Rapport nr. **180**

Inaktivering av patogene mikroorganismer i fiskebiprodukter

Delprosjekt Salmonella

RUBIN

Oppdrett

RAPPORTTITTEL

Inaktivering av patogene mikroorganismer i fiskebiprodukter. Delprosjekt Salmonella

RAPPORTNUMMER	180	PROSJEKTNUMMER	4517
UTGIVER	RUBIN	DATO	Juni 2009

UTFØRENDE INSTITUSJONER

Nofima Ingredients
Kjerreidviken 19, 5141 Fyllingsdalen

Kontaktperson: Halvor Nygaard

SAMMENDRAG OG KONKLUSJONER

EUs Biproduktforordning krever høytrykkssterilisering av kategori 2 materiale, dvs. dødfisk fra oppdrett, før det kan anvendes som råstoff til biogassproduksjon eller jordforbedring. Dette kravet er både uproporsjonal i forhold til farer som skal kontrolleres og kostbart. Det ødelegger videre nærings-verdien, begrenser anvendelsesmuligheter og dermed muligheten for verdiskapning.

Målet må være at regelverket aksepterer at biprodukter fra fiskeoppdrett, både kategori 2 og 3, kan behandles med metoder som Norge har god erfaring med over flere tiår. Videre må det åpnes for at prosessert materiale kan anvendes til flest mulige formål innenfor regelverket.

Næringen har derfor søkt om godkjenning av den eksisterende metoden for hygienisering av kategori 2 materiale fra fisk i henhold til biproduktforordningen. Dette innebærer ensilering med påfølgende varmebehandling ved 85°C. Under søknadsprosessen har prosessanleggene en midlertidig godkjenning i hht. følgende krav: "Den ensilerte massen skal varmes opp til en kjernetemperatur på minst 85°C i minst 25 minutter og tidligst 24 timer etter tilsetting av maursyre".

Det har vært nødvendig å gjennomføre nye forsøk for å få godkjent den eksisterende metoden. Resultatene kan brukes både i forhold til å dokumentere effekt av ensilering og varmebehandling. Det vil dessuten være viktig grunnlag for søknad om å få godkjent ensilering av kategori 3 materiale som metode og få anerkjent flere anvendelsesmuligheter.

I første omgang er det kjørt inaktiveringsforsøk på Salmonella (denne rapporten). I neste omgang planlegges forsøk med IPN- virus, som regnes som det mest hardføre fiskevirus.

Resultatene fra forsøkene, der Salmonella er tilsatt oppmalt biråstoff av laks, viste at konsentrasjonen av Salmonella var redusert med mer enn 4 $\log_{10}$ til under 3/gram, dvs. under deteksjonsgrensen med den aktuelle analysemetoden, etter kun ensilering der pH er redusert til 4,0-4,1 i 24 timer.

I et analyseprogram for Salmonella i ensilasje og ensilasjeprodukter, basert på både tidligere og nye analyser, var alle prøvene negative. Dette indikerer at forekomst av Salmonella er lav og at forholdene for overlevelse og vekst er ugunstig.

Stiftelsen RUBIN
Pirsenteret
7462 Trondheim

Telefon 73 54 56 30
Telefax 73 51 70 84
E-mail rubin@rubin.no
www.rubin.no

RUBIN

Nofima Ingredients
Kjerreidviken 16, NO-5141 Bergen, Norway
Tel.: +47 55 50 12 00, fax: +47 55 50 12 99
ingredients@nofima.no
www.nofima.no

Business reg. no.: NO 964 441 898 VAT

Report

	Accessibility: **Confidential** **K-354**
Title: Inactivation of pathogenic microorganisms in fish by-products Sub-project *Salmonella*	*Date:* 22.05.2009
	Number of pages and appendixes: 16
Author(s): Halvor Nygaard	*Project no.:* P 20781
By agreement with: FHL and RUBIN	*Employers ref.:* G.H.Knutsen and S.Bekkevold

Three keywords:
Fish by-products, formic acid, thermal inactivation

Summary:

The aim of the present project was to determine the inactivation effect on *Salmonella*, of an alternative processing method for category 2 material of fish origin. The method includes reduction of particle size to ≤ 10 mm, mixing with formic acid to pH ≤ 4.0, storage for ≥ 24 hours and heating to ≥ 85 °C for ≥ 25 minutes.

Gamma irradiated (10.3 kGy) fish suspensions spiked with Salmonella Senftenberg (CCUG 19369) were used in the laboratory trials. Experimental conditions (acid dosage, temperature and exposure times) did not exceed the minimal levels described for the method.

After exposure to formic acid at pH 4.0 - 4.1 for 24 hours, the concentration of viable *Salmonella* was reduced by more than 4 $\log_{10}$ cycles to below 3/gram, which is the lower detection limit for the analysis method. Hence, the effect of further heat treatment could not be determined.

Much literature is available on the heat resistance of *Salmonella*. Typical values reported are Z = 5 °C and D_{65} = 1 minute. From this, D_{85} = 0.0001 minute can be predicted. Increased thermal inactivation rates at reduced pH have been reported. Therefore, *Salmonella* in fish silages at pH ≤ 4.0 will probably have a D_{85} < 0.0001 minute.

