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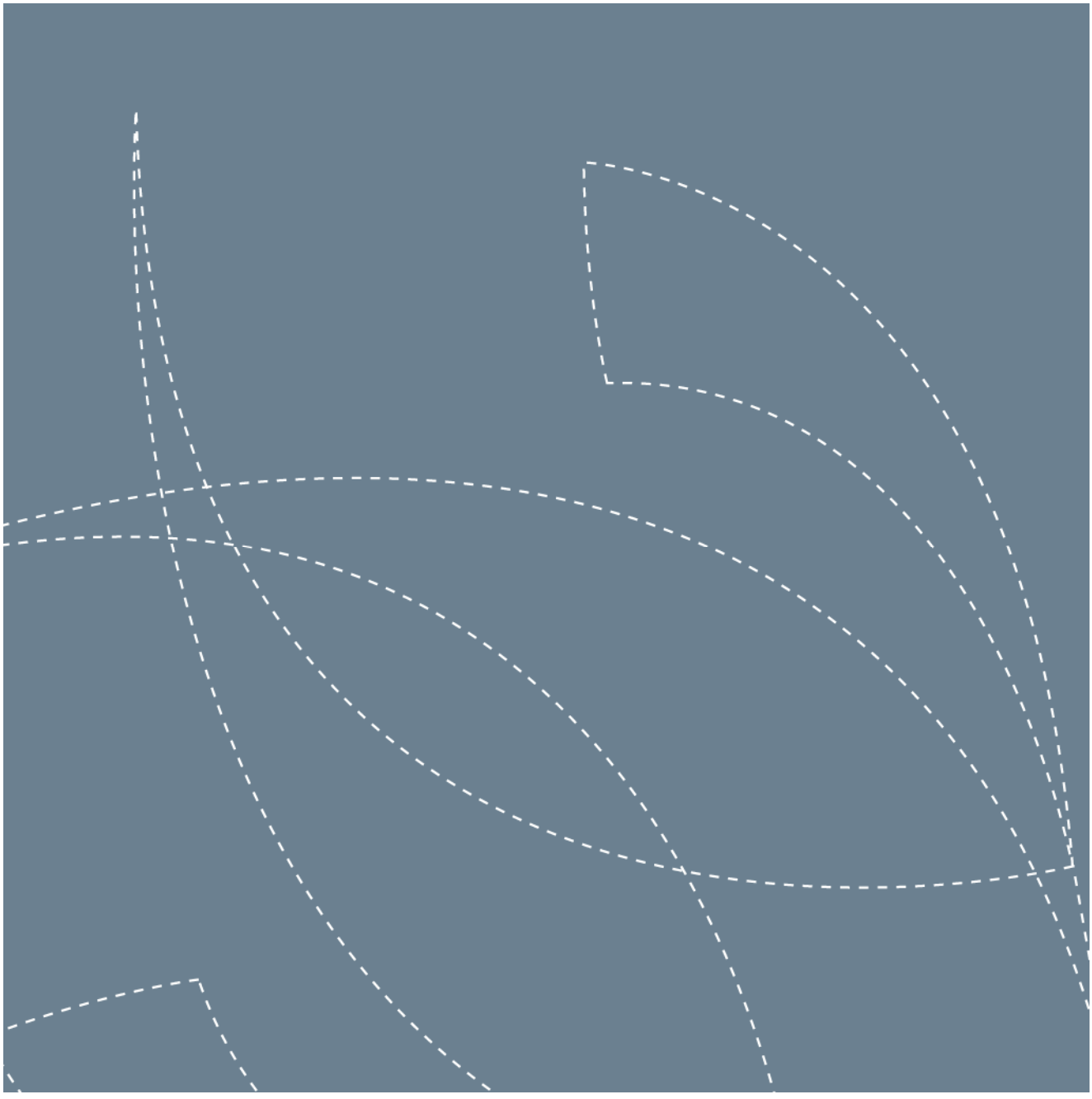
Inactivation of *Clostridium sporogenes* spores in fish by-products by a new processing method

Halvor Nygaard and Kari M. Lie



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Summary:

The aim of the study was to determine the hygienization effect of a new processing method intended for category 2 material of fish origin. The method includes storage (≥ 24 hours) and heat treatment (≥ 85 ºC/≥ 25 minutes) of grinded fish material with added formic acid to pH ≤ 4,0.

