The United Nations University
FISHERIES TRAINING PROGRAMME
PO Box 1390, Skulagata 4
120 Reykjavik, Iceland

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The Occurrence of *Listeria monocytogenes* and Microbiological Quality of Cold Smoked and Gravad Fish on the Icelandic Retail Market

Lorena Noriega Orozco

CIAD - Guaymas
Mexico

Supervisors: Hannes Magnússon and Birna Guðbjörnsdóttir, Icelandic Fisheries Laboratories

ABSTRACT

Cold smoked and gravad fish products can be naturally contaminated with low numbers of *Listeria monocytogenes.* This could represent a serious hazard for susceptible people. This studied was conducted to determine and quantify the occurrence of this pathogenic bacteria on the Reykjavik retail market, estimate the remaining shelf life using the Pathogen Modelling Program (PMP) and assess the microbiological quality of cold smoked salmon and trout and gravad salmon products. Evaluation of pH, Aw, aerobic plate count (APC), MPN of total and faecal coliforms, pos/neg and MPN of *L. monocytogenes* were conducted in 38 samples (14 cold smoked salmon, 10 cold smoked trout and 14 gravad salmon). Aw and pH values were lower for cold smoked trout. APC values were found higher than the ISFT specifications, but in accordance with other works. *L. monocytogenes* was found more frequently in gravad salmon than any other product. There was found positive, four gravad salmon and one wood cold smoked salmon samples. In most of the samples *L. monocytogenes* was found at low levels, and high counts were found only in samples stored at high temperatures (retail level). Predictions of the remaining shelf life were higher than the producer specification on the label, except in temperature abused samples. Therefore, the levels of *L. monocytogenes* found in cold and gravad salmon represent a potential hazard if temperature abuse can occur. The importance of the cold chain at retail and consumer level must be emphasised.

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1 INTRODUCTION

Fish production is one of the major economic activities in Iceland. Smoked products are traditionally consumed, even nowadays when fresh fish is more popular in supermarkets and seafood shops. One of the most common smoked products is salmon. During 1998 and 1999 a total harvest of whole salmon of 5,000 and 4,000 tons respectively was reported by the Fish Information Services (FIS 2000). However, the total production of smoked salmon is difficult to estimate. There are several small companies producing smoked salmon to supply local markets and that production is not registered.

In 1999 the Icelandic salmon exports were 1,357.4 tons. Approximately 15% of this was smoked salmon, second after fresh farmed salmon (Hagstofa Islands 2000). Icelandic per capita consumption of fish and fishery products is one of the highest in the world (FAO 2000), and has increased from 35,7 to 45,1 during the past 20 years (Hagstofa Islands 1999). Considering that most of the population lives in or around Reykjavik, we can assume that most of the fish products are marketed for local consumption in this area.

Cold smoked salmon and trout dominate the market for smoked products, although hot smoked products are also found. Consumers prefer fresh and juicy flesh appearance. Therefore other forms such as gravad products can easily be found in the market.

Ready-to-eat seafood including cold smoked fish and other food products has been linked to a number of outbreaks by *Listeria monocytogenes* (Farber *et al*. 2000, Brett *et al*. 1998, Ericsson *et al*. 1997). Different species of *Listeria* can cause illness in animals, but only *L. monocytogenes* has been recognised as pathogenic to humans. It was first described and linked to infected animals in 1926 (Beumer 1997). Since then, different isolation methods have been described and studied but until recently, no methods were simple enough or fully recognised to be used for routine analysis. Today, the methods issued by the Food and Drug Administration (FDA) and United States Department of Agriculture (USDA) are usually accepted worldwide. These methods were developed in response to the two main listeriosis outbreaks in the United States during the 80´s (Beumer 1997).

Cold smoked salmon and other ready-to-eat fish products could be naturally contaminated with low numbers of *Listeria* and, therefore are good carriers of *L. monocytogenes.* This could represent a serious hazard for susceptible individuals or "YOPI" (young, old, pregnant and immuno-compromised people). In medium shelf stable refrigerated foods, where storage temperatures are high enough, the slow growth of these pathogenic bacteria represents the greatest risk. Studies have shown the occurrence of *L. monocytogenes* in different foods, but more attention is given to meat and dairy products, since the most recent and serious outbreaks have been associated with those products (Dillon and Patel 1992, Samelis and Metaxopoulus 1999). Studies on smoked salmon and trout have been conducted in several countries, including Iceland. A study done by Hartemink and Georgsson (1991), showed evidence of the incidence of *Listeria* species including *L. monocytogenes* in cold smoked and gravad fish, but as in most studies, the number of organisms present in the samples was not quantified. This is not surprising, since the international criterion, including the European regulation available at the time of the study, did not allow the presence of *L. monocytogenes* in any ready-to-eat products. Nowadays, some countries adhere to this policy, but others have changed their policy and only consider ready-to-eat products hazardous that allow the growth of *L. monocytogenes* up to the

levels of 10^2 - 10^3 organisms per gram (IFST 1999). Therefore, one of the main objectives of this project was to determine and, if possible, to quantify the occurrence of *L. monocytogenes* in cold smoked and gravad fish on the Reykjavik retail market. At the same time to assess the microbiological quality and use the Pathogen Modelling Program (PMP) (Buchanan and Whiting 1995) to calculate the remaining shelf life of *L. monocytogenes* positive samples.

2 LITERATURE REVIEW

Bacteria present on the fish are normally associated with those found in their natural environment and influenced by the season and the harvesting conditions. The proportion of the initial population can easily be changed after the harvesting process depending on the ability of those bacteria to adapt to the new conditions (ICMSF 1998).
Spoilage bacteria are predominant on newly caught fish, but some pathogenic bacteria could also be present in the skin, gills or guts. The type and number of pathogenic bacteria found in seafood can be divided in two groups: indigenous and non-indigenous bacteria (Huss et al. 1995, Nickelson and Finne 1992).

- **Indigenous pathogenic bacteria**: are commonly found in the aquatic environment, they are present on the live fish and their presence in the final product is predictable (e.g. *Clostridium botulinum*, *L. monocytogenes*, *Aeromonas hydrophila* and *Vibrio* sp.)
- **Non - indigenous pathogenic bacteria**: are normally associated with human or warm-blood animals and their faeces, and not naturally present in fish or seafood products (e.g. *Salmonella*, *Escherichia coli* and *Stapylococcus aureus*). When found they are a consequence of process contamination or mishandling.

The presence of indigenous pathogenic bacteria does not represent a hazard in itself, because they are normally at very low levels. The problems can arise when handling, processing or storing conditions provide favourable conditions for the growth of the pathogenic bacteria. This is the case in cold smoked vacuum packed fish products, in which refrigeration temperatures and anaerobic condition only reduce the growth rate of *L. monocytogenes*.

2.1 Listeria monocytogenes

Listeria monocytogenes is a small non-spore forming gram-positive and catalase positive rod shape bacterium, difficult to identify in old cultures because of coccoidal appearance (BAM 1995). It can grow under anaerobic or microaerophilic conditions and under a wide temperatures range (0 - 45°C) with an optimum range of 30 - 37°C. Because it can grow at low temperatures it is considered as psychrotrophic organism that can easily adapt and grow under the conditions of most foods. Its capacity to grow at refrigeration temperatures can be one of the most important factors that make them present at the end of the shelf life of non-sterile refrigerated products. For example, growth of *L. monocytogenes* at 0 °C has been reported in beef with generation times varying from 5 to 7 days (Beumer 1997) and shows a higher growth rate on fish and shrimp tissue than in beef or chicken at 4°C and under anaerobic conditions (Shineman and Harrison 1994). The limiting growth conditions for *L. monocytogenes* are summarised in Table 1.

