/100 g) is below detection limit in the amino acid analysis, and maximum methionine concentration detected in desalted roe is more than 500 times this value (one mole of methionine leads to one mole of methanethiol, see section 4.5.5). As earlier mentioned part of the methionine is oxidised during analysis, and detected concentrations may be too low. In addition liberation of methionine was shown to occur during storage of heavily salted roe (section 3.5.2), and could be proceeding in the products, as suggested for cyst(e)ine.
Regarding the remaining sulphur compounds listed in table 4.4, their exact identities needs to be determined. In future studies it should also be investigated whether cyst(e)ine and methionine are in fact the precursors of all the volatile sulphur compounds detected during storage of lightly salted lumpfish roe products. The sulphur containing compound taurine, that is present in the roe in far higher concentration, than any regular amino acid, was not attacked by the fish spoilage bacteria examined by Herbert and Shewan (1975), but can be utilised by *Escherichia coli* as a sulphur source (Ploeg et al. 1996). It should be examined, whether members of the Enterobacteriaceae flora of lightly salted lumpfish roe are able to utilise this compound and if so, which end products are produced.

Future studies should first of all aim to identify the volatile sulphur compounds important in spoilage odours. Indications could be obtained by odour assessments of roe products with known standards of volatile sulphur compounds. The use of quantitative analysis is also needed in order to allow direct comparison to odour thresholds of the compounds. Based on the obtained results, it is however likely, that other volatile compounds are contributing to the sulphidy, cabbage-like, green/hay, rotten and sour spoilage odours detected in spoiled roe products.
Stutz et al. (1991) concluded that the putrid, sulphidy spoilage odours of ground beef rather than one specific compound, best could be ascribed to a "volatile compound profile", including volatile sulphur compounds, esters, ketones and aliphatic hydrocarbons. Several different combinations of compounds were described as having a sour odour.

4.9 Summary and conclusions

Lightly salted lumpfish roe, that spoiled during storage at 5°C, did so due to off-odours resulting from bacterial activity. The overall quality of the product was however influenced by rancidity, developing during storage of heavily salted roe prior to production.

Off-odour development was highly variable among batches of lightly salted roe. After storage both: 1) practically unchanged roe products, 2) borderline and rejected roe products (sour, marinated, rotten) and 3) heavily spoiled roe products (sulphidy or cabbage-like in different combinations with rotten, sour, green/hay, fruity and ammonia-like) were noted.

The total viable counts of stored lightly salted lumpfish roe were in general around 7 log cfu/g, while 8 cfu/g rarely occurred. The microflora was shown to be dominated by LAB or LAB and Enterobacteriaceae, while *Vibrio* spp. composed a minor part.
The LAB flora included a wide range of organisms able to grow in the products. Probably due to the combination of pH and salt concentration, the Enterobacteriaceae flora were more uniform, and dominated by *Serratia* spp. (*Ser. liquefaciens* and *Ser. plymuthica*), with less frequent occurrence of different subspecies of *Morganella morganii.* Other genera of Enterobacteriaceae were rarely observed. A salt concentration of 4 % WPS was shown to be close to the limit of growth in roe extract of most of the *Serratia* spp. In roe products with > 4 % WPS, Enterobacteriaceae were only occasionally able to grow (> 5 log cfu/g).

Several potential chemical spoilage indicators have been analysed. The following compounds are concluded to be without value as indicators of spoilage in lightly salted lumpfish roe: Trimethylamine (TMA) (no increase, low initial level of TMAO), total volatile bases/ammonia (TVB) (uniform increase among spoiled and non-spoiled roe batches), nucleotides (low level after storage), lactic acid (only slight increase), acetic acid (low level after storage), formic and propionic acid (undetectable).
Volatile sulphur compounds were produced during storage of lightly salted lumpfish roe (H_2S, probably CS_2, CH_3SH and CH_3CH_2SH or CH_3SCH_3). Different profiles of the compounds (semi-quantitative measures) were obtained among roe batches, and more compounds or larger amounts were detected in spoiled compared to borderline/non-spoiled roe. Volatile sulphur compounds are concluded to be likely contributors in spoilage odours.

Two different *Morganella morganii* isolated from spoiled products, were shown to produce several volatile sulphur compounds during growth in a lightly salted pasteurised roe product made from normal desalted roe, and in lightly salted roe made from fresh sterile roe. The roe was rejected at *Morganella morganii* counts below 6 log cfu/g, but volatile sulphur compounds were not detected until counts of 6 -7 log cfu/g were reached. The off-odours

produced were described as sulphidy, sewer-like, faecal and cabbage-like. At original isolation from naturally spoiled roe (cabbage-like odours) the counts of one of the strains were 6.4 log cfu/g, and *Morganella morganii* is concluded to act as a spoilage organism, when present in lightly salted lumpfish roe. One suggestion for future studies aiming to explain the faecal off-odours produced by *Morganella morganii*, is the analysis of indole/skatole. It has been calculated, that well over sufficient amounts of free tryptophane is available in the roe, for the odour threshold of indole to be exceeded.

Serratia liquefaciens and *plymuthica* isolates were likewise tested in pasteurised roe, and produced weak or no off-odours. In fresh roe one *Serratia liquefaciens* produced sour, sulphidy and faecal off-odours, but at count of 8 log cfu/g not observed in naturally contaminated products. Only small amount of an unidentified sulphur compound, not occurring in naturally spoiled products, was detected. Among normal roe products stored for three months, roe batches with an Enterobacteriaceae flora consisting of *Serratia liquefaciens* and/or *Serratia plymuthica*, were all close to borderline and described as sour, marinated and rotten.
It is concluded that *Serratia* spp. are likely to contribute in spoilage of lightly salted lumpfish roe, but the end products responsible for the off-odours are yet to be identified.

Among the LAB only two *Carnobacterium* spp. produced off-odours in pasteurised roe. The odours were described as sour, sweet, malty, bread and fruity, and were not sufficiently strong to cause rejection of the roe.
Among normal products, roe batches with domination by LAB (Enterobacteriaceae < 5 log cfu/g) have in no case been rejectable after three months of storage. Still unusually high counts (8 log cfu/g) of LAB have been observed in two spoiled roe batches with Enterobacteriaceae counts below 6 log cfu/g. Those results are however difficult to interpret due to the choice of agar, and due to the fact that the %-rejections were as high as 100 %, and the roe may have been spoiled for some time already.
LAB are concluded to be less important in spoilage of lightly salted lumpfish roe than the Enterobacteriaceae. As a wide range of LAB are able to grow in the products, other LAB than those tested in this project might have other properties, including different spoilage potentials. The role of LAB in spoilage, including their interaction with Enterobacteriaceae, is not clear. LAB may contribute directly in spoilage reactions by utilising substrates liberated by Enterobacteriaceae, or indirectly by acting as competitors for available substrates.

Vibrio spp. comprising a minor part of the flora, is concluded to be unimportant in spoilage of lightly salted lumpfish roe.

It is concluded from the results discussed in this chapter, that a fully satisfactory sterile model system imitating lightly salted lumpfish roe is yet to be developed. The product differ from most other lightly preserved fish products by the long storage of the roe prior to production. The significance in spoilage reactions of the variation among roe batches in concentration of various soluble compounds introduced during storage and processing, is unknown. Likewise the role, if any, of autolytical and/or bacterial proteolysis/breakdown of peptides occurring during storage of the final products needs to be examined.
Knowledge on the range and order of substrates utilised by different parts of the microflora in natural spoilage, is necessary to obtain an understanding of the spoilage mechanism.

Chapter 5. Potential safety hazards of lightly salted lumpfish roe

5.1 Introduction

In this chapter safety hazards of lightly salted lumpfish roe will be evaluated, with special emphasis on pointing out pathogenic bacteria, which pose a risk to the consumer, if the product is not additionally preserved. It is also intended with this chapter to provide information of general use for the industry in the production of lumpfish roe products.

Lightly salted roe was defined in chapter 1 as having a salt concentration of 4 % WPS and a pH of 5.4 (0.1 % lactic acid). The product is vacuum packed in (air-proof) glass jars, and stored at 5°C. The intended shelf life was defined to be three months. As with other lightly preserved fish products, the long shelf life creates an increased risk of growth of psychrotrophic pathogenic bacteria. In addition the product is a "ready to eat product", that receives no heat treatment before consumption.

Lightly salted lumpfish roe is produced by the standard procedures illustrated in figure 2.2. Briefly repeated those includes removal of roe sacs - screening into loose eggs - heavy salting - storage in plastic barrels at ÷5°C (≤ one year) - desalting by water addition and drainage - addition of acid - packaging. When this entire line of processing is considered regarding possible sources of pathogenic bacteria, it is noted, that both indigenous bacteria from the marine environment, and non-indigenous bacteria from multiple sources during several handling/contact steps can be introduced in the roe. It is also noted, that the roe receives no bactericidal treatment during processing. Only a few of the processing steps listed above may slightly influence the size of the population already present. This is that some bacteria may not survive the long storage of heavily salted roe, and during desalting a reduction in numbers can be expected.
Consequently the evaluation of safety hazards in the following sections is based on the general assumption, that pathogenic bacteria, once introduced, are present in the final product. It is also based on the assumption, that their initial numbers are low, and occasional cases of excessive contamination with specific bacteria or excessive temperature abuse (lack of cooling) are not considered.

In the last sections of this chapter other hazards due to viruses, parasites and biogene amines are briefly evaluated.

5.2 Indigenous bacteria

As lumpfish are caught in a colder climate along the coasts of the North Atlantic, potentially pathogenic, psychrotrophic bacteria are of special concern. Their initial numbers can be greatly reduced by good hygienic and time/temperature practice during the initial processing steps until salting. The roe is sterile until removed from the fish (Trust 1974, paper 3). Presence of low numbers of indigenous pathogenic bacteria in the final products must however be considered unavoidable in practice. Consequently control of these bacteria depends on the prevention of their growth in the final products.

5.2.1 *Clostridium botulinum*

Clostridium botulinum type E and non-proteolytic type B and F are of special concern due to their psychrotrophic nature. Type E is the most prevalent in our part of the world, and has been reported in marine sediments along the coast of Greenland and Canada (Atlantic), in Scandinavian waters and in the North Sea, thus at catching locations for lumpfish. Type E has also been shown to dominate largely among positive samples of marine fish from waters around Scandinavia (Huss and Pedersen 1979, Huss 1981).
Clostridium botulinum causes botulism, which is a serious, sometimes lethal (5 - 15 % in most countries) intoxication by preformed toxin (Hauschild 1989).

Non-proteolytic *Clostridium botulinum* (type E, B and F) grow at a minimum temperature of 3.0 - 3.3°C, at a minimum pH of 5.0 and at maximum salt concentrations of 3 - 5 % (Graham et al. 1997, Hauschild 1989, Hazzard and Murell 1989, Huss 1981, Huss 1994, Kim and Foegeding 1993). With the combination of 10°C and 3 % WPS in hot-smoked trout, toxin production by *Clostridium botulinum* type B, E and F (2 log cfu/g) could be avoided during storage for 30 days (Cann and Taylor 1979). In a recent study Graham et al. (1997) defined growth/no growth boundaries for *Clostridium botulinum* type B, E and F (inoculated to 4 log cfu/g in a strictly anaerobic medium) based on growth at different combinations of temperature, pH and salt concentration. According to their results, at 5°C the combination of 4 % WPS and pH 5.4 in lightly salted lumpfish roe is well beyond the limits of growth (visually observed turbidity) in a three months period. Storage at 10°C would apparently also be safe, but the margin is narrow, and much dependent on the pH (growth and toxin production occurred in 7 weeks by type B at pH 5.7 and 4 % WPS).
The authors question the margin of safety in the recommendation by the UK Advisory Committee on the Microbiological Safety of Food (at least 3.5 % salt in products stored at below 10°C for more than 10 days), as growth in their study was detected at 4 % salt in 11 weeks at 5°C (type F, 12 weeks for type B) and in 2 weeks at 8°C (type F, 3 weeks for type B

and E). At 4.5 % salt growth was detected in 6 weeks at 8°C (type E and F, 9 weeks for type B) (all at neutral pH). In several of these cases, toxin production was also detected.

Predictions of growth of non-proteolytic *Clostridium botulinum* has been obtained using Food Micromodel (Anon. 1996). Predicted growth curves at various conditions are shown in figure 5.1. It is seen from the figure, that at 4 % WPS, variations in pH alone (5.9) or temperature alone (10°C) are not hazardous during storage for three months. In the case of small variations of pH (5.5 and 5.6) and mild temperature abuse (10°C) *Clostridium botulinum* is predicted to reach counts of just below 5 and 7 respectively in a three months period from initial counts of 1 log cfu/g. This is in agreement with the importance of the pH level indicated by Graham et al. (1997).

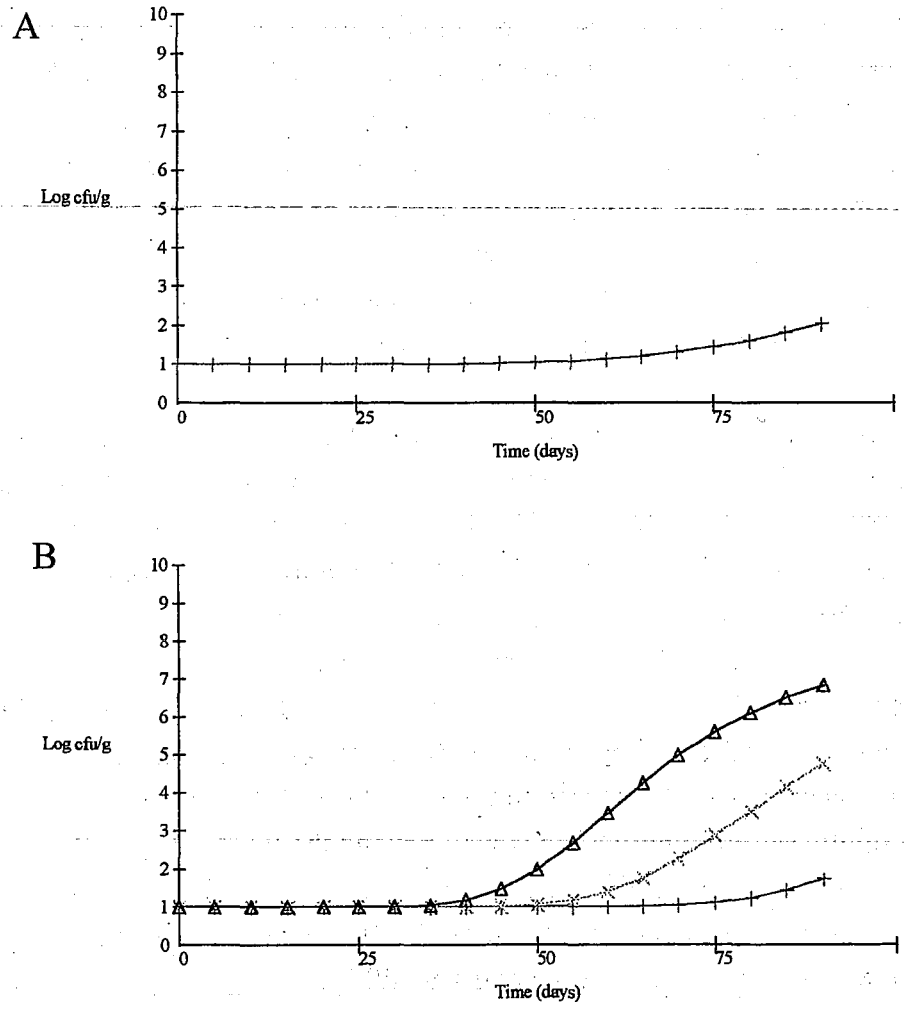


Figure 5.1 Predicted growth curves for non-proteolytic *Clostridium botulinum* at 4 % WPS, using Food Micromodel (Anon. 1996). A: 5°C, pH 5.9 (no growth for 90 dys at pH 5.7). B: 10°C. +: pH 5.4. ×: pH 5.5. Δ: pH 5.6.

Conclusion

Lightly salted lumpfish roe can be concluded botulinum-safe at 5°C. Thus the control points are thorough adjustment of salt concentration and pH, which includes appropriate mixing to avoid deviations locally in the roe. The product is probably also safe when stored at 10°C (not unlikely to occur in supermarket coolers in the summertime or in consumers homes). In the case of such temperature abuse, safety seems to be largely dependent on the lowered pH, which adds to the importance of controlling this parameter.

5.2.2 *Listeria monocytogenes*

Listeria monocytogenes is the *Listeria* species by far most frequently involved in human disease (listeriosis). *Listeria monocytogenes* is a ubiquitous organism, strains of which are pathogenic to humans, and have been the cause of a number of outbreaks due to contaminated foods. The infective dose is not known, and immunosuppressed persons including pregnant women seem to be at particular risk (Lovett 1989, Sutherland 1989). *Listeria monocytogenes* is isolated from soil, plants and a wide range of foods, in the latest years also from seafoods, including ready-to-eat products (Ben Embarek 1994, Dillon et al. 1994, Farber 1991, Fuchs and Nicolaides 1994, Fuchs and Reilly 1992, Hartemink and Georgsson 1991, Motes 1991, Rørvik and Yndestad 1991). However only a few documented or suspected cases of seafood involvement in disease have been reported (Huss 1994). The evaluation of *Listeria monocytogenes* as a potential hazard is complicated by the difficulties in separating pathogenic and non-pathogenic strains. Also the method of analysis can influence which specific *Listeria monocytogenes* are detected in seafood (and other foods, sediments, water etc.) as reported by Loncarevic et al. (1996). These authors analysed (restriction enzyme analysis) 151 *Listeria monocytogenes* isolates obtained from nine foods by plating on Oxford agar, either directly or after an enrichment procedure. The results showed, that some clones were lost during the enrichment procedure, and a larger number of different clones were obtained from a specific food by the direct plating method.

The presence of *Listeria monocytogenes* in live fish most likely depends on its presence in the surrounding waters. *Listeria monocytogenes* has been found in coastal waters including sediments (Motes 1991). Possible sources of sea water contamination are effluents, rivers and seabirds (Ben Embarek 1994). Thus the catching location may determine the presence or absence of *Listeria monocytogenes* on live lumpfish.

Listeria monocytogenes has been shown to survive for 34 - 68 days in a medium with 12 % salt at 20 - 24°C, and for more than 60 days in meat in 30 % brine at 4°C as reviewed by Marth (1993). Thus it is possible that *Listeria monocytogenes* can survive in some barrels of heavily salted roe (14 - 20 % WPS, stored for two months to one year at around ÷5°C, see chapter 2). *Listeria monocytogenes* can, however, also be introduced from the environment

during processing, as reported for cold smoked salmon by Rørvik et al. (1995), who found the salmon smokehouse to be colonised by one type (ET, multilocus enzyme electrophoresis), while sea water, slaughterhouse and slaughtered fish contained other ET's. Obviously good hygienic practice and efficient sanitation must be applied in order to minimise contamination during processing. Still, regardless of the source of *Listeria monocytogenes*, the bacteria should not be allowed any possibility of growth during storage of the final products.

Strains of *Listeria monocytogenes* have been reported to grow at a minimum temperature of ÷1 - ÷4°C (Walker et al. 1990). Growth at minimum pH of 5.2 at 4°C and pH 4.6 at 7°C and 10°C (4.7 log cfu/ml in inoculated laboratory medium) was reported by George et al. (1988). In a laboratory medium with pH 5 at 5°C (inoculum 4.9 log cfu/ml) Ben Embarek and Huss (1992) found visible growth after 12 days at 3 % salt and no growth for 41 days at 6 % salt. At the combination of 4 % salt and pH 6 growth was observed after 10 days, and a delay of only two days occurred when the salt concentration was increased to 5 %. Farber et al. (1989) found that at 4°C, minimum pH for growth was in the range of 5.0 - 5.5 (lowered with lactic acid).

Thus according to the literature, the combination pH 5.4, 4 % WPS and 5°C of lightly salted lumpfish roe, cannot be concluded to be safe regarding growth of *Listeria monocytogenes*. The Food Micromodel programme predict, that *Listeria monocytogenes* will grow to high numbers at these conditions (initially 1 log cfu/g), as illustrated in figure 5.2. In case of temperature abuse (10°C) the maximum level will be reached much sooner.

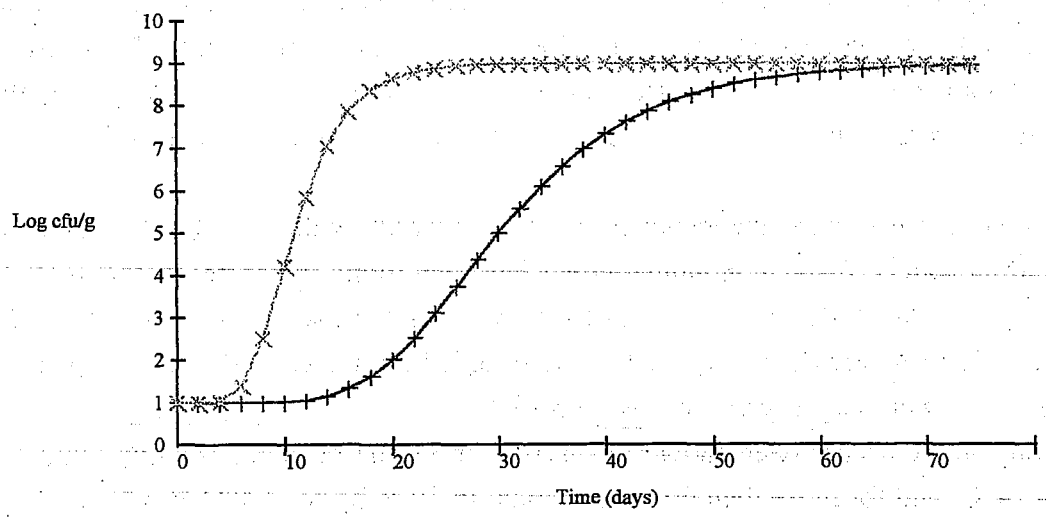


Figure 5.2 Predicted growth curves for anaerobic growth of *Listeria monocytogenes* at 4 % salt and pH 5.4 using Food Micromodel (Anon. 1996). +: 5°C. × : 10°C.

Several factors may influence the actual growth in naturally contaminated lumpfish roe products, including the composition of the roe, the remaining flora, the size of the initial population and the strain of *Listeria monocytogenes*. Consequently an inoculation experiment was performed in this project, in which lightly salted lumpfish roe was inoculated with *Listeria monocytogenes* in low numbers (< 1 log cfu/g) (note 4). Two mixtures of strains originating from lightly preserved fish products/productions, were used for the experiment. The strains were preincubated at 10°C as preincubation at low temperature has been reported to result in a shortened lag phase during growth at low temperatures (Walker et al. 1990). Barbosa et al. (1994) found major differences in lag phase duration among 37 equally treated *Listeria monocytogenes*, with Scott A having the longest lag phase at low temperature.

The results, illustrated in figure 5.3, show that the inoculated *Listeria monocytogenes* was perfectly able to grow in lightly salted lumpfish roe also at 5°C, and better growth was observed than predicted from the programme (figure 5.2). From the prediction at 5°C, a μ_{max} of 0.031 h^{-1} was obtained, while the calculated μ_{max} for mixtures A and B were 0.038 h^{-1} and

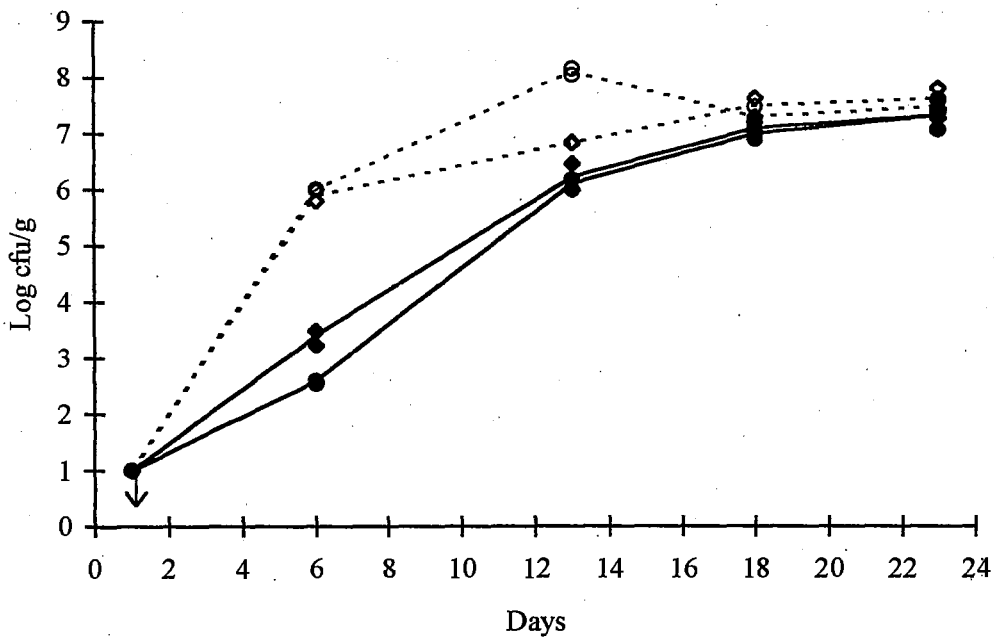


Figure 5.3 Growth of *Listeria monocytogenes* inoculated in lightly salted lumpfish roe (4 % WPS, pH 5.4, vacuum packed in glass jars) stored at 5°C and 10°C. ◆: Mixture of strains 411B1 and 411B5, inoculated to ~ 0.3 log cfu/g, ●: Mixture of strains O57 and H018, inoculated to ~ 0.5 log cfu/g. Closed symbols: 5°C, open symbols and punctured line: 10°C. (Data from note 4).

0.048 h^{-1} (μ_{max} = (logN - log N_0) x 2.303 / t - t_0), which is 22 % and 35 % higher than predicted respectively. As a considerable lag phase duration was also part of the prediction, differences in both parametres contributed to the better growth observed in lightly salted lumpfish roe. The pre-culturing at low temperature is likly to be important in the differences in lag phase duration. Thus when instead time to 5 log cfu/g is used for comparison, the Food MicroModel programme predicts a time of 30 days, while the mixtures A and B reached counts of 5 log cfu/g in 10 and 11 days respectively. The two strains of mixture B (H018 and O57) were also included in other experiments in this project, where similar roe products (4 % WPS, pH 5.4 at 5°C) were inoculated with < 1 log cfu/g. In these experiments it was observed, that time to 5 log cfu/g for strain H018 alone was 10 days equal to that of mixture B, but 20 days for strain O57 (table 1 of note 6). In another experiment O57 reached 5 log cfu/g in 30 days, equal to that of the prediction (table 10 of note 6). In a third experiment O57 only reached 4 log cfu/g (in 20 days) and then declined (table 4 of note 6). Thus large differences in growth potential among strains (all originating from lightly preserved fish products or production environments) were noted. At least two strains (H018 and 411B1/5) grew much better than predicted, while the prediction was much closer to the growth of O57. Differences among experiments with the same strain (O57) may be influenced by at least two factors. One is a possible difference in composition of the LAB flora (development in total LAB counts were equal in experiments yielding 5 log cfu/g *Listeria monocytogenes* in 20 and 30 days). The other is a possible difference in substrate composition among roe bathces in terms of different levels of soluble compounds as discussed in chapter 3 (development of total LAB counts were slower in non-inoculated roe of the same batch, that yielded poor growth of *Listeria monocytogenes*, than in most other experiments during this project (see section 7.4.1).

Conclusion

In spite of the pronounced variation among strains and roe batches, it can be concluded, that if *Listeria monocytogenes* is present in newly produced lightly salted lumpfish roe, even at very low numbers, this bacterium may grow to high numbers during storage for three months at 5°C. Thus *Listeria monocytogenes* must be considered a risk, as pathogenic strains are contained in the species. In order to control *Listeria monocytogenes*, an additional hurdle must be applied.

5.2.3 *Vibrio* spp.

Vibrio cholerae and *Vibrio parahaemolyticus* are well known as pathogenic bacteria associated with foods of marine origin, causing diarrhoea of varying seriousness. Both are ubiquitous occurring in the marine environment, preferably in warmer waters (Desmarchelier

1989, Bryan 1987, Twedt 1989). They are only occasionally isolated from waters with temperatures below 15°C (Gibson 1992).
Another *Vibrio* that can be pathogenic to humans causing septicaemia are *Vibrio vulnificus.* The bacterium has been associated with raw oysters (Bryan 1987, Oliver 1989, Cook and Ruple 1992), and has been isolated from marine water, sediments and shellfish at many locations along the coasts of USA, where examinations have been made (Oliver et al. 1982 and 1983, Kelly 1982). During the winter (January and February, water temperatures 11 - 13°C) *Vibrio vulnificus* could often not be isolated from water, sediment and oysters, while more frequently from the intestines of bottom living fish (DePaola et al. 1994).

Survival of vibrios in different foods at low temperature has been examined by several workers. Corrales et al. (1994) found, that *Vibrio cholerae* survived in meat for up to 90 days at ÷5°C, while in milk it was not detectable after 34 days. Muntada-Garriga et al. (1995) inoculated 10^2- 10^7 cfu/ml *Vibrio parahaemolyticus* in oyster meat homogenate. They concluded that at all storage temperatures (÷24°C - 4°C) the numbers of surviving *Vibrio parahaemolyticus* were a logarithmic function of log time, and that inactivation of this organism by low temperature is only a question of sufficient storage time. Southcott et al. (1976) inoculated salmon roe with high levels (10^6 - 10^7 log cfu/g) of *Vibrio parahaemolyticus*, and examined the development in counts during processing of the roe. After washing in 3 % saline, dipping in saturated brine for half an hour, draining and standing for a week, the counts of the organism were in the range of < 20 - 790 cfu/g. Likewise *Vibrio vulnificus* have been shown to tolerate low storage temperatures poorly, as demonstrated by Oliver (1981) in oyster homogenate at 4°C, and by Cook and Ruple (1992) during storage of naturally contaminated raw oysters, however the organism was successfully cultured from oysters after storage at ÷20°C for 12 weeks.

Thus even if pathogenic *Vibrio* spp. are occasionally present in fresh lumpfish roe, their numbers are likely to be greatly reduced during the storage of heavily salted roe, either by entering a non-culturable state or by actually dying out. Low numbers of surviving organisms in the final products do not pose a risk, as growth conditions in the products are sub-optimal or inhibitory. At the low storage temperature (5°C), the numbers of pathogenic vibrios are more likely to decrease than to increase, in addition the pH of the products are lowered to 5.4. Abundantly optimal growth of *Vibrio vulnificus* occurs at high oxygen tension (Oliver 1989).

Conclusion

Vibrio cholerae, V. parahaemolyticus and *V.vulnificus* are not considered a risk in lightly salted lumpfish roe processed and stored at standard conditions, and no additional control means are required.

5.2.4 *Aeromonas* spp.

Aeromonas spp. are primary aquatic microorganisms occurring preferably in fresh water, but also in marine environments. Motile *Aeromonas* spp. are of concern in aspects of public health, as they have been associated to foodborne diarrhoeal disease. Species of this group of microorganisms are pathogenic to different animals including fish. *Aeromonas* spp. are not restricted to aquatic environments, but have been recovered from a wide range of foods, including fish and seafood. (Fricker and Tompsett 1989, Kirov 1993, Kirov et al. 1993, Knøchel 1989, Merino et al. 1995, Majeed and Mac Rae 1989, Stelma 1989). Further epidemiological studies are, however, necessary for the public health significance of infections caused by *Aeromonas* spp. to be elucidated. Only few cases have been reported, where *Aeromonas* spp. were associated with foodborne disease. A well established methodology for identification of pathogenic *Aeromonas* spp. has, however, not been available, and it is suspected that many cases may have passed unreported (Merino et al. 1995, Kirov 1993).
According to the recent review by Merino et al. (1995) the enteropathogenic strains are mainly represented by *Aeromonas veronii sobria* (formerly *A. sobria*) and *Aeromonas hydrophila* belonging to the serotypes O:11 and O:34. These serotypes are usually detected in very low numbers in foods compared to the detected level of *Aeromonas* spp. in general (Kirov 1993). Recently Hänninen et al. (1997) identified *Aeromonas* spp. in fish (93 % of samples), fish-eggs (100 %), shrimp (16 %) and fresh water (100 %). *Aeromonas hydrophila* hybridization group (HG) 1 and *Aeromonas veronii* subspp. *sobria* or *veronii* HG 8/10, known to be associated with human diarrhoea, were uncommon in all samples, as the majority of strains belong to other HG's (ribopattern analysis of *Sma*I digested DNA). By examination of *Aeromonas hydrophila* strains of aquatic (sea sediments) and clinical origin, Krovacek et al. (1994), however, found that isolates from both the sources produced all or some of the virulence factors, which may be involved in the pathogenesis of *Aeromonas* infections. The role in pathogenicity of the different virulence factors are still being investigated.

Large variations occur among different strains of motile *Aeromonas* spp. in their ability to grow at various conditions for example in terms of pH and temperature. Knøchel (1990) found that the ability of strains to grow at 5°C and 37°C reflected the temperature of the environment, from which they were isolated (< 15°C or > 25°C). However many clinical strains could grow at 5°C, and all strains grew at 10°C. Theoretical minimum temperatures for growth in the range of 4.4°C - 6.0°C have been reported for clinical or food isolates as reviewed by Kirov (1993). A strain of *Aeromonas hydrophila* originating from cooked mussels has been reported to grow at 3°C and ÷1.5°C when inoculated on hot-smoked blue cod packed under vacuum (pH 6.2 approaching 7.0 during storage) (Bell et al. 1995). The

bacterium was inoculated at a level of 5 log cfu/g and reached counts of 8 log cfu/g in 10 and 35 days at the two temperatures respectively. The competing microflora (LAB and *Moraxella* spp.) reached the same level. The cod was immersed in brine for 20 min. before smoking, but no information on the resulting salt concentration was given.
Knøchel (1990) found that among 80 strains of motile *Aeromonas* spp. from different environments, most were at 5°C inhibited by either 4 % salt or pH 5.3. At their optimum temperature a few strains tolerated 4 % salt. 90 % of the isolates were able to grow at pH 5.1 at 25°C, but were inhibited at sub-optimal temperatures. Thus with a combination of 4 % WPS, pH 5.4 and 5°C growth of motile *Aeromonas* spp. is not likely to occur.

Conclusion
Potentially pathogenic *Aeromonas* spp. is not considered a risk in lightly salted lumpfish roe processed and stored at standard conditions, and no additional control means are required.

5.2.5 *Plesiomonas* sp.

Plesiomonas containing one species *Plesiomonas shigelloides* belongs to the family Vibrionaceae. It is isolated from different animals including both pets, domestic animals and reptiles, but a primary reservoir of this organism seems to be fresh water/fresh water fish. It is a known pathogen of fish and reptiles, and in humans it can be the cause of different diseases including enteritidis as reviewed by Koburger (1989). Seasonal variations occur in the number of organisms detectable in surface water, the number declining after onset of the cold season (water 3 - 7°C) (Schubert 1984).
In an experiment including forty strains (environmental and clinical isolates) *Plesiomonas shigelloides* was, at otherwise optimal conditions, reported to initiate growth at pH 4.0 (58 % of the strains) and capable of growth in the presence of 5 % salt (65 % of the isolates) in laboratory medium. None of the strains however grew at 5°C, and at 8°C only 25 % was able to grow when observed for two weeks (Miller and Koburger 1986).
The risk of contamination of roe from lumpfish caught in colder waters is probably low, but is theoretically possible to occur during processing. The ability to grow at low temperatures even at optimal pH and salt concentration is however limited.

Conclusion
Plesiomonas shigelloides is not considered a risk in lightly salted lumpfish roe processed and stored at standard conditions, and no additional control means are required.

5.3 Non-indigenous bacteria

There may be multiple sources of bacterial contamination of the roe during processing into heavily salted roe and later during further processing. Such sources could involve all equipment including barrels for fresh roe, screening and mixing devices and barrels for salted roe; humans (diseased persons or healthy carriers) and animals ranging form pets to seabirds. As earlier mentioned, the hygienic level and available sanitising equipment is highly variable among catching locations, and not easily controlled. During further processing at the production plant, the routine programme for sanitation and personal hygiene has been applied to ensure, that the contamination frequency and level is minimised. Even though the important part is to avoid contamination with pathogenic non-indigenous bacteria, some of which have low infection doses, the possibilities of their proliferation in the products should still be considered. In normal processing, an accidental contamination for example from a person during handling, is likely to be mixed/distributed in a 105 kg portion of roe. Thus some jars of final product may be without contamination, while others may contain a few cells.
Growth potentials of a few bacteria of the Enterobacteriaceae family and *Staphylococcus aureus* are briefly considered below. In lumpfish roe production the source of possible contamination is most likely to be humans. As people more and more often travel to distant countries, other pathogenic bacteria may occasionally be brought home, which are not included in this chapter.

5.3.1 Enterobacteriaceae

Among an abundance of *Escherichia coli* serotypes of different pathogenic groups, *E.coli* O157:H7 has received recent attention due to several foodborne outbreaks most frequently associated with improperly cooked hamburgers, but also raw milk and a few other foods, as reviewed by Molenda (1994).
Palumbo et al. (1995) examined 16 strains including *E.coli* O157:H7 in broth culture at various temperatures. They concluded, that since all stains grew at 10°C and verotoxin was produced at all conditions supporting growth, the organism poses a risk in temperature abused refrigerated foods. The ability of *E.coli* O157:H7 to grow at various conditions was examined by Buchanan and Klawitter (1992a), who found no growth in 17 days at 5°C (0.5 - 2.0 % salt and pH 5.5 - 7.5) in broth culture (2 log cfu/ml) neither aerobic nor anaerobic. At 10°C no growth occurred at pH 5.5 and 2 % salt. By comparison to previous reports, the authors concluded that growth kinetics of *E.coli* O157:H7 is similar to those of non-pathogenic strains.

Salmonella occurs in more than 2000 serovars, and is common in domestic animals and birds, and many are asymptomatic excreters (Huss 1994). Growth of different *Salmonella* spp. in the presence of NaCl was reviewed by Marth (1993). This author reported that at 8°C growth of *S. heidelberg* occurred at ≤ 2 % salt, of *S. thyphimurium* at ≤ 1 % salt and *S. derby* failed to grow when 1 % salt was added to the medium. At 12°C the three strains grew at ≤ 4 % salt. Still in the cases of mild temperature abuse, the lowered pH of lightly salted lumpfish roe introduces an additional hurdle.