In a surveillance program for *Salmonella* in silages and silage products, based on both filed analysis data and new analyses, all samples were negative. This indicates that the incidence of *Salmonella* is low, and that the conditions for microbial survival or growth in such materials are unfavourable.

In light of the demonstrated effect of formic acid on *Salmonella*, knowledge of its heat tolerance and the results of the surveillance program, it can be concluded that the processing method described will be suitable for inactivation of *Salmonella* and similar bacteria in fish by-products.

Table of contents

1 Introduction ... 3

2 Materials and methods ... 4
2.1 Preparation of fish suspension ... 4
2.2 Preparation of *Salmonella* culture ... 4
2.3 Experimental setup ... 4
2.4 Analysis methods ... 5
2.5 Surveillance of *Salmonella* in raw silages and silage products ... 5

3 Results and discussion ... 6
3.1 Characterization of fish suspension ... 6
3.2 Effect of alternative processing method ... 7
3.3 *Salmonella* in raw silages and silage products ... 9
3.4 Conclusion ... 10

4 References ... 11

Appendix I. Estimation of MPN-indexes and confidence intervals ... 13

Appendix II. Temperature logger report from the period of heat treatment ... 16

1. Introduction

The project was defined by Norwegian Seafood Federation (FHL) and funded by RUBIN, a foundation working for increased and more profitable utilization of by-products from the fisheries and fish farming in Norway.

The aim of the present sub-project was to determine the inactivation effect on *Salmonella* of an alternative processing method for category 2 material of fish origin. In a parallel sub-project accomplished by the Norwegian National Veterinary Institute, the effect of the same processing method on fish pathogenic microorganisms was examined. The two sub-projects were coordinated, but reported separately.

Category 2 materials of fish origin are mainly derived from dead fish collected at aquaculture farms on a daily basis as part of good hygiene practice. The mortality could be due to infectious or non-infectious diseases. Another major source is dead fish from disease outbreaks, whether the cause is listed or non-listed diseases. This may include fish without clinical signs of disease, killed in order to eradicate epizootic diseases. Category 2 material can also contain dead fish collected after mass mortality caused by e.g. sudden changes in water quality or jellyfish invasions. Less common is fish containing residues of veterinary drugs exceeding permitted levels.

According to our own experiences from previous inactivation studies with *Salmonella*, and from quality control of products from the silage industry, it is considered unlikely that viable *Salmonella* could be present in formic acid preserved fish material at pH 4, or in this material after heat treatment at 85 °C. However, *Salmonella* may be suitable as a model organism for non-sporeforming bacteria for the validation of inactivation methods. In Regulation (EC) 1774/2002, *Salmonella* together with *Enterobacteriaceae* and *Clostridium perfringens*, are used as indicators for the inactivation effect from different processing methods.

2. Materials and methods

2.1 Preparation of fish suspension

By-products from slaughtering of Atlantic salmon and sea trout were provided by a local manufacturer of feed grade ingredients. Head/backbones were coarsely minced and subsequently mixed with equal parts of viscera before new mincing and homogenisation using an Ultra-Turrax knife homogenizer. Finally, the suspension was passed through a metal sieve ASTM 8 (mesh 2.36 mm opening), distributed in 500 ml capacity screw capped polyethylene bottles and frozen at ≤ -20 °C.

The material was sent in a frozen condition to Institute for Energy Technology, N-2027 Kjeller, Norway, and exposed to 10.3 kGy of gamma irradiation, in order to eradicate its indigenous microbial flora. After irradiation, the fish suspension was stored frozen until use. According to The Codex General Standard for Irradiated Foods (CAC/RS 106-1979) the overall average dose absorbed by a food subjected to radiation processing can be up to 10 kGy. The treatment is considered to affect nutritious properties less than thermal processing and it should neither affect other properties of relevance to our experiments.

2.2 Preparation of Salmonella culture

Freeze-dried culture of Salmonella Senftenberg[1] (CCUG 19369) was purchased from Culture Collection, University of Gothenburg, Sweden. The content of one ampoule was reconstituted in peptone-salt solution (ISO 6887-1), inoculated on NA (Nutrient Agar, Oxoid CM 0003) and incubated at 37 °C over night. One pure colony from NA was transferred to NB (Nutrient Broth, CM 0001) and incubated at 37 °C over night. 100 ml of the NB culture was finally mixed with 20 ml of 60 % autoclaved glycerol, distributed in cryo-tubes (Nunc 363401) and freeze-stored at ≤ 20 °C.

Fresh late exponential phase cultures to be used in our experiments were prepared by transferring some ice crystals from the frozen stock-cultures to NA and incubated at 37 °C for about 18 hours.

2.3 Experimental setup

<u>Pre-incubation</u>
100 gram of decontaminated fish suspension was inoculated with 10 µl fresh *Salmonella* culture (ca. 1x10E9 bacteria/ml). The Inoculated suspension was incubated at 1.2 ± 0.2 °C for 24 hours. The temperature in the cooling incubator was logged during the whole experiment using an accredited calibrated logger (EBRO EBI-125A. S/N: 10046769).