Table 1: Limiting growth factors and heat resistance for *Listeria monocytogenes* (Donnelly et al. 1992, Huss et al. 1997, Beumer 1997, FAO 1999).

PARAMETER	RANGE
Aw	>0.92
Temperature (°C)	-0.4 - 45 (optimum 30 - 37)
pH	4.5 - 9.6
NaCl	<0.5 -10
Heat resistance	D_{60} = 2.4 -16.7 min in meat D_{60} = 1.95 - 4.48 min in fish

Increased attention has been paid to *L. monocytogenes* since it was recognised as a food borne pathogen being responsible for human listeriosis (Jemmi and Keusch 1994). Listeriosis is not a common illness in healthy persons, but it can cause meningitis or septicaemia in elderly or immuno-compromised persons. It may also affect pregnant women where it may cause abortion or illness in the newborn (McLauchlin 1996). More recently, *L. monocytogenes* has been implicated in a new form of disease, causing mild gastrointestinal symptoms (FAO 1999).

2.2 Occurrence

L. monocytogenes has been isolated from many natural environments, such as water, soil, sewage, mud, birds and faeces (Donnelly et al. 1992, Nickelson and Finne 1992), and it is considered an environmental contaminant.
The incidence of *L. monocytogenes* in fresh water (rivers, lakes, ponds) and sea water has been reported in several studies (Colburn et al. 1990, Dillon and Patel 1992). However, Jemmi and Keusch (1994) have reported just the isolation of *Listeria* spp. in water samples from three Sweden trout farms, without detecting *L. monocytogenes*. Most of the reports of *L. monocytogenes* in water refer to polluted samples (near populated areas), without detecting it in unpolluted ones (Huss et al. 1995).
The hypothetical infection cycle of *Listeria* to humans (Figure 1) indicates that for fish and shellfish, water is the main contamination vector. Jemmi and Keusch (1994) suggested that birds could cause water or even fish contamination in fish farms. In both cases, when *L. monocytogenes* is naturally present in water and fish, it is always in low numbers, sometimes not even detectable. Duffes (1999) related the fish contamination to sewage effluents, animal faeces and run-off from agricultural land that can contaminate fresh or sea water.

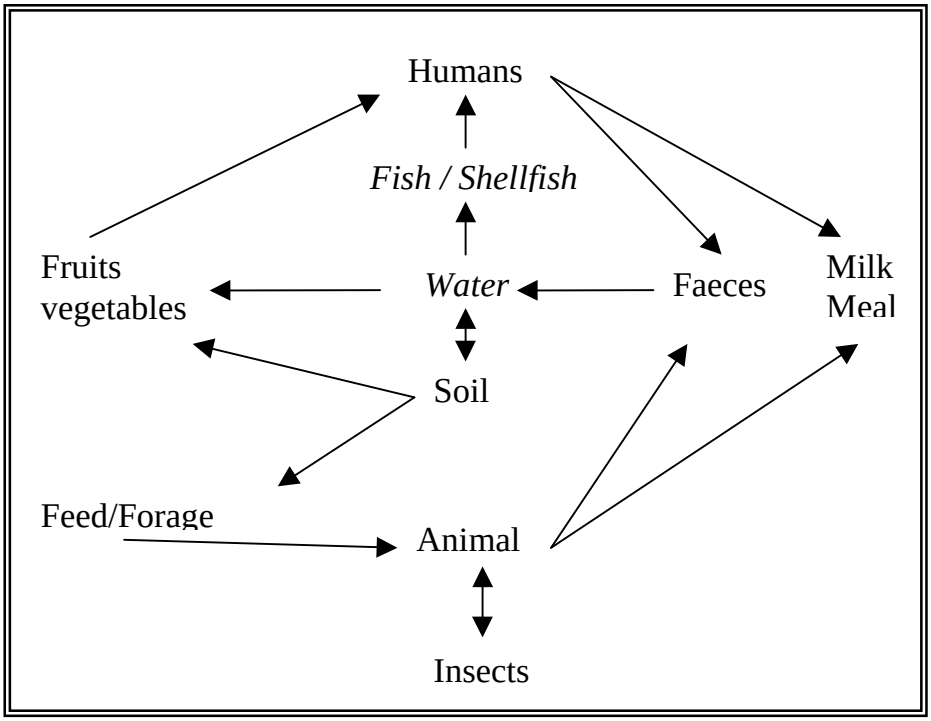


Figure 1: The hypothetical cycle infection of *Listeria* to humans (Dillon and Patel 1992).

2.3 Smoked fish products

There are several studies reporting incidences of *Listeria* spp. and *L. monocytogenes* in fish products, farmed salmon and trout (Rørvick et al. 1995, Heinitz and Johnson 1998) and smoking processing plants (Autio et al. 1999, Rørvick et al. 1997). The initial levels of *Listeria* species found in fish or seafood can be influenced by many factors such as origin (wild or farmed), season, fishing technique, handling and storage conditions. Specifically in salmonid fishes, the origin could be one of the most important factors. A greater incidence of *Listeria* species including *L. monocytogenes* has been reported in fresh water (81%) than in sea water (30%) (Colburn et al. 1990). The source of *L. monocytogenes* in the processing facilities is the fish itself. Once inside the processing facilities it is not always easy to get rid of. Eklund et al. (1995) found that fish can be exposed to water that is contaminated (18.6%) with *L. monocytogenes* during transport. The contamination is easily transferred to the equipment (slicing machines, tables, cutting surfaces) and adheres to it within short period of contact time (Lundén et al. 2000). Therefore, a subsequent re-contamination of fish during the first stages of processing is possible. Appendix 1 shows the general flow diagram for cold smoked and gravad salmon process. The reported incidence in freshly processed cold smoked salmon varies form 10 to 60% with levels below 100 cfu/g (FAO 1999).
According to Beumer (1997) once present in fish (smoked or not), the ability of *L. monocytogenes* to grow will depend on:

a) intrinsic factors: e.g. pH, water activity, preservatives in the food, etc.,
b) extrinsic factors: e.g. storage temperature, atmosphere in the package, etc.,
c) implicit factors: e.g. competition with other micro organisms.

The process for cold smoked fish does not provide the conditions that will stop the growth of *L. monocytogenes*. If it is present in the product after cutting the fish, salting or cold smoking steps do not reduce or eliminate *L. monocytogenes* because the water phase salt (WPS) is not high enough (Table 1). Prevention of growth in cold smoked salmon by NaCl has been reported at 6% (WPS) (Nilsson 1999). The added salt reduces the water activity (up to 0.95), but this is not sufficient to inhibit the growth of *L. monocytogenes*. When salting is done by soaking or needle injection, the risk of re-contamination may even increase (Duffes 1999).
Normal temperature at the cold smoking step (approximately 28°C) is too low to destroy these pathogenic bacteria (Huss *et al*. 1995) and refrigeration and vacuum packaging do not stop its growth, but it has been suggested that the smoke has an inhibitory effect on *L. monocytogenes* (Loncarevic *et al.* 1996). In cold smoked products the salt concentration (2.5-4%) in conjunction with refrigeration temperatures can reduce the growth rate of *L. monocytogenes* significantly (Thurette et al. 1998). Even though anaerobic conditions allow its growth in vacuum packaging a slower growth is often observed, probably due to the competitive or inhibitory effect of the lactic acid bacteria (Nilsson 1999).
European legislation forbids the use of additives like antibacterial chemical substances or antioxidants in cold smoked fish products. Therefore, all the control of *L. monocytogenes* must be done by the processing practices and/or processing parameters. Huss, and co-workers (1995) propose the GMP (good manufacturing practices) to control the presence of *L. monocytogenes* in cold smoked products, and since temperature during storage of the final product can not be properly controlled (<5°C), the storage time should be reduced to three weeks.
New preservation techniques for cold smoked fish products are under study. Most of them focus on protective cultures that help to inhibit the growth of *L. monocytogenes*, such as the inhibitory effect of different LAB (lactic acid bacteria) and its bacteriocins as bio preservatives (Duffes 1999; Nilsson et al. 1999).