Yersinia enterocolitica is a psychrotrophic organism most frequently found in cooler climates, and the serotypes O:3 and O:8 are the dominant human pathogens. Animals especially swine are the most important reservoir of pathogenic strains. Outbreaks after contamination of food by humans have however been reported, and strains have on several occasions been isolated from apparently healthy individuals. Yet it has been questioned whether these strains are clinically significant. (Kapperud 1991, Kraft 1992, Prpic and Hughes 1989, and Schiemann 1989). *Yersinia enterocolitica* can be isolated from several foods including fish and shellfish, but the public health significance of these environmental isolates (*Yersinia enterocolitica*-like) is not certain either (Peixotto et al. 1979, Kapperud 1991).
Yersinia enterocolitica is able to grow at temperatures approaching 0°C (Prpic and Hughes 1989, Kapperud 1991), but the ability to compete with other psychrotrophic organisms may be poor (Schiemann 1989, Stern et al. 1980). At 5°C *Yersinia enterocolitica* has been reported to reach 9 log cfu/g in one month (initially 2 log cfu/g) in mussel homogenate, both aerobically and anaerobically (Hudson and Avery 1994). Growth of *Yersinia enterocolitica* to high numbers during storage at ÷1.5°C of sliced roast beef and smoked blue cod along with the natural microflora has been reported by Hudson et al. (1994) and Bell et al. (1995) respectively. Hanna (1977) reported little or no growth at pH 5 (25°C), while Stern et al. observed growth at pH 4.6 (25°C, 2 to 8 log cfu/ml in two days). The last author also reported growth at 3°C in the presence of 5 % salt (two log cycles in ten days). Thus *Yersinia enterocolitica* is capable of growing at less supportive salt and temperature combinations than present in lightly salted lumpfish roe, and at pH values below 5.4. In vacuum packed cold smoked salmon slow growth was observed at 5°C by Hudson and Mott (1993). *Yersinia enterocolitica* grew from am initial number of just below 6 log cfu/g to around 8 log cfu/g in three weeks. Conditions regarding salt concentration (3 %) and pH (6.1) was however less severe than in lightly salted lumpfish roe, and the inoculum was rather high.
Whether the combination of temperature, salt and pH in lightly salted lumpfish roe, together with the remaining microflora and the nature of the substrate can support growth of this organism in a three months period is, however, inconclusive.
As it is also uncertain, whether the risk of contamination has any significance in practical lumpfish roe production, and several strains as mentioned above probably are non-

pathogenic, further investigations on growth potential in lightly salted lumpfish roe has not been initiated in this project.

Conclusion

The risk of contamination with pathogenic Enterobacteriaceae mentioned in this section (*Escherichia coli*, *Salmonella* and *Yersinia enterocolitica*) is probably low, and should be minimised by good hygienic practice. As some of the bacteria have low infection doses, avoiding contamination is central. In the case of accidental contamination, an increase in numbers during storage of the final products is not likely to occur, maybe with the exception of *Yersinia enterocolitica.*

5.3.2 *Staphylococcus aureus*

The principal source of *Staph. aureus* during production is humans with wound or throat infections, but the organism is ubiquitous, and present in the environment such as dust, air and surfaces, in fact all equipment handled by man, where it survives well. Among healthy persons 30 - 50 % sometimes accommodate *Staph. aureus*, while 15 - 35 % are constant carriers. Symptoms are normally limited to nausea, vomiting and sometimes diarrhoea, and are caused by preformed toxins (enterotoxin A, B, C1, C2, D, E and F) (Bergdoll 1989, Dick et al. 1989, Eyles 1989). Consequently, unlike some Enterobacteriaceae described in the previous section, growth of the organism in the food is required to cause disease. However it is frequently occurring, and is not realistic to eliminate contamination completely, but it can be reduced by following good hygiene and sanitation procedures. Thus low numbers do not pose a risk, provided growth of *Staph. aureus* in the products is inhibited.
Staph. aureus is at otherwise optimal conditions able to grow down to 6°C, pH 4.2 and at up to 15 % salt (Eyles 1989, Bergdoll 1989). Toxin production is however not likely to occur below 10°C, and is reduced at anaerobic conditions (Dick et al. 1989).
In aerobic broth culture (~ 5 log cfu/g) Notermans and Heuvelman (1983) reported no growth at 8°C, but at 12°C and < 1 % salt growth occurred at pH 4.9. At increased salt concentration (5.7 %) growth at 12°C did not occur below pH 5.5. The authors reported that enterotoxin A, responsible for around 80 % of staphylococcal intoxication, was produced at nearly all conditions allowing growth, while production of enterotoxin B, C and F was more susceptible to reduced water activity.
Anaerobic enterotoxin B production on ham submersed in brine of various combinations of salt, pH and temperature was examined by Genigeorgis et al. (1969). With a high initial inoculum (6.3 log cfu/g) they found anaerobic toxin production at 10°C in maximum 8 weeks at pH values ≥ 5.9 (2.5 - 5.8 % salt), while at pH 5.6 toxin production was not detected until after 16 weeks (2.3 - 4.4 % salt).

Conclusion

At 5°C growth and enterotoxin production by *Staph. aureus* present in low numbers in lightly salted roe does not pose a risk. Even at mild temperature abuse (10°C) growth to high numbers is unlikely, particularly due to the lowered pH. The sub-optimal growth at anaerobic conditions and the presence of a competing flora creates an additional safety margin.

5.4 Viruses

There is a lack of knowledge on the incidence of seafood transmitted viral infections, and sufficient information is not available, to allow evaluation of the risk of enteric or other viruses being present in lumpfish roe. Hepatitis A virus and Norwalk-like agents are thought to be the greatest threat among viruses. Reliable methods for their detection in food are however still under development (Wekel et al. 1994).

Though some catching locations for lumpfish are situated near cities, lumpfish roe cannot be considered a high risk seafood regarding virus contamination, as are shellfish, which concentrate viruses during filtering of surrounding water. With a few exceptions those have been the cause of all reported cases of seafood associated viral infections (raw or improperly cooked) (Huss 1994).

Direct or indirect faecal contamination of the roe in the many handling steps during processing probably poses the greatest risk of viral contamination of the roe. Thus good personal hygiene is very important in preventing such contamination, as already stated for non-indigenous bacteria.

There is also a lack of data on how viruses will respond to factors as salt, pH and temperature of lightly salted lumpfish roe, and there is no evidence that the combination of factors is a guarantee against survival of viruses in the products. Thus the only means of control is in practice to keep the level of contamination as low as possible.

5.5 Parasites

Among the most important parasites with regard to public health, only two are associated with marine fish from the northern hemisphere as reviewed by Fayer (1992) and Huss (1994). Those are the nematodes *Anisakis simplex* (herring worm) and *Pseudoterranova dicipiens* (cod worm) occurring in the North Atlantic. Literature reviewing the prevalence of parasites of public health significance, does not mention the possibility of nematodes being transmitted to man through fish roe. There is, however, always a theoretical possibility of nematodes being transferred to the roe, when the fish is cut open during removal of the roe.

Experienced workers in the production of lumpfish roe products have, however, never encountered nematodes in the roe, and the risk is probably low.
The storage of roe prior to production at salt concentrations in the range of 14 - 20 % WPS at ÷5°C for a duration of two months up to one year is likely to influence the survival of nematodes, and reduce the risk even further. In marinated fish, safety can be assured by a certain holding time in brines containing salt and acetic acid, which causes the death of *Anisakis* larvae. The mortality rate of nematodes is a function of time and depends mainly on the salt concentration (Karl et al. 1995, Huss 1994). However in reported experiments on the mortality rate of *Anisakis* larvae at various salt concentrations in herring marinades, 2.5 - 3.0 % acetic acid was also applied, and the acid is part of the traditional recipes. At an acetic acid concentration of 2.6 %, maximum survival time between 5 and 17 weeks at salt concentrations ranging from 9 % down to 4.3 % was noted (Karl et al. 1995). A certain part of the survival time is, however, required to obtain appropriate salt concentrations in the flesh of marinated fish through diffusion from the brine, and equilibration times of 2 - 4 weeks depending on the salt concentration of the brine was observed. During salting of fresh lumpfish roe, the high salt concentration is obtained immediately. Karl et al. (1995) was unable to demonstrate a marked effect of different acetic acid concentrations (0.2 - 0.7 %) similar to the effect of different salt concentrations, but 0 % acetic acid was not included in the study. A survival time of maximum 12 weeks was observed with 0.2 % acetic acid at salt concentrations of 2.1 - 3.7 %. When the equilibration time is withdrawn, and the high salt concentration in lumpfish roe is considered, lumpfish roe processing is probably safe, but the lack of acid addition hinders an indisputable conclusion.

5.6 Biogenic amines

Several biogenic amines can be produced in foods by bacterial decarboxylation of amino acids as recently reviewed by Silla Santos (1996) and Rawles et al. (1996). The most toxic amine detected in foods is histamine (from histidine decarboxylation), which causes dilatation of peripheral blood vessels resulting in hypotension, flushing and headache, while contraction of intestinal muscle may account for abdominal cramps, diarrhoea and vomiting. The toxic effect depends on the consumed amounts, the presence of other amines interfering with histamine oxidase, alcohol consumption and the intestinal physiology of the individual. Therefore an exact toxicological level is difficult to determine (Silla Santos 1996, Rawles et al. 1996). As reviewed by Huss (1994) a "hazard action level" of 50 mg/100 g of histamine has been established in the USA, while a "maximum allowable limit" of 20 mg/100 g exists in the EEC.

Bacterial formation of biogenic amines in food depends on the availability of relevant free amino acids, the presence of decarboxylating bacteria and growth conditions that allow decarboxylase synthesis and activity.
The former prerequisite may limit the risk of histamine formation in lightly salted lumpfish roe, as the concentration of free histidine in the roe is low. Concentrations of free histidine in ten lightly salted roes analysed in this project were in the range of 0.7 - 1.8 mg/100 g (calculated from data shown in table 5 note 1). Thus during decarboxylation of histidine into equimolar amounts of histamine a maximum of 2.5 mg/100 g of histamine could be formed, which is far below the accepted limits mentioned above. Yet, free histidine was released during storage of heavily salted roe (section 3.5.2) demonstrating, that autolytic enzyme/-s releasing histidine from proteins/peptides is/are present in the roe, and may be active in the final products. As mentioned in chapter 4, the extent of bacterial proteolysis is not known either. Thus additional histidine may be made available during a shelf life period of three months.

Among the bacteria comprising the microflora of lightly salted roe, *Morganella morganii* is a well known histamine producer (Frank 1985, Klausen and Huss 1987). This organism was shown to approach counts of 7 log cfu/g during storage at 5°C of lightly salted lumpfish roe (section 4.4.1). Yet, even if sufficient amounts of histidine were made available, and even if decarboxylation would occur at 5°C, histamine production will not pose a risk in the final products. As *Morganella morganii* was identified as a spoilage organism in lightly salted lumpfish roe, this bacterium should not be allowed to grow in the products, and will be a target of preservation to be considered in chapter 6 and 7 along with *Serratia* spp.

Some *Lactobacillus* spp. have also been associated with histamine decarboxylation (Silla Santos 1996). Leisner et al. (1994) examined the incidence of histamine producing LAB in different vacuum-packed, sugar-salted fish products, and found none, indicating that LAB may not be important in accumulation of histamine in these products.
Another biogenic amine, tyramine (from decarboxylation of tyrosine) is however produced by LAB. Leisner et al. (1994) found the majority of tyramine producing isolates to belong to *Carnobacterium* spp. One or more *Carnobacterium* spp. were also detected (6.3 - 7.1 log cfu/g) in all five batches of lightly salted roe, of which the flora composition was reported in section 4.4.1. The amount of tyramine detected by Leisner et al. (1994) was, however, reduced when the temperature was lowered from 9°C to 4°C.
Values of 10 - 80 mg/100 g of tyramine have been reported as toxic doses in food, as reviewed by Brink et al. (1990). The tyrosine concentration in lightly salted roe is in the range of 0.8 - 3.1 mg/100 g (note 1), and consequently a maximum concentration of 4.1 mg/100 g of tyramine can be obtained. The situation is, however, similar to that of histidine regarding possible release of additional tyrosine during storage. As the storage temperature

of lightly salted lumpfish roe additionally is low (5°C), production of toxic amounts of tyramine by carnobacteria is not likely to pose a risk.

Thus for several reasons (low concentrations of histidine and tyramine, low incidence of biogenic amine producing bacteria and low storage temperature) lightly salted lumpfish roe, properly preserved to obtain a shelf life of three months, can be considered a low risk product regarding biogenic amine poisoning. Regarding the histamine hazard, the product may even be "fail-safe", as growth of *Morganella morganii* to high numbers will reveal itself by production of offensive off-odours, that ought to prevent consumption of the roe.

5.7 Conclusion

The following potential health hazards of lightly salted lumpfish roe (pH 5.4, 4 % WPS) have been evaluated in this chapter: Non-proteolytic *Clostridium botulinum*, *Listeria monocytogenes*, *Vibrio* spp., *Aeromonas* spp., *E. coli*, *Salmonella* spp., *Yersinia enterocolitica*, *Staphylococcus aureus*, viruses, parasites and biogene amines.

Additional means of control are required to eliminate the possibility of lightly salted lumpfish roe posing a risk to the consumer due to high numbers of *Listeria monocytogenes*. Strains of this organism were shown to be able to proliferate in the product at 5°C, and additional preservation is necessary.

Chapter 6. Biopreservation of lightly salted lumpfish roe

6.1 Introduction

The results obtained in the project so far has shown, that in order to obtain a shelf life of three months, additional preservation of lightly salted lumpfish roe is needed. The preservation should prevent or strongly inhibit growth of Enterobacteriaceae (see chapter 4) and *Listeria monocytogenes* (see chapter 5) without negatively influencing the sensory quality of the products. Any food additive also needs approval by regulatory authorities, in which process the lack of toxicity to humans is central. The latter aspect is briefly discussed in section 6.6.

As described in chapter 1, biopreservation (biological preservation) of lightly salted lumpfish roe was expected by the industry to be way of fulfilling wishes among consumers for more "naturally" preserved foods. Therefore at initiation of this project it was a wish, that the possibility of biopreservation using LAB cultures was evaluated as the first option. For this purpose, LAB culture/mixtures of cultures without significant impact on the sensory characteristics of the roe products were to be searched for, as opposed to a fermented product. LAB should preferably originate from the roe products them selves. This was due to both the wish of "natural" preservation, and to the fact, that such cultures would already have proven their ability to grow in the roe products at relevant storage conditions.

Thus the requirements to be fulfilled by the LAB culture, as also earlier reviewed in general terms by others (Holzapfel et al. 1995, Huss et al. 1995) were:

- No health risk
- Capability to grow and compete well in vacuum-packed lightly salted lumpfish roe at 5°C
- Consistent antibacterial effect in the roe products on growth of Enterobacteriaceae and *Listeria monocytogenes*
- No negative influence by the LAB culture on sensory characteristics of the roe products

A definition of biopreservation has been presented by Ray (1992a) as "the use of antimicrobial compounds of plant, animal or microbial origin to enhance the safety and extend the shelf life of foods". The distinction between biopreserved food products and preserved food products in general is however difficult, using this definition. For example the chemical preservatives benzoic and sorbic acids, naturally present in various plants, may also be claimed to be included in this broad definition. Preservation by addition of lactic acid/sodium lactate, produced by fermentation (described in chapter 7), would also fit the definition of biopreservation. In this thesis the term biopreservation is restricted to describe

preservation using live cultures of microorganisms. At least with this narrow definition the existence of a separate term is justified, and a meaningful distinction to other means of preservation by addition into foods of (non-living) compounds/molecules is obtained. Consequently fermented products are also included in this definition.
In this chapter the group of LAB is defined (section 6.2). Their antagonistic activities towards other microorganisms are briefly described, and the potential usefulness in preservation of lightly salted lumpfish roe is evaluated (section 6.3). Then own experiments aiming to isolate antagonistic LAB from roe products (section 6.4), and inoculation experiments with selected LAB strains in lightly salted lumpfish roe are presented (section 6.5). In the last section (6.6) future possibilities and alternative strategies in biopreservation of roe products are discussed.

6.2 Taxonomy of LAB

LAB is not a taxonomically well-defined group of bacteria. LAB are Gram-positive, non-sporeforming, catalase-negative, microaerophilic bacteria producing lactic acid as their main fermentation product from carbohydrates (Kandler 1983). Kandler included cocci of the genera *Streptococcus, Pediococcus* and *Leuconostoc* and the rods *Lactobacillus* in the group of LAB. In the last decade significant changes has occurred in the taxonomic classification of LAB. From the genus *Streptococcus* the following has been separated: *Lactococcus* (former Lancefield group N) (Schleifer et al. 1985), *Enterococcus* (former part of Lancefield group D) (Schleifer and Kilpper-Bälz 1984), and later the genus *Vagococcus* of (generally motile) cocci (Collins et al. 1989). The genus *Carnobacterium* of non-aciduric *Lactobacillus* has been defined (Collins et al. 1987), and the genus *Weissella* containing five heterofermentative former *Lactobacillus* species (Collins et al. 1993). The genus *Tetragenococcus* resulted from an investigation of the phylogenetic relationships of the tetrad forming genera *Pediococcus* and *Aerococcus* (Collins et al. 1990). The genus contains one species *Tetragenococcus halophilus,* which is halophilic. Other minor changes including only a few species has occurred in addition (Schleifer and Ludwig 1995).
There are slight differences in the range of genera included in LAB by different authors. Stiles and Holzapfel (1997) however reviewed the current taxonomy of lactic acid bacteria, and listed species of the following genera to be important in food: *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus , Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella.*

6.3 Antagonism of LAB

Apart from influencing the growth of other bacteria by competing for nutrients and occupying niches/space, LAB can, depending upon the raw material/substrate and the specific strain, produce many types of antimicrobial compounds, enabling us to utilise selected LAB with certain properties, for preservation purposes. The inhibitory substances include both non-peptide compounds and protein-containing molecules (bacteriocins).

6.3.1 Non-peptide inhibitors

The range of antimicrobially active metabolites produced by LAB have in the last decade been reviewed by several authors, due to the much increased interest in utilising these bacteria and their metabolites. The basic information on antimicrobial compounds produced by LAB described in this section, is extracted from Daeschel and Penner (1992), Davidson and Hoover (1993), Holzapfel et al. (1995), Lindgren and Dobrogosz (1990), Ray (1992b) and Ray and Sandine (1992).

Organic acids primary lactic acid, are as already mentioned characteristic products of carbohydrate fermentation by LAB. Acetic acid can be produced, and propionic acid in smaller amounts by various LAB. Production of formic acid may occur at certain conditions of hexose limitation. The effect (prolonged lag phase, reduced growth rate, bacteriostatic or bactericidal effect) of organic acids on bacteria depend on pH, the particular acid, the concentration of acid and the kind of organism to be inhibited. The sensitivity to organic acids vary among spoilage and pathogenic bacteria, and is influenced by other factors characterising the food product, such as salt concentration and temperature. The mode of action of organic acids are described in more detail in section 7.2.
The possibilities of utilising acid production by LAB as antimicrobial principle in lightly salted lumpfish roe may be limited by adverse effects of low pH on the sensory characteristics of the product. It may not be appropriate to decrease the pH below 5.0 (lactic acid) in lightly salted lumpfish roe (see section 7.5 and note 7). Furthermore the amount of carbohydrates in lightly salted lumpfish roe is limited, and during growth to around 7 log cfu/g of LAB in naturally contaminated roe products, only small amounts of acids were produced (chapter 4). Thus addition of appropriate amounts of fermentable carbohydrate would be necessary. The perspectives in such an alternative strategy, producing a fermented roe product, are discussed in section 6.6.

Hydrogenperoxide is produced by a number of LAB in the presence of oxygen, and may accumulate to antimicrobial concentrations due to the general absence of catalase in LAB. Due to the vacuum packaging in air-tight glass jars, hydrogenperoxide production is not

considered relevant in lightly salted lumpfish roe. In addition it is questionable, whether hydrogenperoxide production should be appreciated, as the roe products have already been subject to lipid oxidation (chapter 3), and should not be charged further.

Carbondioxide is toxic to a number of aerobic bacteria, but may promote the growth of others. Production of carbondioxide, primary by heterofermentative LAB fermenting hexoses, is however not wanted in lightly salted lumpfish roe. In lightly salted lumpfish roe carbondioxide production is regarded as spoilage of the product. Also the lit of a jars is not tight, if vacuum in the jar is lost.

Diacetyl (2,3-butadione) is produced by strains in several genera of LAB. It is produced in significant amounts at conditions other than hexose fermentation. Citrate can be converted to diacetyl via pyruvate, which is the reason why citric acid was not chosen for pH regulation of the roe products. Diacetyl creates the characteristic aroma of butter, which is not appreciated in lightly salted lumpfish roe. As high concentrations is in general needed for inhibition, the sensory characteristics of the compound limits its use to certain food products.

Reuterin (3-hydroxypropionaldehyde) is produced by *Lactobacillus reuteri* from glycerol (glyceraldehyde) at anaerobic conditions. It is inhibitive to a broad spectrum of microorganisms (antibacterial, antimycotic and antiprotozoal effects), probably through inhibition of ribonucleotide reductase. Reuterin may be a promising candidate for applications as food preservative, which however largely depend on information on toxicity to humans. On this background, reuterin addition was not considered a preservation method, that could fulfil the wishes for a "natural" preservation. In using live cultures of *Lactobacillus reuteri,* there is an additional obstacle, as this bacterium is obligate heterofermentative.

6.3.2 Bacteriocins

Bacteriocins are antimicrobial proteins produced by a wide range of microorganisms. Six criteria defining bacteriocins, originally suggested by Tagg et al. (1976), were based on studies of the colicins produced by Gram-negative bacteria, and included the criteria bactericidal mode of action and activity against a narrow spectrum of closely related organisms. Based on the later increased knowledge on bacteriocins of LAB and other Gram-positive organisms, Montville and Kaiser (1993) however stated, that only few bacteriocins meet all the criteria. They presented a redefinition including only two criteria for bacteriocins: Their proteinaceous nature and their lack of lethality to cells, which produce them. Opposed to peptide antibiotics such as bacitracin A and polymyxins, many

bacteriocins are true proteins made ribosomally, and not by enzymatic condensation reactions of amino acids (Montville and Kaiser 1993).

An overwhelming amount of research on bacteriocins of LAB have been reported in the last 5 - 10 years. Still many questions remain to be answered in order to understand all aspects of bacteriocin production and the response of exposed organisms, which can be expressed by citing Klaenhammer (1993): "Studies on the genetics and biochemistry of bacteriocins produced by LAB, suggest that we have only begun to understand the nature and complexity of these interactions". In the following, basic information is presented on characteristics of bacteriocins and their mode of action, which has been extracted from Abee et al. (1995), Bruno and Montville (1993), De Vyust and Vandamme (1994), Jack et al. (1995), Hill (1995), Hoover and Steenson (1993), Klaenhammer (1988 and 1993) and Schillinger (1990).

Bacteriocins have a peptide/protein component, that is essential for their activity, and most known bacteriocins are small, heat-stable peptides. Some have however been reported to have a lipid or carbohydrate moiety or consist of combinations of proteins. In 1993 Klaenhammer defined four classes of bacteriocins of LAB:

- I. Lantibiotics. Small membrane-active peptides (< 5 kD) containing the unusual amino acids lanthionine, β-methyl lanthionine and dehydrated residues (dehydroalanine and dehydrobytyrine).
- II. Small heat-stable, non-lanthionine containing, membrane-active peptides (< 10 kD). The class was further divided into: IIa. *Listeria*-active peptides with a specific sequence in the N-terminus. IIb. Complexes of two peptides for poration activity. IIc. Thiol activated peptide(s).
- III. Large heat-labile proteins (> 30 kD)
- IV. Complex bacteriocins with one or more lipid/carbohydrate moieties required for activity.

This is however currently revised, as new knowledge gets available. De Vyust and Vandamme (1994) do no recognise the existence of class IV, and the subdivision of class II (containing the majority of known bacteriocins) differs. Apart from the group of bacteriocins requiring two peptides the class was divided into Pediocin-like and Lactococcin-like bacteriocins, each sharing some amino acid sequence homology in their N-terminus.
In a resent report, Martinez et al. (1996) describes the finding of a bacteriocin (Lactococcin 972), that cannot be properly included in any of the four classes described by Klaenhammer (1988). Also this bacteriocin seem to have a different mode of action, than previously reported, as its primary target is not the cytoplasmatic membrane.

Bacteriocins can be either chromosomally or plasmid encoded. Many bacteriocins are produced as pre-peptides, with extensions of 18 - 24 amino acids (leader-peptide) at the N-terminus, the amino acid sequence of which is known for several bacteriocins. The leader peptide is supposed to be cleaved off prior to excretion of the mature bacteriocin into the environment.
Little is known on the mechanisms of immunity of the producing LAB strain to its own bacteriocins. It has been assumed, that the immunity of the producing cell is due to the production of an immunity protein. For a majority of bacteriocins, immunity proteins have however not been purified. For some bacteriocins it has been suggested, that the gene for immunity protein production is part of the bacteriocin operon. Many of the Pediocin-like bacteriocins show cross-immunity, suggesting similarities at the level of both action and immunity. Lactoccocin A immunity protein was examined by Nissen-Meyer et al. (1993). The exposure of sensitive cells to an excess of the immunity protein did not affect the sensitivity, and exposing immune cells to an antiserum against the immune protein did not sensitise the cells to Lactococcin A. The authors concluded, that the immunity protein did not act simply by binding to the bacteriocin, and may in fact not act extracellularly.

Control of bacteriocin production by LAB is in general not well understood. Resent studies on sakacin P has however indicated, that bacteriocin production is controlled by an autoinduction pathway, in which a bacteriocin-like molecule (inducing factor) may function as a cell density signal. The inducing factor triggered the production of sakacin P, immunity to sakacin P (and related bacteriocins) and the inducing factor itself. This could be interpreted in the manner that, when certain cell densities and certain levels of inducing factor is reached and competition therefore may increase, the bacteriocin production is initiated (Eijsink et al. 1996).

The range of inhibitory activity by bacteriocins of LAB can be either narrow, inhibiting only those strains closely related to the producer organism, or wider (less common) inhibiting a diverse group of Gram-positive organisms (other LAB, *Listeria, Clostridium* etc.). The latter makes bacteriocin production especially attractive in refrigerated, minimally processed and raw foods.

In their mode of action, bacteriocins of LAB seem to be membrane active. Pore formation by different mechanisms in the cytoplasmatic membrane is common of those LAB bacteriocins, for which the mode of action has been determined. This generally leads to disturbance of membrane transport, loss of proton motive force and thus inhibiting energy production and biosynthesis. Some bacteriocins that have a narrow spectrum of inhibition, may specifically interact with membrane receptors, while many appear to exhibit relatively little adsorption specificity. Lack of adsorption specificity indicates, that sensitivity or resistance to a

bacteriocin is not solely determined by the presence or absence of receptors on the cell surface. The existence of proteinaceous receptors, their specific or non-specific nature and role in bacteriocin action on cells is a debated area.

Others have reported successful inhibition of *Listeria monocytogenes* in food products (for example Buchanan and Klavitter 1992b, Choi and Beuchat 1994), and it might be possible to ensure the safety of lightly salted lumpfish roe regarding this hazard, by the use of LAB, producing *Listeria*-active bacteriocins.

6.4 Isolation of LAB from lightly salted lumpfish roe

As indicated in section 6.3, several obstacles may hinder a successful preservation of lightly salted lumpfish roe using LAB cultures. This is due to the prerequisite for the desired product to be sensory unaffected by the preserving culture, which would make the use of bacteriocinogenic LAB the most appropriate, and secondly to the conclusion reached during this project, that apart from *Listeria monocytogenes* target organisms include Gram-negative spoilage bacteria (Enterobacteriaceae). Yet it was not wanted to rule out any possibilities in advance, and in the light of the many unanswered questions on LAB antagonism, and the possible existence of undiscovered capabilities of LAB, attempts have been made to isolate LAB from roe products, antagonistic to relevant target organisms.

Bacteriocin production by LAB is influenced by environmental factors, which are being investigated by an increasing number of workers. Bavaricin A was produced at 4°C by a *Lactobacillus bavaricus* from sour dough, but increasing the salt concentration from 1 to 3 % caused a loss of bacteriocin production at the low temperature (Larsen et al. 1993). Mørtvedt-Abildgaard et al. (1995) reported the production of Lactococin S by a *Lactobacillus sake* to be 10-fold higher at pH 5 than at pH 6. The production increased in the presence of 1 % ethanol, and production was almost eliminated by aeration of the culture. Kaiser and Montville (1993) showed that glucose-limitation in continuos culture of a *Lactobacillus bavaricus* resulted in an increased production of Bavaricin MN. De Vyust et al. (1996) also reported that bacteriocin production by a *Lactobacillus amylovorus* was stimulated by unfavourable growth conditions such as low temperature, presence of ethanol and oxygen. They found bacteriocin production to be enhanced by slow growth rates. On the contrary Yang and Ray (1994) found that supplementation of the medium (TGE-broth) with tryptone, glucose and yeast extract or with different vitamins increased Pediocin AcH production (*Pediococcus acidilactici*) by about 27 %. These authors also concluded that growing cells under an optimum environment for 16 hours facilitated high bacteriocin production. Nisin production by a *Lactococcus lactis* subsp. *lactis* was shown to be affected

by nutritional factors in the manner, that nisin biosyntheses was strongly dependent on the presence of a sulphur source (De Vyust 1995). The amino acids serine, threonine and cysteine highly stimulated nisin production without affecting the final cell yield.

Thus in the detection of antagonistic activity in the present study, the aim was to mimic product conditions as closely as possible, in order to select for strains producing bacteriocins (or other antimicrobial compounds) at product relevant conditions. Unspecific antagonism assays were performed in roe agar.
Naturally occurring LAB of six different batches of lightly salted lumpfish roe (3.5 - 4.8 % WPS) stored for two and a half months was screened for antagonistic activity against 10 Enterobacteriaceae (roe isolates) and *Listeria monocytogenes* strain O57. 3 *Vibrio* spp. from roe products were included as well (experiment 1 of note 5). Replica plates on roe agar was made from primary NAP-agar plates, incubated at 5°C until visible LAB colonies had formed, and overlaid with soft roe agar seeded with target organisms (pre-cultured in roe extract at 5°C). Incubation conditions for the plates were 5°C and oxygen limitation. As only a few extremely weak inhibition zones against *Vibrio* spp. developed, a well diffusion assay was performed instead. A total of 43 LAB from stored roe products, was included (experiment 2 of note 5). A traditional well diffusion assay was performed in soft roe agar, seeded with target organisms (6 Enterobacteriaceae, 2 *Listeria monocytogenes* and in addition 3 *Vibrio* spp.). All strains were precultured in roe extract at low temperature, and incubation of the agar plates were as described above. Using this method, inhibition zones of varying size was produced by 27 of the 43 LAB against 2 *Vibrio* spp. None of the 43 LAB produced inhibition zones against any of the Enterobacteriaceae (presumptive *Serratia liquefaciens* and *plymuthica*). *Morganella morganii* and *Listeria monocytogenes* failed to grow in the roe agar.

Thus at the conditions 5°C and oxygen limitation *Vibrio* spp. were inhibited by several LAB in a substrate close to lumpfish roe with pH 5.4 (buffered) and 4 % salt. The mechanism of inhibition was not further investigated, but at these conditions, neither acid production nor production of hydrogenperoxide was likely to have caused the antimicrobial effect. Though not very relevant in terms of lightly salted lumpfish roe preservation, future investigation of the nature of inhibition of *Vibrio* spp. by LAB would be interesting from a scientific point of view, and it may be possible, that inhibition of *Vibrio* spp. occur in the roe products as well.

Though closer than a nutritious laboratory medium, roe agar is still not identical to the roe products. As a dilution step and heat treatment is part of the processing of roe into roe agar, growth conditions must be expected to be less supportive than those of the roe products. This may have caused the lack of growth of the two target organisms in the roe agar. As tested in a trial, *Listeria monocytogenes* was able to grow in roe extract, but not in roe agar

(regardless of the pre-culturing medium, experiment 3 of note 5). The lower concentration of nutrients, combined with reduced diffusion of nutrients in the agar, may have caused insufficiency of substrates for growth of *Listeria monocytogenes.*
Consequently it was considered necessary to go back to using laboratory media, and a non-specific well diffusion assay with 31 LAB roe isolates were performed in APT-agar with *Listeria monocytogenes* O57 as the target organism (incubation conditions were 5°C and oxygen limitation) (experiment 3 of note 5).
A total of 8 of 31 LAB produced inhibition zones against *Listeria monocytogenes*, 4 of those however only very narrow zones (≤ 1 mm).

In summary the yield of the isolation experiments were sparse in terms of strains with biopreservation potential. Apart from 27 of 43 LAB producing inhibition zones against *Vibrio* spp., only 4 LAB producing significant inhibition zones (6 - 12 mm) against *Listeria monocytogenes* and no LAB producing inhibition zones against Enterobacteriaceae was obtained.
Thus the good intentions to mimic product conditions was not entirely successful, and illustrates the importance of developing an optimal substrate for use in antagonism assays (and for other studies of bacterial isolates from lightly salted lumpfish roe as well). It can be repeated here, what was stated in chapter 4, that a sterile, non-heated model substrate of desalted (lightly salted) lumpfish roe composition is highly desirable in this regard.

6.5 Effect of LAB inoculated in roe products

6.5.1 Effect on *Listeria monocytogenes*

The effect of selected LAB on growth of *Listeria monocytogenes* has been determined in normal roe products. Three LAB strains were selected in lightly salted lumpfish roe, which in the antagonism assay had produced inhibition zones of 11 - 12 mm (strain L31), 6 - 7 mm (strain F61-1) and 0 mm (strain F44). A mixture of strains F61-1 and L31 was also included, and the LAB (5 - 7 log cfu/g) were inoculated with two strains of *Listeria monocytogenes* (< 1 log cfu/g) (experiment 1 of note 6).
The results showed, that the LAB strain with the most pronounced effect on growth of both *Listeria monocytogenes*, was strain F44, not producing inhibition zones in the laboratory medium. The effect of inoculation with LAB F44 on growth of the two *Listeria monocytogenes* is shown in figure 6.1. At least the only known difference between roe batches with good or poorer growth of *Listeria monocytogenes* was the addition of F44.

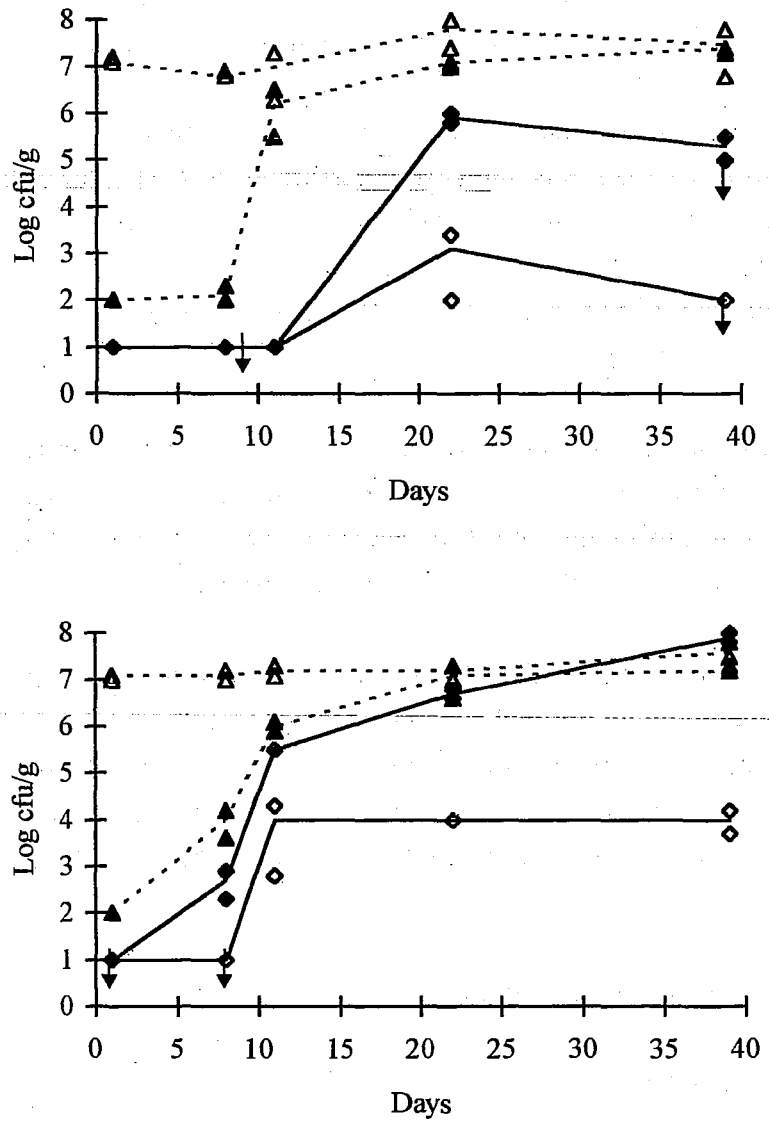


Figure 6.1 Effect of inoculation with LAB F44 on growth at 5°C of two *Listeria monocytogenes* strains in lightly salted lumpfish roe (4 % WPS, pH 5.4, vacuum packed). ◆: *Listeria monocytogenes*, ▲ (punctured lines): LAB. Closed symbols: roe inoculated with *Listeria monocytogenes* alone, open symbols: roe inoculated with *Listeria monocytogenes* and LAB F44. ↓ : Below detection limit (data from note 6, experiment 1).

LAB F44 was inoculated at a level of 7 log cfu/g, and the LAB counts only changed slightly during storage. Inhibition of *Listeria monocytogenes* by a non-growing *Pediococcus* sp. inoculated at 6 log cfu/g in sausage mix has previously been reported (Berry et al. 1990). According to Ray (1992c) live cells of non-growing LAB, inhibiting pathogenic and spoilage

bacteria, is assumed to either act as carriers of or produce antimicrobial compounds other than fermentation acids, and both bacteriocins and hydrogenperoxide was suggested.
As shown in figure 6.1, counts of the best growing *Listeria monocytogenes* H018 were around 4 log units lower after 39 days of storage compared to roe not inoculated with LAB F44, while the poorly growing *Listeria monocytogenes* O57 decreased to < 2 log cfu/g. It is not known whether prolonged storage would have resulted in further inhibition of *Listeria monocytogenes*, or renewed growth as earlier reported by Ben Embarek et al. (1994).
The effect of LAB F61-1, L31 and their combination on growth of H018 was weaker (similar for all three), and counts of H018 after 39 days were only ≤ 2 log units lower (around 6 log cfu/g) than those of H018 alone. The inoculum of LAB L31 turned out to be only 5 log cfu/g, which however apparently increased to above 7 log cfu/g after 11 days. After 39 days there was no effect of LAB F61-1, L31 or their combination on *Listeria monocytogenes* O57. The growth curve of O57 was however changed, as the LAB inoculum seemed to have enhanced growth of O57 during the first part of the storage, while slower growth thereafter resulted in unchanged final counts (note 6, experiment 1).

The experiment once again underlined the importance of the availability of an appropriate substrate, as the antagonistic activity, which F44 seem to have against *Listeria monocytogenes* in the roe products would have passed undetected, if only an antagonism assay in laboratory medium had been performed.
The experiment also showed, that LAB F44 (belonging to group B2 of presumptive *Carnobacterium* sp. defined in paper 3) may have biopreservation potential in terms of *Listeria monocytogenes* inhibition in lumpfish roe products.
LAB of group B2 produced weak off-odours in pure culture in pasteurised lightly salted lumpfish roe (section 4.6.2). LAB F44 have however also been included in another storage experiment as a mixture with LAB V6 in lightly salted lumpfish roe preserved by addition of 2.8 % (w/w) sodium lactate (experiment 3 of note 6). The inoculum of around 7 log cfu/g remained unchanged throughout storage of the roe (116 days), and no sign of spoilage developed.
The addition of 2.8 % sodium lactate totally inhibits growth of *Listeria monocytogenes* (see section 7.4.1), but in preserved roe with F44 and V6 it was noted, that after one month at 5°C, *Listeria monocytogenes* was detected in two roe jars at a level of 3 log cfu/g, after which the count decreased to undetectable levels (table 10 of note 6, batch 2). It is not known whether the inoculated LAB metabolised sodium lactate present in the product. Such effects should however be carefully examined if a biopreserving culture is to be used in combination with other antimicrobials such as sodium lactate.