<u>Acid treatment</u>
Pre-incubated inoculated fish suspension was added 0.6 ml 80 % formic acid. The silage was stored at 18 - 20 °C for 24 hours. pH was measured to 4.0 10 minutes after addition of acid and to just below 4.1 after 24 hours.

<u>Heat treatment</u>
Portions of 4.5 ml silage was transferred to glass test tubes (diameter 15 mm, length 100 mm) and placed in a water bath set to 85 °C. The tubes were kept in continuous movement

[1] Salmonella enterica subsp. enterica serovar senftenberg

during the first 5 minutes to facilitate heat transfer from water bath to samples. Heat treatment was terminated after predetermined heating times, by transferring tubes from the water bath to a mixture of water and melting ice. The temperature of the water bath during the experiment was 84.7 ± 0.2 °C (Appendix II) according to accredited calibrated logger (EBRO EBI-125. S/N: 10046765).

2.4 Analysis methods

All chemical and microbiological analyses were conducted by the accredited testing laboratorium, Nofima Ingrediens, Biolab Analyse, N-5141 Fyllingsdalen.

Salmonella analysis

Salmonella was analysed by a combined method consisting of a conventional method, NMKL[2] 71 and a PCR[3] method. The conventional method includes pre-enrichment in BPW (Buffered Peptone Water, Merck 1.07228.500) at 37.0 ± 1.0 °C for 20 ± 4 hours. Pre-enrichment cultures are analysed by real-time PCR[4]. Analysis of PCR positive pre-enrichment cultures was completed according to NMKL 71, ending with biochemical and serological confirmation of pure isolates.

The method is qualitative, and the results are "*Salmonella* detected" or "*Salmonella* not detected" in a specified amount of sample. In order to quantify *Salmonella*, 3 parallel analyses were conducted in a decimal dilution series of each sample. Initial suspensions (10^{-1} dilution of samples) and the appropriate number of further decimal dilutions were prepared according to ISO[5] 6887-1. The final result is estimated using the MPN (Most Probable Number) statistical method (U.S. FDA, 2006).

In the experiments described, 1 gram samples (normally 25 gram) were analysed. Therefore 9 ml BPW was used (normally 225 ml) for each analysis in order to maintain the 1:10 ratio between sample and medium. Tubes with BPW intended for pre-enrichment of undiluted silage samples were added 80 µl 1 N NaOH to achieve an initial pH of 7.4 (ISO 6887-3).

Other analyses

Aerobic microorganisms	Internal method based on Petrifilm
Enterobacteriaceae	ISO 21528-2
Anaerobic sulphite reducing bacteria	NMKL 56
Fat (Ethylacetate extraction)	NS 9402
Raw protein Kjeldahl N*6,25	ISO 5983
Total dry matter	ISO 6496
Ash	ISO 5984

2.5 Surveillance of Salmonella in raw silages and silage products

The fish silage processing industry provided analysis data from internal product quality control, and new samples of raw silages received from different aquaculture establishments.

[2] NMKL: Nordisk Metodikkommitte fôr Livsmedel (Nordic Committee on Food Analysis)

[3] PCR: Polymerase Chain Reaction.

[4] PCR analysis includes sub-cultivation in BHI (Brain Heart Infusion, Merck 1.10493.0500) for 3 hours, DNA-extractjon by Foodproof Salmonella ShortPrep Kit, Biotecon Diagnostics) and real-time PCR analysis in Light Cycler (Roche Diagnostics) by Foodproof Salmonella Detection Kit, Biotecon Diagnostics).

[5] ISO: International Organization for Standardization

The samples (25 g in 225 ml BPW) were analysed by the method described above. pH was adjusted to 7.0 - 7.5 with 1 N NaOH shortly after sample addition in BPW, in accordance with ISO 6887-3.

3. Results and discussion

3.1 Characterization of fish suspension

The results in Table 1 show that the fish suspension has a high content of fat and a low content of protein and ash. The main reason for this is probably that sieving of homogenized material removed more bone fragments than other constituents. In industry scale silages, bone sedimentation is frequently encountered. The composition of the fish suspensions used in our experiments is therefore probably not very different from industrial silages after bone sedimentation.

In spite of a high content of dry matter, the suspension has a low viscosity, allowing effective blending following addition of bacteria and acid, as well as during the heat treatment.

By-products from farmed fish may contain a microbial flora able to interfere with quantitative analyses of the added model organisms. The suspension was intended for use in both the described experiments with *Salmonella*, and for additional experiments with fish pathogenic bacteria and viruses. The suspension was therefore exposed to low dose gamma irradiation to minimize the inherent microbial flora while simultaneously retaining other properties of relevance to our experiments.