2.4 Outbreaks

L. monocytogenes has been recognised as the only *Listeria* species that can infect humans, even though *L. ivanovii* has been implicated in a small number of cases (Gellin and Broome 1989). Special attention has been given *to L. monocytogenes* since the three majors outbreaks in North America during the early 80´s (Table 2). During the Canada and USA incidents in 1981 and 1985 respectively, mainly pregnant woman and their foetus or newborn were affected.
In addition to these large registered outbreaks, sporadic cases can occur. In 1986 an estimate of 1700 sporadic cases of listeriosis occurred in the United States alone (7.1 cases in a million). During the same year the Council of State and Territorial Epidemiologist in the United States, strongly advised to include it as a reportable disease (Gellin and Broome 1989). Recently, a wide range for the annual incidence of listeriosis in Europe (0.1 to 11.3 per million) was reported by Notermans and co-workers (1998) and FAO (1999) estimates that this disease fluctuates from 2 to 10 cases per million with a fatality rate of 20 - 30%.

Table 2: *Listeria monocytogenes* cases registered in different countries since 1981 (Gellin and Broome 1989, Dalton et al. 1997, Ericsson et al. 1997, Brett et al. 1998, FAO 1999, Nilsson 1999, Farber et al. 2000).

Place	Year	Implicated Food	No. Cases	Fatality
New Zealand	1980	Raw shellfish	29	31%
Canada	1981	Coleslaw	41	41%
USA	1983	Pasteurised milk	56	29%
USA	1985	Mexican style cheese	142	33%
USA	1989	Shrimp	9	11%
Italy	1989	Fish	1	0
Switzerland	1983-1987	Soft cheese	122	28%
Australia	1991	Smoked mussels	4	Not Known
New Zealand	1992	Smoked mussels	4	0
USA	1994	Milk	52	0
Sweden	1994-1995	Cold smoked and gravad trout	9	25%
Canada	1996	Imitation crab meat	2	Not Known

Most of the listeriosis cases, in which seafood has been implicated, are not considered large outbreaks (less than 10 cases per outbreak) (Table 2). Even if most of the incidents of *L. monocytogenes* are reported from industrialised countries, the low or nil incidences reported form Africa, Asia and Latin America does not necessarily mean that it is not present in those continents (Destro 2000), although it has been suggested the incidence may be lower due to the climate conditions (Ababouch 2000). It has been mentioned by FAO (1999), that combination of factors such as consumption patterns, dietary habits, host susceptibility or lack of testing facilities may be the reason for the low reporting incidence in undeveloped countries.
The level of contamination of *L. monocytogenes* needed to produce listeriosis is a matter of debate, but most of the reports agree that there is a need for more information and documentation of the cases. The foods responsible for both sporadic and epidemic cases of listeriosis have shown low (<100 cfu/g) and high (>1,000 cfu/g) levels of *L. monocytogenes* (Rocourt and Cossart 1997). Therefore, there is not enough data to establish an infection dose at low limits, but it seems to be lower for immuno-compromised and patients with reduced gastric acidity. The resistance for a healthy person can vary from 10^3 to 10^4 cfu/g (Gellin and Broome 1989, FAO 1999). One of the main reasons to clearly establish the infection dose became of the difficulty to trace back listeriosis cases to a specific food product, and the further bacterial isolation from the patient. This is mainly due to outbreaks that can extend several months or years and the long incubation period in humans that can be up to 91 days (Farber et al. 2000). The variable incubation period may be due to the combination of different factors such as variation in the number of pathogenic cells ingested, differences in the host susceptibility or differences in the virulence of the strains (FAO 1999).

2.5 International criteria for Listeria

Since it is not possible to determine an effective dose response for *L. monocytogenes* for the entire population, some countries have set a zero tolerance policy not just for ready-to-eat or sensitive products (Table 3). Considering the available information, the zero tolerance could be used for specific markets, group of products but it is hardly a realistic option for foods in general. Some countries such as the Netherlands

do not have specific legislation for *Listeria* species in fish or fishery products only refer to a non-detectable level for pathogenic bacteria or their toxins. A more realistic position is taken by those countries, which consider that the harmonisation of the fish and seafood product regulations based on the HACCP principles should offer adequate protection for the consumer. In those cases the type of product, risk category and time of consumption must be taken into account to develop a complete risk assessment and the implications for international trade.

Nørrung (2000) mentions that concentrations not exceeding 100 cfu/g of *L. monocytogenes* when consumed do not represent a high risk to a normal healthy person or even for the immuno-compromised portion of the population (1 in a million). This assumption was made based on two previous works reported for the German immuno-compromised population assuming that all the listeriosis cases reported in that country were caused by smoked salmon.

At present, there is no international agreement regarding the acceptable levels for *L. monocytogenes* in fish or seafood products (Huss et al. 2000), but the first step has been taken as most of the evidence supports the idea that low levels do not represent a health risk. Harmonisation and mutual recognition between countries is in the process. Examples of this are the trade agreements between countries and the tendency to work together in a similar Codex standard base. This tendency is driven by the increased trade in fish and seafood products that emphasises the effect of sanitary and phytosanitary regulation (Caswell 2000).

Table 3: Microbiological criteria for fish products in some countries or organisations and their legal status (Elliot and Kvenberg 2000, WHO 2001)

Country	Food	Micro organisms and other information	Numerical values (CFU/g or ml if not specified)	Sampling plans	Point of application	Legal status
Australia	Fish, mussels smoked products	*L. monocytogenes*	m = 0 in 25 g	n=5, c=0	Not specified	standards
Canada	Ready to eat (RTE)	Supporting growth of *L. monocytogene*s with refrigerated shelf-life <10 days and all RTE foods not supporting growth, produced under GMP	ND in 25 g >100 <100	n = 5 n = 5 n = 5	Manufacturing Manufacturing Manufacturing	Class I recall (retail level) Recall / stop sale Allow sale and recall / stop sale
Denmark	Foods raw	*L. monocytogenes*	m = 10, M = 100	Sampling size 25 g, n and c not specified	Retail	Guidelines
France	Foods with no listericidal treatment	*L. monocytogenes*	Objective = ND in 25g; unacceptable > 100	n = 5, c = 0	Production / Consumption	Not specified
Ireland	Crustaceans, fish, raw oysters, shellfish, pate, cooked, herring/roll map, smoked pickled fish	*L. monocytogenes* and *Listeria* spp. (not *L. monocytogenes)*	Satisfactory- ND in 25g, Borderline-in 25g - <200, Unsatisfactory- 200 - <1000, Unacceptable- >1000	Not specified	Retail	Guidelines
Israel	Fish hot and cold smoked Fish products	*L. monocytogenes*	ND in 25g	M = value of standard, n=1 c=0	Not specified	Voluntary

	(breaded frozen)		ND in 25g	M = value of standard, n=1 c=6	Not specified	Mandatory
IFST	Fish and seafood pickled, smoked, marinated, raw; frozen or chilled; RTE	*L. monocytogenes*	GMP ND in 25 g Maximum 1000	Not specified	Not specified	Recommended
Italy	Raw and frozen foods	*L. monocytogenes*	m =12 M= 110	1g; n =3 c=2	Manufacturing	Mandatory
New Zealand	RTE, processed shellfish	*L. monocytogenes*	m=0 per 25g	n=5 c=0	Not specified	Guidelines

Table 3 (Cont.): Microbiological criteria for fish products in some countries or organisations and their legal status (Elliot and Kvenberg 2000, WHO 2001).