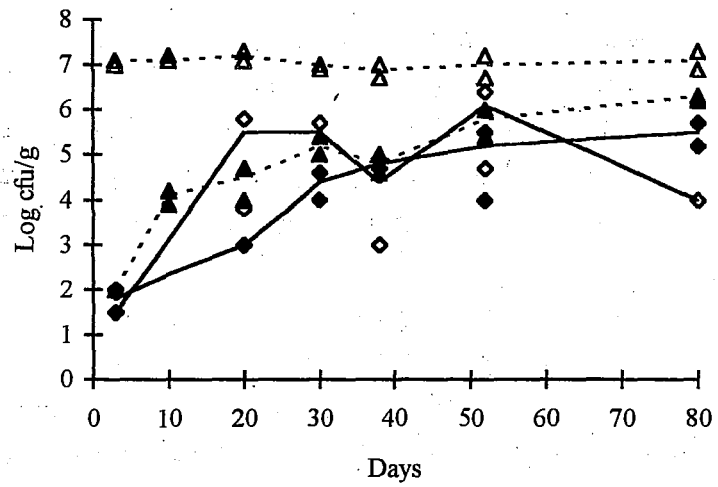
6.5.2 Effect on Enterobacteriaceae

As no LAB candidates for biopreservation directed against Gram-negative spoilage organisms was obtained from the assays in roe agar, experiments have been performed using the strain V6 mentioned above. The strain (*Leuconostoc* sp.) originated from another lightly preserved fish product (non-spoiled sugar-salted herring) stored at 10°C, and have in a disc assay been shown to have antagonistic activity against a range of bacteria including Gram-negatives such as *Shewanella putrefaciens, Serratia* spp., *Morganella morganii, Aeromonas* spp. and *Yersinia enterocolitica* (Jeppesen and Huss 1993a). The inhibitory principle(s) was not identified, but the authors stated, that acidification by V6 was less pronounced, than that of other examined LAB exhibiting weaker antagonistic activity. Hydrogensulphide production may have played a role, but is not likely to occur in lightly salted lumpfish roe products. Prevention of growth of low (2 log cfu/g) but not higher (4 log cfu/g) numbers of *Yersinia enterocolitica* by V6 was later also demonstrated in shrimp extract at 5°C (pH 5.8, 3 % WPS) (Jeppesen and Huss 1993b).

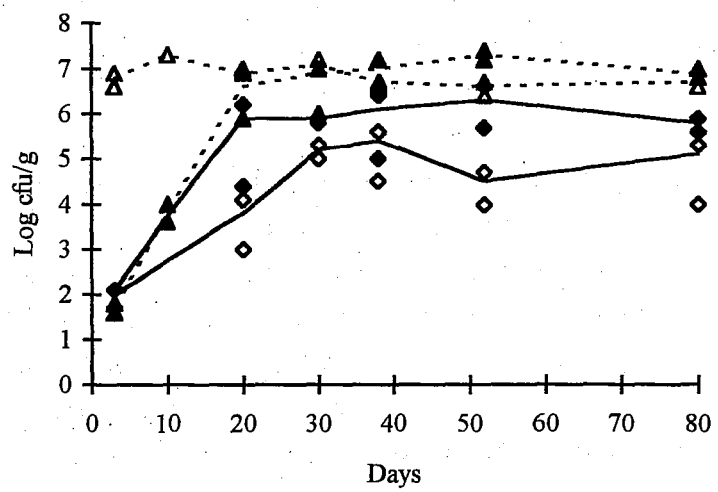
Lightly salted lumpfish roe has been inoculated with V6 at a level of 7 log cfu/g. Both naturally contaminated roe product and roe product, which in addition were inoculated with *Morganella morganii,* were added V6 and stored at 5°C. The development of Enterobacteriaceae and LAB during storage for 80 days is shown in figure 6.2.
As Enterobacteriaceae counts vary among double determinations, the results should be interpreted with precaution. No systematic inhibition of naturally occurring Enterobacteriaceae by V6 could be noted, while in roe with *Morganella morganii* there seemed to be a slight inhibition, which however became less pronounced, when the storage time approached three months.
It should also be noted, that when only roe without V6 is considered in the figure, the naturally present LAB flora seem to be affected by the presence of *Morganella morganii,* and slower LAB growth occurred in roe without *Morganella morganii.* In fact this apparent effect was equally strong as the effect of V6 on Enterobacteriaceae observed in roe with *Morganella morganii.* Still the effect may not be consistent, when the variation among double determination (different development in individual jars of roe) is considered.
The conclusion from the experiment is, that the inhibition of Enterobacteriaceae by V6 do not offer good prospects for a convincing ensurance of a three months shelf life.

An additional aspect of LAB inoculation should be mentioned. In a preserved roe product (2.8 % (w/w) sodium lactate), growth of Enterobacteriaceae was totally inhibited (se section 7.4.1), but when LAB V6 + F44 were added (7 log cfu/g), growth of Enterobacteriaceae occurred to a level of 4.5 - 5 log cfu/g after one month. At the end of storage (116 days) 4.1 log cfu/g Enterobacteriaceae were still detected (table 9 of note 6, batch 1). Eight randomly

picked isolates (116 days) belonged to Enterobacteriaceae group 1, presumptive *Serratia liquefaciens* (table 13 of note 6). This seemed to be similar to the effect of V6 + F44 on *Listeria monocytogenes* in roe products preserved using 2.8 % (w/w) sodium lactate, mentioned in the previous section.



Naturally contaminated roe product



Roe product with *Morganella morganii*

Figure 6.2. Effect of inoculation with LAB V6 on growth at 5°C of Enterobacteriaceae in lightly salted lumpfish roe (4 % WPS, pH 5.4, vacuum packed). ◆ Enterobacteriaceae, ▲ (punctured lines): LAB. Closed symbols: roe without V6, open symbols: roe inoculated with LAB F44 (data from note 6 experiment 2).

6.6 Perspectives in biopreservation of salted lumpfish roe products

As discussed in this chapter the prospects of biopreservation of lightly salted lumpfish roe using live LAB cultures, active against both *Listeria monocytogenes* and Enterobaceriaceae, is not good. In the literature, there are some reports on unidentified compounds produced by LAB active against Gram-negatives. Among these some authors have noted inhibition of Gram-negatives, which could be reduced or eliminated by proteolytic enzymes (Spelhaug and Harlander 1989, Vignolo et al 1993, Simonetta et al 1997), and Santos et al. (1996) reported an inhibitory effect on *Aeromonas hydrophila* by a *Lactococcus lactis* both in agar assay and mixed culture. The effect could not be eliminated by catalase or proteolytic enzymes, and could only be reduced (not eliminated) by neutralisation. Yet until these inhibitory substances are purified, identified and their mode of action established, it is not possible to evaluate the future potential of such compounds for preservation of foods.

Still alternative strategies could be considered in obtaining a biopreserved lumpfish roe product. It was evaluated in this project, whether a product may be developed with somewhat lowered pH (around 5.0) obtained by LAB fermentation of appropriate amounts of glucose. Bacteriocin producing LAB were to be used, providing an additional hurdle against *Listeria monocytogenes*. In a storage trial 1 % (w/w) glucose was added to lightly salted roe, and a roe batch was also inoculated with the previously described strain V6 (note 6 experiment 4). The pH dropped below 5, and glucose concentration needed adjustment. The strategy was however not at all proven successful, as the glucose addition resulted in gas production, both in roe with and without V6. Therefore the idea was not further pursued.

Thus if a fermented product is ruled out, it is necessary to stay with the sensory unchanged products, as originally intended. Based on the possible antagonistic principles discussed in this chapter, it seems that biopreservation of lumpfish roe products using LAB cultures, may be limited to those products in which the Gram-negative flora is eliminated, and inhibition of Gram-positive spoilage organisms can be based on bacteriocin producing strains. It can be suggested to examine the possibility of biopreservation of those roe products existing today, which have higher salt concentrations (and longer shelf lives), than the lightly salted lumpfish roe examined in this project. Such products seem to be dominated and eventually spoiled by LAB, as earlier reported by Huss et al. (1984) ($\geq$ 5.7 % WPS). Control of spoilage LAB by bacteriocinogenic LAB in vacuum-packed beef has been reported by Leisner et al. (1996) and bacteriocinogenic LAB has been suggested for control of spoilage LAB (and pathogenic bacteria) in vacuum-packed/MAP meats by McMullen and Stiles (1996).

Even if potential biopreserving cultures of LAB can be found, which produce bacteriocins at product relevant conditions, active against the relevant target organisms of the chosen roe

product(s), without negatively influencing the sensory characteristics, some possible obstacles needs consideration in addition. These are briefly discussed below and are of general concern in application of bacteriocin producing LAB for preservation of foods.

While the safety of many LAB strains has been accepted after a long history of use in traditional foods, the pathogenic potential of LAB are debated, as new strains are being introduced. Data from animal studies indicate, that most LAB does not possess acute toxicity when ingested in large amounts (Donohue et al. 1993). However it is important to test new strains for their ability to cause infections including bacteremia, as some LAB have been associated with a variety of clinical conditions (Aguirre and Collins 1993, Harty et al. 1994). Yet Adams and Marteau (1995) reported for the LABIP (Lactic Acid Bacteria Industrial Platform) workshop on the safety of LAB, that apart from the enterococci, the overall risk of LAB infections is very low. They also stated that in all known cases, patients had underlying conditions, and no LAB infections have been reported in otherwise healthy people.

Bacteriocins may not be sufficiently effective in the food, even if produced by well growing LAB at product relevant conditions. This can be due to inactivation or to the emergence of resistant target organisms. Regarding the former both regular destruction (e.g. non-specific degradation by naturally present proteolytic enzymes, oxidation) and inactivation by binding to food components or additives, may compromise the efficiency. Also phage infections or loss of bacteriocin producing ability may occur (Daeschel 1993). Food pH is also important, an example of which is the reduced solubility of nisin above pH 5, limiting its use (Stiles 1994).
The mechanisms of acquired resistance to bacteriocins is not well known. Nisin resistance has been studied the most, and the production of nisinase (nisin degrading enzyme) by several bacteria has been revealed (Daeschel 1993). Still other mechanisms seem to exist. Davies and Adams (1994) suggested resistance of *Listeria monocytogenes* to nisin to be acquired by adaptation of either the cytoplasmic membrane or cell wall. Later Mazzotta and Montville (1997) demonstrated that nisin induced changes in the fatty acid composition of the membrane in nisin-resistant *Listeria monocytogenes* regardless of the growth temperature. Ming and Daeschel (1995) found a significant decrease on the amount of three individual phospholipids in resistant *Listeria monocytogenes*, and the cell surface were less hydrophobic compared to sensitive cells. Strains spontaneous resistant to other bacteriocins also occur (Daeschel 1993), and the problem needs further investigations in order to evaluate the possible consequences in food applications. In order to reduce the risk, combination of strains producing different bacteriocins have been suggested. This strategy may even enhance the antibacterial effect as reported by Hanlin et al. (1993). These authors noted a greater antibacterial spectrum and activity of the combination of nisin and pediocin AcH, compared to each of the bacteriocins alone.

Chapter 7. Preservation using lactic acid/lactate

7.1 Introduction

As already discussed in section 6.1 the primary targets of inhibition are Enterobacteriaceae and *Listeria monocytogenes*, and the shelf life of lightly salted lumpfish roe should be at least three months. Lactic acid and lactates are GRAS (*generally recognised as safe*) additives (Code of Federal Regulations no.21, U.S. Gov. § 184.1061, § 184.1639, § 184.1768 c.f. Shelef 1994), and no technical problems have to be solved in the production, as technical application only requires thorough mixing, which is already a part of roe processing. Lactic acid/lactate used in this project is sold under the description "natural lactic acid", meaning that L(+) lactic acid is produced by fermentation, while chemical synthesis would yield a racemic mixture (Den Uijl and van Burik 1990).
In this chapter the mode of action and use as a food preservative of lactic acid and lactates are described. Results are presented from storage experiments carried out with roe products preserved by the use of lactic acid/sodium lactate as an alternative to biopreservation using live LAB cultures

7.2 Mode of action

Organic acids including lactic acid have for a long time been used to ensure microbial stability of foods, either directly added or as the result of food fermentation. The antimicrobial effect depends as mentioned in section 6.3.1 upon several factors, which include the effect of the pH itself, the concentration of undissociated acid, the kind of acid and the kind of organism to be inhibited. It is generally accepted that the antimicrobial activity is primarily ascribed to the undissociated form of short chain carboxylic acids such as acetic and lactic acid, which are able to enter the bacterial cell. Once inside, the molecule dissociates according to the internal pH and eventually causes acidification of the cytoplasm, when the capacity of energy consuming cellular mechanisms for maintaining internal pH is exceeded. Lowering of internal pH may have numerous effects on the cell function interfering with the cell membrane and metabolic enzymes (Adams and Hall 1988, Baird Parker 1980, Corlett and Brown 1980, Grau 1981, Kabara and Eklund 1991).
Regarding the effect of external (food) pH in itself, a high proton (H_3O^+) concentration may interfere with essential activities such as transport of nutrients by affecting conformation and activity of proteins located outside the membrane. High proton concentration in itself does not affect the internal pH to the same extent as permeant weak acids (Brown and Booth 1991). Acidification of the cell interior governed by pH alone relies on very low pH, that

may be unacceptable in most foods (Corlett and Brown 1980). A sole pH effect may optimally be observed by controlling pH with a strong acid.

Rosso et al. (1997) analysed data from the literature reporting minimum pH for initiation of growth (pH_{min}) in the presence of several different acids (at otherwise optimal growth conditions). They found a simple relationship between the pK_a of the acid used and the pH_{min}, and proposed a model for predicting pH_{min} values in the presence of any other acid for a given strain in a given growth medium. They found the relationship to consist of two phases, and interpreted this in the manner, that for acids with pK_a values below a certain threshold, pH_{min} seemed to be constant (absolute pH minimum - for the analysed data around pH 4) corresponding to the maximum proton concentration in the medium. Above the threshold pK_a value the bacteria would behave in a pattern that suggest a concentration effect. This upper phase must be considered the relevant one in most foods.

The effect of the concentration of undissociated organic acids on spoilage and pathogenic bacteria (23 different Enterobacteriaceae) from silage was examined by Östling and Lindgren (1993) in a pH interval between 4.2 and 5.4 in a liquid medium incubated aerobically or anaerobically at 30°C. They demonstrated that the minimum inhibitory concentration (MIC) of undissociated lactic acid remained fairly constant within the defined pH-range, although a higher total concentration of acid was required to inhibit the bacteria at the higher pH levels (more distant to the pK_a value). They concluded that the inhibitory effect of the acids in this pH interval, was caused mainly by the undissociated acids. The same conclusion has been reached by Adams and Hall (1988), who studied the effect of lactic and acetic acids on growth of *Escherichia coli* and *Salmonella enteritidis*, and by Grau (1981) who studied the growth of four Gram-negative fermentative bacteria on meat and in broth at various combinations of pH, lactic acid concentration and oxygen availability.

Though the inhibitory effect on growth of bacteria is mainly caused by diffusion into the cell of undissociated acid, the effect obtained is also influenced by the kind of acid and the organism to be inhibited.
Young and Foegeding (1993) reported the inhibitory effect on *Listeria monocytogenes* Scott A (in terms of reduced growth rate) of undissociated acids to be in the order citric > lactic > acetic acid, when the effect of equimolar amounts of undissociated acid at similar pH values (pH 5 and 6) were compared. This could be due simply to the difference in pK_a values of the acids, since similar amounts of acids diffusing into the cell, would dissociate to a different degree once inside, thus affecting the internal pH differently. However other factors such as active transport of acids into the cell, excretion or ability of the cell to metabolise acids may also play a role in the observed differences in inhibitory effect (Young and Foegeding 1993).

Influence by such mechanisms would also govern differences in response to specific acids among different bacteria.
Diez-Gonzales and Russel (1997) reported different response to acetate by two *Echerichia coli* strains; *E. coli* O157:H7 tolerated much higher levels than *E. coli* K-12. The tolerance could be explained by fundamental differences in metabolism and intracellular pH regulation. By decreasing the intracellular pH and producing large amounts of lactate, O157:H7 was able to decrease the pH gradient over the membrane, and prevent toxic accumulation of acetate. In his review Russel (1992) also concluded, that accumulation of potentially toxic concentrations of acid anions does not occur in bacteria, that maintains a constant pH gradient across the cell membrane rather than a constant internal pH. According to Russell (1992) the effect of undissociated acids at low pH, which dissociates after entering the cell, is not just explained by the effect on pH, but also the accumulation of acid anions. There may however be good reasons why all bacteria do not allow internal pH to decrease and maintain a constant pH gradient. Only certain carriers are pH sensitive such as those for glutamate in different LAB. Those bacteria for which glutamate/glutamine is essential, try to maintain internal pH, while others for which glutamate is not essential, allow internal pH to decrease. As *Strep. bovis* allows internal pH to decrease, certain metabolic enzymes are inhibited, and this is coped with by a shift in fermentation pattern resulting in different end products (Russel 1992).
Thus the different effects on different organisms seem to depend upon numerous factors determining how a specific microorganism can cope with the acids.

A low pH resulting from acid addition may change the sensory characteristics of food in an unacceptable manner in terms of taste and texture. Sourness may in some products be acceptable, while in others it may be equated with spoilage and thus be unwanted. In proteinaceous foods, texture may be affected by acid addition, because the proteins coagulate around their isoelectric point and thus limit the use of acids (Brown and Booth 1991). Due to these limitations other strategies have been developed. These include the use of the acid salt such as sodium lactate or the use of buffered systems. The latter have been applied in products like mayonnaise, sauces, dressings and salads (Debevere 1987).
In spite of acid addition to pH levels of 3.7 - 5.0, spoilage problems due to *Lactobacillus* spp. and yeasts occurred frequently in those products. Debevere (1987) used buffering to a certain pH, and demonstrated a greater microbial stability of industrial salads buffered to pH 4.9 containing lactic and acetic acid, than unbuffered salads with the same pH. By the use of buffering, the system simply allowed the addition of higher concentration of acid without a subsequent drop to very low pH levels.
The system was in another experiment also proven effective in a buffered medium against *Salmonella blockley*, *Echerichia coli*, *Staphylococcus aureus* and *Streptococcus faecalis* (Debevere 1988).

The use of the acid salts such as sodium lactate serves a similar purpose in terms of increasing the concentration without decreasing the pH. As the pH at equilibrium after lactate addition is higher, than after lactic acid addition, the concentration of undissociated acid relative to dissociated acid is lower, which can be observed from the basic relationship described by the equation (C indicates the concentrations of dissociated and undissociated acid) (modified from Dillard and Goldberg 1971):

$$pH = pK_a + \log \frac{C_{diss}}{C_{undiss}}$$

Therefore in order to obtain a certain absolute concentration of undissociated acid, larger total amounts are required when using sodium lactate compared to lactic acid.

Den Uijl and Van Burik (1990) discussed the preserving effect of lactic acid and lactate. The effect of lactate was attributed to a water activity lowering effect and a "specific lactate ion effect". The nature of the "specific lactate ion effect" was not explained, but the existence of such an effect was suggested due to results showing a greater inhibition by sodium lactate compared to sodium chloride added to similar water activities. Wit and Rombouts (1990) reported an experiment in which the antimicrobial effect of sodium lactate was compared to the effect of sodium chloride at similar water activities (0.958) in terms of increased lag-phase and decreased growth yield of *Staphylococcus aureus, Salmonella thyphimurium* and various LAB (inoculated to 4 - 5 log cfu/g in laboratory medium pH 7.2). They concluded that in spite of a low concentration of undissociated acid (0.2 mM) in the medium, there was an antimicrobial effect of sodium lactate, that could not be ascribed to its water activity lowering effect, and several factors were suggested to influence the effect. Among these were the possibility, that the membrane permeability to lactic acid may be higher at higher pH (around neutral), and the accumulation of lactate ions may be counteracted by carrier mediated efflux, which requires energy and results in a reduced growth rate.

Buchanan et al. (1993) found that the inactivation by both lactic acid/lactate and acetic acid/acetate was strongly correlated to the concentration of undissociated acids also at near neutral pH. In their experiment inactivation of *Listeria monocytogenes* (initially 8 log cfu/g) was observed in media with different combinations of pH (4, 5, 6 and 7) and lactic acid/lactate or acetic acid/acetate concentration (0.1, 0.5, 1.0 and 2.0 M). The effect on *Listeria monocytogenes* was expressed as time to a 4-D (99.99 %) inactivation. They found a linear relationship between log 4-D inactivation times and the square root of undissociated acid concentration. The authors stated, that the antilisterial activity of both acids remained attributable to the concentration of undissociated acid, and argued against alternative

hypotheses of separate inhibitory mechanisms at higher pH-values. According to their conclusion increases in antimicrobial activity can be expected if the pH is lowered even to a small extent, to become closer to the pK_a of the acid.

Results reported by Houtsma et al. (1996) illustrate the different modes of action of sodium lactate at various conditions, and the different effects on different bacteria. The authors analysed MIC values for sodium lactate at different pH levels (5.7 - 7.0) to Gram-positive and Gram-negative spoilage and pathogenic bacteria relevant in meat, and compared the results to the MIC values for NaCl at similar pH. The bacteria could be divided in two groups according to their response. For one group (*Carnobacterium piscicola, Listeria monocytogenes, Salmonella enteritidis* and *Pseudomonas fragii*) MIC values for sodium lactate were reduced as pH decreased, and the difference between the MIC values and those for NaCl increased. However calculation of undissociated acid concentrations revealed, that at low pH more undissociated acid was present at inhibition than at high pH values. The authors explained this phenomenon by a shift in the relative importance of inhibition due to undissociated acid and inhibition due to lowering of water activity. At high pH the MIC values for total acid approached the MIC values for NaCl indicating that lowering of water activity by high concentrations of lactate ions was the main effect. At inhibitive concentrations at low pH lactate ion concentration was much lower, the effect on water activity was reduced, and the main effect may be ascribed to the undissociated acid. For the second group of bacteria (*Staphylococcus aureus* and LAB other than *Carnobacterium* spp.) MIC values for sodium lactate were unaffected by pH. As these organisms were able to grow at rather high NaCl concentrations (unaffected by pH in the range of 5.7 - 7.0), the MIC values for sodium lactate were considerably lower than those for NaCl. The general lack of decrease of MIC values for lactate at decreasing pH was, by the authors, explained by the ability of these organisms to cope with undissociated lactic acid at low pH by maintaining a constant pH-gradient over the cell membrane instead of maintaining a high internal pH, as previously mentioned.

In her review Shelef (1994) noted that the antimicrobial effect of lactates, in addition to the possible effects described above (undissociated acid, water activity), may also include an effect due to its chelating properties. Shelef suggested that chelation of iron in meat may contribute to the antilisterial activity of lactate. Such properties may also explain the effect on TBA-values during storage of Catfish fillets reported by Williams et al. (1995). TBA values were significantly lower in fillets treated with 2 % sodium lactate compared to untreated fillets.

Thus the mechanisms of inhibition by lactate seem to be complex, and are still under investigation and debate. Factors governing the different responses among microorganisms to lactate are yet just beginning to be elucidated.
Briefly summarised the antimicrobial effect of lactic acid, lactates or their combinations for preservation purposes seem to be comprised of at least three effects: An effect of pH, a water activity lowering effect and an effect of the undissociated acid entering the cell, causing an increase in internal concentration of H_3O^+ and acid anions. The relative importance of these effects on a microorganism depend upon the combination of pH and added amount of acid and/or acid salt in the preserved food.

7.3 Lactic acid/lactate in meat and fish

Many papers have in the recent years been published on treatment of especially meat and meat products, but also fish by dipping in lactic acid or lactate solutions resulting in increased shelf lives or reduced growth/reduced contamination by pathogenic bacteria, for example Greer and Dilts (1995) (3 % lactic acid solution for pork), Williams et al. (1995) (≤ 2 % sodium lactate for catfish fillets), Papadopoulos et al. (1991) (≤ 4 % sodium lactate for cooked beef) and Palumbo and Williams (1994) (5 % lactic acid for frankfurters). Quantitative comparison to lightly salted lumpfish roe is however difficult in many of the reported experiments, as the treatments are most often specified in terms of spraying or dipping for a certain time in lactic acid/lactate solutions containing certain concentrations.

Several reports on the addition of lactic acid/lactate into the food in known concentrations, are however also available, especially with the aim of inhibiting *Listeria monocytogenes*. Shelef and Yang (1991) reported reduced growth of *Listeria monocytogenes* (inoculum 2 log cfu/g) in sterile comminuted beef at 5°C. When 4 % potassium lactate was added, the counts were 3 log cycles lower after one month. Pothuri et al. (1995) demonstrated a steady decline of *Listeria monocytogenes* (initially 4 log cfu/g) in heat sterilised vacuum packed Crawfish tail meat homogenate at 4°C in the presence of 2 % lactic acid. With 1.5 % counts increased less than 1 log cycle in 20 days. Growth of *Listeria monocytogenes* (initially 2.5 log cfu/g) was inhibited by 2 % sodium lactate in bologna-type sausages at 5°C for 28 days. The addition was reported to only slightly lower the sensory score compared to control sausages (Qvist et al. 1994). In comminuted raw vacuum packed salmon stored at 5°C, growth of *Listeria monocytogenes* (1 log cfu/g) was totally inhibited by the combination of 2 % (w/w) sodium lactate and 3 % WPS for 50 days. At 10°C growth was totally inhibited for 35 days by 3 % sodium lactate and 3 % WPS (Pelroy et al. 1994). Thus the effect is influenced by other hurdles of the particular product.

Inhibition of 178 Enterobacteriaceae (59 *Serratia liquefaciens*, 14 *Ser. marcescens*, 55 *Enterobacter aerogenes* and several other meat species) by lactic acid/lactates was examined by Gill and Newton (1982), who found that only around 20 % of the strains were unable to grow at pH 5.5 (lowered with lactic acid) at 2°C (aerobically), and the inhibited strains included only 3 of the 59 *Serratia liquefaciens*.
Östling and Lindgren (1993) found MIC values for lactic acid to be slightly lower at anaerobic than aerobic conditions, and MIC values (undissociated acid) were 2 to 5 times higher for 23 examined Enterobacteriaceae than for *Listeria monocytogenes*.
Shelef (1994) reviewed reports from the literature on inhibitive levels of lactate in meat products, and the levels reported to inhibit growth of the aerobic or anaerobic microflora, growth of *Listeria monocytogenes* or delay toxigenesis by *Clostridium botulinum* (proteolytic and non-proteolytic) were in the range 1.2 - 4 % (w/w).

The reports available from the literature represent a range of different concentrations of lactic acid or lactate, different pH values in the foods/media and different temperature conditions. The antimicrobial effect has been reported to be enhanced at decreasing temperatures (Shelef and Yang 1991, Grau 1981). However in a study of 30 strains of Gram-positive and Gram-negative environmental and type strains Houtsma et al. (1996) found the MIC values of sodium lactate in laboratory medium to be unaffected by temperature for most strains (4° to 37°C). When MIC values were affected, they were reduced at decreasing temperature. The nature of the food itself may also influence the effect of lactic acid/lactate as noted by Shelef and Yang (1991), who found *Listeria monocytogenes* to be more sensitive to lactate in beef than in chicken. Obviously in product formulations, where lowering of water activity is contributing to inhibition of the bacterial flora, the additional presence of NaCl will also influence the effect.

7.4 Preservation of lightly salted lumpfish roe using lactic acid/sodium lactate

7.4.1 Effect on spoilage and on growth of *Listeria monocytogenes*

In this project the effect of 2.8 % (w/w) sodium lactate on growth of *Serratia* roe isolates in roe extract, and the effect on microbial development during storage of lightly salted lumpfish roe has been determined.
In this connection it should be explained, that the aim was to examine the effect of 2 % (w/w) sodium lactate, that is mentioned as a general recommendation for meat products in the review by Shelef (1994). The product used in this project was Purasal®S/SP 60, a 60 % solution of sodium lactate. As the concentration was not further specified at delivery, the

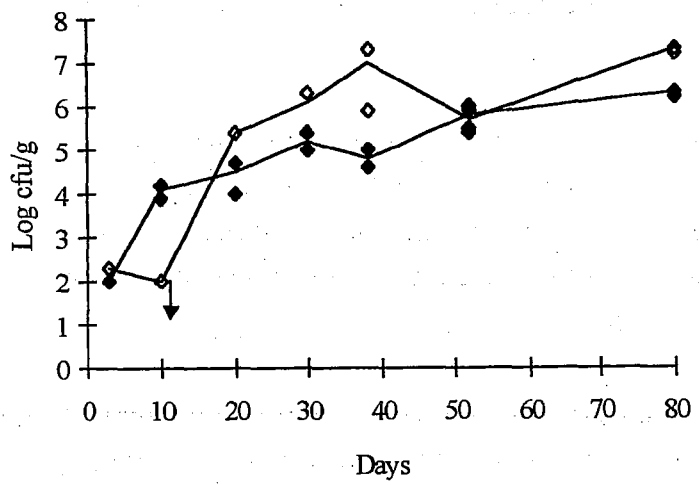
supplier was asked, and gave the erroneous answer that the concentration was given on volume basis. Later this turned out to be incorrect, and the concentration was now specified to be on weight basis (60 % w/w). This was experienced only after most of the experiments had been performed.
Consequently due to the high density of the sodium lactate solution, the resulting concentration of sodium lactate except for one experiment (note 7) was in fact 2.8 % (w/w) instead of the intended 2 % (w/w).

In roe extract with the standard pH 5.4 and 4 % NaCl, no growth occurred by three *Serratia liquefaciens* and two *Serratia plymuthica* in the presence of 2.8 % sodium lactate, when incubated at 5°C for 67 days (note 2). In a preliminary experiment, it had been noted, that 1.4 % (w/w) sodium lactate in roe extract was not sufficient to inhibit growth of all *Serratia* isolates originating from roe products (data not shown).

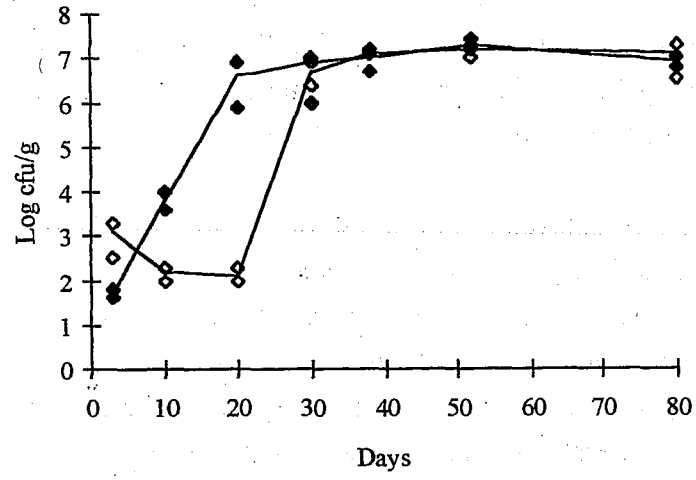
In lightly salted lumpfish roe produced from heavily salted roe by the procedure described in note 6 (pH 5.4, 4.0 % WPS) the addition of 2.8 % sodium lactate affected the development of the LAB and Enterobacteriaceae flora as illustrated in the upper parts of figure 7.1 and 7.2. As the spoilage organism *Morganella morganii* cannot always be expected to develop naturally (chapter 4), roe was inoculated with this bacterium (lower parts of figures 7.1 and 7.2). Roe inoculated with *Listeria monocytogenes* was also included in the experiment. As standard lightly salted lumpfish roe with a pH of 5.4 have already been added 0.1 % lactic acid, the total concentration of lactic acid/lactate in the roe was 2.9 % (w/w).

It is seen from figure 7.1, that lactate addition delayed the initiation of growth of LAB, but did not affect the maximum counts obtained. It has not been examined, whether a shift in LAB flora occurred due to selection of different strains in roe with lactate addition. Growth of Enterobacteriaceae was inhibited by the lactate addition, both naturally occurring bacteria and the inoculated *Morganella morganii* (figure 7.2). Only in a few roe jars did their counts reach 3 - 4 log cfu/g. The better growth of Enterobacteriaceae observed in roe with *Morganella morganii* (figure 7.2) seemed to enhance growth of LAB (figure 7.1), as earlier observed (section 6.5.2), but again considerable variation in Enterobacteriaceae counts among roe jars was noted. The enhanced growth of LAB is however the most typical growth curve of the two in lightly salted lumpfish roe (reaching counts of 7 log cfu/g in three weeks and remaining at this level, see chapter 4).
In roe inoculated with *Listeria monocytogenes* no growth occurred in the presence of sodium lactate (< 1 log cfu/g) during 80 days at 5°C. Counts of *Listeria monocytogenes* in roe without lactate reached a maximum of 4 log cfu/g after three weeks (table 4 of note 6), which is rather poor growth compared to previously obtained results (section 5.2.2). Thus both LAB and *Listeria monocytogenes* grew worse in this experiment, than earlier observed.

One possible explanation for this may lie in the available nutrients, since the concentrations of soluble compounds in different roe batches vary considerably (chapter 3).



Naturally contaminated roe product



Roe product with *Morganella morganii*

Figure 7.1. Growth of LAB at 5°C in lightly salted lumpfish roe (vacuum packed, pH 5.4 - 5.6, 4 % WPS). Closed symbols: No lactate addition. Open symbols: 2.8 % (w/w) sodium lactate. ↓: ≤ 2 log cfu/g. (Data from table 3 of note 6, roe batch 1, 4, 7 and 8).

In order to further confirm the suitability of sodium lactate addition as preservation method for lightly salted lumpfish roe, a storage experiment of four months duration, which included odour assessments, was performed. The experiment covered combinations of 2.8 % lactate/no lactate with normal lightly salted roe, roe inoculated with *Listeria monocytogenes* and roe inoculated with *Morganella morganii.*

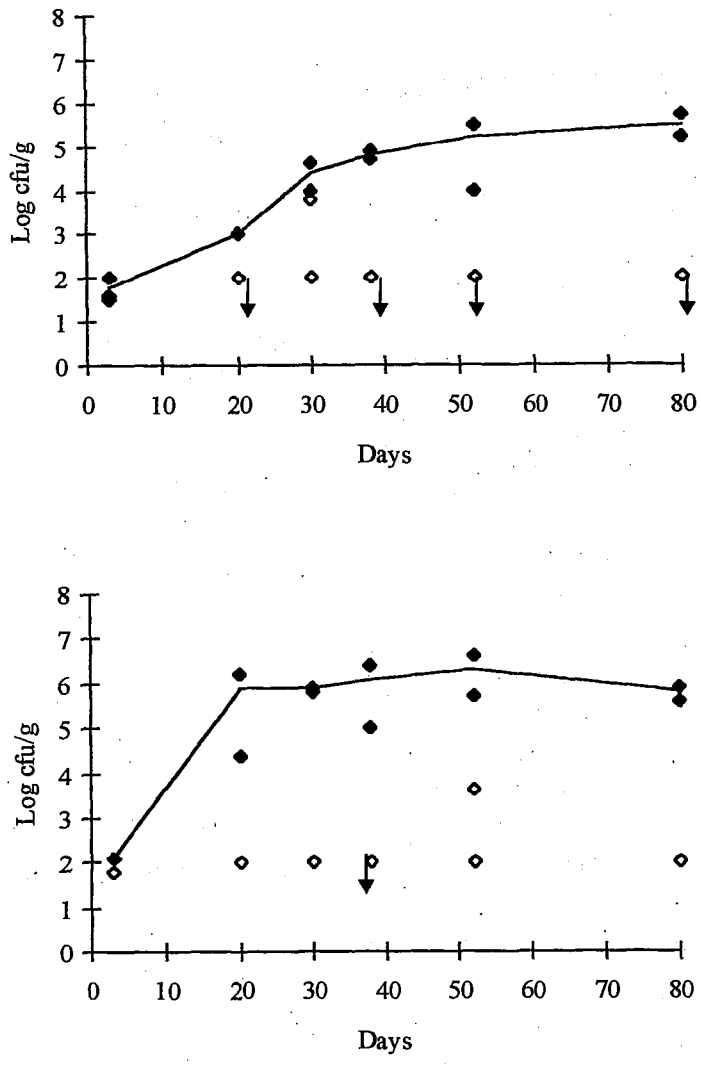


Figure 7.2. Growth of Enterobacteriaceae at 5°C in lightly salted lumpfish roe (vacuum packed, pH 5.4 - 5.6, 4 % WPS). Closed symbols: No lactate addition. Open symbols: 2.8 % sodium lactate. ↓: < 2 log cfu/g. (Data from table 5 of note 6, roe batch 1, 4, 7 and 8).