C.sporogenes (ATCC 19404) spores replaced *C.perfringens* spores in D-value determinations and in examinations of hygienization effect during all phases of the new processing method. In order to minimize interference from the indigenous flora on the analysis of added spores, the fish minces were pre-treated with gamma irradiation (10 kGy).

Average D-value for heat treatment of *C.sporogenes* spores at 85 ºC in fine fish mince adjusted to pH 7 was 580 minutes. Acidification of fish mince with formic acid to pH 4,0 had no inactivation effect per se, but heat treatment at 85 ºC in the presence of the same acid dosage resulted in a markedly reduced D-value; 8,9 minutes.

The inactivation effect encountered during the complete new processing method was at least 3 Log_{10} reductions in fine as well as in coarse fish mince. This is regarded as satisfactory, considering the low incidence of *C.perfringens* spores in fish silages.

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1 Introduction

The aim of the present study was to demonstrate the hygienization effect of a new processing method intended for category 2 material of fish origin. The method includes storage (≥ 24 hours) and heat treatment (≥ 85 ºC/≥ 25 minutes) of grinded fish material with added formic acid to pH ≤ 4,0.

According to Regulation (EC) 1774/2002, approval of new processing methods requires that samples taken directly after heat treatment comply with a microbiological standard where the heat resistant bacterium *C.perfringens* is absent.

In order to determine the effect of the new processing method on the viability of *C.perfringens*, highly standardized spore preparations, traceable to recognized reference culture collections, should preferably be used. However, when searching for suitable preparations, it became evident that sufficiently dense *C.perfringens* spore preparations were not commercially available. Therefore, it was decided to use a surrogate micro-organism (*C.sporogenes*) for the determination of both decimal reduction time (D-value) at the temperature defined for the heating step of the new process and the effect of the complete process.

A similar but less comprehensive study was reported in 2009 (Nygaard, 2009). EFSA's comments[1] to this report were taken into account during the planning of the present project.

[1] E-mail of 25. November 2010 from EFSA to the Norwegian Food Safety Authority.

2 Materials and methods

2.1 Matrices for acid- and heat treatment

Samples of fresh fish by-products were provided by Biomega AS.

2.1.1 Fine mince of fish by-products

Head/backbones from Atlantic salmon and sea trout 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 kGy of gamma irradiation, in order to inactivate its indigenous microbial flora. After irradiation, the fish suspension was stored frozen until use.

2.1.2 Coarse mince of fish by-products

Head/backbones, whole fish and viscera from Atlantic salmon were separately minced in a meat grinder with coarse screen (11 mm) and equal amounts of each fraction were mixed together and distributed in 500 ml capacity screw capped polyethylene bottles and frozen at ≤ -20 °C.

The material was gamma sterilized (cfr. 2.1.1).

2.1.3 Phosphate buffer

0,1 M phosphate buffer pH 7,0 was sterilized by filtration (0,2 µm syringe filters).

2.2 Test organism

Clostridium sporogenes (ATCC 19404)

Spore suspensions with 2,5 x 10^7 ml^{-1} heat chocked[2] spores in 40 % ethanol were purchased from SGM Biotech, Inc., Bozeman, MT 59715, USA.

[2] Maximum recovery of spores requires a preliminary exposure to heat. Heat shock will also kill any vegetative microbes that might have been accidentally introduced into the spore suspension.

2.3 Heat treatment

For D-value determinations in fish by-products, fine mince of neutral or acidified fish by-products were added 1 % (v/v) *C.sporogenes* spore suspension. 4,5 ml portions of inoculated suspension were distributed in 14x100 mm glass reagent tubes.

For D-value determinations in phosphate buffer, the buffer was added 10 % (v/v) *C.sporogenes* spore suspension. 2,2 ml portions of inoculated buffer were distributed in 14x100 mm glass reagent tubes.