Jamdar og Harikumar (2007) demonstrated that 5 and 10 kGy reduced the number of viable bacteria in poultry viscera by 4 og 6 $\log_{10}$ cycles, respectively, while 20 kGy resulted in sterility. Even 20 kGy had little effect on other examined organoleptic and biochemical parameters. Similar doses are also known to be effective against viruses (De Benedictis et al. 2007). Hwang og Hau (1995) showed that 10 kGy had little effect on the activity of proteolytic enzymes and neither resulted in any structural changes of myofibrils.

The irradiation dose used (10 kGy) are the maximum allowed in foods, according to The Codex General Standard for Irradiated Foods (CAC/RS 106-1979).

Table 1: Analyses in fish suspension before and after gamma irradiation (10,3 kGy)

		Results	
Analysis	Unit	Before irradiation	After irradiation
Raw protein Kjeldahl N*6.25	%		6,6
Fat (Ethylacetate extraction)	%		54,4
Total dry matter	%		60,2
Ash	%		0,8
Aerobic microorganisms	Number per. G	1.000.000	< 250
Enterobacteriaceae	Number per. G	1.500	< 10
Anaerobic sulphite-red. bacteria	Number per. G	890	< 10
Salmonella	Detected/Not det. in 25 g	Not detected	Not detected

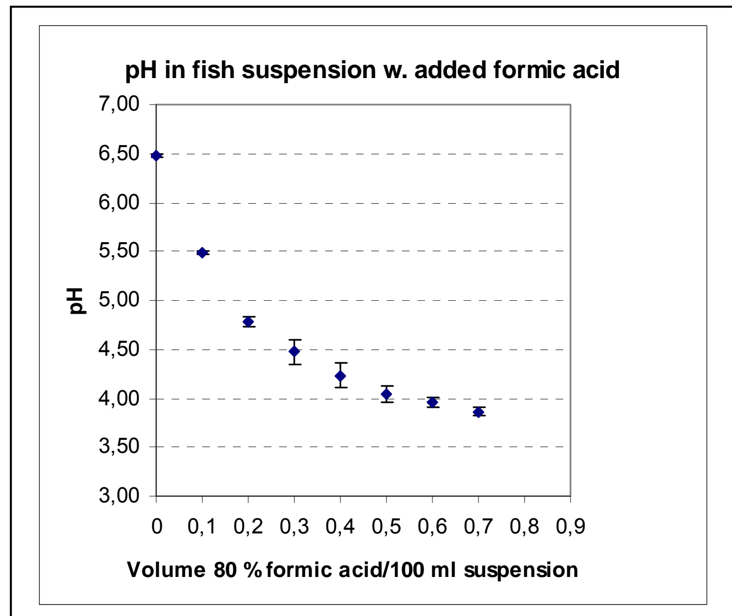


Figure 1: pH in fish suspension versus amount of added 80 % formic acid. The points represent average and standard deviation for three parallel experiments.

In an separate examination (Figure 1) it was demonstrated that the addition of 0.6 ml 80 % formic acid to 100 ml fish suspension resulted in a pH 4.0. This dosis, 0.6 %, is low compared to present practice in the industry which is 1.0 – 3.5 %, depending on the composition of the fish material (RUBIN, 1993). The low consumption of acid is probably due to a low content of bones and a high content of fat.

Furthermore, it was found that the pH in 90 ml BPW with 10 ml of the acidified fish suspension added, could be restored to the original pH (7.4) by adding 800 µl 1N NaOH, corresponding to 80 µl 1N NaOH in 9 ml of the same mixture.

In previous experiments where similar silages were heat treated with exactly the same method, 85 °C were reached after 2 minutes. Temperature exceeded 80 °C in 1.5 minutes. By cooling the silage in ice/water mixture, the temperature decreased from 85 °C to below 30 °C in 0.5 minutes.

3.2 Effect of alternative processing method

The alternative processing method for the treatment of category 2 material of fish origin, includes reduction of particle size to ≤ 10 mm, mixing with an organic acid (usually formic acid) to pH ≤ 4.0 and storage for ≥ 24 hours, pending further treatment. The mixture is finally heated at ≥ 85 °C for ≥ 25 minutes.

When examining the effect of the processing method on *Salmonella*, experimental conditions (acid dosage, temperature and exposure times) did not exceed the minimal levels described for the method. The effect obtained can therefore be considered minimum of what can be expected from an industrial scale process where the conditions has to be equal to or exceed the minimum levels described.

Salmonella Senftenberg was chosen as a test organism. Even if other serovars are more frequently the cause of human and animal salmonellosis, its incidence is high in feed processing plants (Zoonoserapporten 2007) as well as in marine environments and seafoods (Martinez-Urtaza et al. 2004).

In heat resistance studies with *Salmonella*, the extreme tolerance of S.Senftenberg strain 775W is often emphasized. However, this strain is not representative for either the genus *Salmonella* or the serovar S.Senftenberg (Ng et al., 1969).

Salmonella was added to the fish suspension prior to the acid, in order to mimic a real situation where the fish material can be contaminated before ensiling.

The results (Table 2) show a *Salmonella* concentration of 43.000/gram shortly after inoculation, and approximately the same concentration after storage for 24 hours at 1.2 °C.