Apart from examining the effect on spoilage and growth of *Listeria monocytogenes* by sodium lactate, the experiment also had the purpose of examining the development of microflora and off-odours during storage of normal roe with and without the presence of *Morganella morganii,* and if possible further confirm the conclusions reached in chapter 4. Figure 7.3 illustrate the development of microflora and off-odours in unpreserved roe with and without the addition of *Morganella morganii.* The results confirm the conclusion reached in chapter 4, that LAB and Enterobacteriaceae (as determined on selective agars) are

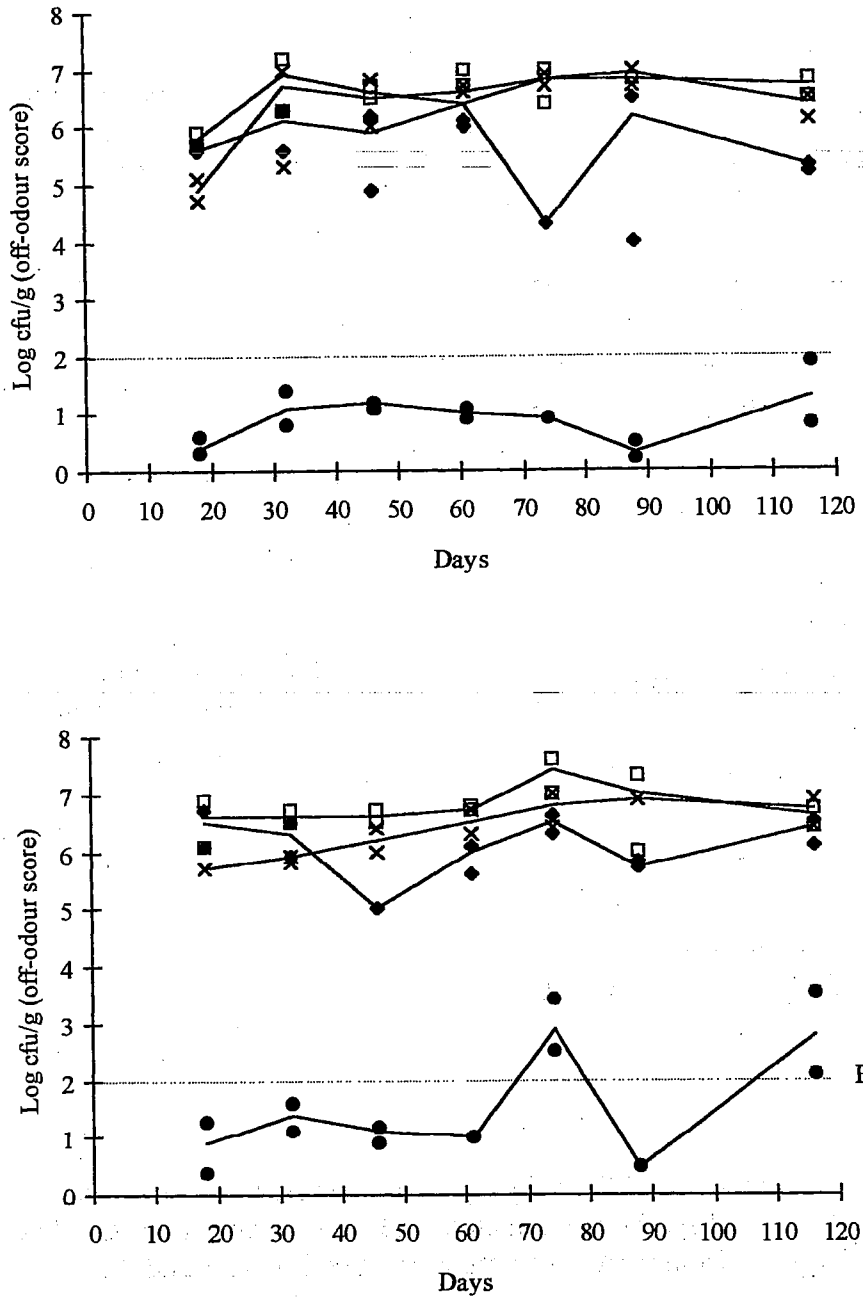


Figure 7.3. Development of microflora and off-odours in lightly salted lumpfish roe (pH 5.4, 4 % WPS, vacuum packed) during storage at 5°C. □ Total viable counts (10°C). × LAB. ◆ Enterobacteriaceae. ● Off-odour score (0 = no off-odour, 1 = weak off-odour, 2 = borderline, 3 = significant off-odour, 4 = strong off-odour) (data from table 7 - 9 and 11 of note 6, roe batch 7 and 8).

the major groups of bacteria comprising the microflora. In both roe batches presented in figure 7.3, LAB counts approached 6 log cfu/g in one month and remained at a level of 6 - 7 log cfu/g throughout storage. Enterobacteriaceae counts of naturally contaminated roe had reached 6 log cfu/g in 3 - 4 weeks. Lower counts were observed after 74 days, after which counts returned to a level of 5 - 6 log cfu/g. This may indicate a shift in Enterobacteriaceae population, but on the other hand considerable variation occurred among double determinations (for example after 45 and 88 days). The roe was not rejected due to off-odours, and only approached borderline after 116 days (figure 7.3). Among 7 isolates picked up from the RVG-G agar plates at the end of storage, one belonged to Enterobacteriaceae (possibly *Serratia* sp., table 13 of note 6). The remaining 6 isolates were oxidase-positive, as also reported in previous experiments (see table 1 of paper 2, non-spoiled or borderline batches 1, 3 and 5). Thus in this particular roe batch, with its inherent combination of available nutrients and initial microflora, preservation was in fact not needed to prevent spoilage in a shelf-life period of three months.

In roe with *Morganella morganii* fluctuations of Enterobacteriaceae counts were also observed, but less variation occurred among double determinations (figure 7.3). After the decrease of counts after 46 days, off-odour development seemed to follow the development of Enterobacteriaceae counts. Slightly higher LAB counts, however, also occurred in one of the jars after 74 days. Both roe jars examined after 74 days were rejected due to off-odours (score 2.5 faecal, sulphidy, sour - score 3.4 faecal, putrid, sulphidy, sour) and after 116 days (score 2.1 faecal, sour - score 3.5 faecal, cabbage-like, sour, old cheese) (table 11 of note 6). The Enterobacteriaceae counts were then just above 6 log cfu/g. After 88 days two non-spoiled jars were examined with counts just below 6 log cfu/g). Among 12 isolates picked up from the RVG-G agar plates at the end of storage, 6 belonged to Enterobacteriaceae and 5 of those gave biochemical reactions similar to those of *Morganella morganii* inoculated into the roe (table 13 of note 6). The other half of the isolates were oxidase-positive as in roe without *Morganella morganii*. Thus *Morganella morganii* were present at a level of 6 log cfu/g in the spoiled roe, and is the likely cause of the different off-odour development compared to non-inoculated roe. In non-inoculated roe with 2.9 % lactic acid/sodium lactate, total viable counts remained in general below 5 log cfu/g during storage, except for 5.6 log cfu/g obtained in one roe jar after 74 of storage (figure 7.4). Maximum LAB counts in the presence of lactate were lower in this roe batch compared to that shown in figure 7.1 A, which may be explained by differences in LAB floras among batches. Enterobacteriaceae counts remained below 3.2 log cfu/g for 116 days at 5°C, and the roe was still non-spoiled at the end of storage.

Results were similar for roe with lactate and *Morganella morganii*. Total viable counts did not exceed 5 log cfu/g and Enterobacteriaceae counts did not exceed 3.5 log cfu/g. This roe also remained unspoiled with an off-odour score below 1 at the end of storage (table 7 - 9 and 11 of note 6, roe batch 5).

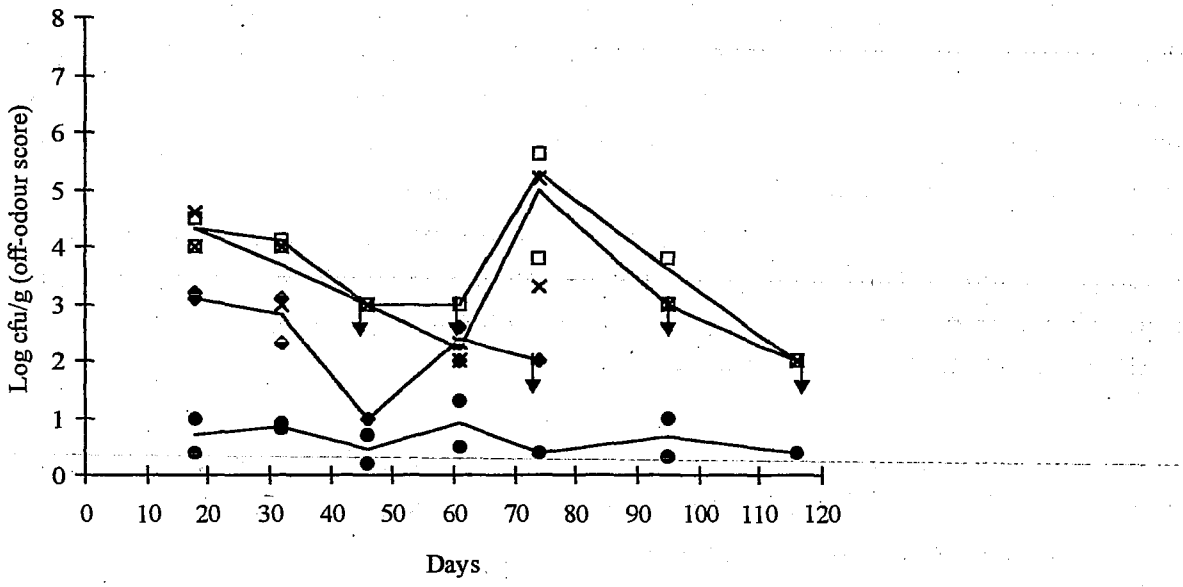


Figure 7.4. Development of microflora and off-odours in lightly salted lumpfish roe with 2.9 % lactic acid/lactate (pH 5.5, 4 % WPS, vacuum packed) during storage at 5°C.
□ Total viable counts (10°C). × LAB. ◆ Enterobacteriaceae (counts remained below 3 log cfu/g from 74 to 116 days of storage). ● Off-odour score. ↓ : Below detection limit. (Data from table 7 - 9 and 11 of note 6, roe batch 4).

In roe without sodium lactate *Listeria monocytogenes* reached a maximum level of 6 log cfu/g, thus higher than in the previous experiment. In roe with sodium lactate no growth occurred (< 1 log cfu/g) in 116 days, and no *Listeria* were detected in non-inoculated roe (table 10 of note 6, roe batches 3, 6 and 7). Lactic acid (undissociated) have previously been reported to be two to five times more efficient in inhibiting *Listeria monocytogenes* than Enterobacteriaceae (Östling and Lindgren 1993).

As the concentration of undissociated acid in the preserved roe unknown, the magnitude of the influence by this parameter on the observed effect on the microflora is also unknown. When attempting to adjust pH of the roe to around 5.4 by addition of lactic acid during this project, a natural buffering could be observed in the manner that the pH increased when the roe were left to stand after pH adjustment (5.4). The amount of acid needed to obtain a stable pH of around 5.4 was 0.1 % (w/w), an amount which has been used as a standard addition

throughout the project. Half an hour of standing/mixing time before pH measurement has also been used as standard procedure.
When titrating the roe with lactic acid solution, a steady slow decrease of pH was observed down to pH 4.5 and even slower when approaching pH 4 (data not shown). No jumps of the curve could be observed, and many different molecular groups are likely to participate in the buffering effect.
The addition of 0.1 % lactic acid (i.e. 0.011 moles/kg or around 0.015 M in the water phase) resulted as described in a pH decrease of half a pH unit (pH 5.9 to 5.4). At pH 5.4 the H_3O^+ ion concentration is 4×10^{-6} M. The buffering effect can be illustrated by comparing this to the effect on pH of similar amounts of lactic acid in pure aqueous solution. A 0.015 M solution of lactic acid in water would have a pH of 2.8 (pH = ½ (pK_a - log total acid), Dillard and Goldberg (1971), pK_a for lactic acid 3.86, (CRC 1988)). The corresponding H_3O^+ ion concentration would then be 1.6×10^{-3} M. Thus the vast majority (around 998/1000) of H_3O^+-ions formed during lactic acid addition were consumed by the corresponding dry matter of the roe, and removed from the water phase (H_3O^+ ion concentration at the initial pH 5.9 is neglected in the calculation).
The subsequent addition of 2.8 % (w/w) sodium lactate only caused a slight change of pH, and the resulting preserved product contained 2.9 % (w/w) lactic acid/lactate and had a pH of 5.5 (table 12 of note 6).
Due to the influence of the natural buffering on the equilibrium between lactic acid and lactate-ions, the relation between pH and concentration of dissociated and undissociated acid is disturbed, and the equation mentioned in section 7.2 is no longer valid. Therefore attempts have not been made to calculate the concentration of undissociated acid in the preserved roe.

The experiments reported in this section showed, that 2.8 % sodium lactate is in lightly salted lumpfish roe, able to keep Enterobacteriaceae (or rather Gram-negative) counts at ≤ 3 log cfu/g, and prevent spoilage odours from developing for more than three months. Some LAB seemed to be inhibited as well, while others reach maximum counts similar to those obtained without lactate, but at a later stage. An indirect effect on the LAB flora due to the lack of Enterobacteriaceae growth, may contribute. Addition of 2.8 % sodium lactate is also able to totally prevent growth of *Listeria monocytogenes* present in low numbers.

7.4.2 Effect on sensory quality

Sodium lactate Purasal®S/SP 60 is described by the purchaser as having a mild saline taste, while bitterness may accompany the use of potassium lactate. As the roe produced from heavily salted roe has elements of both slight bitterness, rancidity and obviously salt taste, the choice of sodium or potassium salt of lactic acid may not be crucial. However due to the

known bitterness of potassium lactate, sodium lactate was chosen for the preservation of lightly salted lumpfish roe.
The sensory characteristics of potassium lactate in combination with sodium chloride was examined in a gelatine based model system by Brewer et al. (1995). They found that as potassium lactate concentration increased (0 - 3 %), saltiness and bitterness increased. Bitterness was masked by 2 % NaCl, while both lower and higher NaCl concentrations were less effective in masking bitterness of potassium lactate. Kim and Brewer (1996) studied sensory characteristics of sodium lactate in a similar model system, and found increasing saltiness and increasing soapiness at increasing concentrations (0 - 3 %). Bitterness was also recognised at higher concentrations, while reduced bitterness was observed at 1 % compared to 0 %. Combinations with NaCl were not included in that experiment.

The effect on sensory characteristics of lightly salted lumpfish roe was determined by the internal taste panel at ABBA Seafood, Thisted, DK, consisting of 9 persons occupied at the production company. Roe with 2 % (w/w) sodium lactate was compared to roe without lactate in triangle tests, i.e. three samples were served at a time, and the judges asked to point out the one sample different from the other two (either 0 or 2 % lactate). Samples with and without lactate were served in different combinations and order, and a total of six answers were obtained from each judge, yielding 54 answers in total. Among those, 17 gave correct identification of the differing sample among three (table 1 of note 7). A score of 17 correct answers out of a total of 54 is sufficiently low to conclude, that the samples with and without 2 % sodium lactate were identical regarding their taste. The critical number (maximum) of correct answers, at which the conclusion "identical" should still be accepted is 18 of 54 (confidence levels 0.01 - 0.1 %) (Meilgaard et al. 1991).
Therefore it can be concluded that negative influence on the taste of lightly salted lumpfish roe is no obstacle in using 2 % sodium lactate for preservation purposes.

7.5 Perspectives in preservation of lightly salted lumpfish roe by lactic acid/sodium lactate

In order to avoid "overpreservation" of lightly salted lumpfish roe, it should be examined whether lower concentrations than 2.8 % sodium lactate would be sufficient to ensure a shelf life period of three months, in which neither spoilage nor growth of *Listeria monocytogenes* occurs. The effect of 2 % sodium lactate, which have been shown not to influence the taste of the product, could be examined. If shown to be efficient after the above criteria, the application can proceed without additional obstacles. The efficiency should be tested in several different roe batches originating from different barrels of heavily salted roe. This is due to the variation among roe batches revealed during this project in terms of both chemical

composition (chapter 3) and microflora (chapter 4) governing large variations in off-odour development (chapter 4) within the defined shelf life period.

It can, however, be recommended to consider whether alternative combinations of pH, salt concentration and sodium lactate concentration would be preferred in terms of sensory quality. It may be possible to adjust the three factors in a manner that maintains the antimicrobial effect demonstrated in this chapter (pH 5.4 by 0.1 % lactic acid, 2.8 % sodium lactate and 4 % WPS), and at the same time improve the sensory quality of the product.
In the future process of choosing the optimal combination of factors, it should also be ensured, that safety is not jeopardised, regarding other hazard than *Listeria monocytogenes* (see chapter 5).

It is a wish in the industry to reduce the saltiness of the products, which may for example be achieved by maintaining a high lactate concentration, and reduce the salt concentration instead. Still in terms of weight, more sodium lactate than NaCl is needed to lower the water activity due to its higher molecular weight. Lowering the salt concentration also means lowering the concentration of other soluble compounds, which may influence growth of the microflora. Yet it may also negatively influence the taste of the product, which should be considered.
Lowering the pH is another possibility, which have been demonstrated to affect growth of the *Serratia* spp. (figure 4.1). In work earlier performed in this laboratory a pH below 5.1 was however experienced to negatively influence the texture of the roe in terms of softness and stickiness (Huss et al. 1984).
The internal taste panel at ABBA Seafood AB, Kungshamn (9 persons) was asked to evaluate the effect on appearance, taste and texture of pH levels in the roe of 5.2, 5.0 and 4.8 (lowered with lactic acid) compared to the standard 5.4. The results showed, that the roe with pH 4.8 had an acid, acrid taste and a more watery, softer, less crispy consistency. Some judges however found the taste to be milder. The roe was rejected by 33 % of the judges (table 2 of note 7). The colour of the roe may be affected by pH/lactic acid addition, but the detected differences were very slight, and the judges disagreed on whether roe with low pH was stronger or more lightly coloured. The majority of judges detected no consistency differences in roe with pH 5.0 and 5.2 compared to the normal roe with pH 5.4.
Thus the major effect causing rejection by some judges was the acid, acrid taste, increasing with increasing acid addition, and at the lowest pH value (4.8) also an effect on consistency. Based on these indications, it may not be appropriate to lower the pH further than 5.0. Decreasing pH to this level could allow a reduction of sodium lactate (and/or NaCl) concentration.

Chapter 8. Conclusions and future work

Lightly salted lumpfish roe, which is produced by desalting of stored heavily salted roe, spoils due to bacterial activity. Autolytical and chemical changes however also occur influencing the overall quality of the products.
Changes taking place during storage of heavily salted roe for up to a year prior to production, include lipid hydrolysis and oxidation as measured by increased levels of free fatty acids, peroxide values and TBA-values. Rancid odours were detected in newly produced products, but did not develop further during vacuum packed storage. Thus in this regard the damage is already done before the products are produced. No solutions are readily available to completely avoid the development of rancidity. Alternative means of processing are difficult to find, as such means should be applicable to a large number of local fishermen working at distant geographic locations at sometimes primitive conditions. However it is recommended to give increased priority to limitation of oxygen availability, and search for suitable antioxidants may also be worthwhile.
During the storage amino acids are to a varying extent liberated from proteins/peptides, and then reduced somewhat during desalting of the roe. The combined effect is an increased concentration and a changed composition of free amino acids in most roe batches, and a pronounced variation among desalted roes. The direct influence on sensory quality of these changes has not been examined, but may include both positive and negative (bitter) influences on the taste.

During storage at 5°C a microflora developed, that with few exceptions only slightly exceeded 7 log cfu/g, and remained at a practically unchanged level throughout storage. The microflora was dominated by LAB or LAB and Enterobacteriaceae, with less numerous *Vibrio* spp. sometimes occurring. Spoilage in terms of off-odour development varied greatly among batches of lightly salted lumpfish roe. Some of this variation may be explained by the presence or absence of bacteria with high spoilage potential i.e. Enterobacteriaceae, which as one factor seem to be influenced by variations in salt concentration. Counts of Enterobacteriaceae is however too unspecific a measure to provide a good spoilage indicator for lightly salted lumpfish roe, even if a more accurate method for its determination were available. Spoilage potential among Enterobacteriaceae vary, and strains of *Morganella morganii* were the only roe isolates identified as spoilage organisms, when both the characteristics of produced off-odours, chemical endproducts (volatile sulphur compounds) and their counts at spoilage are considered.
An understanding of the spoilage mechanism(s) of lightly salted lumpfish roe still requires a considerable amount of research. Future work should continue at aiming to identify the volatile compounds responsible for off-odours detected at spoilage, the producing organisms and the precursors for their production. In investigations of the probably complex

mechanisms governing the eventual profile of volatile compounds causing spoilage of the roe, it can be suggested to focus on the significance of variation in available substrates. Variation in concentration of amino acids have been demonstrated, but all soluble compounds probably vary due to the desalting process. Both the influence of availability of specific precursors for production of volatile compounds, and the impact on the composition and metabolism of the microflora should be considered. Thus knowledge on the range and order of substrates utilised by different parts of the microflora and the significance of competition for nutrients in the development of spoilage is desirable. In future investigations of spoilage reactions of desalted lumpfish roe it is necessary to eliminate from the experiments the uncontrolled influence of the variation in available substrates among roe batches experienced in this project, and the development of a suitable sterile model substrate is crucial in this regard.

In preservation of lightly salted lumpfish roe the targets of inhibition are Enterobacteriaceae and *Listeria monocytogenes.* The latter has been concluded to be the only microbially derived hazard, that pose a risk, if the products are not immoderately temperature abused. Different approaches in biopreservation using LAB cultures have been tested without success. An antagonistic principle which combines an efficient inhibition of Enterobacteriaceae with absence of negative influence on sensory quality, have not been found. Antilisterial LAB however occur in the products, and also the use of biopreservation in products with higher salt concentrations might be possible. It could be interesting to reveal the nature of the *Vibrio* inhibition noted among roe LAB, and examine the potential for use in other fish products, where inhibition of vibrios is needed.
It is instead suggested to use sodium lactate for preservation of lightly salted lumpfish roe. The additive is produced by fermentation of LAB and still holds some elements of "natural preservation". Efficient inhibition of Enterobacteriaceae and *Listeria monocytogenes* has been obtained using 2.8 % (w/w) sodium lactate. Lower concentrations may be sufficient, and 2 % has been shown not to influence the taste of the products. It is recommended to investigate whether alternative combinations of salt, pH and sodium lactate concentration are preferred in terms of sensory quality.

As a final summarising remark, it can be stated that deterioration of lightly salted lumpfish roe, viewed over its entire line of existence, i.e. from the fresh roe till the eventually spoiled product, is influenced by numerous factors. Quality deterioration is governed by the combined effects of autolytical (enzymatic), chemical and microbiological changes, some of which affect the sensory quality directly, others indirectly. Some quality changes is absolutely critical, while others may seem of minor importance. On the bottom line, the decision is made by the consumers, as to which sensory quality is appreciated at a certain price. The latter aspect has not been addressed in the present project, at it is left for future investigations.

Paper 1. Chemical composition of fresh and salted lumpfish (*Cyclopterus lumpus*) roe

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Abstract

Fresh, stored heavily salted and lightly salted lumpfish roe was examined. These represent different steps in standard processing procedures.
Proximate composition was not influenced by processing. Fresh and lightly salted roe had similar compositions of fatty acids and total amino acids. The following differences were noted: Lightly salted roe had increased levels of free fatty acids, peroxide values, thiobarbituric acid values and free amino acids. Composition of free amino acids also differed. The results indicated that lipid hydrolysis, lipid oxidation, and liberation of amino acids occurred during storage of heavily salted roe prior to desalting. Trimethylamine and trimethylamineoxide concentrations were low or undetectable in all roes.

Keywords: Lumpfish roe, proximate composition, fatty acid composition, amino acid composition.

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Introduction

Roe from many fish species is utilised for food, either as whole roe sacs or as loose eggs, processed and preserved by different means of technology including salting, smoking, chilling, freezing, drying, canning or combinations of these (Zaitsev et al. 1969).
In Scandinavia salted lumpfish roe products (lumpfish caviar) have been produced for many years. These include both raw and pasteurised products, with various combinations of salt concentration, preservatives, colouring and flavour components.
The roe is removed from the fish right after catch, screened into loose eggs by passing it through a screen, and dry-salted in 105 kg plastic barrels to a salt concentration of 11 - 16 % (w/w) (heavily salted roe), and if necessary topped up with saturated brine. The roe is stored in the barrels at the production factory at ÷5°C until needed for production, normally not more than a year. During processing the roe is desalted to the salt concentration wanted in the final product (3.3 - 7.0 % w/w). This is done by addition of water and subsequent drainage (lightly salted roe). The relevant preservatives, colouring, flavouring and stabilisers are added, and the roe is packed under 65 - 75 % vacuum in glass jars (lumpfish caviar). Some products are then pasteurised while in the jars, by the use of hot water. The processing steps are shown in figure 1.

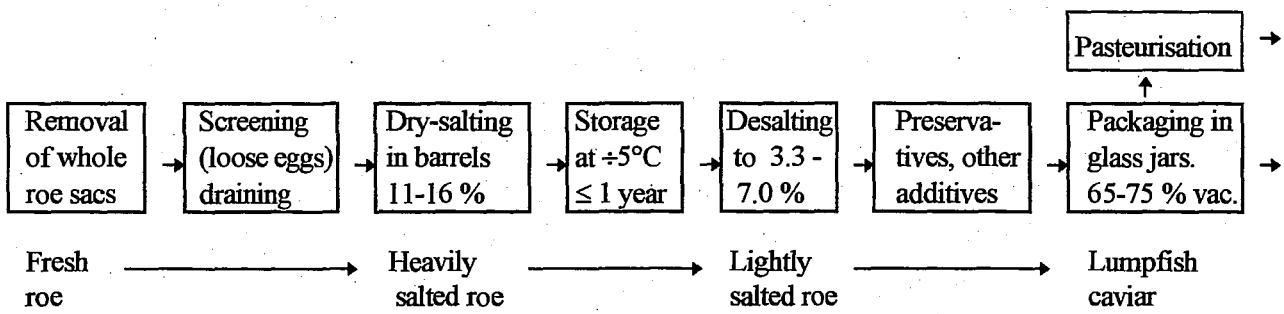

Figure 1. Processing of lumpfish roe into salted, preserved roe products (lumpfish caviar).

A number of papers have been published on proximate composition of roe from different fish species. The data shows, that pronounced variation occurs among species, regarding both moisture content and the proportion of protein and lipids in the dry matter (table 1).
Because of the unsaturated nature of marine lipids and the relatively high lipid content of roe of several fish species, a number of studies have been carried out with special emphasis on changes in the lipid fraction during storage at different conditions. It has been established that development of rancidity is an important factor in quality deterioration during storage of

Table 1. Proximate composition of fresh roe from different fish species utilised for human consumption.

Fish species	Moisture % (w/w)	Crude protein % (w/w)	Lipid % (w/w)	Reference
Sturgeons (different species)	51.5-55	26-28	14-16	Zaitsev et al. 1969
Whitefish (*Coregonus albula*)	69.3	18.7	9.9	Vuorela et al. 1979
Baltic herring (*Clupea harengus*)	78.4	17.8	2.6	Vuorela et al. 1979
Rainbow trout (*Salmo gairdneri*)	63.1	26.6	7.6	Vuorela et al. 1979
Chum salmon (*Oncorhynchus keta*)	57.6	27.0	14.1	Iwasaki and Harada 1985

fish roe (Linko et al. 1979, Kaitaranta 1981 and 1982, Linko et al. 1983, Shaban et al. 1984, Huynh 1985).

The aim of the present study was to obtain basic information on chemical characteristics of lumpfish roe used as the raw material for production of lumpfish caviar. Proximate compositions have been determined for fresh roe, lightly salted roe and heavily salted roe of various geographic locations and storage times. Examination of fatty acid and amino acid composition and measures of lipid oxidation have been carried out on fresh roe and roe after storage and desalting. The data are intended to provide basic information for the industry and for further studies on quality and deteroration during chill storage of a lightly salted lumpfish roe product, produced by the means of processing shown in figure 1.

Materials and methods

Roe samples

Two 5 kg portions of fresh roe were delivered frozen to the laboratory (ABBA Seafood, Canada). The roe had been screened into loose eggs and drained by the normal procedure at two catching locations in Canada. In addition fresh roe was removed from five lumpfish, landed in Copenhagen, Denmark, and killed imidiately before sampling. The roes were removed from the fish at the laboratory, and screened into loose eggs. From each fish 0.8 - 1 kg was obtained.

Samples (5 kg) of heavily salted roe (see figure 1) were taken from 105 kg barrels, making sure that roe from different levels of the barrels was represented (ABBA Seafood A/S, Denmark). The barrels included three barrels stored at ÷5°C for 2 months and three barrels stored for one year.
Samples (5 kg) of lightly salted roe (see figure 1) were taken after desalting by the normal industrial procedure (ABBA Seafood A/S, Denmark). Desalted barrels included three barrels stored at ÷5°C for one year and ten barrels stored for 8 months. Heavily and lightly salted roe originated from different catching seasons and locations in Canada.
All samples were vacuum packed in portions of 200 g and frozen at ÷ 40°C as recommended by Shaban et al. (1984). Double sampling was performed for all analysis except amino acid analysis, where a large sample (app. 200 g) was homogenised, before material was taken for the analysis.

Analytical methods

Dry matter/moisture content was determined by weighing before and after drying at 105°C for 20-24 hours.
Ash content was determined by weighing after ashing of the dried samples for 20 - 24 hours at 600°C.
Salt concentration was calculated from the amount of chloride as determined in duplicate by boiling in nitric acid with excess of $AgNO_3$ followed by titration with 0,1 M KSCN solution (AOAC 1995, no. 937.09).
pH was measured using a PHM 82 (Radiometer, Copenhagen, Denmark).
Total volatile bases (TVB), trimethylamineoxide (TMAO) and trimethylamine (TMA) were determined by a modified Conway micro-diffusion method (Conway and Byrne 1933).
Crude protein (N x 6.25) was determined by the Kjeldahl method following the principles described by AOAC (1995) no. 981.10, using Kjeltabs (K_2SO_4, $CuSO_4$, TiO_2) in the destruction step.
Lipids were determined by extraction using the method of Bligh and Dyer (1959). Extracts also prepared by this method, were covered with nitrogen and frozen at ÷80°C, until used for the analysis of free fatty acids, peroxide value and fatty acid composition.
Free fatty acids (FFA) were determined on the lipid extract as concentration of oleic acid (% (w/w) of lipid) after titration according to the method no. 41.1.21 of AOAC (1995).
Peroxide value (PV): CO_2-gas was bubbled through the lipid extract for 5 min., and saturated potassium iodide and glacial acetic acid were added. The peroxide value was determined (meq./kg lipid) by titration according to the method no. 41.1.16 of AOAC (1995).
Fatty acid composition of extracted lipids was determined following the principles described in method no. Ce 1b-89 of AOCS (1993), with a $C_{17:0}$ internal standard. Column and chromatographic conditions were as described by Staby et al. (1993) (method 6).

Thiobarbituric acid (TBA) *value*: A trichloroacetic acid extract of roe was prepared, and heated with TBA solution as described by Wyncke (1975). The absorbance at 530 nm was measured and the TBA value (µmoles malonealdehyde/kg roe) calculated on the basis of a standard curve.

For *amino acid* analysis the roe was comminuted in a stainless steel blender after addition of liquid nitrogen, and freeze dried. For determination of total amino acids, 20 mg of this roe powder was hydrolyzed after addition of 6 N hydrochloric acid, evacuation, and heating at 110°C for 24 hours. The liquid was removed by evaporation under vacuum. The hydrolysate was dissolved in 1 ml A-buffer (Barkholt and Jensen 1989), diluted x 100 with A-buffer and used for analysis. For determination of free amino acids 150 mg of roe powder was added 10 ml 0.1 M hydrochloric acid. Of this suspension 0.5 ml was filtered, diluted x 10 with A-buffer and used for analysis. During analysis the column, instrumentation and chromatographic system were as described by Barkholt and Jensen (1989). Injection volumes were 160µl and 20 µl for total and free amino acids respectively.

Results and discussion

Proximate composition

Proximate composition of fresh, heavily salted and lightly salted roe is shown in table 2. The moisture content of fresh lumpfish roe was rather high (77.4 - 81.0 %) compared to the roe of several other fish species. The moisture content was at the same level as in the roe of Baltic herring, but the lumpfish roe contained more lipid and less protein than Baltic herring roe (see table 1). The dry matter was apart from ash (salt), composed of crude protein and lipid in the proportion 3:1. Variations were, however, observed in lipid and crude protein among roes of individual lumpfish (Danish roe shown in table 2). Vourela et al. (1979) examined the proximate composition of roe from 10 rainbow trout (*Salmo gairdneri*) landed during a four months period, and reported a pronounced variation among individual fish, including fish landed on the same day. Variation was observed in both crude protein (26.1 - 32.6 %), lipid (7.1 - 9.7 %) and moisture (57.8 - 63.7 %) content.

The heavily salted roe originated from two catching seasons, and had been stored in the barrels in the form of heavily salted roe for either a short (two months) or long (one year) period of time. Normal raw material for industrial production falls within this range of storage times. The roe stored for one year was desalted (4.8 ± 0.3 % salt) and analysed again. The results in table 2 show that apart from a slight increase in moisture content during desalting, the proximate composition (extra salt withdrawn) of heavily and lightly salted roe was rather similar to that of fresh roe. Rehbein (1985) also reported that differences in proximate composition between fresh roe of sturgeon and the corresponding caviar products were very slight. The variations in proximate composition resulted in both higher (16.2 %)

and lower (14.8 %) content of crude protein than observed for the fresh roe, while both similar (5.7 %) and lower (5.1%) lipid contents occurred. Kaitaranta (1980) also reported that no systematic influence by the year of catch occurred in the lipid content of whitefish (*Coregonus albula*) roe (9.1 - 11.4 %).

Table 2. Proximate composition (mean ± standard deviation) of fresh roe of different catching locations, stored heavily salted roe of different catching seasons/storage times, and lightly salted lumpfish roe.

Roe	Salt % w/w	Moisture % w/w	Lipid % w/w	Protein [1] % w/w	Ash % w/w
Fresh roe					
Denmark [2]	0.9±0.05	81.0±1.3	5.5±0.4	15.4±2.3	1.0±0.04
Canada [3]	1.0±0.07	77.4±0.1	6.1±0.4	15.5±0.4	1.1±0.00
Heavily salted roe					
Actual season [4]	12.9±0.06	67.5±0.1 (76.7) [5]	4.5±0.5 (5.1)	13.0±0.5 (14.8)	13.8±0.5
Previous season [6]	11.4±0.4	68.4±0.2 (76.5)	5.1±0.1 (5.7)	14.5±0.5 (16.2)	12.3±0.5
Lightly salted roe					
Previous season [7]	4.8±0.3	77.8±1.2 (80.9)	5.0±0.2 (5.2)	13.3±0.2 (13.8)	4.6±0.1

1) Crude protein, N x 6.25. 2) Roe of five lumpfish analysed separately in duplicate. 3) Screened roe from two geographic locations analysed separately in duplicate. 4) Stored for two months at ÷5°C, three barrels (105 kg) were analysed in duplicate. 5) Mean values shown in parentheses are calculated as concentration in roe with 0.9 % salt. 6) Stored for one year at ÷5°C, three barrels (105 kg) were analysed in duplicate. 7) Made by desalting of three barrels stored for one year, the barrels were analysed in duplicate.

Total volatile bases (TVB), trimethylamine (TMA) and trimethylamineoxide (TMAO)

The TVB concentration of both heavily salted roe in barrels and fresh roe was in the range of 24 - 33 mgN/100 g. In ten lightly salted roes (3.0 - 5.8 % salt) TVB were after desalting lowered to 4 - 12 mgN/100 g, generally being lowest in roe with the lowest salt concentrations (data not shown). TMAO and TMA concentrations were low in all roes, ≤ 4.4 mgN/100 g and ≤ 1.3 mgN/100 g respectively. In several roes the compounds were not detectable (data not shown). TMAO can be converted by fish spoilage bacteria to the malodorous compound TMA, and is therefore an important factor in fish spoilage (Gram et al. 1987). Equally low levels of TMAO as detected in this study, were reported in herring roe by Huss and Larsen (1979) and Linko et al. (1979). In the same studies a TVB level of 15 mgN/100 g and 10 - 20 mgN/100 g respectively was reported for herring roe.

Fatty acid composition

Fatty acid composition of fresh roe of five lumpfish was determined, and 27 fatty acids were identified (table 3). The portion of around 5 % unidentified acids only contained acids present at concentrations ≤ 1 % of total fatty acids. The lipid was dominated by four fatty acids: Palmitic (16:0), oleic (18:1(n-9)), eicosapentaenoic (20:5(n-3)) and docosahexaenoic acid (22:6(n-3)) comprised 73.5 %, and the latter alone comprised 25.8 % of total fatty acids.

Table 3. Fatty acid composition (% w/w of total fatty acids) of fresh and lightly salted lumpfish roe (made by desalting of heavily salted roe stored for 8 months at ÷5°C). Salt concentration is shown in table 4.

Fatty acids	**Fresh roe** [1]		**Lightly salted roe** [2]	
	Mean ± SD	Range	Mean ± SD	Range
14:0	1.3 ± 0.16	1.1 - 1.5	1.5 ± 0.09	1.3 - 1.6
15:0	0.4 ± 0.05	0.3 - 0.4	0.3 ± 0.00	0.3
16:0	14.4 ± 0.46	13.8 - 15.0	14.4 ± 0.10	14.3 - 14.6
17:0	0.4 ± 0.05	0.4 - 0.5	0.3 ± 0.04	0.3 - 0.4
18:0	3.2 ± 0.09	3.1 - 3.3	3.8 ± 0.08	3.6 - 3.9
14:1 (n-5)	0.1 ± 0.00	0.1	0.1 ± 0.00	0.1
16:1 (n-7)	1.4 ± 0.09	1.3 - 1.5	2.1 ± 0.09	2.0 - 2.3
18:1 (n-9)	15.9 ± 0.86	14.7 - 16.6	14.5 ± 0.51	14.0 - 15.4
18:1 (n-7)	3.0 ± 0.32	2.5 - 3.4	4.8 ± 0.04	4.8 - 4.9
20:1 (n-11)	0.4 ± 0.19	0.2 - 0.6	0.1 ± 0.10	n.d.[3] - 0.3
20:1 (n-9)	2.9 ± 0.29	2.5 - 3.1	2.2 ± 0.17	1.9 - 2.5
20:1 (n-7)	0.5 ± 0.09	0.4 - 0.6	0.7 ± 0.06	0.6 - 0.8
22:1 (n-11)	0.7 ± 0.19	0.4 - 0.9	0.4 ± 0.04	0.4 - 0.5
22:1 (n-9)	0.5 ± 0.08	0.4 - 0.6	0.4 ± 0.03	0.4 - 0.5
24:1	0.2 ± 0.09	0.1 - 0.3	0.2 ± 0.06	0.1 - 0.3
16:2	0.6 ± 0.04	0.5 - 0.6	0.5 ± 0.05	0.4 - 0.6
18:2 (n-6)	1.0 ± 0.16	0.8 - 1.2	0.6 ± 0.00	0.6
20:2 (n-6)	0.2 ± 0.04	0.2 - 0.3	0.2 ± 0.04	0.1 - 0.2
16:3	0.3 ± 0.05	0.3 - 0.4	0.3 ± 0.00	0.3
18:3 (n-3)	0.4 ± 0.13	0.3 - 0.6	0.3 ± 0.00	0.3
18:4 (n-3)	0.7 ± 0.16	0.5 - 0.9	0.6 ± 0.07	0.5 - 0.7
20:4 (n-6)	0.8 ± 0.04	0.8 - 0.9	0.4 ± 0.05	0.4 - 0.5
20:4 (n-3)	0.9 ± 0.13	0.7 - 1.0	0.7 ± 0.03	0.7 - 0.8
20:5 (n-3)	17.4 ± 0.75	16.4 - 18.3	19.6 ± 0.19	19.3 - 19.9
21:5 (n-3)	0.4 ± 0.09	0.3 - 0.5	0.5 ± 0.05	0.4 - 0.5
22:5 (n-3)	1.4 ± 0.14	1.3 - 1.5	1.6 ± 0.05	1.6 - 1.7
22:6 (n-3)	25.8 ± 0.64	24.7 - 26.3	24.6 ± 0.69	22.9 - 25.2
Not id. [4]	4.9 ± 0.53	4.5 - 5.8	4.6 ± 0.51	3.6 - 5.2
Calc. i.v. [5]	248 ± 2.1	245 - 250	248 ± 2.6	242 - 250

1) Roe of five lumpfish analysed separately in duplicate. 2) Roe of ten 105 kg barrels after desalting analysed separately in duplicate. 3) n.d.: Not detected. 4) Not id.: Not identified. 5) Calc. i.v.: Calculated iodine value (g iodine/100 g identified fatty acids).