Country	**Food**	**Micro organisms and other information**	**Numerical values (CFU/g or ml if not specified)**	**Sampling plans**	**Point of application**	**Legal status**
Norway	Salmon trout ground marinated	*L. monocytogenes*	Not specified	n = 5, c = 0	Not specified	Guidelines
Norway	Cold or hot smoked fish, "gravlax"	*L. monocytogenes*	ND in 25g	n = 5, c = 0	Not standardized	Guidelines
Norway	Surimi-products, frozen	*L. monocytogenes*	ND in 25g with use-by-date of more than 15 days	n = 5, c = 0	Use-by date	Guidelines
Switzerla	All RTE foods	*L. monocytogenes*	ND in 25g	Swiss food manual	Swiss food	Mandatory

nd	Fish, cold smoked		100	Swiss food manual	manual Swiss food manual	Mandatory
USA	RTE	*L. monocytogenes*	ND in 25 g	Not specified	Not specified	Class I recall

Where M = Acceptability threshold, the result is considered unsatisfactory if one or more units give values = or > M
m = threshold below which all results are considered satisfactory
n = number of units making a sample
c = number of units in the sample giving values between m and M; the sample being considered acceptable if the values of the other sample units are = or < m.
ND = not detectable
RTE = Ready to eat foods

3 METHODS

3.1 Sampling

Vacuum packed cold smoked and gravad salmon and trout were purchased in six stores in the Reykjavik area. These were selected on the basis of a survey of commercially available brands. Sets of at least two packages from the same production day from each product from 10 different companies were obtained.
The fish storage temperature in the shops was measured using a TFX 492 thermometer from Ebro. After purchasing, the samples were placed in an insulated box with gel-ice to keep the samples at 4°C or below while transported to the laboratory. In the laboratory a code number was given to each sample considering the date of production to assure that the analysis will be conducted before the stated shelf life expire. A total of 38 packages were collected and analysed. The time between the analysis and expiration date was 5 to 10 days.

3.2 Sample analysis

All the samples were tested for water activity (Aw), pH, and microbiological analysis (total aerobic plate count, most probable number -MPN- total coliforms, MPN faecal coliforms, pos/neg test for *L. monocytogenes*, MPN of *Listeria monocytogenes*, and identifications of isolated *Listeria* species).
Water activity (Aw): a sample of ten to twenty grams of sample, were placed in a Aw - Wert - Messer (Durotherm) capsule at 22°C and kept in an incubator for at least four hours before a reading was taken. Calibration and temperature corrections were made according the manufacturers instructions.
pH: a duplicate sample of approximately five grams of minced tissue was mixed with the same amount of water (weight), and pH measurement was made using the PHM 80 Portable Radiometer Analytical Copenhagen, with an immersed electrode according to instructions of the manufacturer's manual.
Microbiological analysis: Aerobic plate count and most probable number (MPN) of total and faecal coliforms was conducted according the procedures of the Compendium of Methods for the Microbiological Examination of Foods (APHA 1992). The enumeration and detection of *Listeria* spp. and *L. monocytogenes* was carried out using the two-steps enrichment method described by Cook (1998) and McClaine and Lee (1989).
All the sample packages were disinfected with ethanol (70% w/w), aseptically opened and the fish flesh was grinded. Two sub-samples of 25 g were taken for the microbiological analysis. The first sub-sample plus 225 g of buffer (Butterfield´s phosphate) were stomached for one minute and five 10-fold serial dilutions were prepared for the analysis of the samples. The aerobic plate count was determined by the pour plate method (plate count agar + 0.5% NaCl) after 48 hours incubation at 30°C. Total and faecal coliforms were estimated using the three tube MPN method. The second sub-sample was mixed with 225 ml of UVM and incubated at 30°C, after 24 hours 0.1 ml were transferred to 10 ml Fraser broth plus 0.1 ml of ferric ammonium citrate (second enrichment) and incubated for 1 to 2 days at 35°C. All the black tubes were streaked on MOX agar (24 - 28 hours at 35°C). All black colonies were tested for haemolysis, catalase and Gram stain. Suspected *L. monocytogenes* colonies were identified using the API-*Listeria* test (BioMérieux™). The *Listeria*

quantification was determined using the sequence and media mentioned above but using a three tube MPN technique using UVM broth.

3.3 Pathogenic modelling program (PMP)

The remaining shelf life of positive *L. monocytogenes* samples was calculated using the PMP version 5.1 (Buchananand Whiting 1995) available from USDA. Samples where quantification of *L. monocytogenes* was possible, the MPN obtained was used. Samples which tested positive but negative in MPN enumeration method, the minimal detectable level for both methods (0.04 cfu/g and 0.3 cfu/g) were used. Finally samples where quantification was not possible because the dilution factor was low, the value corresponding to the highest dilution (110) were used.
The input data needed for the PMP for *L. monocytogenes* were: a) temperature, b) pH, c) water activity, d) sodium nitrite, e) initial level (Log (CFU/g)), and f) level of concern. Those values were obtained as follows:

a) Temperature: the retail temperature.

b) pH: the mean value of two measurements per sample obtained in the laboratory.

c) Water activity: the sample value obtained during the experiment.

d) Sodium nitrite: none of the sample packages declares the use of sodium nitrate, so this variable was given a value of 0.0 mg/kg.

e) Initial level: the Log of the MPN for *L. monocytogenes* obtained in the laboratory for each sample. In positive samples but negative for the MPN method the value of -1.4 (0.04 cfu/g) and -0.5 (0.3 cfu/g) was used.

f) Level of concern: the fixed level of concern used for all the positive samples was 1000 cfu/g, that corresponds to international standards (IFST 1999) as a non acceptable ready-to-eat fish and seafood product.

3.4 Data analysis

Analysis of variance (ANOVA) was used to test for differences in pH and Aw. Aerobic plate count data were analysed using a generalised linear model with a Poisson distribution (S-PLUS Version 5.1). A multiple comparison test by product, doing all possible comparisons, was conducted and where a difference ($\alpha = 0.05$) was detected, the Tukey´s test was done.

4 RESULTS

A total of 38 packages of fish, 14 cold smoked salmon, 10 of cold smoked trout and 14 gravad salmon were sampled. Results are grouped according to types of products: a) wood cold smoked salmon (WSS), b) manure cold smoked salmon (MSS), c) wood cold smoked trout (WST), d) manure cold smoked trout (MST) and e) gravad salmon (GrS).