Salmonella's minimum temperature for growth is approximately 5 °C (Kapperud and Lassen, 1996). Storage below this temperature (cold shock) is not considered to affect the heat resistance of *Salmonella* (Wesche, 2005).

After exposure to formic acid at pH 4.0 - 4.1 for 24 hours, the concentration of viable *Salmonella* was reduced by more than 4 $\log_{10}$ cycles to below 3/gram, which is the lower detection limit of the analysis method used.

Salmonella was not quantified between 0 and 24 hours of acid treatment. It is generally accepted that *Salmonella* can grow at pH down to 4.5 (Kapperud and Lassen, 1996). Inactivation effect from formic acid and similar carboxylic acids are however not only due to pH, but also to toxic effects of undissociated acid. The pK_a of formic acid is 3.75 implying that barely half of the acid exists in its undissociated form at pH 4.0.

Table 2. Quantification of Salmonella in fish suspension by MPN estimates at different phases of the alternative processing method.

		95 % confidence limits	
	MPN/gram	Low	High
Fish suspension prior to inoculation with *Salmonella*	< 3	-	9,5
After inoculation w. *Salmonella*	43.000	9.000	180.000
After inoculation w. *Salmonella* and 24 hours cold storage	38.000	8.700	110.000
After inoculation w. *Salmonella*, 24 hours cold storage and 24 hours acid treatment	< 3	-	9,5
After inoculation w. *Salmonella*, 24 hours cold storage and 24 hours acid treatment. Heat treated for 1 minute	< 3	-	9,5
After inoculation w. *Salmonella*, 24 hours cold storage and 24 hours acid treatment. Heat treated for 2 minutes	< 3	-	9,5
After inoculation w. *Salmonella*, 24 hours cold storage and 24 hours acid treatment. Heat treated for 5 minutes	< 3	-	9,5
After inoculation w. *Salmonella*, 24 hours cold storage and 24 hours acid treatment. Heat treated for 10 minutes	< 3	-	9,5
After inoculation w. *Salmonella*, 24 hours cold storage and 24 hours acid treatment. Heat treated for 25 minutes	< 3	-	9,5

The effect of the heat treatment in the presence of formic acid could not be determined, because the acid treatment alone had decreased the concentration of viable *Salmonella* to below detection limit.

However, a large quantity of literature data is available on the heat resistance of *Salmonella*.

According to U.S. FDA (2000), D-value (decimal reduction-time[6]) for Salmonella Senftenberg at 65 °C is 1.1 minute and Z-value (temperature coefficient[7]) is 5.6 °C. Those are typical values for Salmonella, and can be used to predict D-values at temperatures other than 65 °C. If Z = 5 °C and D_{65} = 1 minute, D_{70} = 0.1 minute and D_{85} = 0.0001 minute can be estimated.

Van Asselt og Zwietering (2006) processed over 1141 published D-values for *Salmonella ssp*. and estimated an average D-value at 70 °C to 0.1 minute. The only significant product effect observed was a protective effect from high fat content.

The mechanisms involved in bacterial heat resistance are not fully understood. The majority of studies deal with the role of heat shock proteins which is activated when bacteria is exposed to sub-lethal heat shock prior to thermal processing (Foster and Spector, 1995).

There is also found a link between the membrane fatty acid composition and heat resistance. Alvarez-Ordonez et al. (2009) reported that *Salmonella* adapted to acidic conditions during growth, had increased heat resistance owing to a low ratio of unsaturated/saturated fatty acids in cell membrane. Cold stress prior to heat exposure had an opposite effect. The article says nothing about the influence of pH during heat exposure.

Heat exposure in the presence of lethal concentrations of formic acid, will probably contribute to a more rapid inactivation than at neutral pH.

Gabriel et al. (2008) found that inactivation rate at 72 °C for Salmonella Typhimurium in a citrus product, was directly related to pH. Increased thermal inactivation at reduced pH was explained by detrimental effects of low pH on nutrient transport and other essential enzymatic processes. Similar results were reported by Manas et al. (2002). When the pH of a buffer system was lowered from 6 to 4, the D_{56} value for Salmonella Typhimurium decreased from approximately 2.0 to 0.8 minutes.

Taking into consideration the cited literature data on thermal inactivation of *Salmonella* and the influence of pH on heat inactivation, it must be concluded that *Salmonella* in fish silages at pH ≤ 4.0 will probably have a D_{85} below 0.0001 minute.

3.3 *Salmonella* in raw silages and silage products

The data material provided by the industry (Table 3) contains no positive results. All analysis reports were issued by accredited laboratories.

In this material however, both raw silages (not heat treated) and category 2 materials were poorly represented. Therefore, on our request, the silage processing industry supplied us with new samples received from local aquaculture farms during the last few days.

When analysing those new samples, pH in pre-enrichment cultures was adjusted to 7.0 - 7.5 shortly after sample addition, in order to eliminate the risk of false negative results due to low

[6] D-value is the exposure time (min) needed to reduce the number of viable bacteria by 1 Log_{10} cycle or 90 %.