18:0, 18:1(n-7) and 20:1(n-9) were present at a level of 2 - 5 %, while the remaining 20 fatty acids comprised less than 2 % each (table 3). The domination by the four fatty acids listed above was less extreme in whitefish (*Coregonus albula*) roe, for which Kaitaranta (1980) reported the four acids to comprise only about half the total fatty acids, and several other acids were present at concentrations above 4 %. In cod (*Gadus morhua*) roe Ackman and Burgher (1964) found 85 % of the fatty acids to consist of 16:0, 18:1, 20:5 and 22:6, while 6.4 % was 16:1 and 2.4 % was 20:4. All remaining acids were present at concentrations below 1 %.

Fatty acid composition was determined after desalting of roe of ten barrels of heavily salted roe. The roe had been stored in the barrels for 8 months prior to desalting, and represented average raw material for lumpfish caviar production. Only slight differences in fatty acid composition of the ten lightly salted roes were observed compared to the fresh roes. The iodine value was calculated as a measure of the degree of unsaturation. Iodine values of fresh (245 - 250) and lightly salted roe (242 - 250) lipids were identical, and slightly higher than the value of 210 reported for whitefish (*Coregonus albula*) roe (Kaitaranta 1980).

Lipid hydrolysis and oxidation

As a measure of the degree of lipid breakdown, free fatty acids (FFA), peroxide value (PV) and thiobarbituric acid (TBA) value were determined on the same fresh and lightly salted roes, for which fatty acid composition was reported above. The results are shown in table 4. FFA concentration in fresh roe ranged from 2.5 - 4.3 % of lipid, while the concentration in all lightly salted roes was higher (4.8 - 10.8 %), indicating that lipid hydrolysis occurs during the storage. The influence of the desalting process was not determined, and higher levels of FFA may have been present in the heavily salted roe prior to desalting. The results however show that FFA concentrations in the range of 4.8 - 10.8 % must be expected in newly produced lumpfish caviar. The pH of fresh roe was in the range of 5.9 - 6.2 while the pH of desalted roe was slightly lower and in the range of 5.7 - 5.9 (data not shown).

Several studies have been conducted on lipid hydrolysis in fish roe stored at different conditions. Significant increases in FFA concentration during storage of salted fish roe have been reported for rainbow trout (*Salmo gairdneri*) and whitefish (*Coregonus albula*) roe (14 % salt, one year at 2°C) by Kaitaranta (1982), for perch (*Perca fluviatilis*) roe (16 % salt, one year at 2°C) by Linko et al. (1980) and for Baltic herring (*Clupea harengus*) roe (6 and 16 % salt, one year at 5°C) by Linko et al. (1979). Initial concentrations of FFA in the different fish species varied in the range of around 2 - 7 %, perch roe having the highest concentration. Linko et al. (1979) concluded that lipid hydrolysis in herring roe occurred enzymatically and could be prevented by pasteurisation or freezing (÷20°C for one year). The rate of the process was concluded to depend on the fish species. Huynh (1985) similarly demonstrated that liberation of FFA in herring roe stored in saturated brine decreased with decreasing storage temperature (2°, ÷12°, ÷18° and ÷28°C).

Table 4. Characteristics of the lipid fraction of fresh and lightly salted lumpfish roe (made by desalting of heavily salted roe stored for 8 months at ÷5°C).

	Fresh roe [1]		Lightly salted roe [2]	
	Mean ± SD	Range	Mean ± SD	Range
Salt % w/w	0.9 ± 0.05	0.9 - 1.0	3.8 ± 1.0	3.0 - 5.8
Lipid [3] % w/w	5.5 ± 0.4	4.9 - 5.9	4.9 ± 0.2 (5.0)	4.6 - 5.1 (4.8 - 5.3)
Free fatty acids [4] % w/w of lipid	3.6 ± 0.8	2.5 - 4.3	6.3 ± 1.7	4.8 - 10.8
Peroxide value meq./kg lipid	n.d. [5]	n.d.	20.4 ± 7.2	9.4 - 31.8
TBA value [6] µmoles/kg roe	2.6 ± 1.7	1.2 - 5.4	21.8 ± 5.4	11.9 - 29.1

1) Roe of five lumpfish analysed separately in duplicate. 2) Roe of ten 105 kg barrels after desalting analysed separately in duplicate. 3) Values shown in parentheses are calculated as concentration in roe with 0.9 % salt. 4) Free fatty acids as oleic acid. 5) n.d.: Not detected. 6) TBA: Thiobarbituric acid.

In fresh roe hydroperoxides could not be detected, while low TBA-values were observed (1.2 - 5.4 µmoles/kg roe) (table 4). The TBA-value is presented as µmoles malonaldehyde/kg roe, but other food components such as sugars and amino acids decomposed during the analytical procedure can react with TBA, and may contribute to the low levels detected in fresh roe (Gray and Monahan 1992, St. Angelo 1996). Increased but varying PV levels (9.4 - 31.8 meq./kg lipid) were detected in lightly salted roe (table 4). In a preliminary study in this laboratory (unpublished) six commercial barrels of heavily salted roe of different age and origin were examined, and in all barrels higher levels of PV and TBA-values were detected in the top layer than in roe from the deeper levels of barrels. During desalting the roe is mixed to a certain extent, and the original location in the barrels of roe samples analysed in the present study cannot be determined. However the data reflects the variation among lumpfish caviar products, produced by the normal procedure. In perch roe (16 % salt) stored at 2 °C in wooden boxes submersed in 50 % saturated brine, the PV reached a maximum after 9 months, and then decreased towards the end of storage (one year) (Linko et al. 1980). Decrease of the elevated levels of PV occurs due to decomposition of hydroperoxides into secondary oxidation products (Gray and Monahan 1992). The present study showed that secondary oxidation products had been formed in the lumpfish roe, as increased TBA-values (11.9 - 29.1 µmoles/kg) were detected in the stored desalted roe (table 4) (slightly higher values would have been obtained, if calculated per kg of roe with 0.9 % salt).

Through several reports it has been established that lipid hydrolysis and oxidation are important factors in quality of fish roe, and are influenced by both storage conditions and fish species, the latter due to differences in lipid composition (Kaitaranta 1982, Linko et al. 1979, Linko et al. 1980, Huynh 1985). Linko et al. (1979) concluded that in long-term storage (one year) of Baltic Herring roe, heavy salting (16 % w/w dry or brine salted) caused a rapid development of hydrolytic and oxidative rancidity in the products as measured by increasing FFA and PV. In that regard, frozen storage in air-proof vacuum package proved to be the best method.

The changes in the lipid fraction demonstrated in the present study had occurred even before the roe products had been produced. This is a consequence of the seasonal supply forcing the industry to preserve the roe until needed for production. The traditional solution to this problem has been the use of heavy salting, and alternative processing means are not simple to find. Such means should be applicable to the production of small quantities of salted roe by a large number of local fishermen at very distant geographic locations along the coasts of Norway, Iceland and Canada.

Amino acid composition

Composition of total and free amino acids in fresh and lightly salted roe is shown in table 5. In fresh roe the total amount of amino acids varied among five fish in the range of 87.3 - 108.7 mg/g. The amino acid composition was rather uniform, and among 16 amino acids the major were glutamic acid (12.8 - 13.1 %), leucine (9.8 - 10.0 %) and aspartic acid (9.6 - 9.8 %). The results differed only slightly from the data reported by Rehbein (1985), who found a higher concentration of glutamic acid (16.1 % of total amino acids). The samples examined in that study were commercial roe products, detailed ingredients of which were not stated.

The total amount of free amino acids was in the range of 21.5 - 28.4 mg/100 g roe, comprising 0.28 % of total amino acids. Composition of free amino acids varied more than total amino acids, and the composition was different in that glutamic acid dominated (29.1 - 36.7 %), while the following places were occupied by alanine (9.9 - 12.0 %), aspartic acid (5.4 - 12.7 %), lysine (4.2 - 8.2 %) and glycine (4.4 - 7.4 %). Tryptophane, not detected among total amino acids (lost during the hydrolytic step), was present among free amino acids at a concentration of 0.3 - 1.5 %.

Taurine (2-aminoethanesulphonate) concentration was in the range of 44.4 - 63.1 mg/100 g roe, thus around double the total concentration of free amino acids. In the analysis of total amino acids a similar level of taurine (0.5 - 0.6 mg/g) was detected, as taurine is not a regular amino acid and not incorporated into protein.

Gjessing (1963) showed that the great changes occurred in the fraction of free amino acids in hard herring roe (*Clupea harengus*) during the last few weeks before spawning. For each of six quantified amino acids, large increases were observed both on dry matter and wet weight basis, though the moisture content also increased towards spawning. As the rate of the

Table 5. Composition of total and free amino acids (% w/w of identified amino acids) of fresh lumpfish roe and of lightly salted lumpfish roe (made by desalting of heavily salted roe stored for 8 months at ÷5°C). Salt concentration is shown in table 4.

Amino acids	Total amino acids [1]				Free amino acids [2]			
	Fresh roe [3]		Lightly salted roe [4]		Fresh roe [3]		Lightly salted roe [4]	
	Mean±SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Aspartic acid	9.6±0.09	9.6 - 9.8	9.6±0.08	9.5 - 9.7	9.8±2.86	5.4 - 12.7	7.0±0.95	4.6 - 8.0
Threonine	5.5±0.03	5.4 - 5.5	5.5±0.06	5.4 - 5.6	5.5±0.86	4.8 - 6.6	4.9±0.40	4.2 - 5.5
Serine	6.5±0.12	6.3 - 6.6	6.1±0.20	5.7 - 6.3	3.9±0.65	2.9 - 4.6	4.9±0.41	4.5 - 6.0
Glutamic acid	12.9±0.10	12.8 - 13.1	13.1±0.18	12.7 - 13.3	32.2±2.86	29.1 - 36.7	14.2±1.08	12.6 - 16.2
Proline	5.4±0.09	5.3 - 5.5	5.5±0.20	5.2 - 5.8	4.3±0.82	3.2 - 5.1	4.9±0.77	3.7 - 5.8
Glycine	3.0±0.04	3.0 - 3.1	3.0±0.04	3.0 - 3.1	5.6±1.19	4.4 - 7.4	2.1±0.31	1.6 - 2.7
Alanine	5.6±0.07	5.5 - 5.7	5.7±0.14	5.5 - 5.9	11.0±0.82	9.9 - 12.0	6.2±0.33	5.6 - 6.7
Cyst(e)ine	1.5±0.25	1.1 - 1.7	1.4±0.35	1.0 - 2.0	1.0±1.05	n.d.[4] - 2.2	n.d.	n.d.
Valine	6.6±0.08	6.5 - 6.7	6.9±0.08	6.8 - 7.1	3.7±0.57	2.9 - 4.3	4.6±0.13	4.4 - 4.8
Methionine	2.6±0.03	2.6 - 2.6	2.4±0.32	1.5 - 2.7	n.d.	n.d.	0.1±0.08	n.d. - 0.2
Isoleucine	5.5±0.10	5.4 - 5.6	5.6±0.08	5.4 - 5.7	2.1±0.31	1.8 - 2.6	6.4±0.54	5.5 - 7.1
Leucine	9.9±0.10	9.7 - 9.9	9.9±0.16	9.7 - 10.2	4.3±0.30	3.9 - 4.6	10.9±0.38	10.3 - 11.4
Tyrosine	5.0±0.07	4.9 - 5.1	5.1±0.08	5.0 - 5.3	2.0±0.26	1.7 - 2.3	5.9±0.41	5.1 - 6.7
Phenylalanine	4.7±0.05	4.6 - 4.8	4.7±0.06	4.6 - 4.8	1.8±0.19	1.6 - 2.1	4.3±0.21	4.0 - 4.7
Histidine	3.1±0.11	3.0 - 3.2	3.2±0.11	2.9 - 3.3	2.7±0.45	2.3 - 3.5	3.4±0.65	2.6 - 4.5
Tryptophane [6]					0.9±0.52	0.3 - 1.5	4.2±0.62	3.2 - 5.5
Lysine	6.5±0.10	6.3 - 6.6	6.3±0.14	6.0 - 6.5	5.3±1.68	4.2 - 8.2	8.3±0.48	7.4 - 8.8
Arginine	6.1±0.04	6.1 - 6.2	6.1±0.06	6.0 - 6.2	3.9±0.77	3.1 - 5.1	7.9±1.07	6.7 - 10.1
Total mg/g (total aa) or mg/100g (free aa)	93.6±8.8	87.3 - 108.7	94.1±5.6 (97.0±5.4)[7]	84.5 - 102.1 (88.8 - 104.3)[7]	25.8±2.82	21.5 - 28.4	38.6±13.3 (39.8±14.0)[7]	14.8 - 51.9 (15.2 - 54.4)[7]
Free % of total [8]					0.28±0.03	0.24 - 0.30	0.41±0.14	0.16 - 0.61
Taurine mg/g (total aa) or mg/100g (free aa)	0.6±0.05	0.5 - 0.6	0.1±0.05[7]	0.1 - 0.2[7]	53.0±7.2	44.4 - 63.1	14.0±5.3[7]	6.6 - 22.0[7]
Taurine/free aa [9]					2.1±0.19	1.8 - 2.3	0.35±0.05	0.3 - 0.4

1) The chromatograms also contained small amounts of ammonia, oxidised methionine and small unidentified peaks at retention times around 30, 49 and 55 minutes.
2) The chromatograms also contained small amounts of ammonia, oxidised methionine and small unidentified peaks at retention times around 36 and 49 min. and a peak at around 55, probably ethanolamine. 3) Roe of five lumpfish analysed separately. 4) Roe of ten 105 kg barrels after desalting analysed separately. 4) n.d.: Not detected.
6) Tryptophane is lost during the hydrolysis, and can only be detected among free amino acids. 7) Calculated as concentration in roe with 0.9 % salt. 8) Free amino acids as % of total amino acids. 9) Taurine concentration/ total concentration of free amino acids.

increase was not identical for all amino acids, the composition of free amino acids changed considerably during ripening. Vuorela et al. (1979) showed that the degree of ripening of rainbow trout (*Salmo gairdneri*) roe as measured by a maturity index, was not correlated to the catching date. Fish with ripe and less ripe roes could be caught both at beginning and end of the catching season. Thus the composition of free amino acids of roe of just caught fish is not uniform.

The amount and composition of total amino acids in stored desalted roe was similar to that of fresh roe. The total concentration of free amino acids in lightly salted roe varied between 15.2 and 54.4 mg/100 g (extra salt withdrawn). In spite of the washing out likely to occur during desalting, higher concentrations of free amino acids than in fresh roe were detected in eight of ten desalted roes (not shown), indicating that liberation of free amino acids occurred during the storage of heavily salted roe in barrels. Free amino acids comprised 0.41 % (range 0.16 - 0.61 %) of total amino acids, compared to the 0.28 % for fresh roe. A similar situation was reported by Chiou and Konosu (1988), who noted a large increase in mullet (*Mugil cephalus*) roe during processing. In Alaska pollac (*Theragra chalcogramma*) roe a smaller increase during processing was reported by Chiou et al. (1989a). Chiou et al. (1989b) demonstrated, that caseinolytic, acid protease and aminopeptidase acitivities at different levels were present in the roes throughout processing. The composition of free amino acids in lightly salted roe also differed from that of fresh roe. Some amino acids were present at a lower concentrations (glutamic acid, glycine and alanine), while others made up a greater part of free amino acids than in fresh roe (isoleucine, leucine, tyrosine, phenylalanine, arginine and tryptophane).

Taurine concentration in lightly salted roe had decreased to 6.6 - 22.0 mg/100 g. The reduction roughly equals the reduction in salt concentration during desalting. When the concentration of taurine is calculated relative to the concentration of total free amino acids, a rather constant relation is obtained of taurine being around one third (factor 0.3 - 0.4) the amount of free amino acids in the ten lightly salted roes, while in fresh roe a taurine concentration of double the concentration of free amino acids was detected. Thus proportionally a greater reduction of taurine than free amino acids occurred. This confirms the indication that while both taurine and free amino acids are lost during desalting, certain amino acids are supplemented by breakdown of peptides and proteins, which could explain the altered composition of free amino acids in lightly salted roe.

Thus the amount of free amino acids in lightly salted roe available for bacterial growth during chill storage of the final products varies considerably. The highest concentration detected in this study was more than three times the lowest concentration, probably resulting from the combined effects of liberation of amino acids during storage, and washing out during desalting.

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Paper 2. Spoilage of lightly salted lumpfish (*Cyclopterus lumpus*) roe at 5°C.

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Abstract

Lumpfish roe (3.5 - 4.8 % waterphase salt, pH 5.4, vacuum-packed) was stored at 5°C. In roe with antimicrobials (sodium-nitrite, actidion, polymyxin-B-sulphate, oxytetracycline) added, spoilage off-odours failed to appear. Different degrees of spoilage occurred among eleven roe batches after 2½ or 3 months of storage, ranging from very weak odours to strong sulphidy, sour odours. The microflora consisted of Lactic Acid Bacteria, Enterobacteriaceae and *Vibrio* spp. Concentration of lactic acid, acetic acid, trimethylamine and total volatile bases were unrelated to spoilage odours. Volatile sulphur compounds (H_2S, probably CS_2, CH_3SH and CH_3CH_2SH or CH_3SCH_3) were produced during storage, and may contribute in spoilage.

Keywords: Lightly salted lumpfish roe, microbiology, spoilage indicators, off-odours, volatile sulphur compounds.

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Introduction

Scandinavia has a long tradition for the production of salted lumpfish (*Cyclopterus lumpus*) roe products, the main part of which is exported. The products are produced from heavily salted lumpfish roe. Lumpfish are caught in the spawning season along the coasts of the North Atlantic, from early spring till summer depending on the geographic location. Immediately after catch the roe is removed from the fish and dry salted in 105 kg plastic barrels (14 - 20 % waterphase salt (WPS)). The heavily salted roe is stored at ÷5°C until needed for production (normally ≤ one year). During production the roe is partly desalted by addition of water and subsequent drainage, to obtain the salt concentration wanted in the product (4 - 9 % WPS). The products are preserved by acidification and addition of chemical preservatives and/or pasteurisation.

As industry wishes to meat consumers' demands for a reduced use of salt and chemical preservatives, there is a need to investigate spoilage reactions and assess shelf life of a lightly salted roe product with acidification as the only additional type of preservation. The industry estimates a shelf life of three months to be necessary in practical production and marketing.

While bacterial spoilage of fresh unpreserved fish is well investigated, the bacterial spoilage of lightly preserved fish products has not been studied as extensively, particularly not salted roe products, and spoilage organisms have not been identified.

In a study of spoilage of vacuum-packed "gravad" fish (4 % salt, not heat processed, no acid but sugar added) From and Huss (1988) concluded, that organisms likely to act as spoilage organisms were H_2S producers as *Shewanella putrefaciens*, Enterobacteriaceae and Vibrionaceae. Yeast, *Brochotrix* spp. and Lactic Acid Bacteria (LAB) may contribute to spoilage, but were judged as less important. Huss et al. (1984) found, that the microflora of lumpfish roe with salt concentrations in the range of 5.7 % - 9.5 % WPS stored at 10°C, was dominated by presumptive *Lactobacillus* spp. (6 - 7 log cfu/g). Truelstrup Hansen (1995) found LAB, Enterobacteriaceae, *Vibrio* spp. and *Photobacterium* spp. to constitute the microflora of spoiled, vacuum packed cold smoked salmon, but the microflora was highly variable among production batches and among different processing plants.

The aim of the present work was to study the spoilage of lightly salted lumpfish roe (salt concentrations in the range of 3.5 - 4.5 % WPS and pH 5.4 obtained by addition of lactic acid). Chemical, microbiological and odour characteristics during storage at 5°C has been investigated.

Materials and methods

Roe products

Lightly salted lumpfish roe was produced by a local producer (ABBA Seafood A/S, Denmark) using standard processing procedures. Eleven different barrels (i.e. batches) of heavily salted roe, that had been stored for six months at ÷5°C, were desalted to obtain a salt level in the final products in the approximate range of 3.5 - 4.5 % WPS. The pH was lowered from initially around 5.9 to 5.4 by addition of lactic acid (0.1 % w/w) (PURAC®SP 80, Nordisk Droge Handel A/S, Denmark). In order to examine whether spoilage were caused by microbial activity, a batch of roe with app. 4 % WPS was prepared with and without addition of the following antimicrobial substances: Sodium nitrite 0.69 g/kg (Merck 6549), actidion/cycloheximide 0.11 g/kg (Sigma C-6255), polymyxin B sulphate 28600 IU/kg (Sigma P-1004) and oxytetracycline 0.1g/kg (Oxoid SR 073). All roe was packed in non-sterile commercial glass jars. The jars were closed with twist-off caps under approximately 65 - 75 % vacuum and stored at 5°C.

Assessment of spoilage

The odours of stored roe products was assessed after two and a half or three months by a panel of 10 instructed persons. Frozen roe products (vacuum packed and stored at ÷40° C) of the same batch were used as non-spoiled controls. An overall assessment of the roe as either "acceptable" or "spoiled/rejectable" was given by each panellist. The %-rejection was calculated for each batch. Rejection by more than 50 % of the judges were regarded as rejection of the batch. The odours were further characterised as ammonia-like, sulphidy, putrid, sour and rancid. The panellists were asked to use other descriptive words if appropriate.

Microbiological analysis

Flora composition after three months of storage, corresponding to the shelf life period wanted by the industry, was determined in five roe batches (batch 1 - 5). Samples of 10 g of roe were diluted ten-fold with peptone saline (PS) (0.85 % NaCl, 0.1 % peptone (Difco 0120.01) and homogenised for 30 seconds in a Colworth Stomacher 400. Further ten-fold dilutions were prepared in PS before spread plating. Direct phase contrast microscopy (1000 x magnification) was carried out on the first ten-fold dilution of samples, to estimate the number and types of microorganisms.

Samples were spread on: 1) Blood agar (BA) pH 6.8 (10 g/l meat extract (Oxoid L29), 10 g/l peptone (Difco 0120-01), 2 g/l yeast extract (Difco 0127-01-7), 5 g/l NaCl and 15 g/l agar). Sterile, defibrinized calf blood (50 ml/l) (Danish Veterinary Laboratory, Copenhagen, Denmark) was added after autoclaving for 15 min. at 121°C, 2) Blood agar with 3 % salt (BA3%) and 3) All Purpose Tween agar (APT broth, (Difco 0655-01-7) and 13 g/l agar), as

an elective agar for Lactic Acid Bacteria. The APT agar were incubated at aerobic and anaerobic conditions (Oxoid anaerobic jars with gas-generating kit BR38, catalyst BR42 and indicator BR55). All plates were incubated at 10°C for 6 days.
The colonies were differential counted giving one count for each morphological type of colonies. No influence of salt concentration on counts on blood agar was observed. A dilution was chosen with no more than 250 colonies, from which isolation was carried out. For comparison among agars, three colonies of each observed type were taken from the BA3% and aerobic APT plates, while one was taken from the anaerobic APT plates. No influence on counts of incubation atmosphere or type of agar was observed, and counts on BA3% are presented.
Enterobacteriaceae and LAB counts were determined of 6 roe batches. LAB were determined by spread plating on Nitrite Actidione Polymyxin (NAP) agar (Davidson and Cronin 1973) modified to pH 6.7 (Jeppesen and Huss 1993). Enterobacteriaceae counts were determined by pour plating in Tryptone Soya Agar (TSA) (Oxoid CM131), left for one hour at room temperature (NMKL 1984, no. 105) and overlaid by Violet Red Bile Glucose agar (VRB-G) (Oxoid CM485) (NMKL 1992, no.144). The plates were incubated at 25°C for two days (VRB-G) or three days (NAP). Preliminary experience had established, that similar counts could be obtained at 10°C and 25°C.
During storage total viable counts of roe with added antimicrobials were determined using spread plating on Tryptone Soya Broth-agar (Tryptone Soya Broth (TSB) (Oxoid CM 129) and 13 g/l agar) incubated at 10°C for 6 days.

Characterisation of isolates

Culturing of isolates was carried out in TSB and on TSB-agar. Unless otherwise stated the incubation temperature was 25°C, also in the tests described below.
The following tests and reactions were used for characterisation of isolates: Shape and motility were examined using phase contrast microscopy 1000 x magnification after incubation at 10°C and 25°C. Gram-reaction was tested using the KOH-method (Gregersen 1978). Gram-positive isolates were transferred to APT-agar before performing the catalase test. Catalase-test was done using 20 % H_2O_2 (Wilkinson and Jones 1977).
Cytochromoxidase reaction was tested with DrySlide™ Oxidase (Difco) based on Kovacs (1956). Glucose metabolism was examined by the OF-test of Hugh and Leifson (1953) using OF-test medium (Merck 10282).
Gram-negative isolates that were catalase- and oxidase-positive, fermentative rods were cultured and tested at 15°C, because cells cultured at 25°C were densely filled with granules and had a rather pleomorphic appearance. These isolates were additionally examined for their sensitivity to vibriostaticum O/129 (150 mg tablets, Rosco Diagnostica, Denmark). The test was done on TSB agar, the first 2 hours at 5°C followed by 3 days at 15°C, before reading inhibition zones.

Chemical analysis

Salt concentration (w/w) was calculated from the amount of chloride as determined in duplicate by boiling in nitric acid with excess of $AgNO_3$ followed by titration with 0,1 M KSCN solution (AOAC 1995, no. 937.09).

Dry matter content (DM) was determined in duplicate by weighing before and after drying at 105°C in 20-24 hours.

pH was measured using a PHM 82 (Radiometer, Copenhagen, Denmark).

Total volatile bases (TVB), trimethylamineoxide (TMAO) and trimethylamine (TMA) were determined by a modified Conway micro-diffusion method (Conway and Byrne 1933).

To determine the concentration of organic acids five grams of roe were blended with 25 ml 0.6 M perchloric acid and filtered. 10 ml extract was neutralised (pH 6.5 - 6.8) with KOH, frozen over night, filtered again and diluted to 20 ml. The extracts were analysed by HPLC on a BIORAD HPX-87H Organic Acids Column (300x7,8mm) using 0.008 M H_2SO_4 as an eluent and Refractive Index detection. Identification and quantification was done on the basis of relative retention times of standards (lactic, formic, acetic and propionic acid).

Volatile sulphur compounds were determined using the method described by Dalgaard et al. (1993). Ten grams of sample were kept in a 50 ml headspace bottle at 30°C in a water-bath for 30 minutes before 3 ml of headspace was injected into the gaschromatograph.

Results

Microbial and sensory characteristics

Very different off-odour development was noted among the eleven batches of lightly salted roe (table 1). After two and a half and three months of storage three different situations regarding off-odour had occurred: Heavily spoiled batches (batch 2, 6, 7 and 10) had sulphidy or cabbage-like odours combined with sour, rotten and green/hay-like odours; batches close to borderline (batch 3, 4, 5 and 8) had sour, fruity, rotten and marinated odours, while some batches (batches 1, 9, and 11) had very weak odours, and were still acceptable. Occasionally judges rejected the roe due to rancid odours. This type of rejection remained at a constant level of 10 - 15 % and did not increase during storage. Therefore rejections due to rancidity were withdrawn from the data before %-rejections due to other off-odours were calculated.

Both roe with and without addition of antimicrobials were rejected after three months of storage. Roe without antimicrobials had sulphidy, sour, rotten, faecal and ammonia-like odours. The total viable counts increased to 7.6 log cfu/g in three weeks and were 7.5 log cfu/g after three months. Roe with antimicrobials had odours described as chemical, varnish/paint and malt/soy-sauce/caramel, and the total viable counts remained below 4 log cfu/g throughout storage (data not shown). Thus off-odours developing without microbial

growth showed no similarity to odours in natural spoilage, and were most likely caused by the additives.

In batch 1 - 5 total viable counts increased from initially < 2 - 3.5 log cfu/g to around 7 log cfu/g after four weeks (data not shown). The counts after three months of storage were still at a level of 7 log cfu/g and very uniform among batches (table 1). The number of cells observed in the direct microscopy corresponded to the counts on agar plates. Based on basic testing of 108 isolates, the microflora (counts > 5 log cfu/g) of the five batches could be divided into three groups defined by the following criteria:

LAB (70 isolates): Gram-positive, catalase- and oxidase-negative, fermentative and except for one group non-motile rods and cocci.

Enterobacteriaceae (30 isolates): Gram-negative, catalase-positive, oxidase-negative, fermentative, motile rods.

Vibrio spp. (8 isolates): Gram-negative, catalase- and oxidase-positive rods, fermentative without gas production, motility was absent or very weak. The isolates were sensitive to vibriostaticum O/129 (150 µg).

Table 1. Salt concentration, microbiological data and odour characteristics of 11 batches of lightly salted lumpfish roe (pH 5.4). The roe was vacuum packed in glass jars and stored at 5°C. Batch 1 - 5 was stored for three months, and counts were obtained from Blood-agar with 3 % of salt. Batch 6 - 11 was stored for two and a half months, and counts of LAB and Enterobacteriaceae were obtained from selective agars.

Salt[1)] % WPS	**Batch no.**	**%-rejection**	**Odour characteristics**	**LAB log cfu/g**	**Enterobacteriaceae log cfu/g**	***Vibrio* spp. log cfu/g**	**Total viable counts log cfu/g**
3.5	6	100	Sulphidy, green/hay, sour, rotten	8.1	5.3		
3.5	7	100	Sulphidy, sour, rotten	8.0	5.8		
3.6	4	43	As batch 3, but weaker	6.7	6.9	≤ 5	7.1
3.7	2	73	Cabbage-like, NH_3, fruity, rotten	7.0	6.9	≤ 5	7.3
3.7	8	50	Sour	7.7	5.5		
3.8	5	37	As batch 3, but weaker	7.1	6.6	5.5	7.3
3.8	3	53	Sour, marinated, fruity, rotten	7.2	6.5	6.1	7.3
4.2	1	10		6.9	≤ 5	6.1	7.0
4.5	10	75	Sulphidy, rotten	7.3	5.8		
4.7	9	25		7.6	≤ 5		
4.8	11	25		7.3	≤ 5		

1) Calculated from mean values of total salt concentration and dry matter content. Standard deviations in these analysis were ≤ 3 % and ≤ 2 % respectively.

In batch 1 - 5 and 8 - 11 LAB counts after storage were around 7 log cfu/g, while higher counts (8 log cfu/g) were observed in batch 6 and 7 (table 1). LAB counts were not systematically affected by the salt concentration. The lowest (batch 4) and the highest (batch 6) LAB counts were obtained in batches with a very small difference in salt concentration. Enterobacteriaceae counts obtained in the eleven batches were affected by the salt concentration in the manner, that at salt concentrations above 4 % WPS Enterobacteriaceae were only in one case (batch 10) able to grow to levels above 5 log cfu/g (table 1) (by basic testing of colonies from VRB-glucose agar plate of batch 10, growth of Enterobacteriaceae was confirmed). Enterobacteriaceae counts of the remaining batches ranged from 5.3 to 6.9 log cfu/g. The counts of batch 6 - 11 determined by the use of selective agar, tended to be lower than those of batch 1 - 5.

Chemical changes

The concentration of lactic acid of batch 1 - 5 did not exceed the added amount (0.1 %). Only slight changes in pH and lactic acid concentration occurred during storage. Small amounts of acetic acid were formed, and final values between 3.24 ± 0.05 (batch 1) and 6.30 ± 0.23 (batch 3) µmol/g were reached after three months of storage (data not shown).
The TVB concentration of batch 6 to 11 showed a uniform increase during storage, regardless of the degree of spoilage (figure 1). The initial level varied (2.8 - 7.0 mgN/100g), which could be due to different degrees of washing out of compounds during desalting, as the lowest TVB concentrations were detected in low-salt batches. Initial concentrations of TMAO and TMA were < 4 mgN/100g and < 1 mgN/100g respectively. TMA concentration did not increase during storage (data not shown).

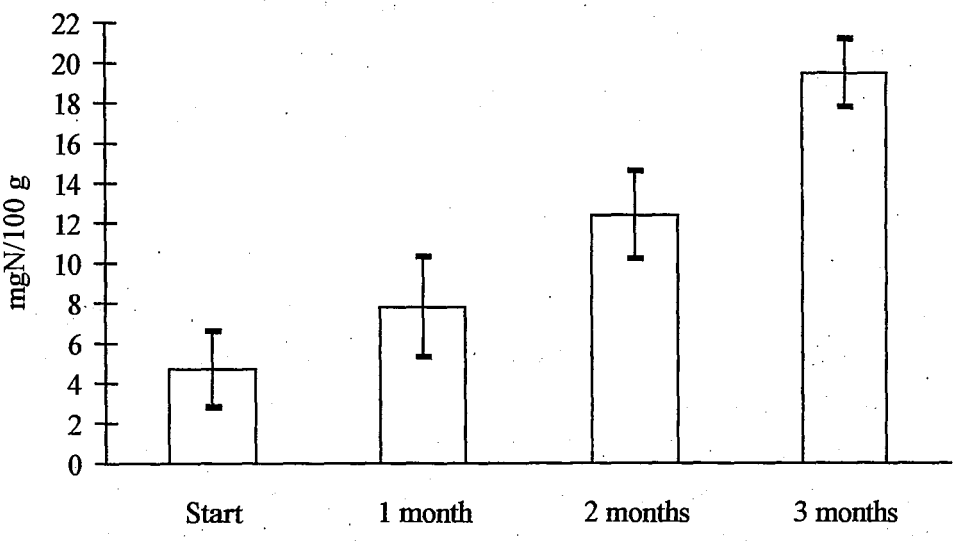

Figure 1. Changes of total volatile bases (TVB) in six batches (6 - 11) of lumpfish roe (pH 5.4 and 3.5 - 4.8 % WPS) during vacuum packed storage at 5°C.

In the three heavily spoiled batches 6, 7 and 10 with off-odours described as sulphidy (see table 1), three or four volatile sulphur compounds were detected in significant amounts, while no or smaller amounts of some of the compounds were detected in non-spoiled (9, 11) and the borderline (8) batches (table 2). The results are however not unambiguous, and the two non-spoiled batches 9 and 11 have rather different profiles, several compounds being detected in the latter.

Table 2. Volatile sulphur compounds of six batches of lumpfish roe (pH 5.4 and 3.5 - 4.8 % WPS) after vacuum packed storage for two and a half months at 5°C.

Batch	**6**	**7**	**8**	**9**	**10**	**11**
H_2S	+[1)]	+	(+)	-	+	(+)
CS_2 [2)]	+	-[3)]	+	-	+	(+)
CH_3SH	+	+	(+)	-	+	+
CH_3CH_2SH/ CH_3SCH_3 [4)]	+	+	(+)	-	+	+

1) +: The compound detected, (+): Peak area max. 25 % of the largest peak area of the same compound, - : Peak area below detection limit. 2) Likely identity. Retention time 0.64 - 0.71 min. 3) Overflow of the coloumn by H_2S might have hidden the peak. 4) Retention times of the two standards equally close, identity inconclusive.

The method of analysis does not allow direct quantification of sulphur compounds, but for each compound there is comparability among batches. The compounds can only be quantified relative to the amounts of the same compound in the other batches of roe. Thus the results shown in table 2 is a rough profile of volatile sulphur compounds for each batch of stored roe, witch shows some relation to the degree of spoilage. In the frozen controls only trace amounts of sulphur compounds were detected.

Discussion

Spoilage odours, microflora and potential chemical indicators of lightly salted lumpfish roe, not earlier investigated, was examined in the present study. Based on 108 isolates, the microflora after three months of storage at 5°C was shown to consist of LAB and Enterobacteriaceae with *Vibrio* spp. as a minor part. These groups of bacteria have been shown to be part of the flora during chill storage of similar products like vacuum packed cold-smoked salmon (Truelstrup Hansen 1995) and "gravad" fish (From and Huss 1988). *Shewanella putrefaciens* and *Brochotrix* spp. also reported part of the flora of "gravad fish" by the last authors, were absent (< 5 log cfu/g) in the present study, which is probably due to

the lower pH (5.4) of lightly salted lumpfish roe (Gill and Newton 1979, Campbell et al. 1979).

LAB were present at counts of 6.7 - 8.1 log cfu/g in the eleven batches of roe (table 1). This is in accordance with the findings of other workers, reporting LAB to grow well in fish products with salt addition (≤ 6 % WPS) (Jeppesen and Huss 1993, Magnusson and Traustadottir 1982, Mauguin and Novel 1994). Many LAB tolerate much higher salt levels than used in the present study (Axelsson 1993), and no systematic influence by salt concentrations was seen on LAB counts. Growth of Enterobacteriaceae was apparently inhibited by salt concentrations above 4 % WPS (counts ≤ 5 log cfu/g after storage). In one high-salt batch (batch 10, 4.5 % WPS) however, at least one member of the Enterobacteriaceae family was able to grow. *Vibrio* spp. constituted only a minor part of the flora (max. 13 %), which is likely to be due to the relatively low pH at which they do not compete well (Walker 1992).

The results showed, that off-odours were caused by bacterial activity, and very unevenly distributed among eleven batches of roe with salt concentrations in the range of 3.5 - 4.8 % WPS. High salt batches without growth of Enterobacteriaceae (batch 1, 9 and 11) exhibited very week off-odours, though LAB counts were at a similar level (around 7 log cfu/g) as in spoiled and borderline batches (2 - 5, 8 and 10). Members of the Enterobacteriaceae family must be considered organisms with a high spoilage potential as reviewed by Dainty et al. (1983) and Gill (1986) due to formation of H_2S and malodorous amines from amino acid breakdown. In the spoiled batches 6 and 7 high LAB counts of 8 log cfu/g were however observed, while Enterobacteriaceae counts in those batches and the spoiled batch 10 were below 6 log cfu/g. Yet comparison among batches was complicated by the use of selective agar in some batches. Further differentiation and characterisation of the LAB and Enterobacteriaceae flora is necessary to evaluate the role in spoilage by these organisms.

Rancid odours were also detected in the products. Non-microbial changes is taking place during storage of heavily salted roe (16 - 20 % WPS at ÷5°C), resulting in increased levels of peroxide and thiobarbituric acid values (Basby et al. 1997a). Thus rancid odours formed prior to production does influence the overall product quality, but no further development was observed during vacuum packed storage at 5°C.

Production of TMA, that takes place in many fish products (Gram and Huss 1996), did not occur in lightly salted lumpfish roe. TMAO concentration of fresh, heavily salted and lightly salted lumpfish roe has earlier been examined in this laboratory, and were shown to be low (≤ 4.4 mgN/100 g) and often undetectable (Basby et al. 1997a). The TVB, thus consisting of ammonia, increased during storage, but he concentration were at the same level in all analysed batches despite very different odour characteristics of the roe. Lactic acid concentration changed only slightly during storage, and the detected concentration of acetic acid (3.2 - 6.3 µmoles/g) were low compared to the values reported by Truelstrup Hansen et

al. (1995) during storage at 5°C of vacuum packed cold smoked salmon. Levels of 12 - 23 µmol/g were detected at sensory rejection of the salmon.
Several volatile sulphur compounds (H_2S, probably CS_2, CH_3SH and CH_3CH_2SH or CH_3SCH_3) were produced during storage of lightly salted lumpfish roe, and may contribute to spoilage off-odours, as previously reported for both fresh fish and meat (Herbert et al. 1971, Dainty et al. 1979, Edwards and Dainty 1987). A rough profile of the four volatile sulphur compounds of spoiled, borderline and non-spoiled roe batches exhibited some relation to the off-odours detected. More sulphur compounds and/or larger amounts were detected in spoiled batches with odours described as sulphidy (6, 7 and 10), compared to borderline and acceptable batches (8, 9 and 11).
In conclusion lightly salted lumpfish roe stored at 5°C spoiled due to bacterial activity, and spoilage organisms are to be found among LAB and/or Enterobacteriaceae. The overall quality of the products was however influenced by rancidity developing prior to production. Products with salt concentrations above 4 % WPS spoiled less frequently, but additional preservation is nevertheless required.
Further examination of the isolated bacteria with special emphasis on their spoilage potential and ability to produce volatile sulphur compounds in lightly salted lumpfish roe is being conducted in this laboratory.