4.1 Temperature, Aw and pH

Temperature: the maximum and minimum retail temperatures per type of product and producers are given in Table 4. Most of the packages had been kept below 4 °C. There were just two establishments that showed non acceptable temperatures (>5°C)

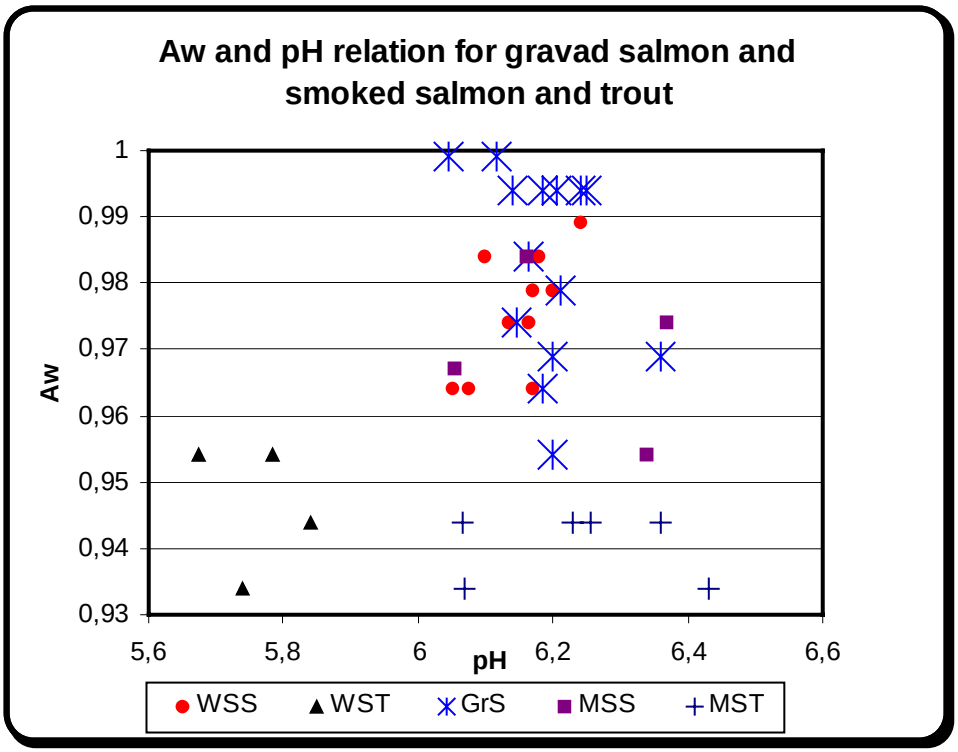
for refrigerated vacuum packed fish (9.0 and 13.2°C), which were affecting 10 packages (26.3%) from three different companies.
All the producers were given a single letter code, which is shown in Table 4, making it possible to identify the number of producers by type of product.

Table 4: Number of samples per product, maximum and minimum retail temperature (°C) and the number of packages that were stored at temperatures >5°C.

Product	No. of Samples	Retail Temperature (°C)	No. of Samples stored at > 5°C	Producer
		Minimum/Maximum	No. (%)	
Wood cold smoked salmon	10	3.2 / ***13.2***	4 (10.5)	A, B, D, E, G
Wood cold smoked trout	4	3.2 / 4.3	0 (0)	C, I
Manure cold smoked salmon	4	2.5 / 2.7	0 (0)	E, H
Manure cold smoked trout	6	2.5 / ***9.0***	2 (5.3)	H, I, J
Gravad salmon	14	2.5 / ***13.2***	4 (10.5)	A, B, E, F, G
Total / Average	**38**	**5.3**	**10 (26.3)**	**10**

pH and Aw: the pH and Aw values obtained in the 38 samples are shown in the Figure 2, and the statistical analysis for those parameters is shown in Appendix 2. The pH values were significantly lower for wood cold smoked trout than any other product (α= 0.05). The Aw values were lowest for cold smoked trout and gravad salmon samples show the highest values, but there is no significance difference between salmon products. Aw is significantly higher (α = 0.05) in salmon, than in both types of cold smoked trout. There is also a significant difference between cold smoked trout (wood and manure) with wood cold smoke salmon, but only manure cold smoke trout was significantly lower than for all types of salmon (α=0.05). Wood cold smoked trout was significantly lower than woods cold smoked salmon, gravad salmon (α= 0.05) and manure cold smoke salmon (α= 0.06).

Figure 2: Aw and pH values for WSS (wood cold smoked salmon), MSS (manure cold



smoked salmon), WST (wood cold smoked trout), MST (manure cold smoked trout) and GrS (gravad salmon).

4.2 Microbiological analysis

The microbiological results are summarised in Table 5, and the statistical analysis is shown in Appendix 2. The mean values were calculated by type of product. The APC counts found for wood cold smoked trout were significantly lower than all the other products (= 0.05). APC counts for gravad salmon were significantly higher than for wood cold smoked salmon.
The MPN of total coliforms was expressed as a range showing the minimum and maximum values. The MPN of faecal coliforms is not included in the table, because they were not detected in any sample.

Table 5: Total bacterial count, MPN of total coliforms and *L. monocytogenes* (MPN and presence/absence) by type of product and international specifications (IFST 1999).

Type of product	**No. Sample**	**TPC cfu/g**	**MPN Total Coliforms**	**L. mono (pos / neg)**	**L. mono/g (MPN)**
Int. Specification "GMP"		$<10^6$	---	ND / 25 g.	---
Int. Specification (Maximum)	---	10^7	---	---	10^3
Wood Cold smoked Salmon	10	4.9 x 10^6	<0.3->11,000	1 (12.5%)	<0.3
Manure Cold smoked Salmon	4	2.1 x 10^7	2.3 - >74.9	Negative	<0.3
Wood Cold smoked Trout	4	1.8 x 10^5	< 0.3-0.9	Negative	<0.3
Manure Cold smoked Trout	6	5.7 x 10^7	0.4 - 9.3	Negative	<0.3
Gravad Salmon	14	3.8 x 10^7	0.3 - >2,150	4 (28%)	<0.3->110
Total of Products	**38**	x 10^6	**<03->11,000**	**5 (13%)**	**<0.3->110**

ND = not detectable

Five of the 38 samples analysed tested positive for *L. monocytogenes* (Table 5); four of them of gravad salmon and one of wood cold smoked salmon. The four positive gravad salmon samples all came from the same producer (Table 6), and three of them from the same production day, but were bought in different stores. In one of the four *L. monocytogenes* positive gravad salmon samples, the dilution used in the three tube MPN method were not sensitive enough (<0.3 MPN/g) to allow its quantification.

Table 6: *Listeria monocytogenes* positive samples, producer and retail temperature (°C).

Type of product	**Producer**	**Retail Temperature (°C)**	**L. mono (pos /neg)**	**L. mono/g (MPN)**
Wood Cold smoked Salmon	D	4.3	Positive	< 0.3
Gravad Salmon	B	2.5	Positive	110
Gravad Salmon	B	13.2	Positive	< 0.3
Gravad Salmon	B	13.2	Positive	> 110
Gravad Salmon	B	4.3	Positive	24

4.3 Pathogenic modelling program (PMP)

The remaining shelf life of the five *L. monocytogenes* positive samples was predicted by the USDA predicting model, using the values obtained in the experiment for pH, Aw, initial population level and temperature (Table 7). The obtained growth curves for those samples are shown in Appendix 3.

Table 7: Parameters used in the Pathogenic Modelling Program (Buchanan and Whiting 1995) for *L. monocytogenes* positive samples under anaerobic conditions (WSS - sample code = wood cold smoked salmon and GRS- sample code = gravad salmon).

Parameter	SAMPLE - Code number						
	WSS-5B	WSS-5B	GRS-1B	GRS-2A	GRS-2A	GRS-2B	GRS-4B
Temperature (Retail °C)	4.3	4.3	4.0*	13.2	13.2	13.2	4.3
pH	6.2	6.2	6.2	6.3	6.3	6.1	6.2
Aw	0.97	0.97	0.984	0.994	0.994	0.974	0.994
Sodium Nitrate (ppm)	0	0	0	0	0	0	0
Initial population ((Log (cfu/g))	-1.4	-0.5	2.0	-1.4	-0.5	2.0	1.38
Level of concern ((log(cfu/g))	3	3	3	3	3	3	3
Remaining label shelf life - days	6	6	4	4	4	4	5
Estimated time to reach the level of concern (days)	9.5-15.5	8.5-13.8	4.4-6.4	1.9-2.3	1.6-2.0	1.4-1.9	5.2-4.1

* The retail temperature was lower, but 4.0°C is the minimum value accepted for the model.