[7] Z-value is the temperature increase needed to reduce D-value by a factor of 10 (to obtain 10 times faster killing).

pH restricting *Salmonella* proliferation. Salmonella was not detected in any of the samples (Table 4).

Table 3. Analysis data from internal product quality control, obtained from the fish silage processing industry. Data is grouped according to product type and source. Numbers in brackets are category 2 material.

				SALMONELLA IN 25 GRAM	
PRODUCT TYPE	SOURCE	PERIOD	SAMPLES	DETECTED	NOT DET.
Raw silage	A	2004-2008	21 (0)	0	21
Silage oil	A	2004-2008	37 (1)	0	37
Silage concentrate	A	2004-2008	80 (14)	0	80
Silage concentrate	B	2008	27 (0)	0	27
SUM		2004-2009	165 (15)	0	165

Table 4. Analysis results for samples of raw silage, originating from different aquaculture farms. Data is grouped according to source. Numbers in brackets are category 2 material.

				SALMONELLA IN 25 GRAM	
PRODUCT TYPE	SOURCE	PERIOD	SAMPLES	DETECTED	NOT DET.
Raw silage	A	MAY 2009	11 (11)	0	11
Raw silage	B	MAY 2009	15 (15)	0	15
SUM		MAY 2009	26 (26)	0	26

The gathered analysis results (Table 3 and 4), comprising 47 raw silages (of which 26 from category 2 material) and 144 heat treated silage products (of which 15 from category 2) show that *Salmonella* is not commonly encountered in raw fish silages or in silage products. The results probably also reflect that the conditions for microbial survival or growth in such products are highly unfavourable.

For the purpose of assessing the prevalence of Salmonella in the material to be heat treated with the alternative processing method, it is probably not necessary to distinguish between category 2 and 3 materials. This categorisation is based on other criteria than those disposing for *Salmonella* contamination.

3.4 Conclusion

In light of the demonstrated effect of formic acid on *Salmonella*, knowledge of its tolerance towards heating, in particular heating in the presence of acid, and the results of the surveillance program, it can be concluded that the processing method described will be suitable for inactivation of *Salmonella* and similar bacteria in fish by-products.

4 References

Alvarez-Ordonez, A., Fernandez, A., Lopez, M. and Bernardo, A. (2009).
Relationship between membrane fatty acid composition and heat resistance of acid and cold stresses Salmonella senftenberg CECT 4384.
Food Microbiology 26 (3): 347-353.

De Benedictis, P., Beato, M.S. and Capua, I. (2007).
Inactivation of avian influenza viruses by chemical agents and physical conditions: a review.
Zoonoses Public Health 54: 51-68.

Foster, J.W. and Spector, M.P. (1995).
How Salmonella survive against the odds.
Annual Review Microbiology, 49: 145-174.

Gabriel, A.A., Barrios, E.B. Barrios and AzanzaM.P. (2008).
Modeling the thermal death of Salmonella typhimurium in citrus systems.
Journal of Food Process Engineering, 31 (5): 640-657.

Hwang, Hi. and Hau, Lb. (1995).
Effect of ionizing radiation on the enzyme activities and ultrastructural changes of poultry.
Radiation Physics and Chemistry 46 (4-6): 713-716.

ISO 5983 Animal feeding stuffs - Determination of nitrogen content and calculation of crude protein content.
Part 1: Kjeldahl method.

ISO 5984 Animal feeding stuffs -- Determination of crude ash.

ISO 6496 Animal feeding stuffs -- Determination of moisture and other volatile matter content.

ISO 6887-1 Microbiology of food and animal feeding stuffs. Preparation of test samples, initial suspension and decimal dilutions for microbiological examination.
Part 1: General rules for the preparation of the initial suspension and decimal dilutions.

ISO 6887-3 Microbiology of food and animal feeding stuffs. Preparation of test samples, initial suspension and decimal dilutions for microbiological examination.
Part 3: Specific rules for the preparation of fish and fishery products.

ISO 21528-2 Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of Enterobacteriaceae.
Part 2: Colony count method.

Jamdar, S.N. and Harikumar, P. (2008).
Radiation decontamination of poultry viscera.
Radiation Physics and Chemistry. 77 (4): 467-472.

Kapperud, G. and Lassen, J. (1996).
Salmonella ssp. In: Smittsomme sykdommer fra mat, Næringsmiddelbårne infeksjoner og intoksikasjoner. Author: Granum, P.E. Høyskoleforlaget. ISBN nr.: 82-7634-048-2.

Manas, P., Pagan, R., Raso, J. and Condon, S. (2002).
Predicting thermal inactivation in media of different pH of Salmonella grown at different temperatures.
International Journal of Food Microbiology 87: 45-53.

Martinez-Urtaza, J., Peiteado, J., Lozano-Leon, A. and Garcia-Martin, O. (2004).
Detection of Salmonella Senftenberg associated with high saline environments in mussel processing facilities.
Journal Of Food Protection 67 (2): 256-263.