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Paper 3. Characterisation of the microflora of lightly salted lumpfish (*Cyclopterus lumpus*) roe stored at 5°C.

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Abstract

Numeric Taxonomy Analysis of 70 Lactic Acid Bacteria (LAB) and 30 Enterobacteriaceae from lightly salted lumpfish roe, showed that Enterobacteriaceae formed three subgroups: *Morganella morganii*, presumptive *Serratia liquefaciens* and *Serratia plymuthica*. LAB formed three subgroups of presumptive *Lactococcus* spp. and *Carnobacterium* spp. Production of off-odours and volatile sulphur compounds by twelve selected strains and three *Vibrio* spp. of identical origin in pasteurised roe, and of three Enterobacteriaceae in sterile fresh roe, was examined. *Morganella morganii* produced off-odours and volatile sulphur compounds in both pasteurised and fresh roe. One *Serratia liquefaciens* produced strong off-odours but no volatile sulphur compounds in fresh roe.

Keywords: Lightly salted lumpfish roe, Enterobacteriaceae, *Morganella morganii*, Lactic Acid Bacteria, spoilage potential.

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Introduction

Roe from the lumpfish (*Cyclopterus lumpus*) has for many years been used in Scandinavia as the raw material for production of lightly salted lumpfish roe products. The roe is salted as loose eggs (14 - 20 % NaCl in the water-phase (waterphase salt; WPS)) right after catch, and stored in plastic barrels (105 kg) at ÷5°C until needed for production. During production the salt concentration is lowered by addition of water and subsequent drainage. A variety of such products with different salt concentrations (4 - 9 % WPS) and addition of various preservatives and other additives are produced, and a large proportion is exported to other countries. In order to meet consumers demands, the industry now wishes to produce lumpfish roe products with reduced use of salt and chemical preservatives. This emphasises the need for knowledge regarding spoilage organisms in order to choose the proper preservation method to ensure shelf life and safety.

Recently a study was conducted on spoilage of lightly salted lumpfish roe (app. 3.5 - 4.5 % WPS) without further preservation except for lactic acid (0.1 % w/w) added to lower the pH to 5.4. Products were vacuum packed in glass jars and stored for two and a half and three months at 5°C (Basby et al. 1997b). It was shown that spoilage of the products was caused by bacterial activity. The microflora developing during storage consisted of Lactic Acid Bacteria (LAB) (6.7 - 8.1 log cfu/g), Enterobacteriaceae (≤5 - 6.9 log cfu/g), and *Vibrio* spp. (<5 - 6.1 log cfu/g). No or very weak off-odours developed in products with Enterobacteriaceae counts ≤ 5 log cfu/g. Off-odours of varying strength and nature developed in roe with higher Enterobacteriaceae counts. Occasionally high counts (≥ 8 log cfu/g) of LAB also occurred in spoiled roe.

The present study reports work carried out in order to further characterise the microflora of five roe batches with special emphasis on evaluating the spoilage potential of the organisms. LAB (70 isolates) and Enterobacteriaceae (30 isolates) are classified on the basis of biochemical testing to reveal differences in flora composition of roe batches with different sensory characteristics. The ability of selected LAB, Enterobacteriaceae and *Vibrio* isolates to produce off-odours and volatile sulphur compounds, when inoculated in lightly salted lumpfish roe, is examined.

Materials and methods

Bacterial isolates

The bacteria examined in the present study were isolated from five batches of lightly salted lumpfish roe stored at 5°C for three months (Basby et al. 1997b) (table 1). The isolates were taken from blood-agar with 3 % salt and APT-agar (aerobic and anaerobic), as described by Basby et al. (1997b). All plates were incubated at 10°C for 6 days.

Prior to isolation the colonies were differential counted giving one count for each morphological type of colonies. A primary agar plate was chosen with no more than 250 colonies, from which isolation was carried out. For comparison among agars, three colonies of each observed type were taken from blood-agar (3% salt) and aerobic APT plates, while one was taken from the anaerobic APT plates. No influence on counts occurred of type of agar or incubation atmosphere (Basby et al. 1997b). All isolates were included in the present study, and the counts presented are those obtained on blood-agar (3 % salt).

The basic characteristics of the isolates were:

70 LAB: Gram-positive, catalase- and oxidase-negative, fermentative rods and cocci.

30 Enterobacteriaceae: Gram-negative, catalase-positive, oxidase-negative, fermentative rods. 8 *Vibrio* spp.: Gram-negative, catalase- and oxidase-positive rods, sensitive to vibriostaticum O/129 (150 µg). The origin of isolates are shown in table 1.

Table 1. Characteristics of five batches of vacuum packed lightly salted lumpfish roe (4 % WPS, pH 5.4), from which 108 isolates were taken. The roe had been stored at 5°C for three months (Basby et al. 1997b).

Batch	**1**	**2**	**3**	**4**	**5**
Water phase salt %	4.2	3.7	3.8	3.6	3.8
Odour assessment:					
%-rejection [1)]	10	73	53	43	37
Odour	very weak odours	cabbage-like, NH_3-like, fruity, rotten	sour, marinated, fruity, rotten	as batch 3, but weaker	as batch 3, but weaker
Viable counts, log cfu/g [2)]					
Total viable counts,	7.0	7.3	7.3	7.1	7.3
LAB	6.9	7.0	7.2	6.7	7.1
Enterobacteriaceae	≤ 5	6.9	6.5	6.9	6.6
Vibrio spp.	6.1	≤ 5	6.1	≤ 5	5.5
Isolates:					
LAB	F28 1-2, F29 1-3, F32 1-3, F33 1-3, 34, F35	F36 1-3, F37 2-3, F41 1-2, F42 1-3, F44, F45	F 46 1-3, F48 1-3, F54 1-3, F57, F58, F61 1-3	F63 1-3, F64 1-2, F66 1-2, F68, F69, F70, F83 1-2, 84 1-3, F85 1-2	F 72 1-3, F73 1-3, F76 1-3, F77 1-3, F80, F81
Enterobacteriaceae	-	F38 1-3, F39 1-3, F40-1, F43	F49, F50, F52 1-3, F53 1-3, F55, F56	F59 1-3, F65-1, F67	F71 1-3, F75-1, F78, F79, F82
Vibrio spp.	F30, F31	-	F47 1-3, F51	F62	F74

1) A %-rejection of more than 50 is regarded as rejection of the roe. 2) Blood agar with 3 % salt at 10°C.

Characterisation and grouping of isolates

The strains isolated from different morphological types of LAB colonies and the different Enterobacteriaceae colonies by Basby et al. (1997b), were further tested following the procedures described below.

LAB isolates were cultured in All Purpose Tween (APT) broth (Difco 0655-01-7) and on-APT agar (APT broth and 13 g/l agar). Gram-negative isolates were cultured in Tryptone Soya Broth (TSB) (Oxoid CM 129) and on TSB agar (TSB and 13 g/l agar). Long term kept cultures were frozen at ÷80°C as described by Gibson and Khoury (1986).

Unless otherwise stated, the incubation temperature in the tests described below was 25°C.

LAB isolates were examined as follows: Gas production from glucose was tested in De Man-Rogosa-Sharpe (MRS) broth (De Man et al. 1960) without citrate, using Durham vials, that were observed for build up of gas after four and seven days.

Argininedihydrolase was tested in MRS broth containing 3 mg/ml arginine with and without the addition of 20 mg/ml glucose (Schillinger and Lücke 1987). Ammonia was detected after 48 hours of incubation, mixing two drops of culture fluid with two drops of Nesslers reagent (Bie & Bernsen, Denmark) (Merck's Reagenzien Verzeichniss 1929). Formation of orange/rust-coloured precipitate was reported as a positive reaction.

Fermentation of carbohydrates was determined in micro-well plates (InterMed, Nunc, Roskilde, Denmark) using a method described by Jeppesen and Huss (1993) modified after Jayne-Williams (1975). The test sugars were added to the wells as filter sterilised solutions to a final concentration of 0.02 M. Five ml of basic medium (MRS without glucose and meat extract but with 0.005 % chlorophenol red as indicator) was inoculated with 50 µl of a 24 hours culture in APT broth and 200 µl of this mixture were added to the wells. The wells were covered with 50 µl sterile paraffin oil. Change in colour from red to yellow was observed after 2 and 6 days. Fermentation of the following substrates were tested: cellobiose, galactose, lactose, maltose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, trehalose and xylose.

Hydrogensulphide production was tested in modified lead acetate agar described by Shay and Egan (1981) and with 0.8 % cystein added as filter sterilised solution (J.Tidemand, personal communication). The test was done in vials, and the agar covered with sterile paraffin oil before incubation at 5°C for up to 5 weeks. Readings were done once a week as: - negative, + positive (blackening around stitch), ++ strongly positive (also blackening of most of the agar).

Growth on acetate agar (Rogosa et al. 1951) was visually observed by surface inoculation and incubation in 5 % CO_2-atmosphere for 5 days.

Growth at 45°C was visually observed in MRS-broth after incubation for 4 days in a water-bath.

Enterobacteriaceae isolates were tested using API 20E identification system (bioMerieux, France). Single reactions were evaluated following the instructions of the manufacturer. Hydrogensulphide production at 5°C was additionally tested by the same method as used for LAB isolates.

Positive and negative results of the biochemical testing of LAB and Enterobaceriaceae were scored 1 and 0 respectively. The data were analysed using the NT-SYS-pc software package (Rohlf 1993). Similarity between strains were analysed using the simple matching coefficient (S_{SM}), and dendrograms were constructed using the unweighed pair group method with averages (UPGMA) arithmetic algorithm.

Inoculation of roe

The ability of selected bacterial isolates to produce off-odours was examined by inoculation into lightly salted pasteurised or fresh sterile roe.

Roe with approximately 4 % WPS prepared by the normal industrial desalting method (Abba Seafood A/S, Denmark). The pH was lowered from around 5.9 to 5.4 by addition of lactic acid (i.e. 0.1 % (w/w)) (PURAC®SP 80, Nordisk Droge Handel A/S, Denmark). The roe was packed in glass jars containing 350 g. The jars were closed with twist-off caps under 65 - 75 % vacuum and heated in an autoclave (72°C in the centre for 10 minutes).

Fresh, sterile roe was obtained from newly caught live lumpfish. The fish were killed by cutting the neck, and allowed to bleed. The belly was skin washed in 5 % $NaCO_3$ (Herbert et al. 1971) and then in 70 % ethanol, this was repeated twice to remove the slime. The skin was disinfected using 2 % of formaldehyde (Herbert et al. 1971) and allowed to dry in a flow-bench. The skin and roe sacks were cut open with a sterile knife and the roe removed using sterile spoons. Heat sterilised salt and filter sterilised lactic acid solution were added to approximately the same pH and salt concentration as the pasteurised roe. The salted roe was then packed in sterile glass jars containing 100 g.

Before inoculation into pasteurised or sterile roe, Gram-negatives were cultured in Tryptone Soya Broth (TSB) for 48 hours and LAB in APT-broth for 72 hours. Incubation temperature was 10°C. For both types of roe, the jars were inoculated with 1 ml/100 g roe of a 10^{-2} dilution in 4 % saline of the broth culture, to obtain an inoculum of 4 - 5 log cfu/g roe. After inoculation and mixing the jars were closed under 65-75% vacuum, non-inoculated jars were included as controls. All jars were stored at 5°C.

Odour assessments of inoculated roe

Odours of inoculated roe were assessed by a panel of 8 or 9 instructed persons. Off-odours were assessed as none, weak, distinct or strong compared to the non-inoculated controls, and odours were described in words. An overall assessment of the roe as either "acceptable" or "rejected" was given by each panellist. Rejection by more than 50 % of the judges was regarded as rejection of the inoculated roe.

Microbiological analysis of inoculated roe

A sample of 10 g of roe was used for spread plating after dilution. The samples were diluted ten-fold with peptone saline (PS) (0.9 % NaCl, 0.1 % peptone (Difco 0120.01)) and homogenised for 30 seconds in a Stomacher lab-blender 400. Further ten-fold dilution were made in PS before spread plating on TSB-agar (roe inoculated with Gram-negatives and non-inoculated controls) or on APT-agar (roe inoculated with LAB). The plates were incubated at 10°C for 6 days, and checked for purity. The correct identity of inoculated strains causing rejection, was after the experiment confirmed by repeating the biochemical tests earlier performed.

Chemical analysis of inoculated roe

Salt concentration (% w/w) of the roe was calculated from the amount of chloride as determined in duplicate by boiling in nitric acid with excess of $AgNO_3$ followed by titration with 0,1 M KSCN solution (AOAC 1995, no. 937.09).

Dry matter content (DM) (% w/w) of the roe was determined in duplicate by weighing before and after drying at 105°C in 20-24 hours.

Volatile sulphur compounds were determined using the method described by Dalgaard et al. (1993). Ten grams of sample were kept in a 50 ml headspace bottle at 30°C in a water-bath for 30 minutes before 3 ml of headspace was injected into the gaschromatograph.

Results

Characterisation and grouping of isolates

The isolates were grouped according to their similarity, before spoilage potential of different groups could be examined. On the basis of biochemical testing, the 70 LAB isolates could be divided into three groups (A, B and C) separated at 70 % level or lower, and into four subgroups (A1 and A2, B1 and B2) at 86 % level or lower (figure 1). The 30 Entero-bacteriaceae isolates grouped into three groups (1, 2 and 3) separated at 77 % level or lower (figure 2). The isolates of groups A1 and A2 consisted of coccoid bacteria forming no tetrads but most often long chains. The isolates grew at 10°C but not at 45°C and produced no gas (table 2). On this basis the isolates were presumptively identified as *Lactococcus* spp. (Axelsson 1993, Schillinger and Lücke 1987). The presumptive identification was however very much dependent on the cells being cocci.

Groups B1, B2 and C consisted of clearly rod shaped LAB, which were unable to grow on acetate agar (table 2). On this basis these strains were presumptively identified as *Carnobacterium* spp. (Hammes et al. 1992, Schillinger and Holzapfel 1995). Group C however only shared a similarity of 60 % with groups B1 and B2 (figure 1). Group B1

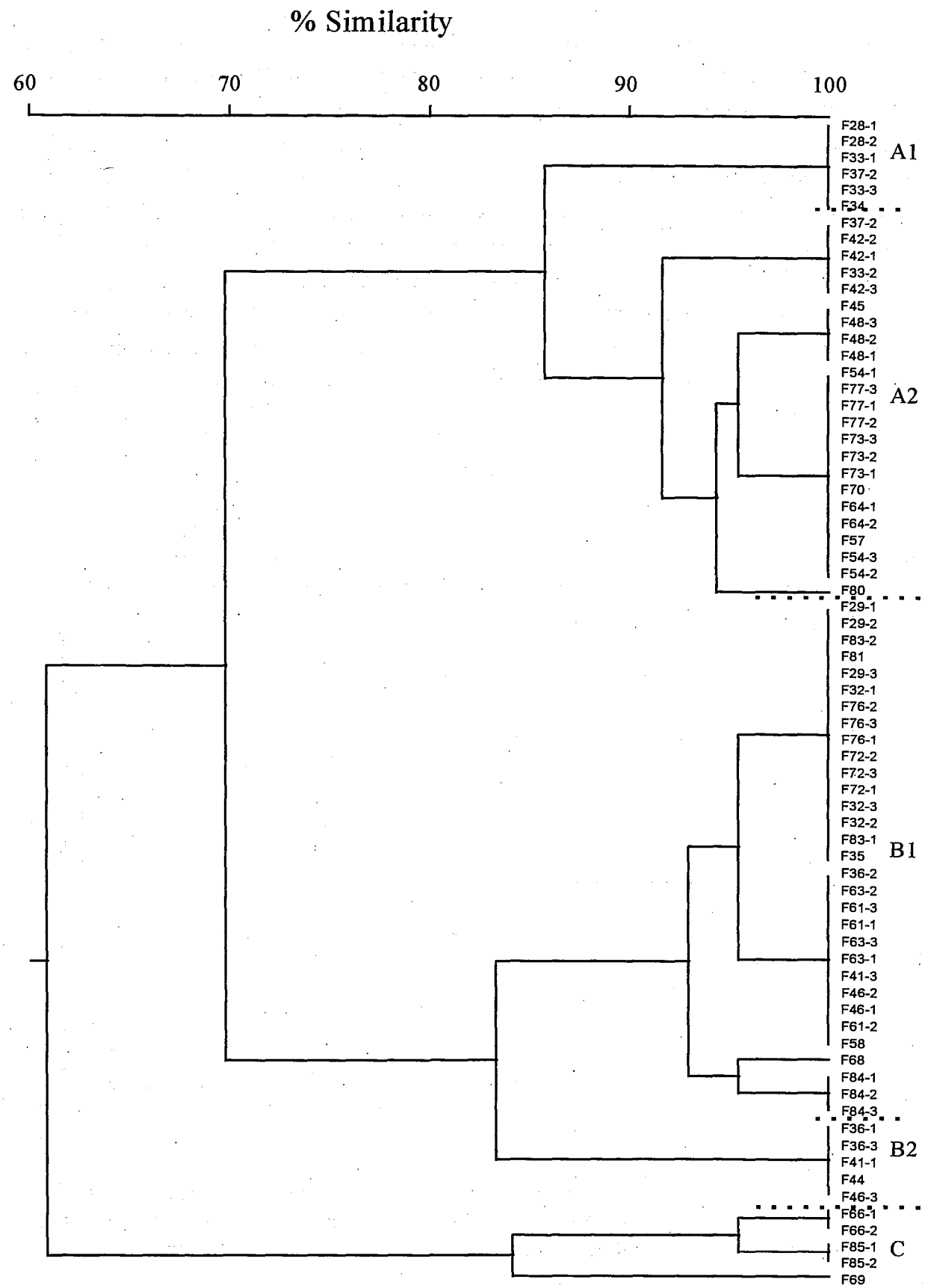

Figure 1. UPGMA dendrogram of the % similarity as determined by the simple matching coefficients (S_{SM}) based on 22 characteristics of 70 Lactic Acid Bacteria strains, isolated from vacuum packed lightly salted lumpfish roe (app. 4 % WPS, pH 5.4) after three months of storage at 5°C.

isolates shared some characteristics with *Carnobacterium divergens* (but all strains were positive in acid production from mannitol) and group B2 with *Carnobacterium piscicola.* According to Schillinger and Holzapfel (1995), all *Carnobacterium* spp. are negative in acid production from raffinose, while Hammes et al. reported some *Carnobacterium piscicola* to be positive, as were the strains of group B2. The group C isolates were motile as are *Carnobacterium mobile, funditum* and *alterfunditum* (Hammes et al. 1992, Schillinger and Holzapfel 1995). None of the isolates could however be identified at species level.
The Enterobacteriaceae strains of group 1 were presumptively identified as different *Serratia liquefaciens* (table 3). The strains of group 2 differed clearly by being negative for lysine and ornithine decarboxylase, and were presumptively identified as different *Serratia plymuthica* (Brenner 1984, Grimont and Grimont 1992). An incubation temperature of 36°C

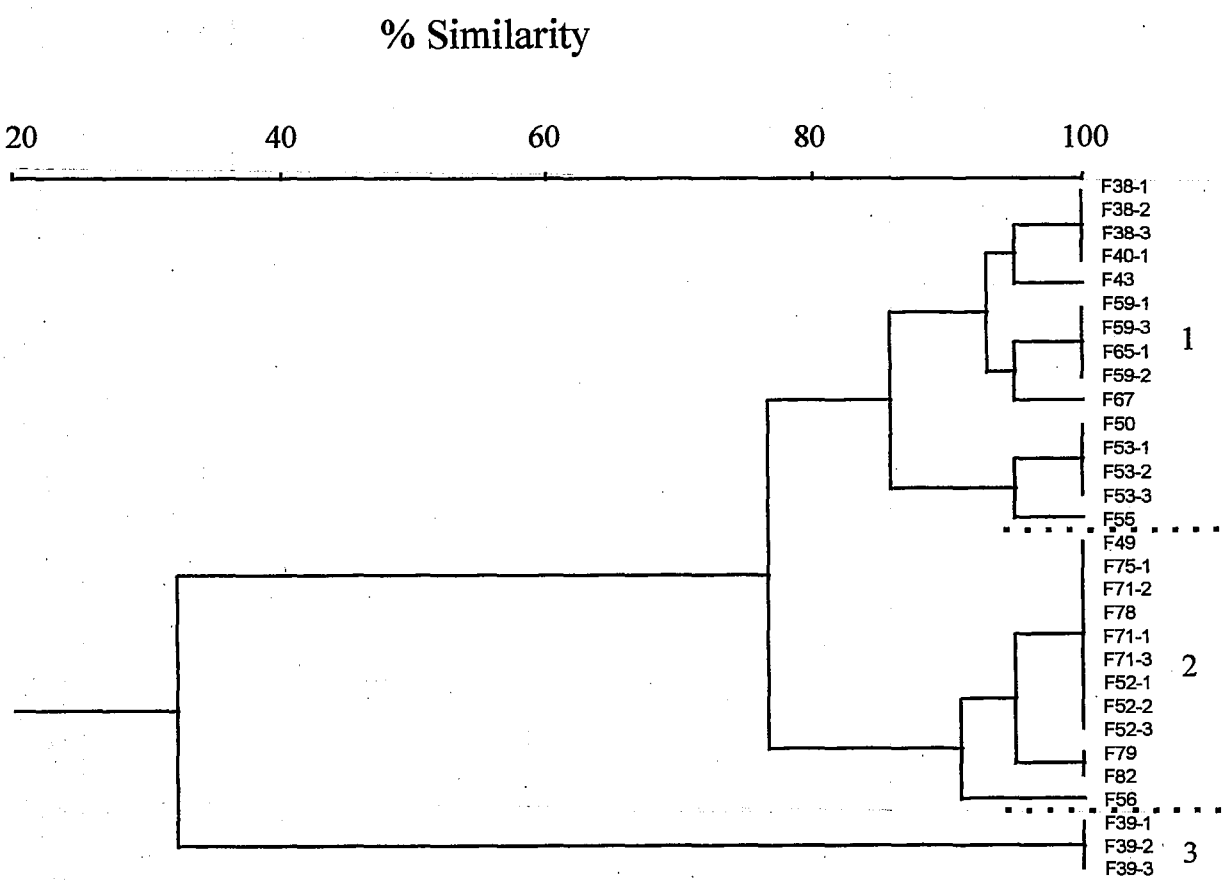

Figure 2. UPGMA dendrogram of the % similarity as determined by the simple matching coefficients (S_{SM}), based of 20 characteristics of 30 Enterobacteriaceae strains, isolated from vacuum packed lightly salted lumpfish roe (app. 4 % WPS, pH 5.4) after three months of storage at 5°C.

was used in the testing of *Serratia* spp. reported by Brenner (1984), and 30°C is in general recommended for *Serratia* spp. by Grimont and Grimont (1992). In the present study testing was carried out at 25°C, as some of the isolates did not grow well even at 30°C. An isolate of group 3 was at Statens Seruminstitut, Copenhagen, Denmark identified by further testing as *Morganella morganii* (subsp. *sibonii*).

Table 2. Results of biochemical testing of 70 Lactic Acid Bacteria (%-positive isolates), isolated from vacuum packed lightly salted lumpfish roe (app. 4 % WPS, pH 5.4) after three months of storage at 5°C. Grouping of isolates originates from figure 1. [1)]

LAB group	**A1**	**A2**	**B1**	**B2**	**C**
Number of strains	6	23	31	5	5
Cell morphology	cocci	cocci	rods	rods	rods
Arginine dihydrolase [2)]	0	0	100	100	0
H_2S production	100	17 [3)]	87	100	0
Motility	0	0	0	0	100
Growth on acetate agar	100	100	0	0	0
Cellobiose	100	100	100	100	80
Galactose	100	100	0	100	0
Lactose	0	96	0	100	0
Mannitol	100	100	100	100	0
Meletzitose	0	0	55	0	100
Melibiose	0	0	0	0	20
Raffinose	0	0	0	100	20
Ribose	100	100	100	100	0
Sucrose	0	39	100	100	0
Trehalose	0	100	100	100	100
Xylose	0	0	0	0	40
Presumptive identification	*Lactococcus* spp. (A1, A2)		*Carnobacterium* spp. (B1, B2, C)		

1) All isolates were negative in: gas production, tetrad formation, growth at 45°C, acid production from rhamnose, and positive in: growth at 10°C and acid production from maltose and salicin. 2) Results were identical regardless of glucose addition. 3) No or very weak growth by a majority of strains.

The distribution among roe batches of the different groups of LAB and Enterobacteriaceae is shown in table 4. The results are individual counts of morphologically different colonies on blood-agar with 3 % salt. The biochemical testing and grouping of strains confirmed, that morphological identical isolates belonged to similar biochemical groups (A1 - C or 1 - 3). Thus the results shown in table 4 is the flora composition of the five roe batches, presented as individual counts of biochemical different LAB and Enterobacteriaceae.

Table 3. Results of biochemical testing of 30 Enterobacteriaceae (%-positive isolates), isolated from vacuum packed lightly salted lumpfish roe (app. 4 % WPS, pH 5.4) after 3 months of storage at 5°C. Grouping of isolates originates from figure 2. [1)]

Enterobacteriaceae group	**1**	**2**	**3**
Number of strains	15	12	3
ONPG	100	100	0
Lysine decarboxylase	100	0	0
Ornithine decarboxylase	100	0	0
Citrate utilisation	100	25	0
Urease	0	0	100
Tryptophane desaminase	0	0	100
Indole	0	0	100
VP	87	92	0
Gelatinase	67	0	0
Mannitol	100	100	0
Inositiol	67	0	0
Sorbitol	100	100	0
Rhamnose	33	0	0
Sucrose	93	100	0
Melibiose	100	100	0
Amygdalin	100	100	0
Arabinose	100	100	0
Presumptive identification	*Serratia liquefaciens*	*Serratia plymuthica*	*Morganella morganii* [2)]

1) All isolates were motile and strongly positive in hydrogensulphide production in modified lead acetate agar at 5°C. All isolates were negative in: Arginine dihydrolase and hydrogensulphide production from thiosulphate.
2) Identification based on further testing at Statens Seruminstitut, Copenhagen, Denmark.

Table 4. Different LAB and Enterobacteriaceae (log cfu/g) in five batches of vacuum packed lightly salted lumpfish roe (app. 4 WPS, pH 5.4) after three months of storage at 5°C. (Blood-agar with 3 % of salt at 10°C).

Batch	**1**	**2**	**3**	**4**	**5**
Lactic Acid Bacteria:					
A1. *Lactococcus* sp.	6.7	-	-	-	-
A2. *Lactococcus* sp.	-	6.0	6.5	6.3 [1)]	7.0
B1. *Carnobac.* sp.	6.5	[2)]	7.1	6.5	6.3
B2. *Carnobac.* sp.	-	7.0	-	-	-
C. *Carnobac.* sp.	-	-	-	5.7	-
Enterobacteriaceae:					
1. *Ser. liquefaciens*	-	6.8	5.7	6.9	-
2. *Ser. plymuthica*	-	-	6.4	-	6.6
3. *Morg. morganii*	-	6.4	-	-	-

1) Group A2 was not recovered from Blood-agar, the count originates from APT-agar.
2) Group B1 present in low numbers, one single isolate was obtained from the ÷5 dilution.

Spoilage potential

In order to assess the ability of strains to produce off-odours and volatile sulphur compounds, pasteurised roe (3.6 % WPS) was inoculated with 5 LAB (one of each of the groups A1, A2, B1, B2 and C), 7 different Enterobacteriaceae (three of the groups 1 and 2 and one of group 3) and 3 *Vibrio* spp. (inoculation level of all strains 4 - 5 log cfu/g). Only *Morganella morganii* caused rejection the pasteurised roe. After one week at 5°C, roe inoculated with *Morganella morganii* (F39-1) was rejected due to off-odour by 63 % of the panellists. The counts were 5.8 log cfu/g, and the off-odours were described as sulphidy, faecal and green/decaying grass. The GC-analysis revealed only very small amounts of volatile sulphur compounds. The analysis was repeated, when the roe with *Morganella morganii* was heavily spoiled (%-rejection of 100 and counts of 7.4 log cfu/g). Significant amounts of methanethiol, dimethyl-disulphide or 1,2 ethanedithiol and an unidentified larger compound (retention time 18.01 min.) was detected, and off-odours were now described as sulphidy, cabbage-like, faecal and sewer-like. Only trace amounts of volatile sulphur compounds could be detected in non-inoculated controls (data not shown).
Jars inoculated with the remaining 14 strains were stored for 103 days in total, and the odour was assessed frequently. The presumptive *Carnobacterium* spp. of group B1 and B2 both produced weak off-odours described as sour, sweet, malty, bread and fruity, after two weeks of storage, but the roe was still judged acceptable (%-rejection 40). The odours did not develop or increase through further storage. The remaining isolates produced even weaker off-odours, which were not stable with time. Counts of most strains within the first 15 days reached a level of 6.3 - 7.9 log cfu/g (data not shown). *Lactococcus* sp. of group A1 and A2 grew poorer (max. 5.8 and 6.0 log cfu/g respectively), while two *Serratia* sp. (group 1 and 2) reached counts of 8.3 log cfu/g. After 103 days counts of all strains were still between 5.8 and 7.3 log cfu/g. Throughout the experiment the total viable counts of non-inoculated roe remained below 4 log cfu/g.

Due to the low incidence of off-odour production among strains, lightly salted roe made from fresh sterile roe was inoculated in order to avoid the heat treatment of the roe.
A low level of contamination (<1 log cfu/g) occurred in several cases, developing into a high level during storage. Therefore only a few results are available, which are shown in table 5. *Morganella morganii* (F39-1) produced off-odours of sulphidy and cabbage-like nature. After one week the roe was rejected, but the odours were transient. After 2 weeks the spoilage was unquestionable, and three volatile sulphur compounds had been produced in significant amounts.

Table 5. Development of off-odours in vacuum packed lightly salted lumpfish roe (pH 5.4) made from fresh sterile roe, inoculated with three Enterobacteriaceae and stored at 5°C.

Strain	Start		1 week		2 weeks			
	Salt % WPS	Inoculum log cfu/g[1)]	log cfu/g	% rejection[2)]	log cfu/g	% rejection	Off-odours	Volatile sulphur compounds
Serratia liquefaciens F38-1	3.3	5.3	6.0	13	6.5	33		None
Morganella morganii F39-1	3.0	5.0	6.3	63	7.3	89	Cabbage-like, sulphidy, faecal, sewer-like	- CH_3SH [3)] - CH_3SCH_3 or CH_3CH_2SH [4)] - CH_3SSCH_3 or $HS(CH_2)_2SH$ [4)]
Serratia liquefaciens F50	3.5	4.8	8.0	75	8.0	67	Sour, sulphidy, faecal	[5)]

1) Analysed value.
2) Roe with F39-1 and F50 rejected at first impression, the odours disappeared very quickly.
3) Similar compounds detected in two determinations.
4) Retention times of two standards very close, identity inconclusive.
5) Very small amounts of an unidentified compound, retention time 25.5 min.

Serratia liquefaciens (F50) also produced transient off-odours after one week. After two weeks, the roe was rejected due to sour, sulphidy and faecal off-odours, but only negligible amounts of volatile sulphur compounds could be detected. Weak off-odours of a different kind (sour, marinated, sweet) were produced by the other *Serratia liquefaciens* (F38-1). The roe was not rejected and production of volatile sulphur compounds were not detected in the roe headspace. The non-inoculated roe remained neutral judged by odour after 14 days of storage, and only trace amounts of volatile sulphur compounds were detected. The salt concentrations were lower than expected, due to difficulties foreseeing the dry matter content of the roe of the different lumpfish.

Discussion

Further characterisation in the present study of LAB and Enterobacteriaceae from lightly salted lumpfish roe isolated by Basby et al. (1997b) showed, that the LAB flora consisted of two groups of presumptive *Lactococcus* spp., and three groups of *Carnobacterium*-like organisms. None of the strains however fitted directly into a species identification. Strains of one group (C) were motile, and differed from strains of the other two groups (B1 and B2) in

a majority of performed tests, resulting in a similarity of only 60 %. Further examination is necessary for proper identification of the isolated LAB strains.

The majority of Enterobacteriaceae isolates were presumptively identified as *Serratia liquefaciens* or *plymuthica.* Both species occur in the natural environment, *Serratia plymuthica* preferably in water (marine and fresh), and can easily be introduced into foods (Grimont and Grimont 1992). *Morganella morganii*, detected in one roe batch, is known in association with fish as a histamine producer likely to be added during handling (Liston 1992).

By comparison of flora composition and off-odours among five roe batches, it was noted that: In roe with Enterobacteriaceae counts $\leq$ 5 log cfu/g, off-odours were not produced (batch 1). Roe with 6.5 - 6.9 log cfu/g Enterobacteriaceae consisting of *Serratia liquefaciens* and/or *Serratia plymuthica*, were close to borderline (37 - 53 % rejection), and off-odours were described as sour, marinated, fruity and rotten (batch 3 - 5). Roe with 6.4 log cfu/g *Morganella morganii* was heavily spoiled due to cabbage-like, ammonia-like, fruity and rotten off-odours (batch 2) (table 1).

Morganella morganii was the only isolate tested in this study, that caused rejection of both pasteurised and fresh roe at 5°C, when inoculated in pure culture. Cabbage-like, sulphidy, faecal and sewer-like off-odours were produced in both types of roe, and volatile sulphur compounds were detected in the headspace of the spoiled roe. Combinations of hydrogensulphide, methanethiol and dimethylsulphide have been shown to be responsible for the sour, sulphidy, cabbage-water odours of stored fish (Herbert et al. 1975), and volatile sulphur compounds are likely to contribute in spoilage off-odours produced by *Morganella morganii.*

The profiles of volatile sulphur compounds produced in the two types of inoculated roe were not identical. Methanethiol and dimethyldisulphide/1,2 ethanedithiol were produced in both roes, dimethylsulphide/ethanethiol only in the fresh roe, and an unidentified larger compound only in the pasteurised roe. There can be many explanations for the different end products observed, like small changes in growth conditions including different salt concentrations, different storage times and differences in available substrates including unknown effects of the pasteurisation. Methanethiol and dimethylsulphide/1,2 ethanedithiol were also detected in naturally contaminated roe products by Basby et al. (1997b).

Off-odours, though transient, were recognised before volatile sulphur compounds could be detected, and other volatile compounds are likely to contribute to the spoilage by *Morganella morganii.* Dainty (1996a) mentioned some unpublished results, where a *Morganella morganii* was shown to cause unusual spoilage of vacuum-packed ham due to production of hydrogensulphide and indole. Indole was also by Giaccone et al. (1994) suggested as indicator of sensory and hygienic quality of bovine tripe. Due to the odour of inoculated roe described as faecal and sewer-like among other words it can be suggested, that indole is included in future studies on spoilage of lightly salted lumpfish roe by *Morganella morganii.*

The counts of *Morganella morganii* at spoilage of inoculated roe were 6 - 7 log cfu/g. Thus at the same level as observed in naturally spoiled roe (6.4 log cfu/g, table 4), and the off-odours produced were of the same nature. It can be concluded, that *Morganella morganii* is a spoilage organism of lightly salted lumpfish roe, producing very offensive odours.
Serratia liquefaciens have been shown to be among the most common Enterobacteriaceae of vacuum-packed DFD meat, and to be able to produce off-odours as reviewed by Dainty et al. (1983). Borch et at. (1996) reported production of slightly sulphurous, acid and fruity off-odours by *Serratia liquefaciens* inoculated into vacuum packed high pH meat at 4°C. In the present study the *Serratia liquefaciens* F50 caused rejection of the fresh roe only, but at counts as high as 8 log cfu/g not observed in the naturally contaminated products (table 4). Though the word sulphidy was used to describe the odours, only small amounts of an unidentified compound (retention time 25.5 min.) were detected, and this compound was not observed in naturally spoiled roe (Basby et al. 1997b). Another *Serratia liquefaciens* (F38-1) produced week off-odours of a different nature (table 5). Since the counts of this organism were lower, it cannot be estimated whether the results were due to regular differences between the two strains regarding their spoilage potential (separated at 86 % level, figure 2). Volatile compounds responsible for off-odours produced by *Serratia liquefaciens* in lightly salted lumpfish roe are yet to be identified.
Two presumptive *Carnobacterium* spp. (group B1 and B2) produced weak non-sulphidy off-odours in pasteurised roe, that did not cause rejection at counts comparable to those of the naturally contaminated products. Due to contamination problems in sterile fresh roe, LAB were only tested in pasteurised roe. The role in spoilage by these bacteria needs further investigation. High (8 log cfu/g) counts of LAB were noted by Basby et al. (1997b) in two spoiled roe batches with Enterobacteriaceae counts below 6 log/cfu/g. It is also possible that LAB contributed to spoilage odours, when growing together with Enterobacteriaceae. Borch et al. (1996) reported that a typical spoilage off-odour developed in sterile vacuum-packed meat (4°C), when a LAB mixture were inoculated together with *Hafnia alvei*. Off-odours of a different nature developed when the LAB mixture was inoculated alone.
The tested *Vibrio* isolates produced no off-odours in pasteurised roe. *Vibrio* spp. constituted a minor part of the flora of stored lumpfish roe. Highest counts (6.1 log cfu/g) were observed by Basby et al. (1997b) in a batch of roe without off-odours after three months. It can be concluded, that *Vibrio* spp. is unimportant in spoilage of lightly salted lumpfish roe.
Basby et al. (1997b) detected hydrogensulphide after storage in several naturally contaminated roe batches. In the present study hydrogensulphide was not produced in the roe model systems by the inoculated Enterobacteriaceae. Though both some LAB and *Vibrio* spp. could have contributed to the hydrogensulphide production observed by Basby et al. (1997b) (Shay and Egan 1981, Gram et al. 1987), hydrogensulphide production by the Enterobacteriaceae was indeed expected. Most Enterobacteriaceae are able to produce hydrogensulphide from cysteine (Cowan 1974). All the Enterobacteriaceae isolated in this

study were strongly positive when tested in modified lead acetate agar with thiosulphate and cysteine at 5°C, and also unable to produce hydrogensulphide from thiosulphate (table 3). The cysteine concentration of the roe is low (Basby et al. 1997a), and it is possible that insufficient amounts of this precursor were present in the two types of inoculated roe. During the long storage period of normal products, autolytical liberation of cysteine could occur, as reported for heavily salted roe by Basby et al. (1997a). This is obviously not possible after pasteurisation, and regarding the fresh roe, the storage period of two weeks was much shorter than in natural spoilage.