5 DISCUSSION

Aerobic plate count and *L. monocytogenes* counts were higher than the maximum specification set by ISFT (1999) by 47% and 2.6% of the samples respectively. Microbiological criteria for APC normally consider the same limits for fresh, frozen or cold smoked and gravad fish. This is also the case for Iceland. Magnússon and Traustadóttir (1982) reported high APC values and good scores from a sensory panel for smoked herring, concluding that total bacterial count has a minor influence on quality assessment of vacuum packed smoked herring. Brining, vacuum packing and low temperatures tend to increase the proportion of lactic acid bacteria that are harmless to humans, and inhibit the growth of Gram negative bacteria (Bell et al. 1995, Cortesi et al. 1997, Jørgensen et al. 2000). A high count of lactic bacteria does not necessarily equate with a hazardous product. This may be the case for most of the vacuum packed smoked fish. Further work should be conducted to develop a specific criterion, such as a chemical quality index based on spoilage of dominant spoilage micro flora as suggested by Jørgensen et al. (2000) for cold-smoked salmon.
L. monocytogenes was found in 13% of the samples. It was found in gravad and wood cold smoked salmon, but not in manure cold smoked salmon, wood or manure cold smoked trout. Gravad salmon shows the highest incidence of these pathogenic bacteria (28%), but there is not enough evidence to relate its occurrence by product, the producer could also have an important effect. Loncarevic et al. (1996) found similar results (20%) when comparing gravad and smoked fish and Dillon et al. (1994) isolated *L. monocytogenes* from cold and hot smoked salmon but not from trout. Loncarevic, et al. (1996) found higher *L. monocytogenes* counts before than

after the cold smoking step, and suggested that smoke may have an inhibitory effect on the bacteria. Rørvik (2000) mentions that the cold smoking process (19–22°C) does not eliminate *L. monocytogenes*, and that during storage time injured cells may recover. The inhibitory effect of lactic acid bacteria on *L. monocytogenes* has also been suggested as a control measure for ready-to eat-products, such as smoked fish (Duffes 1999, Nilsson et al. 1999). Rørvik et al. (1995) mention that the initial micro flora detected in smoked cod is gradually dominated by lactic acid bacteria and *Moraxella* spp. under vacuum packaging and this is more evident under carbon dioxide atmosphere. The combined effect of smoking and lactic acid bacteria may explain the low incidence of *L. monocytogenes* in cold smoked salmon and trout compared with gravad salmon, where the process does not damage or reduce the level of *L. monocytogenes* (Appendix 1).
The minimum Aw reported for *L. monocytogenes* growth is 0.92 (Dillon and Patel 1993), therefore the values for cold smoked salmon (0.96 – 0.99) and trout (0.93 - 0.95) samples do not have a negative effect on its survival. Shineman and Harrison (1994) demonstrated that the pH is a determining factor for the growth *of L. monocytogenes* in raw tissues, showing higher growth rate at higher pH values (closer to 7). The pH and Aw values were lower for wood cold smoked trout samples. Therefore, the combination of low pH and Aw for wood cold smoked trout may contribute to keep *L. monocytogenes* at non detectable levels when incidental contamination occurs if stored below 5°C.
L. monocytogenes can grow in smoked products during storage; Rørvik (2000) reported a higher growth rate at 8 – 10°C than at 4°C. About 40% of the positive samples were temperature abused, and the only sample that showed high levels (>110 MPN/g) was stored at 13.2°C. The incidence and values found for *L. monocytogenes* in cold smoked or gravad products are not high, but temperature abuse improves the conditions for its growth and in worst cases it may reach unacceptable levels.
When comparing the date stamp of the positive samples with the remaining shelf life predicted by the PMP model, two gravad salmon samples were not acceptable. The remaining date stamp was four days but the model predicted a maximum of two days to reach levels of 10^3 organisms per gram. In both cases, the retail storage temperature was high (13.2°C) and the level of *L. monocytogenes* in those samples were <0.3 and >100 bacteria per gram. This emphasis the importance of proper storage conditions for retaining the safety of those products. In the rest of the samples that showed positive values but had not been temperature abused during storage, the shelf life predicted by the model was long from the one stated by the producer, regardless of the initial population (<0.3, 24 and >110 bacteria per gram). Growth of *L. monocytogenes* in vacuum packed smoked salmon can occur at long storage periods or high storage temperature (Cortesi et al. 1997).
Caution should be exercised in drawing conclusions based on predictive models (Thurette et al. 1998). *L. monocytogenes* may grow more slowly in naturally contaminated smoked salmon than in challenge tests or than predicted by models (Dalgaard and Jørgensen 1998, Rørvik 2000). The predicted shelf life of the samples normally exceeds the date stamp with exception of the samples that had suffered temperature abuse. Taking into account only those samples stored below 5°C, the predicted levels of *L. monocytogenes* were acceptable at the end of the shelf life. Therefore, cold smoked and gravad salmon can be considered a safe product to eat when processed under good manufacturing practices. Proper cleaning and disinfecting procedures in the plant facilities will in most cases result in low numbers of this bacterium in the end product.

6 CONCLUSION

The high levels of aerobic counts that are commonly found in cold smoked fish products do not necessarily have an effect of the safety of the products. Therefore other criteria should be developed.
The pH and Aw in conjunction with the cold smoked step may have an inhibitory effect on the growth of *L. monocytogenes* in cold smoked trout.
L. monocytogenes found in cold smoked and gravad salmon should not represent a hazard to healthy individuals, since its presence can be considered as a natural contamination (low levels). The temperature abuse in some of the shops may represent a serious hazard for susceptible and even healthy individuals, by promoting the growth at high levels. Therefore, importance of the cool chain must be emphasised at retail and consumer level.