Ng, H., Bayne, H.G. and Garibaldi, J.A. (1969).
Heat Resistance of Salmonella: the uniqueness of Salmonella senftenberg 775W.
Applied Microbiology 17 (1): 78–82.

NMKL 56 Anaerobe sulfittreduserende bakterier. Bestemmelse i næringsmidler.

NMKL 71. Salmonella. Påvisning i livsmedel.

NS 9402 Atlantisk laks – Måling av farge og fett.

RUBIN (1993). Håndbok I ensilering.
ISBN: 82-993089-0-9.

U.S. FDA. Center for Food Safety and Applied Nutrition (2006).
Bacteriological Analytical Manual Online, Appendix 2: Most Probable Number from Serial Dilutions. Available at: http://vm.cfsan.fda.gov/~ebam/bam-a2.html.

U.S. FDA. Center for Food Safety and Applied Nutrition (2000).
Kinetics of microbial inactivation for alternative food processing technologies. Overacting principles: Kinetics and pathogens of concern for all technologies.
Available at: http://www.cfsan.fda.gov/~comm/ift-over.html.

Van Asselt, E.D. og Zwietering, M.H. (2006).
A systematic approach to determine global thermal inactivation parameters for various food pathogens. International Journal of Food Microbiology 107 (1), 73-82.

Wesche, A.M., Marks, B.P. og Ryser, E.T. (2005).
Thermal resistance of heat-, cold- and starvation injured Salmonella in irradiated comminuted Turkey.
Journal of Food Protection 68 (5): 942-948.

Zoonoserapporten 2007.
Available at: http://www.vetinst.no.

APPENDIX I. Estimation of MPN-indexes and confidence limits

Results from Salmonella analyses and estimation of MPN index w. 95 % confidence interval using tables for 3-tube MPN in Bacteriological Analytical Manual (U,S. FDA, 2006).

Decontaminated fish suspension

						95 % confidence limit	
	A	B	C	Komb. positive	MPN index	Low	High
0	-	-	-				
-1	-	-	-	0-0-0	< 3	-	9,5
-2	-	-	-				
-3	-	-	-				
-4	-	-	-				
-5	-	-	-				
-6							
-7							
-8							

+: Salmonella detected, -: Salmonella not detected

Decontaminated fish suspension, Salmonella added

						95 % confidence limit	
	A	B	C	Komb. positive	MPN index	Low	High
0							
-1							
-2							
-3	+	+	+				
-4	+	+	+				
-5	-	-	+	3-1-0	43.000	9.000	180.000
-6	-	-	-				
-7	-	-	-				
-8	-	-	-				

+: Salmonella detected, -: Salmonella not detected

Decontaminated fish suspension, Salmonella added, stored cold 24 h

						95 % confidence limit	
	A	B	C	Komb. positive	MPN index	Low	High
0							
-1							
-2	+	+	+				
-3	+	+	+				
-4	+	+	+				
-5	-	-	-	3-0-1	38.000	8.700	110.000
-6	+	-	-				
-7	-	-	-				
-8							

+: Salmonella detected, -: Salmonella not detected

Decontaminated fish suspension, Salmonella added, stored cold 24 h, acid treated 24 h

	A	B	C	Komb. positive	MPN index	95 % confidence limit Low	High
0	-	-	-				
-1	-	-	-	0-0-0	< 3	-	9,5
-2	-	-	-				
-3	-	-	-				
-4	-	-	-				
-5	-	-	-				
-6							
-7							
-8							

+: Salmonella detected, -: Salmonella not detected

Same as above, heat treated 1 minute

	A	B	C	Komb. positive	MPN index	95 % confidence limit Low	High
0	-	-	-				
-1	-	-	-	0-0-0	< 3	-	9,5
-2	-	-	-				
-3	-	-	-				
-4	-	-	-				
-5	-	-	-				
-6							
-7							
-8							

+: Salmonella detected, -: Salmonella not detected

Same as above, heat treated 2 minutes

	A	B	C	Komb. positive	MPN index	95 % confidence limit Low	High
0	-	-	-				
-1	-	-	-	0-0-0	< 3	-	9,5
-2	-	-	-				
-3	-	-	-				
-4	-	-	-				
-5	-	-	-				
-6							
-7							
-8							

+: Salmonella detected, -: Salmonella not detected

Same as above, heat treated 5 minutes

	A	B	C	Komb. positive	MPN index	95 % confidence limit	
						Low	High
0	-	-	-				
-1	-	-	-	0-0-0	< 3	-	9,5
-2	-	-	-				
-3	-	-	-				
-4	-	-	-				
-5	-	-	-				
-6							
-7							
-8							

+: Salmonella detected, -: Salmonella not detected

Same as above, heat treated 10 minutes

	A	B	C	Komb. positive	MPN index	95 % confidence limit	
						Low	High
0	-	-	-				
-1	-	-	-	0-0-0	< 3	-	9,5
-2	-	-	-				
-3	-	-	-				
-4	-	-	-				
-5	-	-	-				
-6							
-7							
-8							