Thus in conclusion, when growing in lightly salted lumpfish roe, *Morganella morganii* acted as a spoilage organism, producing off-odours, volatile sulphur compounds and most likely other volatile compounds. *Serratia liquefaciens* produced spoilage odours, but the volatile compounds responsible, were not identified. Spoilage by *Serratia plymuthica* and LAB were not demonstrated, but their role in off-odour production needs further investigation. This includes the significance of interactions between LAB and Enterobacteriaceae.

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Note 1. Detailed chemical data on fresh and salted lumpfish roe.

In this note results on various chemical analyses of roe are presented, that were not found appropriate to incorporate in details in the text. Results shown in table 1 - 6 are discussed in paper 1 and chapter 3 (materials and methods are described in paper 1). Results shown in table 7 and 8 are discussed in paper 2 and chapter 4 (materials and methods are described in paper 2).

Table 1. Proximate composition (mean ± standard deviation, double determinations) of fresh roe of different catching locations, stored heavily salted roe of different catching seasons/ storage times and lightly salted lumpfish roe.

Roe	Salt % w/w	Moisture % w/w	Lipid % w/w	Protein [1] % w/w	Ash % w/w
Fresh roe					
Denmark [2]	0.9±0.04	82.4±0.4	5.4±0.1	12.3±0.9	1.0±0.04
	0.9±0.03	81.4±0.3	5.9±0.1	14.5±1.0	1.0±0.01
	1.0±0.01	78.7±0.6	4.9±0.1	17.4±2.1	1.1±0.01
	0.9±0.01	81.3±2.3	5.9±0.1	18.0±1.1	1.0±0.05
	1.0±0.00	81.1±1.6	5.4±0.1	15.0±1.7	1.0±0.04
Canada [3]	1.0±0.0	77.5±0.2	5.8±0.1	15.2±0.8	1.1+0.1
	0.9±0.0	77.3±0.2	6.4±0.5	15.8±0.2	1.1±0.0
Heavily salted roe					
Actual season [4]	12.9±0.7	67.3±0.1	5.0±0.1	13.5±0.2	14.0±0.2
	13.0±1.0	68.4±0.3	4.1±0.2	12.6±0.3	13.3±0.3
	12.9±0.5	66.7±0.1	4.3±0.1	13.0±0.2	14.2±0.3
Previous season [5]	11.2±1.8	68.2±0.1	5.1±0.3	14.1±0.3	12.0±0.1
	11.9±0.5	68.5±0.4	5.2±0.0	14.4±0.1	12.9±0.0
	11.2±0.3	68.5±0.4	5.0±0.3	15.1±0.6	12.0±0.0
Lightly salted roe					
Previous season [6]	4.8±0.3	77.1±0.7	5.1±0.5	13.2±0.1	4.7±0.0
	4.9±0.5	77.1±0.3	5.0±0.1	13.5±0.5	4.5±0.0
	4.8±0.1	79.1±0.8	4.8±0.0	13.2±0.3	4.5±0.1

1) Crude protein, N x 6.25. 2) Roe of five individual lumpfish. 3) Screened roe from two geographic locations. 4) Stored for two months at ÷5°C, three barrels (105 kg). 5) Stored for one year at ÷5°C, three barrels (105 kg). 6) Made by desalting of three barrels stored for one year.

Table 2. Fatty acid composition (% w/w of total fatty acids) [1] of fresh and lightly salted lumpfish roe (made by desalting of heavily salted roe stored for 8 months at ÷5°C).

Fatty acid	Fresh roe [2]					Lightly salted roe [3]									
14:0	1.4	1.4	1.5	1.1	1.2	1.5 [1]	1.5	1.6	1.6	1.4	1.5	1.3	1.5	1.5	1.5
15:0	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
16:0	13.8	14.1	14.3	15.0	14.6	14.3	14.4	14.3	14.3	14.3	14.6	14.4	14.4	14.5	14.4
17:0	0.4	0.5	0.5	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.4	0.3	0.4	0.3	0.3
18:0	3.3	3.3	3.1	3.2	3.3	3.7	3.7	3.6	3.7	3.8	3.8	3.9	3.8	3.7	3.8
Σ	19.3	19.7	19.8	20.0	19.8	20.1	20.2	20.1	20.2	20.1	20.6	20.2	20.4	20.3	20.3
14:1 (n-5)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
16:1 (n-7)	1.5	1.3	1.5	1.5	1.4	2.0	2.3	2.1	2.1	2.2	2.1	2.0	2.1	2.1	2.0
18:1 (n-9)	16.6	16.3	14.7	16.6	15.3	14.3	15.1	14.0	14.0	15.2	14.2	15.4	14.2	14.5	14.5
18:1 (n-7)	2.9	2.5	3.0	3.4	3.0	4.8	4.9	4.8	4.8	4.8	4.8	4.9	4.8	4.8	4.8
20:1 (n-11)	0.3	0.6	0.6	0.2	0.3	0.1	0.3	0.2	0.2	0.1	n.d.[4]	0.2	n.d.	n.d.	0.1
20:1 (n-9)	3.1	3.1	2.5	2.6	3.0	2.1	2.5	2.1	1.9	2.4	2.2	2.2	2.2	2.2	2.1
20:1 (n-7)	0.4	0.4	0.6	0.4	0.5	0.7	0.8	0.7	0.7	0.6	0.7	0.6	0.7	0.7	0.7
22:1 (n-11)	0.8	0.9	0.6	0.4	0.6	0.4	0.5	0.4	0.4	0.5	0.4	0.4	0.4	0.4	0.4
22:1 (n-9)	0.5	0.5	0.6	0.4	0.4	0.4	0.5	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
24:1	0.1	0.2	0.3	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.3	0.2	0.2
Σ	26.3	25.9	24.5	25.7	24.7	25.1	27.2	25.0	24.8	26.5	25.0	26.3	25.2	25.4	25.3
16:2	0.6	0.6	0.6	0.5	0.6	0.5	0.5	0.6	0.5	0.5	0.5	0.4	0.5	0.5	0.5
18:2 (n-6)	1.0	1.1	1.2	0.8	0.9	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
20:2 (n-6)	0.2	0.2	0.3	0.2	0.2	0.2	0.1	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2
Σ	1.8	1.9	2.1	1.5	1.7	1.3	1.2	1.4	1.3	1.2	1.3	1.2	1.3	1.3	1.3
16:3	0.3	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
18:3 (n-3)	0.5	0.6	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Σ	0.8	1.0	0.8	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
18:4 (n-3)	0.6	0.8	0.9	0.5	0.7	0.5	0.6	0.6	0.7	0.6	0.6	0.7	0.7	0.7	0.7
20:4 (n-6)	0.8	0.8	0.8	0.9	0.8	0.5	0.4	0.4	0.5	0.4	0.5	0.4	0.5	0.4	0.4
20:4 (n-3)	0.9	1.0	1.0	0.7	0.8	0.8	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Σ	2.3	2.6	2.7	2.1	2.3	1.8	1.7	1.7	1.9	1.7	1.8	1.8	1.9	1.8	1.8
20:5 (n-3)	17.1	16.4	17.4	18.0	18.3	19.3	19.9	19.7	19.5	19.6	19.8	19.4	19.8	19.7	19.7
21:5 (n-3)	0.3	0.4	0.5	0.3	0.3	0.4	0.5	0.5	0.4	0.4	0.4	0.5	0.4	0.5	0.5
22:5 (n-3)	1.6	1.3	1.3	1.3	1.5	1.6	1.7	1.6	1.7	1.6	1.7	1.6	1.6	1.6	1.6
Σ	19.0	18.1	19.2	19.6	20.1	21.3	22.1	21.8	21.6	21.6	21.9	21.5	21.8	21.8	21.8
22:6 (n-3)	24.7	26.3	26.2	25.9	25.7	24.6	22.9	24.8	25.0	23.9	25.2	24.8	25.0	24.8	25.0
Not id. [5]	5.8	4.5	4.7	4.6	5.1	5.2	4.1	4.6	4.6	4.4	3.6	3.6	3.8	4.0	3.9
Calc. i.v. [6]	245	247	250	248	250	248	242	250	250	245	250	247	249	249	249

1) Mean of two determinations, standard deviations are omitted for clarity, but were < 3% except occasionally higher for fatty acids present at low concentration (< 2% of total fatty acids). 2) Roe of five lumpfish. 3) Ten barrels (105 kg) after desalting . 4) n.d.: Not detected. 5) Not id.: Not identified.
6) Calc. i.v.: Calculated iodine value (g iodine/100 g identified fatty acids).

Table 3. Salt, dry matter and characteristics of the lipid fraction of fresh and lightly salted lumpfish roe (made by desalting of heavily salted roe stored for 8 months at ÷5°C). Results are mean values ± standard deviation (double determinations).

Roe	Salt % w/w	Dry matter % w/w	Lipid % w/w	Free fatty acids, % (w/w) of lipid [1]	Peroxide value meqv./kg lipid	TBA value [2] µmoles/kg roe
Fresh [3]						
	0.9±0.0	17.7±0.4	5.4±0.1	2.5±0.4	n.d. [4]	1.2±0.1
	0.9±0.0	18.6±0.3	5.9±0.1	3.3±0.4	n.d.	5.4±1.2
	1.0±0.0	21.3±0.6	4.9±0.1	3.7±0.9	n.d.	2.9±0.0
	0.0±0.0	18.7±2.3	5.9±0.1	4.3±0.3	n.d.	1.5±0.1
	1.0±0.0	18.9±1.6	5.4±0.1	4.3±0.2	n.d.	2.1±0.1
Lightly salted [5]						
	5.0±0.0	22.4±0.3	4.9±0.1	5.9±0.4	19.4±0.2	26.8±2.1
	5.8±0.0	22.3±0.1	5.1±0.1	4.9±0.2	23.8±0.1	29.1±1.1
	3.0±0.1	21.1±1.9	5.0±0.1	10.8±0.2	31.8±0.9	20.7±0.9
	3.1±0.1	21.3±0.2	5.0±0.0	5.8±0.5	25.0±1.4	21.4±0.4
	3.5±0.1	20.0±0.2	4.7±0.1	4.8±0.6	26.6±0.5	11.9±0.6
	3.0±0.1	22.4±0.1	5.0±0.1	6.9±0.3	25.6±2.3	24.0±0.1
	3.3±0.2	20.6±0.4	4.9±0.1	5.2±0.2	13.6±5.0	15.3±4.3
	3.1±0.1	21.3±0.1	4.9±0.1	6.1±0.1	9.4±3.0	17.0±3.2
	4.6±0.2	22.8±0.7	4.8±0.1	5.8±0.4	13.0±1.0	25.7±1.3
	4.0±0.0	22.7±0.3	4.6±0.2	7.1±0.3	16.2±1.7	24.7±0.6

1) Free fatty acids as oleic acid. 2) TBA = Thiobarbituric acid. 3) Roe of five lumpfish. 4) n.d.: Not detected. 5) Ten barrels (105 kg) after desalting.

Table 4. Composition of total amino acids (% w/w of identified amino acids)[1)] of fresh lumpfish roe and of lightly salted lumpfish roe (made by desalting of heavily salted roe stored for 8 months at ÷5°C).

Amino acid	Fresh roe [2)]					Lightly salted roe [3)]									
Aspartic acid	9,6	9,8	9,6	9,6	9,6	9,5	9,5	9,5	9,5	9,5	9,7	9,6	9,7	9,6	9,7
Threonine	5,5	5,5	5,5	5,4	5,5	5,4	5,5	5,5	5,4	5,4	5,5	5,6	5,5	5,5	5,6
Serine	6,5	6,6	6,6	6,3	6,4	6,0	6,0	5,9	6,1	5,7	6,2	6,0	6,3	6,3	6,3
Glutamic acid	12,8	12,9	12,9	13,1	12,9	13,3	13,2	13,1	13,2	13,2	13,0	13,1	13,0	12,8	12,7
Proline	5,5	5,3	5,3	5,5	5,5	5,8	5,6	5,5	5,7	5,7	5,4	5,6	5,7	5,4	5,2
Glycine	3,0	3,0	3,0	3,1	3,0	3,0	3,1	3,0	3,1	3,0	3,0	3,1	3,0	3,0	3,0
Alanine	5,6	5,5	5,7	5,6	5,6	5,5	5,7	5,8	5,8	5,8	5,6	5,6	5,5	5,9	5,9
Cysteine	1,7	1,1	1,6	1,7	1,6	1,1	1,6	1,4	1,2	1,6	1,0	1,7	1,1	1,1	2,0
Valine	6,6	6,7	6,5	6,7	6,7	7,0	7,0	7,0	7,0	7,1	6,9	6,9	6,9	6,8	6,8
Methionine	2,6	2,6	2,6	2,6	2,6	2,4	2,4	2,5	2,4	2,5	2,5	2,4	2,6	2,7	1,5
Isoleucine	5,4	5,6	5,5	5,5	5,5	5,6	5,5	5,6	5,5	5,7	5,6	5,4	5,6	5,6	5,7
Leucine	9,8	10,0	10,0	9,8	9,8	9,7	9,7	9,9	9,7	10,0	10,0	9,8	9,9	10,1	10,2
Tyrosine	5,1	5,0	4,9	5,0	5,0	5,1	5,1	5,1	5,1	5,0	5,1	5,3	5,1	5,0	5,0
Phenylalanine	4,8	4,7	4,7	4,7	4,6	4,7	4,7	4,8	4,6	4,8	4,8	4,8	4,8	4,7	4,7
Histidine	3,1	3,0	3,0	3,1	3,2	3,2	3,2	3,2	3,3	2,9	3,2	3,2	3,1	3,0	3,1
Lysine	6,5	6,6	6,5	6,3	6,5	6,4	6,3	6,3	6,4	6,2	6,3	6,0	6,2	6,4	6,5
Arginine	6,1	6,2	6,1	6,1	6,1	6,2	6,1	6,1	6,1	6,0	6,2	6,1	6,1	6,2	6,2
Total, mg/g	88,6	87,3	108,7	89,3	94,3	91,6	84,5	90,0	93,4	90,5	102,1	91,9	100,8	99,2	97,3
Taurine, mg/g	0.5	0.6	0.6	0.5	0.6	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.2

1) The chromatogram also contained small amounts of ammonia, oxidized methionine and small unidentified peaks at retention times around 30, 49 and 55 minutes.
2) Roe of five lumpfish. 3) Ten barrels (105 kg) after desalting.

Table 5. Composition of free amino acids (% w/w of identified amino acids)[1] of fresh lumpfish roe and of lightly salted lumpfish roe (made by desalting of heavily salted roe stored for 8 months at ÷5°C).

Amino acid	Fresh roe[2]					Lightly salted roe[3]									
Aspartic acid	11.5	12.7	10.7	8.7	5.4	7.2	6.8	6.7	6.6	7.5	4.6	7.3	7.0	8.0	7.8
Threonine	4.9	5.0	6.6	6.3	4.8	5.1	4.7	5.4	5.5	4.5	4.9	4.9	4.2	4.6	4.7
Serine	4.2	3.6	4.6	4.0	2.9	4.9	6.0	4.8	4.7	4.7	4.8	4.5	4.8	5.0	5.0
Glutamic acid	36.7	32.0	30.6	32.8	29.1	13.0	14.9	13.7	12.6	14.2	16.2	13.1	14.7	14.5	14.6
Proline	3.2	5.0	3.7	5.1	4.4	5.2	4.3	5.8	5.7	3.9	5.6	3.7	4.5	5.2	5.4
Glycine	6.0	4.7	5.6	4.4	7.4	2.2	2.7	2.3	2.3	2.4	1.9	2.0	1.6	1.9	2.0
Alanine	11.5	9.9	12.0	10.5	11.1	6.3	6.7	6.0	6.1	6.4	6.3	5.6	5.8	6.5	6.2
Cyst(e)ine	n.d.[4]	n.d.	2.0	0.9	2.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Valine	2.9	3.6	3.5	4.3	4.2	4.5	4.4	4.5	4.6	4.5	4.7	4.6	4.4	4.8	4.7
Methionine	n.d.	n.d.	n.d.	n.d.	n.d.	0.2	0.1	0.1	0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Isoleucine	1.8	2.0	2.0	2.6	2.3	5.5	5.7	6.6	7.0	7.1	6.6	7.0	6.3	6.4	6.2
Leucine	4.0	4.4	3.9	4.6	4.4	11.0	10.8	10.8	11.3	10.3	10.4	11.4	10.5	11.2	10.9
Tyrosine	1.7	1.9	1.8	2.3	2.2	5.6	5.1	5.9	6.0	5.7	6.7	5.8	5.9	6.1	6.1
Phenylalanine	1.6	1.8	1.7	2.1	1.8	4.3	4.0	4.5	4.5	4.5	4.3	4.7	4.2	4.2	4.2
Histidine	2.6	2.6	2.7	2.3	3.5	3.5	3.0	3.7	3.3	4.5	3.1	4.4	3.0	2.7	2.6
Tryptophane	0.3	1.5	0.5	1.3	1.1	4.1	3.2	4.2	4.5	4.6	3.7	5.5	4.2	3.8	3.9
Lysine	4.2	5.1	4.4	4.4	8.2	8.4	8.8	8.5	8.2	8.2	8.7	8.1	8.8	7.4	7.6
Arginine	3.1	4.2	3.7	3.5	5.1	8.9	8.8	6.7	7.0	7.0	7.5	7.4	10.1	7.7	8.3
Total, mg/100g	21.5	25.9	28.4	24.9	28.2	51.9	51.8	35.9	35.3	14.8	41.5	19.3	34.9	50.8	49.4
Taurine, mg/100g	49.8	50.7	63.1	44.4	57.2	22.0	20.5	14.3	14.4	6.6	13.8	6.7	9.2	16.6	16.1

1) The chromatogram also contained small amounts of ammonia, oxidized methionine, small unidentified peaks at retention times around 36 and 49 min. and a peak at around 55 minutes, possibly ethanolamine. 2) Roe of five lumpfish. 3) Ten barrels (105 kg) after desalting. 4) n.d.: not detected.

Table 6. Total volatile bases (TVB), trimethylamine (TMA) and trimethylamineoxide (TMAO) (mean ± standard deviation, double determinations) of fresh, heavily salted and lightly salted lumpfish roe.

Roe	TVN mgN/100g	TMA mgN/100g	TMAO mgN/100 g
Fresh roe [1]			
Denmark	27.2±1.6	0.7±0.3	3.0±0.5
Canada	33.1±0.3	1.3±0.8	2.9±0.1
Heavily salted roe			
Actual season [2]	24.5±0.5	n.d.	0.4±0.1
	30.0±0.1	n.d.	1.6±0.7
	24.2±0.2	n.d.	n.d.
Previous season [3]	26.8±0.4	0.7±0.3	2.1±0.1
	31.0±2.7	n.d.	1.9±1.6
	33.1±1.6	1.0±0.2	n.d.
Lightly salted roe [4]	11.7±0.8	0.4±0.6	n.d.
	11.2±0.7	n.d.	4.2±0.7
	7.6±0.1	n.d.	n.d.
	7.9±0.4	n.d.	n.d.
	4.0±0.1	n.d.	0.8±0.4
	12.0±0.7	1.1±0.3	1.1±0.1
	7.6±0.4	1.1±0.5	n.d.
	7.3±0.7	1.0±0.1	3.4±1.1
	11.5±0.1	n.d.	4.4±0.4
	8.6±0.4	n.d.	1.8±0.4

1) Screened roe from several fish. 2) Stored for two months at ÷5°C, three barrels (105 kg). 3) Stored for one year at ÷5°C, three barrels (105 kg). 4) Made by desalting of ten barrels stored for 8 months at ÷5°C, salt concentrations are shown in table 3. n.d.: Not detectable

Table 7. Initial and final concentrations of lactic and acetic acid (mean value ± standard deviation, double determinations) during storage of five batches of lightly salted lumpfish roe. The roe was vacuum packed in glass jars and stored at 5°C for three months (pH 5.4, 3.6 - 4.2 % WPS).

Batch	Lactic acid µmoles/g roe		Acetic acid µmoles/g roe	
	Start	3 months	Start	3 months
1	9.3 ± 0.76	9.5 ± 0.01	n.d.	3.2 ± 0.05
2	9.2 ± 0.18	10.0 ± 0.17	n.d.	4.5 ± 0.15
3	8.6 ± 0.01	10.7 ± 0.03	n.d.	6.3 ± 0.23
4	9.5 ± 0.09	10.0 ± 0.05	n.d.	3.9 ± 0.03
5	9.2 ± 0.04	10.2 ± 0.03	n.d.	4.6 ± 0.12

n.d.: Not detectable

Table 8. Initial and final concentrations of glucose and ribose (mean value ± standard deviation, double determinations) during storage of five batches of lightly salted lumpfish roe. The roe was vacuum packed in glass jars and stored at 5°C for three months (pH 5.4, 3.6 - 4.2 % WPS). Peaks of glucose and ribose was detected in the analysis of organic acids, concentrations of which are shown in table 7 (method of analysis is described in paper 2).

Batch	Glucose µmoles/g roe		Ribose µmoles/g roe	
	Start	3 months	Start	3 months
1	0.07 ± 0.01	0.12 ± 0.03	n.d.	0.06/n.d.
2	0.20 ± 0.07	0.11 ± 0.01	n.d./0.08	0.13 ± 0.02
3	0.36 ± 0.03	0.13 ± 0.01	n.d./0.08	0.12 ± 0.01
4	0.37 ± 0.06	0.09 ± 0.02	0.07 ± 0.01	0.08 ± 0.02
5	0.32 ± 0.03	0.14 ± 0.01	n.d.	0.10 ± 0.01

n.d.: Not detectable

Note 2. Growth of Enterobacteriaceae isolates in roe extract as affected by pH, salt and sodium lactate

The ability of 7 Enterobacteriaceae isolated from lightly salted lumpfish roe to grow at different combinations of pH and salt concentrations with and without sodium lactate was determined by absorbance measurements in roe extract at low temperature. Materials and methods and obtained data are presented in this note.

Materials and methods

Roe extract

Lumpfish roe with added water (0.5 l/kg), boiled for 5 min., cooled and filtered. pH was lowered to 4.9 or 5.4. After centrifugation (49500 x g for 30 min. at 0°C), phosphate buffer was added to the supernatant (KH_2PO_4 7.62 g/l and $K_2H\ PO_4$ 7.66 g/l), and the pH controlled again. Roe extracts of both pH values were prepared with salt concentrations of 4.0, 4.5 and 5.5 %, by addition of NaCl. Roe extract with 2.8 % sodium lactate (Purasal®S/SP 60, 60 % w/w) was also prepared from all combinations of salt and pH. The extracts were in vials of 10 ml sterilised at 100°C for 30 min.

Bacterial cultures and inoculation

The 7 isolates originated from lightly salted lumpfish roe products stored for three months at 5°C (paper 2). Isolates F38-1, F50 and F59-1 belonged the group 1) *Serratia liquefaciens*, isolates F71-1 and F79 to group 2 (*Serratia plymuthica*) and isolates F39-1 and E5 to group 3 (*Morganella morganii*) (see paper 2).

Before inoculation in roe extract the isolates were cultured in Tryptone Soya Broth (TSB) (Oxoid CM 129) at 10°C for 4 days. A 10^{-2} dilution of the cultures in 4 % saline was prepared, and 100 µl inoculated into vials of 10 ml extract. Each of the seven Enterobacteriaceae was inolulated in roe extract of all combinations of pH, salt and lactate. The vials were incubated at 5°C.

Procedures

The viable counts of the cultures before inoculation were determined by spread plating on TSB-agar (TSB and 13g/l agar).

Absorbance at 600 nm (A_{600}) was measured frequently during 67 days incubation using a Ciba Corning colorimeter 254. Non-inoculated extract was used as controls, and measurements were performed in a cold-storage room (10°C).

At the end of the experiment purity of all cultures in roe extract with A_{600} above 0.1 was controlled by phase contrast microscopy at 1000 x magnification. Viable counts of cultures in roe extract with sodium lactate ($A_{600} < 0.1$) was determined by spread plating of 0.1 ml on TSB-agar.

Results

Inoculation level was $\approx 10^5$ cfu/ml.
The isolates F39-1 and E5 failed to grow also in roe extract with low salt and high pH.
Results on the *Serratia* spp. in roe extract without sodium lactate are shown in table 1. Data are presented as number of days until $A_{600} = 0.1$ was reached.

Table 1. Growth of five *Serratia* spp. in roe extract of different pH and salt concentrations.

Strain	pH 5.4			pH 4.9		
	4.0 % salt	4.5 % salt	5.0 % salt	4.0 % salt	4.5 % salt	5.0 % salt
F38-1[1)]	28	67	67	67	67	67
F50[1)]	29	67	67	67	67	67
F59-1[1)]	16	45	67	58	67	67
F71-1[2)]	29	67	67	67	67	67
F79[2)]	9	14	10	15	15	21

1) Belongs to group 1: *Serratia liquefaciens* 2) Belongs to group 2: *Serratia plymuthica*

In roe extract with 2.8 % sodium lactate, none of the strains were able to grow during 67 days of incubation at 5°C. At the end of the experiment viable counts of all strains had decreased. In 50 % of the vials counts were reduced to <10 cfu/g, and counts of all strains were below 1.5 x 10^3 cfu/g.

Note 3. Biochemical testing of 18 Enterobacteriaceae isolates from stored lightly salted roe products

In order to obtain an indication on the variability of the Enterobacteriaceae flora able to grow in lightly salted lumpfish roe, isolates were randomly chosen from stored roe products included in different experiments of this project, and characterised by biochemical testing. Materials and methods and obtained data are presented in this note.

Materials and methods

Roe products

Isolates were obtained from 14 jars of roe products included in 4 different storage experiments. The roe products, from which isolates were taken, had been vacuum packed in glass jars and stored at 5°C for different periods of time. All products had been produced from desalted roe, the normal raw material for production, and the pH adjusted to 5.4 with lactic acid as defined in chapter 1 (section 1.2). Salt concentration and storage time of the roe products at isolation are listed in table 1:

Table 1. Origin of 18 Enterobacteriaceae isolates.

Isolates	**Roe products**		
	Number of jars	Salt concentration % WPS	Storage time months
S1, S4, S6	3	3.6	1½
B10, B11b, A10	2	3.5	2½
B5, A6	2	3.5	1
C6	1	3.7	1
C1b, C1c, C2b, C2c,C2g	2	3.6	3
7B, 8A	2	4.0	3½
C10	1	3.7	2½
E5	1	4.5	1

Testing of bacterial isolates

The isolates were randomly chosen from agar plates representing 5 log cfu/g or higher. The isolates were cultured in Tryptone Soya Broth (TSB, Oxoid CM 129) and on TSB-agar (TSB and 13 g/l agar) at 25°C. The following tests were performed:

Shape and motility was examined using phase contrast microscopy at 1000 x magnification. Gram-reaction was tested using the KOH-method (Gregersen 1978). Catalase test was carried out using 20 % H_2O_2 (Wilkinson and Jones 1977). Cytochromoxidase reaction was tested with DrySlide™ Oxidase (Difco) based on Kovacs (1956). Glucose metabolism was examined by the OF-test of Hugh and Leifson (1953) using OF-test medium (Merck 10282). Finally the isolates were tested using the API 20E identification system (bioMerieux, France). Single reactions were evaluated following the instructions from the manufacturer.

Results

All isolates were Gram-negative, motile rods, that were catalase positive, oxidase negative and fermentative in OF-medium. The results showed the following relations to the three groups of Enterobacteriaceae isolates previously tested (table 4.1 of chapter 4):

- 3 isolates: Results were identical to strains of group 1 (*Serratia liquefaciens*)
- 2 isolates: Results differed in one test (gelatinase) from any strain of group 1.
- 1 isolate: Results differed in two tests (gelatinase and saccarose) from any strain of group 1
- 8 isolates: Results were identical to strains of group 2 (*Serratia plymuthica*).
- 1 isolate: Results differed in one test (rhamnose) from any strain of group 2.
- 1 isolate: Results differed in two tests (rhamnose and inositol) from any strain of group 2.
- 1 isolate : Results differed in four tests (VP, sorbitol, rhamnose and melibiose) from any strain of group 2. This isolate was by the API 20E identification system suggested to be *Enterobacter agglomerans*.
- 1 isolate (E5, obtained from batch 10, paper 2) was at Statens Seruminstitut, Copenhagen by further testing identified as *Morganella morganii* (subsp. *morganii*), and belonged to group 3.

Detailed results are shown in table 1.

Table 1. Results of biochemical testing of 18 Enterobacteriaceae isolates (API 20E). In addition all isolates were negative in arginine dihydrolase and hydrogensulphide production from thiosulphate.

Strain	A6	B5	A10, S4, B10	C6	B11b, S1, S6, C1b, C1c, C2b, C2c, C2g	C10	7B	8A	E 5
ONPG	+	+	+	+	+	+	+	+	-
Lysine decarboxylase	+	+	+	+	-	-	-	-	-
Ornithine decarboxylase	+	+	+	+	-	-	-	-	-
Citrat utilisation	+	+	+	+	+	-	+	-	-
Urease	-	-	-	-	-	-	-	-	+
Tryptophane desaminase	-	-	-	-	-	-	-	-	+
Indole	-	-	-	-	-	-	-	-	+
VP	+	-	+	-	+	+	-	-	-
Gelatinase	-	-	+	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+	-
Inositol	+	+	+	+	-	+	-	-	-
Sorbitol	+	+	+	+	+	+	+	-	-
Rhamnose	+	+	+	+	-	+	+	+	-
Sucrose	+	+	+	-	+	+	+	+	-
Melibiose	+	+	+	+	+	+	+	-	-
Amygdalin	+	+	+	+	+	+	+	+	-
Arabinose	+	+	+	+	+	+	+	+	-

+ : Positive reaction
- : Negative reaction

Note 4. Growth of *Listeria monocytogenes* in lightly salted lumpfish roe

In order to determine the ability of *Listeria monocytogenes* to grow in lightly salted lumpfish roe, roe products were inoculated with *Listeria monocytogenes* in low numbers, and analysed during chill storage. Material and methods and obtained data are presented in this note.

Materials and methods

Roe products:
Lightly salted lumpfish roe was prepared by desalting of heavily salted roe (ABBA Seafood, Denmark) following standard procedures (see chapter 2). When the salt concentration was slightly below 4 % (estimated by direct titration on the brine with 0.1 N $AgNO_3$ and $K_2Cr_2O_7$ as indicator) the roe was drained, weighed and placed at 2°C, while analysis of dry matter and salt concentration of the roe was carried out. Dry matter content was determined by weighing before and after drying at 105°C in 20-24 hours. Salt concentration was calculated from the amount of chloride as determined by the method of AOAC 1995, no. 937.09. Based on these results the exact amount of NaCl needed to obtain a salt concentration of 4 % WPS was calculated. This amount of salt was added to the roe, and the pH lowered to 5.4 by addition of lactic acid (0.1 % w/w) (PURAC®SP 80, Nordisk Droge Handel A/S, Denmark) After thorough mixing, the roe was packed in 100 g glass jars.

Inoculation:
Two mixtures of *Listeria monocytogenes* strains earlier isolated at this laboratory was included: Strain 411B1 and 411B5 isolated from a caviar product (donated by Vibeke From Jeppesen), strain O57 from waste-material in a production plant for "gravad" salmon and strain H018 from sliced cold smoked salmon (donated by Peter Karim Ben Embarek).
The bacteria were cultured in Brain Heart Infusion broth (BHI, Difco) at 10°C for three days. Cultures of 411B1 and 411B5 were mixed (A) and so were cultures of O57 and H018 (B). Of the two mixtures A and B a 10^{-7} dilution was prepared in sterile 4 % saline. 1 ml of this dilution was inoculated in 100 g jars of roe. After finishing all inoculations, 0.1 ml of diluted A and B were spread on BHI-agar for enumeration and control of purity (dilution A: 2.3 log cfu/ml, B: 2.5 log cfu/ml). All jars were closed under vacuum, and stored at 5 or 10°C.

Analysis:
Listeria monocytogenes was determined in duplicate (two jars) by spread plating on Oxford agar (Listeria selective agar, Oxford formulation, Oxoid CM 856 + SR 140). The entire content of a jar was first homogenised (30 s.) in a Colworth Stomacher 400. From the bags 25 g was aseptically taken for ten fold dilution in peptone saline (0.85 % NaCl, 0.1 % peptone) and homogenised again for 30 seconds. Appropriate further ten fold dilutions were plated on Oxford agar. The plates were incubated at 37°C for 48 hours. Provided no growth occurred from non-inoculated roe jars, counts were reported as counts of *Listeria monocytogenes.*

Results

In no case growth on Oxford agar was detected from non-inoculated roe jars. The results on growth of the mixtures A and B of *Listeria monocytogenes* at 5°C and 10°C are shown in table 1.

Table 1. Counts of *Listeria monocytogenes* (log cfu/g) in lightly salted lumpfish roe (4 % WPS, pH 5.4) during vacuum packed storage at 5°C and 10°C. Results from double determinations (two jars) are shown.

Strain mixture	Storage temp.	1 day	6 days	13 days	18 days	23 days
A	5°C	< 1	3.2	6.5	7.0	7.4
		< 1	3.5	6.0	7.2	7.3
A	10°C	< 1	5.8	6.8	7.3	7.5
		< 1	6.0	6.9	7.6	7.8
B	5°C	< 1	2.6	6.0	7.0	7.1
		< 1	2.5	6.2	6.9	7.6
B	10°C	< 1	6.0	8.0	7.5	7.6
		< 1	6.0	8.2	7.1	7.4

1) Results from two jars. 2) Mean value. A: Mixture of strains 411B1 and 411B5, inoculated to 0.3 log cfu/g. B: Mixture of strains O57 and H018 inoculated to 0.5 log cfu/g.

Note 5. Screening for antagonistic activity against *Listeria monocytogenes* or Gram-negative roe isolates among LAB from roe products.

As the idea at initiation of this project was to use live cultures of LAB to preserve lightly salted lumpfish roe (biopreserved), experiments have been performed with the aim of isolating LAB from roe products with antagonistic activity against *Listeria monocytogenes* and potential spoilage organisms.
In experiment 1 and 2 described in this note, the strategy was to ensure as a similarity as great as possible between normal roe products and the experimental conditions at which antagonism were to be detected. Therefore a roe agar was used for the antagonism assays, which were designed as replica plating on roe agar overlaid with soft roe agar (experiment 1) or as well diffusion assays in roe agar (experiment 2). Thus both were unspecific assays intended to detect any type of antagonistic action by LAB at imitated product conditions.
Due to obstacles to be revealed below, a third experiment was performed as a well diffusion assay in a laboratory medium with *Listeria monocytogenes* as the target organism.

Experiment 1

Materials and methods

Roe products
The roe products of which LAB were examined in this experiment were identical to batch 6 - 11 of paper 3 (salt concentrations 3.5 - 4.8 % WPS, pH 5.4). The six batches were prepared by desalting of six barrels of heavily salted roe following the normal procedure. The roe was stored under vacuum in glass jars at 5°C for 2½ months, and spread on NAP agar as described in paper 3.

Roe extract and agar
Heavily salted lumpfish roe was desalted to a salt concentration slightly below 4 %, and placed at 0°C while analysis of dry matter and salt concentration was carried out as described in note 4. From the roe an extract was produced by a method modified after Gram et al. (1987):
The roe was added 0.5 l/kg tap-water, heated, boiled for 5 minutes and allowed to cool. The roe was filtered through double layer gauze. The gauze was transferred to a mechanical press to squeeze the remaining liquid from the roe. To the extract was added 0.1 % (w/w) lactic acid (PURAC®SP 80) and phosphate buffer (7.62 g/l KH_2PO_4 and 7.66 g/l K_2HPO_4). The pH was adjusted to 5.4 (HCL or NaCl).

To reduce the turbidity, the extract was centrifuged (49500 x g) at 0°C for 30 min. The thin upper layer of lipid was removed, and the supernatant collected with a pipette. Based on results on dry matter and salt concentration of the roe and the amount of added water, additional salt needed to obtain a salt concentration of 4 % WPS was calculated, and the salt added.
The extract was sterilised at 100°C for 30 min. For production of roe agar 13 g/l or 7 g/l agar (soft agar) was added (boiled) before sterilisation.

Antagonism assay
After counting of colonies on NAP-agar plates (LAB counts of batch 6 - 11 is shown in table 1 paper 2) an appropriate dilution (10^{-5} or 10^{-6}) was chosen, and the plates copied on roe agar plates (10 ml agar) using dry-sterilised velvet mounted on a plastic cylinder, the size of a petri-dish (Ø 8.5 cm). The plates were incubated at 5°C until visible colonies had formed. Target organisms included 10 Enterobacteriaceae previously isolated from lightly salted lumpfish roe. The groups 1 - 3 were represented: *Morganella morganii* (F39-1, E5) *Serratia liquefaciens* (F38-1, F50, F59-1) and *Serratia plymuthica* (F79, F71-1) (paper 3). A mixture of three *Vibrio* spp. (F30, F47-1, F62) (paper 3) and one *Listeria monocytogenes* (O57, note 4) was also included as target organisms. The target organisms were cultured in roe extract at 5°C and their counts estimated using phase contrast microscopy (x 1000) (level 10^6 cfu/ml).
The roe agar copy plates with LAB colonies were overlaid with soft roe agar seeded with target organisms (1 ml grown broth culture to 10 ml molten soft-agar). One copy plate of each NAP agar plate was not overlaid. The plates were incubated at 5°C in Oxoid Anaerobic jars, reduced to 70 % vacuum and refilled with N_2 and observed for inhibition zones for up to three weeks.

Results

Only hardly visible zones of inhibition of the *Vibrio* mixture were observed around a few LAB colonies. Insufficient growth of some target organisms occurred in addition. Therefore a second experiment was performed (experiment 2).

Experiment 2

Materials and methods

For this experiment 28 LAB were randomly chosen from the non-overlaid copy plates on roe agar described in experiment 1. The isolates were biochemically tested by the methods described in paper 3, the results of which are shown in table 1.
The antagonism assay were changed to a well diffusion assay, and the temperature during culturing of target organisms were increased to 10°C. Still attempting to mimic product conditions the well diffusion assay was carried out in roe agar at low temperature.

Antagonism assay
Inhibitor organisms included the 28 L-strains (table 1) and 15 LAB previously isolated from stored roe products (batch 1 - 5, paper 2), three of each of the groups A1, A2, B1, B2 and C (F28-1, F33-1, F34, F42-1, F45, F77-1, F29-1, F61-1, F76-1, F66-1, F69, F85-1, F36-1, F41-1 and F44) (paper 3).
Target organisms (11 in total) included 2 *Listeria monocytogenes* (O57 and a mixture of 411B1-5 , see note 4), 6 Enterobacteriaceae (F50, F38-1, F59-1, F71-1, F79, F39-1) and 3 *Vibrio* spp. (F30, F47-1, F62) (see experiment 1).
Soft roe agar (0.7 % agar) and roe extract (both with 4 % salt and pH 5.4) was prepared as described in experiment 1. All strains (target and inhibitor organisms) were cultured in roe extract at 10°C for 4 days.
One ml culture of target organisms were added to 10 ml molten (to 45°C cooled) soft roe agar, that were poored into petridishes (Ø 8.5 cm) and allowed to solidify (45 min.) before wells (Ø 3 mm.) were bored. From the cultures of inhibitor organisms 10 µl were added to the wells. A well with 10 µl sterile roe extract was included as control. The plates were incubated for up to 2 weeks at 5°C in Oxoid Anaerobic jars, reduced to 70 % vacuum and refilled with N_2. Inhibition zones were measured from well periphery to zone periphery.
After carrying out the assay all strains were streaked out on agar plates for control of purity.