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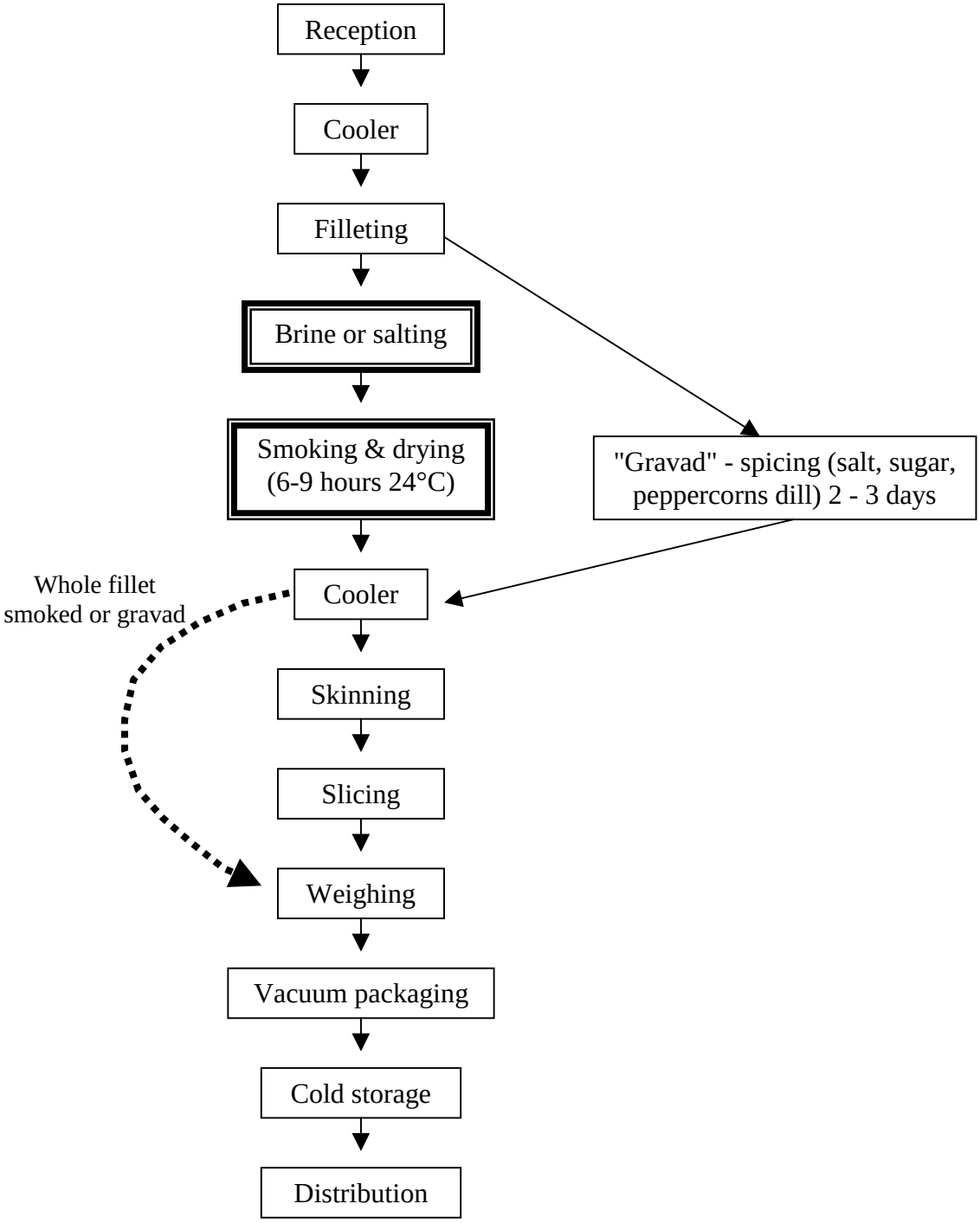
APPENDIX 1: COLD SMOKED AND GRAVAD FISH PROCESS



General flow chart for the cold smoked and gravad salmon.

APPENDIX 2: STATISTICAL ANALYSIS

95% simultaneous confidence intervals for linear combinations, by Tukey´s method.

Product combination	pH	Aw	Aerobic Plate Count
GrS - MSS	No difference	No difference	No difference
GrS - MST	No difference	**Difference**	No difference
GrS - WSS	No difference	No difference	**Difference**
GrS - WST	**Difference**	**Difference**	**Difference**
MSS - MST	No difference	**Difference**	No difference
MSS - WSS	No difference	No difference	No difference
MSS - WST	**Difference**	No difference	**Difference**
MST - WSS	No difference	**Difference**	No difference
MST - WST	**Difference**	No difference	**Difference**
WSS - WST	**Difference**	**Difference**	**Difference**

Where: GrS = gravad salmon; MSS = manure cold smoked salmon; MST = manure cold smoked trout; WSS = wood cold smoked salmon and WST = wood cold smoked trout.

Statistical Analysis: pH values

	Mean	Df	Sum of Sq.	Mean Sq	F value	Pr (F)	
pH-product	6.1449	4	0.6983942	0.1745985	19.8284	2.115895e-08	p_2
Residuals		33	0.2905802	0.0088055			

95 % simultaneous confidence intervals for specified linear combinations, by the Tukey´s method (pH values)

```
critical point: 2.8843
response variable: product
```

Product	Estimate	Std. Error	Lower Bound	Upper Bound	Significant
GS-MS	-0.04230	0.0532	-0.1960	0.111	
GS-MT	-0.04610	0.0458	-0.1780	0.086	
GS-WS	0.04040	0.0389	-0.0716	0.152	
GS-WT	0.42900	0.0532	0.2750	0.582	****
MS-MT	-0.00375	0.0606	-0.1780	0.171	
MS-WS	0.08270	0.0555	-0.0774	0.243	
MS-WT	0.47100	0.0664	0.2800	0.663	****
MT-WS	0.08650	0.0485	-0.0533	0.226	
MT-WT	0.47500	0.0606	0.3000	0.650	****
WS-WT	0.38800	0.0555	0.2280	0.549	****

Aw Values

	Mean	Df	Sum of Sq.	Mean Sq	F value	Pr (F)

Aw-product	0.96908	4	0.00998225	0.00249556	18.5209	4.56031e-08	D2
Residuals		33	0.004446512	0.000134743			

95 % simultaneous confidence intervals for specified linear combinations, by Tukey´s method

```
critical point: 2.8843
response variable: product
```

	Estimate	Std. Error	Lower Bound	Upper Bound	Significant
GS-MS	0.01320	0.00658	-0.005800	0.0322	
GS-MT	0.04230	0.00566	0.025900	0.0586	****
GS-WS	0.00743	0.00481	-0.006430	0.0213	
GS-WT	0.03640	0.00658	0.017400	0.0554	****
MS-MT	0.02910	0.00749	0.007470	0.0507	****
MS-WS	-0.00575	0.00687	-0.025600	0.0141	
MS-WT	0.02320	0.00821	-0.000424	0.0469	
MT-WS	-0.03480	0.00599	-0.052100	-0.0175	****
MT-WT	-0.00583	0.00749	-0.027400	0.0158	
WS-WT	0.02900	0.00687	0.009190	0.0488	****

APC Values

```
Analysis of Deviance Table
Poisson model                    Response: pca and log(pca)
```

	Df	Deviance Resid.	Df	Resid Dev	F Value	Pr(F)	
NULL			37	1499347715			
APC-product	4	696685131	33	802662583	7.640108	0.0001794369	D2

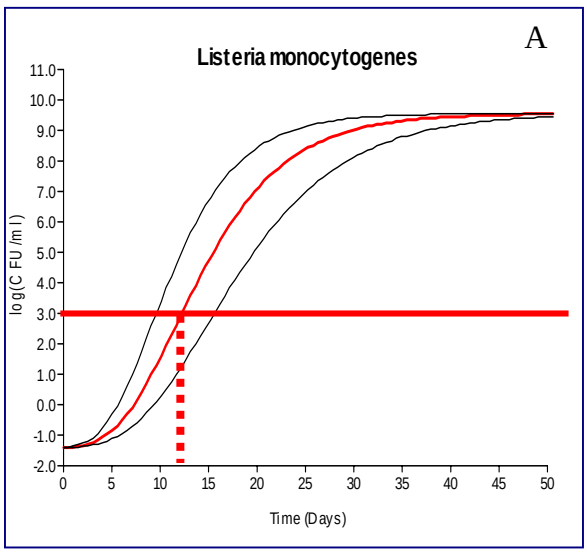
	Df	Deviance Resid.	Df	Resid Dev	F Value	Pr(F)	
NULL			37	11.40934			
log (APC)	4	7.086256	33	4.32308	13.85217	9.835588e-07	D2

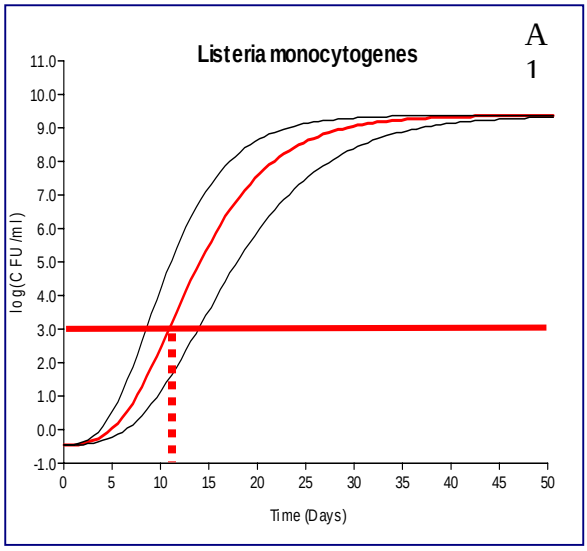
95 % simultaneous confidence intervals for specified linear combinations, by Tukey´s method

```
critical point: 2.8843
response variable: product
```