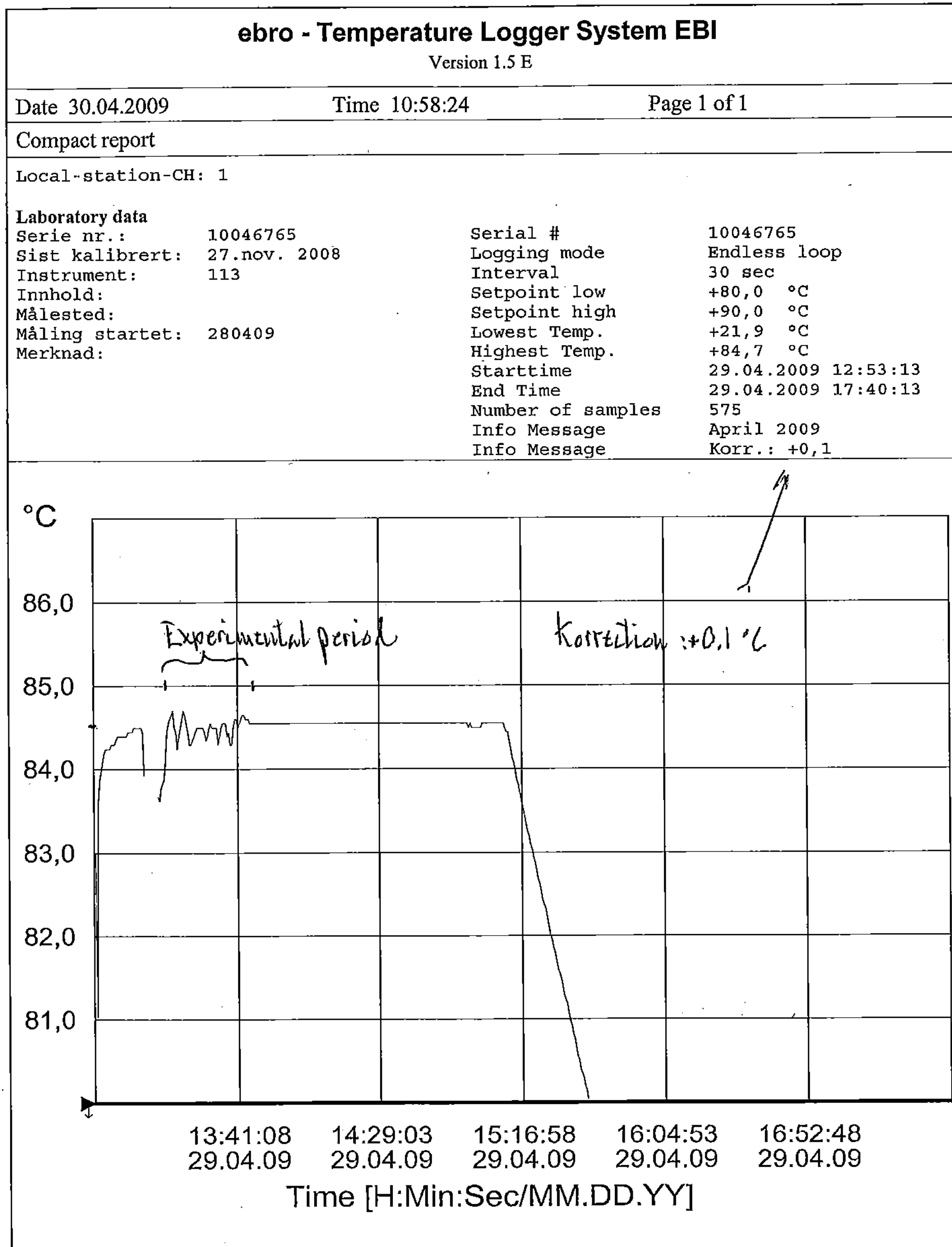
+: Salmonella detected, -: Salmonella not detected

Same as above, heat treated 25 minutes

	A	B	C	Komb. positive	MPN index	95 % confidence limit	
						Low	High
0	-	-	-				
-1	-	-	-	0-0-0	< 3	-	9,5
-2	-	-	-				
-3	-	-	-				
-4	-	-	-				
-5	-	-	-				
-6							
-7							
-8							

+: Salmonella detected, -: Salmonella not detected

APPENDIX II. Temperature logger report from the period of heat treatment



ebro - Temperature Logger System EBI
Version 1.5 E

Date 30.04.2009 Time 10:58:24 Page 1 of 1

Compact report

Local-station-CH: 1

Laboratory data

Serie nr.:	10046765	Serial #	10046765
Sist kalibrert:	27.nov. 2008	Logging mode	Endless loop
Instrument:	113	Interval	30 sec
Innhold:		Setpoint low	+80,0 °C
Målested:		Setpoint high	+90,0 °C
Måling startet:	280409	Lowest Temp.	+21,9 °C
Merknad:		Highest Temp.	+84,7 °C
		Starttime	29.04.2009 12:53:13
		End Time	29.04.2009 17:40:13
		Number of samples	575
		Info Message	April 2009
		Info Message	Korr.: +0,1