Results

One Enterobacteriaceae (F39-1), one *Vibrio* spp. (F30) and *Listeria monocytogenes* (O57 and 411B1-5) did not grow in the roe agar, when observed for two weeks.
None of the remaining Enterobacteriaceae (*Serratia* spp.) were inhibited by any of the 46 LAB tested. The *Vibrio* spp. F47-1 and F62 were inhibited by 27 of the 43 LAB, only 7 of which however produced inhibition zones of 2 mm or more against one or both *Vibrio* spp.. These results are shown in table 2.

Table 1. Results of biochemical testing of 28 LAB randomly chosen from six batches of lightly salted lumpfish roe (batch 6 - 11, paper 2) after 2½ months of storage at 5°C. In addition all strains were negative in tetrad formation and growth at 45°C but positive in growth at 10°C.

Strain	L3, 4, 6, 10, 12, 29	L8, 9, 11, 30	L13	L14 - 17	L18	L19	L20	L21	L22	L23 - 27	L28, 32	L31
Cell morphology	rods	rods	cocci	rods	cocci	rods	rods	rods	rods	rods	rods	rods
Gas production	-	-	+	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	+ [1]	- [2]	- [2]	+	-	- [2]	- [2]	- [2]	-	+	-	-
Motility	-	-	-	-	-	-	-	-	-	-	-	-
Gr.on acetate agar	+	+	+	-	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	-	-	+	-	+	-
Galactose	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	-	-	+	+	-	-
Maltose	+	+	+	+	-	+	+	+	-	+	-	+
Mannitole	-	-	-	+	+	+	+	-	-	-	-	-
Melizitose	-	-	-	+	+	+	+	-	-	-	-	-
Melibiose	+	-	+	+	+	+	+	-	-	+	+	-
Raffinose	-	-	+	+	-	-	-	-	-	-	-	-
Rhamnose	+	-	+	-	-	-	-	-	-	-	-	-
Ribose	+	+	+	+	+	+	+	+	+	+	+	+
Salicine	+	+	+	+	+	+	-	-	+	+	+	+
Sucrose	+	-	+	+	+	+	-	-	+	+	+	-
Trehalose	+	+	+	+	+	+	-	-	+	+	+	+
Xylose	-	+	+	-	-	-	-	-	-	-	-	+

1) Negative when 20 mg/ml glucose was present in the medium (see paper 3 for materials and methods). 2) No or weak growth.

Table 2. LAB (27 of 43) producing inhibition zones against two *Vibrio* spp. in well diffusion assay in roe agar at 5°C. Unless otherwise stated, the zones were fully cleared up with a diffuse rim.

Inhibitors	L18-21	L3,10	F77-1 [1)]	L13	L1, 11, 22-24 [2)]	L4, 12, 25-30, 32, F28-1, 33-1, 34, 42-1, 45 [2)]
Targets						
F47-1	+	+	(+)	÷	(+)	÷
F62	+	(+)	+	+	÷	(+)

÷: No zone, (+): zone < 2 mm, +: zone 2 - 4 mm. 1) Zone incompletely cleared up 2) Rim of zone sharp.

Experiment 3

As described in experiment 1 and 2, *Listeria monocytogenes* failed to grow in the antagonism assays, though growth occurred during culturing in roe extract. Therefore a trial was performed, in which *Listeria monocytogenes* O57 was spread in roe agar and All Purpose Tween agar (APT, Difco) after preculturing in either roe extract or APT-broth. The plates were incubated at 5°C. Growth occurred in both APT-broth and roe extract. On APT-agar plates visible colonies were formed after one weak (precultured in APT-broth) or two weeks (precultured in roe extract). No growth occurred in roe agar regardless of the preculturing medium. Thus in order to detect antilisterial LAB among roe isolates, a traditional unspecific well diffusion assay in APT-agar was performed.

Materials and methods

Antagonism assay

31 LAB (15 F-strains as in experiment 1 and 12 L-strains selected to represent the different LAB shown in table 1) were tested against *Listeria monocytogenes* O57. All strains were cultured in roe extract (4 % salt, pH 5.4) at 10°C for 10 days, and their counts estimated by phase contrast microscopy (x 1000) (around 10^6 and 10^6 - 10^7 cfu/g for *Listeria* and LAB respectively). Of the target organism 1 ml culture were added to 10 ml molten (to 45°C cooled) APT-agar, that were allowed to solidify (45 min.) before wells were bored. Of the LAB cultures 10 µl were added to the wells. A well with 10 µl sterile roe extract was included as control. The plates were incubated for up to 2 weeks at 5°C in Oxoid Anaerobic jars, reduced to 70 % vacuum and refilled with N_2. The plates were frequently inspected, and

after two weeks of incubation, inhibition zones were measured from well periphery to zone periphery.

Results

Among the 31 LAB, inhibition zones against *Listeria monocytogenes* were produced by 8 strains, the 4 of which however only produced very narrow zones:

Narrow zone (≤ 1 mm): F33-1, F36-1, L13 and L14.
Zone 6 - 7 mm: F61-1 and F69.
Zone 11 - 12 mm: L30 and L31.

Note 6. Effect of LAB and/or sodium lactate on growth of *Listeria monocytogenes* and effect on spoilage and microflora of lightly salted lumpfish roe.

In this note materials and methods and obtained results of four experiments are described. In the first experiment LAB isolated from stored lightly salted lumpfish roe products, and previously examined in well diffusion assays (note 5), were inoculated in high numbers in lightly salted lumpfish roe with low numbers of *Listeria monocytogenes*, in order to study their antagonistic effect at normal product conditions.
In the second experiment the effect of sodium lactate and LAB V6 on growth of *Listeria monocytogenes* and Enterobacteriaceae inoculated in low numbers in lightly salted lumpfish roe was examined.
In the third experiment sodium lactate as a preservative in lightly salted lumpfish roe was evaluated during storage for > 3 months. The effect on off-odour development, total viable counts, LAB, Enterobacteriaceae and *Listeria monocytogenes* was determined. LAB V6 and F44 were included in the experiment and observed for any additional effects on the measured parameters.
In the fourth experiment the effect of 1 % glucose addition with or without LAB V6 on spoilage of lightly salted lumpfish roe was determined, in order to evaluate the prospects of producing a fermented lightly salted lumpfish roe product.

Experiment 1

Materials and methods

Roe products
Lightly salted lumpfish roe was prepared from normal heavily salted roe by the method described in note 4 to obtain a salt concentration of 4.0 % WPS and a pH of 5.4.

Preparation of inoculum
Listeria monocytogenes O57 and H018 (originating from lightly preserved fish, note 4) were cultured in Brain Heart Infusion (BHI, Difco) broth at 10°C for three days. A 10^{-7} dilution of the grown cultures were prepared in 4 % sterile saline.
LAB (originating from stored lightly salted roe, note 5) were cultured in All Purpose Tween (APT, Difco) broth at 25°C for two days. Strains L31 and F61-1 previously producing inhibition zones against *Listeria monocytogenes* (O57) in well diffusion assay (note 5, experiment 3), and F44 not producing zones, were included. The grown LAB cultures were centrifuged (10.000 rpm for 10 min.) and the supernatant removed using a sterile pipette.

The cells were suspended in the same amount of 4 % sterile saline. This was done in order to avoid transfer of nutrients, influencing the salt concentration of the roe, and to allow any observed effect of LAB cultures, to be ascribed to their activity in the roe.

Experimental design

Lightly salted lumpfish roe in glass jars containing 75 g, were inoculated with LAB and *Listeria monocytogenes* in all combinations of O57 and H018 with F44, F61-1, L31 and F61-1 + L31. Non-inoculated controls and controls with O57, H018 without LAB were included. 750 µl *Listeria monocytogenes* dilution, and 750 µl LAB suspension in total were used per jar. After inoculation and mixing, the jars were closed with metal twist-off caps under vacuum and stored at 5°C. For control of purity and determination of counts, all suspensions were spread on agar plates (BHI-agar for *Listeria monocytogenes* and APT-agar for LAB) at appropriate dilutions and incubated at 15°C for 2 - 3 days.

Analysis

The entire content of a jar was homogenised (30 s.) in a Colworth Stomacher 400. From the bags 25 g was aseptically taken for ten fold dilution in peptone saline (0.85 % NaCl, 0.1 % peptone) and homogenised again for 30 seconds. Appropriate further ten fold dilutions were plated on Oxford agar as described in paper 2. Counts of LAB were determined by spread plating on Nitrite Actidione Polymyxin (NAP) agar as described in paper 2. In all analysis double determinations were performed by analysing two jars of each combination shown in table 1.

Results

Development in counts of LAB and *Listeria monocytogenes* during storage at 5°C is shown in table 1. Based on counts in suspensions used for inoculation, initial inoculum in the roe was: 0.3 log cfu/g of O57 and H018, 7.0 log cfu/g of F44 and F61-1, 5.0 log cfu/g of L31.

Table 1. Counts (log cfu/g) of LAB and *Listeria monocytogenes* during storage of lightly salted lumpfish roe at 5°C for 39 days. The roe was inoculated with combinations of LAB (F44, F61-1, L31) and *Listeria monocytogenes* (O57, H018). Separate results from double determinations (two jars of roe) are shown. *Listeria monocytogenes* was not detected in non-inoculated controls.

	1 day		8 days		11 days		22 days		39 days	
	List.	LAB	*List.*	LAB	*List.*	LAB	*List.*	LAB	*List.*	LAB
O57										
÷ LAB	< 1	< 2	< 1	< 2	< 1	6.5	5.8	7.0	5.5	7.3
	< 1	< 2	< 1	2.3	< 1	5.5	6.0	7.1	< 5	7.4
F44	< 1	7.2	< 1	6.9	< 1	7.3	< 2	7.4	< 2	6.8
	< 1	7.1	< 1	6.8	< 1	6.3	3.4	8.0	< 2	7.8
F61-1	1.3	6.8	2.8	6.9	4.0	7.7	5.7	7.7	6.3	7.2
	2.3	7.4	2.9	6.9	4.3	7.4	5.4	6.9	6.3	7.4
L31	*	5.2	*	5.9	4.1	7.7	4.1	7.7	6.1	7.3
	*	5.0	*	5.7	4.1	7.6	4.6	7.6	6.1	7.3
F61-1	*	7.1	*	6.7	3.7	7.7	4.7	7.5	5.7	7.4
+L31	*	6.8	*	6.7	3.6	7.7	4.8	7.5	4.7	7.4
H018										
÷ LAB	< 1	< 2	2.3	4.2	5.5	5.9	6.8	6.6	7.8	7.2
	< 1	2.0	2.9	3.6	5.5	6.1	6.6	7.3	8.0	7.3
F44	< 1	7.0	< 1	7.0	4.3	7.1	4.0	7.0	4.2	7.8
	1.0	7.1	< 1	7.2	2.8	7.3	4.0	7.3	3.7	7.5
F61-1	2.6	7.3	2.9	7.1	4.7	6.8	5.4	7.2	6.0	7.2
	2.0	7.3	2.9	7.1	-	-	5.2	7.2	5.8	6.9
L31	*	5.1	*	5.8	4.5	7.8	5.5	7.4	6.5	7.2
	*	5.0	*	6.0	4.8	7.7	5.6	7.2	6.6	7.5
F61-1	*	7.3	*	6.8	4.0	7.8	3.7	7.3	6.4	7.5
+L31	*	7.3	*	6.9	4.2	7.7	5.0	7.5	6.1	7.5

* Counts of *Listeria monocytogenes* obtained were judged as unreliable. Great difficulties were experienced in counting of colonies due to pronounced clotting.

Experiment 2

Materials and methods

Roe products

Lightly salted lumpfish roe was prepared from normal heavily salted roe by the method described in note 4 to obtain a salt concentration of 4.0 % WPS and a pH of 5.4. The roe was divided in two portions, one of which were added 2.8 % (w/w) sodium lactate (35 ml/kg of Purasal® S/SP 60, 60 % w/w, specific gravity (20°C) 1.32 - 1.34 g/ml).

Preparation of inoculum

Listeria monocytogenes O57 was cultured in Brain Heart Infusion (BHI, Difco) broth at 15°C for two days. A 10^{-7} dilution of the grown cultures were prepared in 4 % sterile saline. *Morganella morganii* F39-1 (originating from spoiled lightly salted lumpfish roe, paper 3), was cultured in Tryptone Soya Broth (TSB, Oxoid) at 15°C for two days. A 10^{-5} dilution of the grown cultures were prepared in 4 % sterile saline.
LAB V6 (*Leuconostoc* sp.) (donated by Vibeke From Jeppesen) originated from from non-spoiled sugar-salted herring stored at 10°C. The strain was shown to be antagonistic to various Gram-negative bacteria including *Serratia* spp. and *Morganella morganii* in a disc assay at 5°C, and to *Listeria monocytogenes* and *Yersinia enterocolitica* in addition in shrimp-extract at 5°C at various conditions regarding salt and pH (Jeppesen and Huss 1993a and 1993b). V6 was cultured in All Purpose Tween (APT, Difco) broth at 25°C for two days. The grown culture were centrifuged (10.000 rpm for 10 min.) and the supernatant removed using a sterile pipette. The cells were suspended in the same amount of 4 % sterile saline.

Experimental design

Lightly salted lumpfish roe in glass jars containing 75 g with and without lactate were inoculated with LAB, *Listeria monocytogenes* and *Morganella morganii* in the combinations shown in table 2. 750µl of the three dilutions/suspension of cultures were used per jar. After inoculation and mixing, the jars were closed under vacuum with metal twist-off caps and stored at 5°C. For control of purity and determination of counts, the dilutions/suspension were spread on agar plates (BHI-agar for O57, TSB-agar for F39-1 and APT-agar for V6) at appropriate dilutions in peptone saline, and incubated at 15°C for 2 - 3 days.

Analysis

Analysis of counts of *Listeria monocytogenes* and LAB was performed as described for experiment 1. Counts of Enterobacteriaceae was determined in TSA/VRB-G agar as described in paper 2. In all analysis double determinations were performed by analysing two jars of each combination.

Table 2. Combinations of strains of *Listeria monocytogenes* (O57), *Morganella morganii* (F39-1) and LAB (V6) inoculated in lightly salted lumpfish roe with and without 2.8 % (w/w) sodium lactate.

	V6	O57	F39-1	lactate
1				
2	x			
3	x			x
4				x
5	x	x	x	
6	x	x	x	x
7		x	x	x
8		x	x	

Results

Based on counts in dilutions/suspension used for inoculation, initial inoculum in the roe was: 0.8 log cfu/g for O57, 2.0 log cfu/g for F39-1 and 6.5 log cfu/g for V6.
Development in counts of LAB, Enterobacteriaceae and *Listeria monocytogenes* in the roe during storage at 5°C is shown in table 3 - 5.

Table 3. Counts (log cfu/g) of LAB during storage of lightly salted lumpfish roe (4 % WPS, pH 5.4, vacuum packed) at 5°C for 80 days. Roe with and without 2.8 % (w/w) sodium lactate was inoculated with the combinations of LAB (V6), *Listeria monocytogenes* (O57) and *Morganella morganii* (F39-1) shown in table 2. Separate results from double determinations (two jars of roe) are shown.

	3 days	10 days	20 days	30 days	38 days	52 days	80 days
1	2.0	4.2	4.0	5.0	4.6	6.0	6.2
	2.0	3.9	4.7	5.4	5.0	5.4	6.3
2	7.0	7.2	7.1	7.0	6.7	7.2	7.3
	7.1	7.1	7.3	6.9	7.0	6.7	6.9
3	6.4	6.8	7.0	6.4	7.1	7.1	5.6
	6.7	6.8	6.9	7.0	6.5	6.7	7.3
4	2.3	< 2	5.4	5.0	5.9	5.5	7.2
	2.3	< 2	-	6.3	7.3	5.9	7.3
5	6.9	7.3	6.9	7.0	6.6	6.4	6.6
	6.6	7.3	7.0	7.2	6.7	6.7	6.8
6	6.9	7.0	6.9	6.5	6.8	7.0	7.4
	6.6	6.9	6.8	6.6	6.5	6.8	7.1
7	2.5	2.0	2.3	6.9	7.1	7.0	6.5
	3.3	2.3	2.0	6.4	7.1	7.3	7.3
8	1.6	3.6	6.9	7.0	6.7	7.2	7.0
	1.8	4.0	5.9	6.0	7.2	7.4	6.8

Table 4. Counts (log cfu/g) of *Listeria monocytogenes* during storage of lightly salted lumpfish roe (4 % WPS, pH 5.4, vacuum packed) at 5°C for 80 days. Roe with and without 2.8 % (w/w) sodium lactate was inoculated with the combinations of LAB (V6), *Listeria monocytogenes* (O57) and *Morganella morganii* (F39-1) shown in table 2. Separate results from double determinations (two jars of roe) are shown. *Listeria monocytogenes* was not detected in non-inoculated controls.

	3 days	10 days	20 days	30 days	38 days	52 days	80 days
5	< 1	< 1	3,0	< 3	< 2	< 2	2,0
	< 1	< 1	3,3	< 3	< 2	2,0	2,8
6	< 1	< 1	< 1	< 1	< 1	< 1	< 1
	< 1	< 1	< 1	< 1	< 1	< 1	< 1
7	< 1	< 1	< 1	< 1	< 1	< 1	< 1
	< 1	< 1	< 1	< 1	< 1	< 1	< 1
8	< 1	< 1	-	4,1	3,5	3,3	3,2
	< 1	< 1	4,0	3,0	2,3	2,7	3,1

Table 5. Counts (log cfu/g) of Enterobacteriaceae during storage of lightly salted lumpfish roe (4 % WPS, pH 5.4, vacuum packed) at 5°C for 80 days. Roe with and without 2.8 % (w/w) sodium lactate was inoculated with the combinations of LAB (V6), *Listeria monocytogenes* (O57) and *Morganella morganii* (F39-1) shown in table 2. Separate results from double determinations (two jars of roe) are shown.

	3 days	**20 days**	**30 days**	**38 days**	**52 days**	**80 days**
1	2.0	3.0	4.6	4.9	5.5	5.7
	1.5	< 3	< 4	4.7	< 4	5.2
2	1.5	5.8	4.0	< 3	6.4	< 4
	1.5	3.8	5.7	4.7	4.7	< 4
3	1.7	< 2	< 2	< 2	< 2	< 2
	1.9	< 2	< 2	< 2	< 2	< 2
4	1.6	< 2	3.8	< 2	< 2	< 2
	1.6	< 2	2.0	< 2	< 2	< 2
5	1.7	< 3	5.0	5.6	4.7	4.0
	2.1	4.1	5.3	4.5	< 4	5.3
6	2.2	< 2	< 2	2.0	< 2	< 2
	2.0	2.3	< 2	2.0	< 2	2.0
7	1.8	< 2	2.0	< 2	3.6	2.0
	1.8	2.0	2.0	< 2	< 2	< 2
8	2.1	6.2	5.9	5.0	5.7	5.6
	2.1	4.4	5.8	6.4	6.6	5.9

Experiment 3

Materials and methods

Roe products

Lightly salted lumpfish roe was prepared from normal heavily salted roe by the method described in note 4 to obtain a salt concentration of 4.0 % WPS and a pH of 5.4. The roe was divided in two portions, one of which were added 2.8 % (w/w) sodium lactate (35 ml/kg of Purasal® S/SP 60, 60 % w/w).

Preparation of inoculum

Listeria monocytogenes O57 and *Morganella morganii* F39-1 were cultured and diluted as described for experiment 2. LAB V6 (see experiment 2) and F44 (see experiment 1) were cultured, centrifuged and suspended as described for V6 in experiment 2. Suspensions of the two LAB strains were mixed before inoculation.

Experimental design

Lightly salted lumpfish roe with and without lactate were packed in glass jars containing 75 g or 225 g (roe for odour-assessment). The roe was inoculated with LAB V6 + F44, *Listeria monocytogenes* O57 and *Morganella morganii* 39-1 in the combinations shown in table 6. 750µl of the three dilutions/suspension of cultures were used per jar with 75 g, and 2.5 ml for the bigger jars.

Table 6. Combinations of strains of *Listeria monocytogenes* (O57), *Morganella morganii* (F39-1) and LAB (V6 + F44) inoculated in lightly salted lumpfish roe with and without 2.8 % (w/w) sodium lactate.

Batch	V6+F44	O57	F39-1	lactate
1	x			x
2	x	x		x
3		x		x
4				x
5			x	x
6		x		
7				
8			x	

Analysis

In all analysis double determinations were performed by analysing two jars of each combination. Counts of *Listeria monocytogenes*, LAB and Enterobacteriaceae carried out as described in the two previous experiments. Total viable counts was determined by spread plating on Tryptone Soya Broth agar (TSB, Oxoid and 13 g/l agar). These plates were incubated at 10°C for 6 days. The pH was measured using a PHM 82 (Radiometer, Copenhagen, Denmark).

The odours of stored roe products was assessed by a panel of 9 instructed persons. Non-inoculated roe with or without lactate (vacuum packed and stored at ÷40° C) were used as controls. Off-odours compared to the controls were assessed as none (0), weak (1), borderline (2), significant (3) and strong (4). A score above 2 was regarded as rejection of the roe. The odours were further characterised by descriptive words chosen by the panellists. At the end of storage isolates were taken from TSA/VRB-G agar plates and biochemically tested by the methods described in paper 3.

At the last day of analysis colonies were picked up from TSA/VRB-G agar plates and Enterobacteriaceae were presumptively identified by biochemical testing as described in paper 3.

Results

Development in total viable counts and counts of Enterobacteriaceae, LAB and *Listeria monocytogenes* in the roe during storage at 5°C is shown in table 7 - 10. Results from odour assessments are shown in table 11, and development in pH in table 12.

Table 7. Total viable counts (log cfu/g) during storage of lightly salted lumpfish roe (4 % WPS, pH 5.4, vacuum packed) at 5°C for 116 days. Definitions of roe no. 1 - 8 are shown in table 6. Separate results from double determinations (two jars of roe) are shown.

	18 days	**32 days**	**46 days**	**61 days**	**74 days**	**88 days**	**95 days**	**116 days**
1	7.2	7.1	7.4	7.2	7.3	-	7.0	7.0
	6.8	7.2	7.5	7.1	7.2	-	7.0	7.0
4	4.5	4.0	< 3	< 3	3.8	-	< 3	< 2
	< 4	4.1	< 3	< 3	5.6	-	3.8	< 2
5	4.0	4.3	4.7	3.8	4.0	-	4.2	3.8
	4.3	4.0	3.6	4.0	3.5	-	< 3	4.3
7	5.9	6.3	6.7	6.7	6.4	6.8	-	6.8
	5.7	7.2	6.5	7.0	7.0	6.8	-	6.5
8	6.1	6.7	6.7	6.8	7.6	6.0	-	6.7
	6.9	6.5	6.5	6.7	7.0	7.3	-	6.4

Table 8. Counts (log cfu/g) of Enterobacteriaceae during storage of lightly salted lumpfish roe (4 % WPS, pH 5.4, vacuum packed) at 5°C for 116 days. Definitions of roe no. 1 - 8 are shown in table 6. Separate results from double determinations (two jars of roe) are shown.

	18 days	32 days	46 days	61 days	74 days	88 days	95 days	116 days
1	3.1	4.5	4.1	4.4	4.1	-	4.0	4.1
	3.5	5.0	3.5	< 4	4.2	-	3.7	4.1
4	3.1	2.3	1.0	< 2	< 2	-	< 3	< 3
	3.2	3.1	< 1	2.6	< 2	-	< 3	< 3
5	3.1	2.8	2.2	2.7	3.0	-	< 3	< 3
	3.5	2.9	1.8	2.8	< 3	-	< 3	< 3
7	5.6	6.3	6.2	6.0	4.3	6.5	-	5.2
	5.7	5.6	4.9	6.6	4.3	4.0	-	5.3
8	6.1	6.5	5.0	6.1	6.3	5.8	-	6.1
	6.7	5.9	5.0	5.6	6.6	5.7	-	6.5

Table 9. Counts (log cfu/g) of LAB during storage of lightly salted lumpfish roe (4 % WPS, pH 5.4, vacuum packed) at 5°C for 116 days. Definitions of roe no. 1 - 8 are shown in table 6. Separate results from double determinations (two jars of roe) are shown.

	18 days	32 days	46 days	61 days	74 days	88 days	95 days	116 days
1	7.1	7.1	7.4	7.1	6.8	-	7.1	6.8
	7.0	7.2	7.2	7.1	6.8	-	7.0	6.9
2	6.9	7.1	7.1	7.1	7.0	-	6.2	6.7
	6.9	7.1	7.0	7.0	6.8	-	6.8	6.8
3	< 4	< 3	< 3	2.6	< 3	-	< 3	2.3
	< 4	< 3	< 3	3.0	< 3	-	< 3	< 2
4	4.6	4.0	< 3	< 2	3.3	-	< 3	< 2
	< 4	< 3	< 3	2.3	5.2	-	< 3	< 2
5	< 4	-	3.6	3.0	3.3	-	< 3	2.6
	< 4	-	3.0	3.6	< 3	-	< 3	2.5
7	5.1	7.0	6.0	6.7	6.7	7.0	-	6.1
	4.7	5.3	6.8	6.6	6.9	6.7	-	6.5
8	5.7	5.9	6.4	6.3	7.0	6.9	-	6.4
	5.7	5.8	6.0	6.7	6.4	6.9	-	6.9

Table 10. Counts (log cfu/g) of *Listeria monocytogenes* during storage of lightly salted lumpfish roe (4 % WPS, pH 5.4, vacuum packed) at 5°C for 116 days. Definitions of roe no. 1 - 8 are shown in table 6. Separate results from double determinations (two jars of roe) are shown.

	18 days	32 days	46 days	61 days	74 days	116 days
2	< 1	2.8	< 2	< 1	< 1	< 1
	< 1	3.6	< 2	< 1	< 1	< 1
3	< 1	< 1	< 1	< 1	< 1	< 1
	< 1	< 1	< 1	< 1	< 1	< 1
6	< 2	5.4	5.4	6.4	< 4	< 4
	4.1	4.0	5.6	5.8	6.3	4.0
7	< 1	< 1	< 1	< 1	< 1	< 1
	< 1	< 1	< 1	< 1	< 1	< 1

Table 11. Odour assessment of lightly salted lumpfish roe (4 % WPS, pH 5.4, vacuum packed) stored at 5°C for 116 days. Definitions of roe no. 1 - 8 are shown in table 6. Results from two roe jars are shown, each of which is mean values of assessments by 9 judges. Scores: 0 = no off-odour, 1 = weak off-odour, 2 = borderline, 3 = significant off-odour, 4 = strong off-odour. A score above 2 is regarded as rejection of the roe.

	18 days	32 days	46 days	61 days	74 days	88 days	95 days	116 days
1	1.1	1.3	1.4	1.1	0.4	-	0.7	1.5
	1.1	1.3	0.8	1.0	0.8	-	1.5	1.0
4	1.0	0.8	0.2	1.3	0.4	-	0.3	0.4
	0.4	0.9	0.7	0.5	0.4	-	1.0	0.4
5	1.6	0.4	0.3	0.6	0.6	-	0.2	0.6
	0.5	0.6	0.6	0.6	0.1	-	0.2	0.3
7	0.3	0.8	1.1	0.9	0.9	0.5	-	0.8
	0.6	1.4	1.2	1.1	0.9	0.2	-	1.9
8	0.4	1.1	0.9	1.0	2.5 [1)]	0.5	-	2.1 [3)]
	1.3	1.6	1.2	1.0	3.4 [2)]	0.5	-	3.5 [4)]

Off-odours causing rejection was described by the words: 1) Faecal, sulphidy, sour. 2) Faecal, putrid, sulphidy, sour. 3) Faecal, sour. 4) Faecal, cabbage-like, sour, old cheese.

Table 12. Development of pH during storage of lightly salted lumpfish roe (4 % WPS, pH 5.4, vacuum packed) at 5°C for 116 days. Definitions of roe no. 1 - 8 are shown in table 6. Separate results from double determinations (two jars of roe) are shown.

	18 days	**32 days**	**46 days**	**61 days**	**74 days**	**88 days**	**95 days**	**116 days**
1	5.5	5.5	5.5	5.5	5.5	-	5.5	5.5
	5.5	5.5	5.5	5.5	5.6	-	5.5	5.5
4	5.5	5.5	5.5	5.4	5.5	-	5.5	5.5
	5.5	5.5	5.5	5.5	5.5	-	5.5	5.5
5	5.5	5.5	5.5	5.4	5.5	-	5.5	5.5
	5.5	5.5	5.5	5.5	5.5	-	5.5	5.5
7	5.3	5.3	5.3	5.4	5.5	5.5	-	5.4
	5.3	5.3	5.3	5.4	5.4	5.3	-	5.4
8	5.3	5.3	5.3	5.3	5.3	5.3	-	5.4
	5.3	5.3	5.3	5.3	5.4	5.3	-	5.5

Testing of isolates

At the end of storage colonies of different appearance (e.g. big, small) were picked up from VRB-G agar plates (representing 20 - 25 % of total counts, highest dilution) from selected roes, primarily to confirm growth of *Morganella morganii* in batch 8 (inoculated) and not in batch 7 (non-inoculated). Isolates were also taken from batch 1 (with 2.8 % lactate and V6+F44) in order to examine the identity of bacteria able to grow. The isolates were cultured in TSB and tested by the methods described in note 3. From batch 7 and 8 but not from batch 1, oxidase positive isolates were also obtained. Oxidase positive isolates have previously been isolated from lightly salted lumpfish roe (5.5 - 6.1 log cfu/g), those belonged to *Vibrio* spp. (paper 2).

From batch 1 eight isolates were obtained, all of which were Enterobacteriaceae.

From batch 7 seven isolates were obtained, one of which were Enterobacteriaceae.

From batch 8 twelve isolates were obtained, six of which were Enterobacteriaceae.

Results from biochemical testing of Enterobacteriaceae isolates are shown in table 12.

Table 13. Results of biochemical testing of 14 Enterobacteriaceae isolates (API 20E). In addition all isolates were negative in arginine dihydrolase and hydrogensulphide production from thiosulphate.

Type of isolate/ origin	1 Batch 1	2 Batch 1	3 Batch 7	4 Batch 8	5 Batch 8
No. of isolates	7	1	1	1	5
ONPG	+ [1)]	+	+	+	-
Lysine decarboxylase	+	+	-	-	-
Ornithine decarboxylase	+	+	-	-	-
Citrate utilisation	+	+	+	-	-
Urease	-	-	-	-	+
Tryptophane desaminase	-	-	-	-	+
Indole	-	-	-	-	+
VP	+	+	-	-	-
Gelatinase	+	-	-	-	-
Mannitol	+	+	+	+	-
Inositol	-	-	-	-	-
Sorbitol	+	+	+	-	-
Rhamnose	-	-	+	+	-
Sucrose	+	+	+	+	-
Melibiose	+	+	+	-	-
Amygdalin	+	+	+	+	-
Arabinose	+	+	+	+	-

1) + : Positive reaction, - : Negative reaction

Below the isolated strains are compared to Enterobacteriaceae earlier isolated from lightly salted lumpfish roe in this project (paper 3, note 3):

Isolates obtained from batch 1 (roe with 2.8 % lactate and LAB V6 + F44):
Type 1 isolates (table 12) were identical to strains of group 1 (paper 3) presumptive *Serratia liquefaciens*. Type 2 differed from type 1 only by a negative gelatine reaction, in which reaction 33 % of group 1 *Serratia liquefaciens* were also negative.

Isolates obtained from batch 7 (non-spoiled normal roe product): Type 3 isolates (table 13) differed from group 2 presumptive *Serratia plymuthica* (paper 3) by a positive rhamnose reaction (all of group 2 negative), and a negative VP reaction (92 % of group 2 positive). According to the API20E identification system, another *Serratia* (*ficaria*) is a possible identity. The strain was however not further tested.

Isolates obtained from batch 8 (spoiled roe inoculated with *Morganella morganii* F39-1)
The type 5 isolates (table 13) gave reactions identical to those of group 3 (*Morganella morganii*) (paper 3). The type 4 isolate (table 13) differed in four reactions from strains of group 2 (VP, sorbitol, rhamnose and melibiose), and was by the API20E identification system suggested to be *Enterobacter agglomerans*. The reactions of type 4 isolate was identical to a strain earlier isolated from lightly salted lumpfish roe (note 3).

Experiment 4

Materials and methods

Roe products
Lightly salted lumpfish roe with around 4 % WPS was prepared by desalting of heavily salted roe using the normal procedure, and pH was lowered to 5.4 with lactic acid as previously described. Roe without (batch 1) and with 1 % (w/w) glucose (batch 2 and 3) was prepared. The roe with glucose was divided in two portions, one of which was added LAB V6 (batch 3) by the procedure described below. The roe was vacuum packed in glass jars and stored at 5°C. Roe without extra additions were vacuum packed and frozen to be used as references in the odour assessments. The different combinations of additives are shown in table 14.

Inoculum
LAB V6 was cultured in APT-broth at 21°C for 2 days. The grown cultures were centrifuged at 10.000 rpm for 10 min., and the supernatant removed with a pipette. The cells were

suspended in the same amount of sterile 4 % saline. Of the suspended culture 10 ml/kg was added to the roe.

Analysis

The analysis was carried out in duplicate by examination of two roe jars of each of the combinations shown in table 1. During storage for 119 days, roe jars were taken for analysis 6 times. At each day of analysis the pH of the roe was measured, and odours of the roe was assessed by at panel of 4 persons, members of the internal panel at the roe production company (ABBA Seafood AB, Kungshamn, Sweden). Odours compared to odours of the frozen reference was assessed. If a different odour was detected in the stored roe, the judges were instructed to describe this odour in words.

At the end of storage LAB and Enterobacteriaceae counts were determined by spread plating of appropriate dilutions in peptone saline on NAP and TSA/VRB-G agar respectively, using the methods previously described (paper 2).

Results

Normal lightly salted roe (batch 1)

Roe of batch 1 remained unspoiled during 119 days of storage, and pH remained unchanged. At the end storage Enterobacteriaceae counts were < 5 log cfu/g, and LAB counts 6.4 and 6.6 log cfu/g in the double determinations.

After the first months of storage, gasproduction in an increasing number of jars of batch 2 and 3 occurred. After 49 days half the jars of these batches (evenly distributed among batch 2 and 3) had completely lost their vacuum, and the lits were therefore no longer tightly closed.

Lightly salted roe with 1 % glucose (batch 2)

After the first 14 days pH of the roe had dropped to 4.6 - 4.9, and did not change further. Weak off-odours were detected after 14 days, and after 49 days off-odours were detected by all judges. The odours were described as acid, fruity/sweet and slightly fishy.

Lightly salted roe with 1 % glucose and LAB V6 (batch 3)

After the first 14 days pH of the roe had dropped to 4.5 - 4.6, and did not change further. Slight off-odours occurred after 14 days, and after 49 days 75 % of the judges detected off-odours described as acid and slightly fruity.

Note 7. Effect of sodium lactate or low pH on sensory quality of lightly salted lumpfish roe.

In this note materials and methods and results of two sensory evaluations of lightly salted lumpfish roe are presented.
In the first evaluation (experiment 1) the effect of 2 % sodium lactate on the taste of lightly salted lumpfish roe was examined in order to evaluate the suitability of sodium lactate as a preservative in lightly salted lumpfish roe. In the second evaluation (experiment 2) the effect of low pH on colour, taste and consistency of lightly salted lumpfish roe, in order to evaluate the suitability of using low pH (< 5.4) as an increased hurdle in lightly salted lumpfish roe.

Experiment 1

Materials and methods

Roe products
Lightly salted lumpfish roe with a salt concentration of around 4 % WPS and pH 5.4 was produced from heavily salted roe using the normal procedure (see for example paper 2 and 3). The roe was divided in two portions, one of which received no further treatment (A), while the other was added 2 % (w/w) sodium lactate (B) and mixed thoroughly several times to ensure an even distribution in the roe.

Taste evaluation
A and B roe was distributed in plastic cups containing 10 - 15 g and served to 9 judges. The judges were part of the internal taste panel at the caviar production company (ABBA Seafood A/S, Thisted, Denmark).
The evaluation was performed as triangle tests, in which the six different combinations/ serving orders of three roe samples (two A and one B or two B and one A) were tasted by all judges, who were instructed to point out the one roe sample, that differed from the other two. Three times three samples were served to all judges, and after a break the remaining three times three samples were served (the six combinations/ serving orders are shown in table 1).

Results

The results of the taste evaluation are shown in table 1.

Table 1. Results of taste evaluation in which lightly salted lumpfish roe with and without 2 % sodium lactate was compared (triangle test). A: Roe without lactate, B: Roe with lactate.

Judge no.	Combinations/serving order of three samples					
	ABB	BAA	AAB	BBA	BAB	ABA
1	-	-	-	X	-	-
2	X	-	-	X	-	-
3	X	-	X	-	X	-
4	-	-	-	-	X	-
5	-	-	-	-	-	-
6	X	-	-	X	X	-
7	X	-	-	X	-	-
8		-	X	X	-	-
9	X	-	X	-	-	X
Number of correct identifications. Total = 17	5	0	3	5	3	1

X: Correct identification of the one of three samples, that were different from the other two served.
-: The differing sample among three, was not correctly identified.

Experiment 2.

Materials and methods

Roe products

Lightly salted lumpfish roe with around 4 % WPS was prepared by desalting of heavily salted roe using the normal procedure. The roe was divided in four portions and the pH lowered to 5.4, 5.2, 5.0 and 4.8 in the four portions respectively by addition of lactic acid solution prepared from PURAC®SP 80, 80 % (w/w).

Sensory evaluation

The roe was served in duplicate to 9 judges, members of the internal taste panel at the caviar production company (ABBA Seafood AB, Kungshamn, Sweden).

The roe with pH 5.4 (A) was defined as reference (i.e. normal lightly salted lumpfish roe), and the remaining three roes (B, C and D) were compared to this reference one at a time. The judges were instructed to identify differences in odour, taste and texture of roes B - D compared to roe A, and then to describe the nature of detected differences. Finally an overall assessment of the roe as acceptable or rejectable was given by the judges.

Results

Table 2. Results of sensory evaluation of lightly salted lumpfish roe added lactic acid to different pH levels. Each roe (B - D) was compared in duplicate to roe with pH 5.4 by 9 judges. Only detected differences is indicated.

	Colour	**Taste**	**Consistency**	**% rejection**
B (pH 5.2)	more pink (33) lighter/pale (28)	mild/less salt (28) fishy (6), acid (6)	watery (11)	11 %
C (pH 5.0)	more pink (39) lighter/pale (33)	acid/acrid (17) fishy (11) mild/less salt (6)	soft (11) dry (11)	17 %
D (pH 4.8)	more pink (44) lighter/pale (33)	acid/acrid (28) mild/less salt (22) salt (11)	soft/less crispy (28) sticky (6) watery (17)	33 %

* Numbers in paranthesis indicates the % of assessments, in which a specific difference was detected.

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