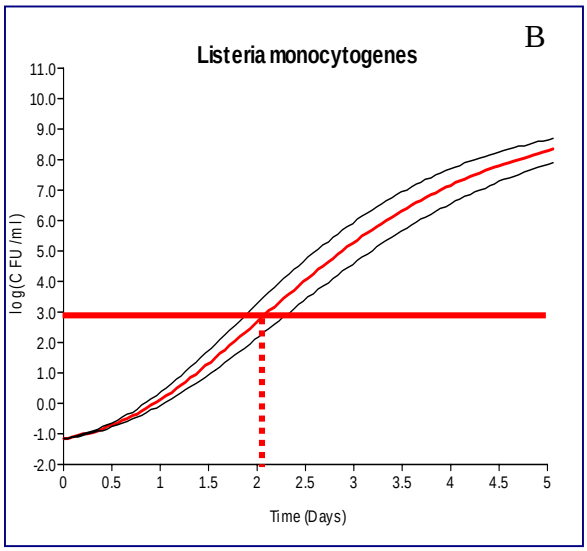
	Estimate	Std.Error	Lower Bound	Upper Bound	
GS-MS	0.0184	0.0495	-0.1240	0.161	
GS-MT	0.0373	0.0429	-0.0864	0.161	
GS-WS	0.1170	0.0372	0.0095	0.224	****
GS-WT	0.3930	0.0577	0.2270	0.559	****
MS-MT	0.0188	0.0567	-0.1450	0.183	
MS-WS	0.0984	0.0526	-0.0532	0.250	
MS-WT	0.3740	0.0686	0.1770	0.572	****
MT-WS	0.0795	0.0464	-0.0542	0.213	
MT-WT	0.3560	0.0640	0.1710	0.540	****
WS-WT	0.2760	0.0603	0.1020	0.450	****

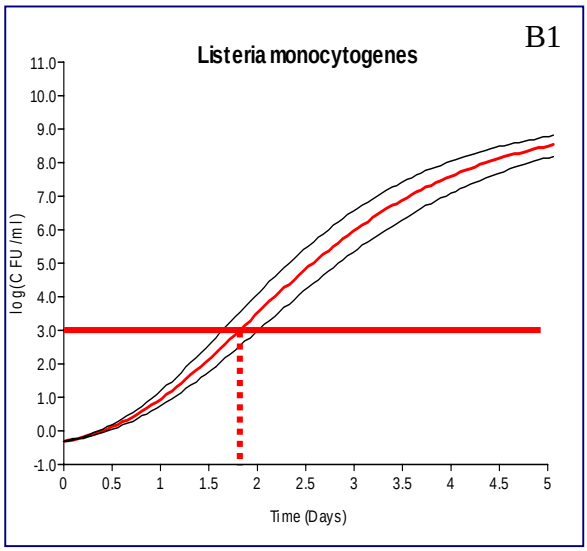
APPENDIX 3: PREDICTED GROWTH CURVES FOR *L. MONOCYTOGENES*



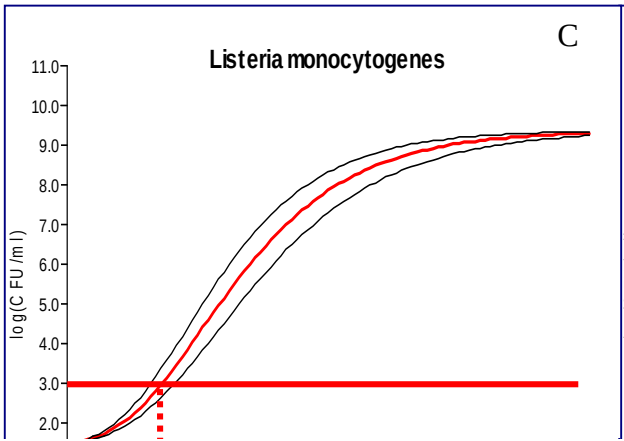


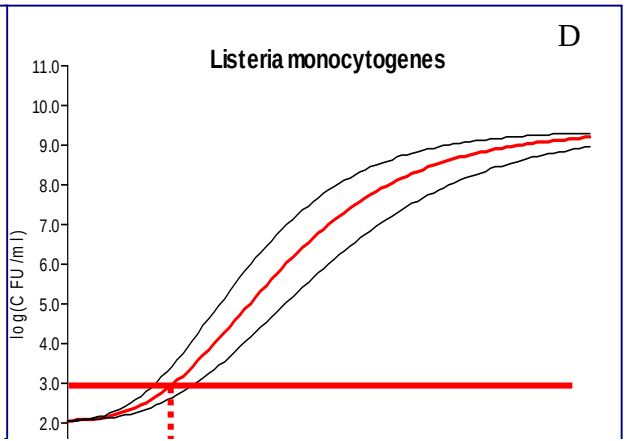
L. monocytogenes predicted growth (Buchanan and Whiting 1995) for wood cold smoked salmon under anaerobic conditions, level of concern = 1000 cfu/g, low levels of contamination (A=0.04 cfu/g and A1=<0.3 cfu/g), low temperature (4.3°C) and pH value = 6.2.

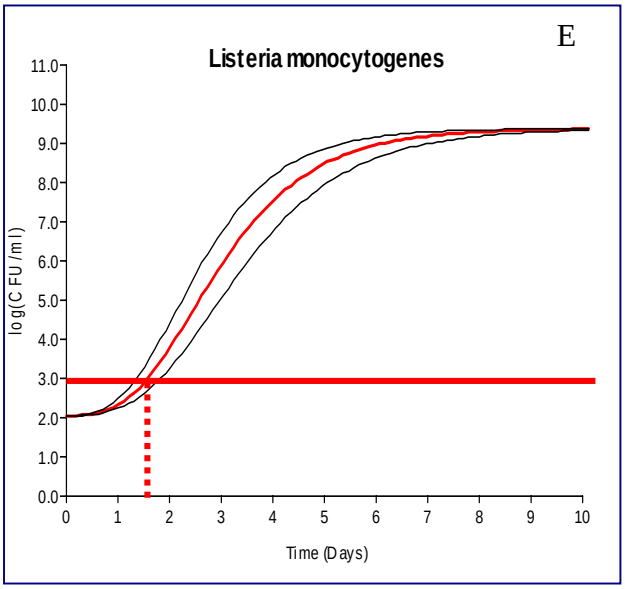




L. monocytogenes predicted growth (Buchanan and Whiting 1995) for gravad salmon under anaerobic conditions, level of concern = 1000 cfu/g, low level of contamination (B=0.04cfu/g and B1=0.3 cfu/g), high temperature (13.2 °C), pH 6.3







L. monocytogenes predicted growth (Buchanan and Whiting 1995) for gravad salmon under anaerobic conditions, level of concern = 1000 cfu/g C = medium level of contamination (24 cfu/g), low temperature (4.3 °C), pH 6.2; D = high level of contamination and (110 cfu/g), low temperature (4.0°C) pH 6.2; E = high levels of contamination (110 cfu/g), high temperature (13.2°C), pH 6.1.