The tubes were submerged in a water bath adjusted to 85.0 ºC and constantly stirred during the first 2 minutes of heat treatment in order to facilitate heat exchange. The portions were heat treated for different periods of time. The temperature of the water bath was monitored, using a calibrated logger system (EBRO EBI-125). After heat treatment, tubes were transferred to an ice/water mixture for rapid cooling.

The pattern of temperature change in the media during heating and cooling was determined (Figure 2 and 3).

2.4 Modelling of new processing method

Pre-incubation

The *C.sporogenes* spore suspension was diluted 1:10 in fine and coarse suspensions of fish by-products and incubated at 5 °C for 24 hours.

Acid treatment

Inoculated and pre-incubated fish suspensions were adjusted to pH 4,0 by adding 80 % formic acid. The acidified suspensions were then stored at 20 ± 1 °C for 24 hours.

Heat treatment

Portions of the acid treated fish suspension, 4,5 ml in 14x100 mm glass reagent tubes, were submerged in a water bath set to 85,0 °C. Temperature monitoring of the water baths, heat treatment and cooling were done according to the procedure outlined in Chapter 2.3.

2.5 Microbiological analysis

Initial suspensions (10^{-1} dilution of sample) and the appropriate number of further decimal dilutions were prepared according to ISO 6887-1.

For enumeration of *C.sporogenes*, 1 ml portions of diluted fish suspension or phosphate buffer were examined by pour plating according to ISO 7218 in RCA (Oxoid CM 0151). Initial suspensions (10^{-1} dilution of sample) were plated on 14 cm Petridishes using 50 ml agar, other dilutions were plated on 9 cm Petri-dishes. Plates were incubated in a modified atmosphere jar (GasPak 150 System, BBL). Anaerobic conditions were created using Anaerogen Sachets (Oxoid AN 0025) and controlled by Dry Anaerobic Indicator Strips (BBL no. 271051). The jars were incubated at 37 ºC for 72 hours. After incubation, typical

C.sporogenes colonies were counted. *C.sporogenes* colonies in RCA are 2-3 mm in diameter and are spherical with a fluffy, cotton-like appearance.

Concentration of organisms in the samples was calculated as weighted mean of the counts from two successive dilutions according to ISO 7218. The lower detection limit was 10 CFU $gram^{-1}$.

The need for neutralization of bacteriostatic residues in the samples was considered. Such residues might inhibit recovery and growth of bacteria, thus causing over-estimation of killing.

The only bacteriostatic agent used in our experiments was formic acid. The antimicrobial activity of formic acid is associated with the undissociated state of the molecule and with low pH (cfr Chapter 3.2). The pKa of formic acid is 3,8, implying that 50 % of the acid is undissociated at pH 3,8. At pH 6,8, only 0,1 % of the formic acid molecule is undissociated.

In the pour plating procedure outlined above, the samples were diluted at least 1:500 and the pH of the agar medium was 6,8 after solidification, even with samples with added formic acid to pH 4,0.

Theoretically, 50 ml agar medium mixed with 1 ml 10-fold diluted sample with 2 % formic acid, will contain only 0,000004 % undissociated formic acid when pH in the agar is 6,8. In our experience this will not inhibit recovery or growth of clostridial spores.

The results of our studies show that 100 % of the added spores are recovered after the acid treatment. Furthermore, comparison of size and appearance of colonies originating from acid treated and non-treated samples, indicate no inhibitory effect of bacteriostatic residues.

2.6 Calculations

Thermal inactivation of micro-organisms at constant temperature is an exponential function. In each equal successive time interval, the same fraction of remaining viable cells is killed. Decimal reduction time (D-value) is the length of time it takes for a population to decrease 10-fold at a given temperature. When the logarithmic number of survivors is plotted versus time, the resulting heat inactivation curve is a straight line and the negative reciprocal of its slope is the D-value. In practice, the points obtained may not fit perfectly into a straight line and the D-value is calculated from the slope of a regression exponential curve.

Similarly, the relationship between Log D-value and the corresponding exposure temperatures is also an exponential function and the negative reciprocal of its slope is the z-value (often referred to as the temperature coefficient).

The relationship between D-values and temperature is expressed by Bigelow's equation (Bigelow, 1921):

Log Dx = (Ty – Tx)/z + Log Dy

Where; Dx and Dy are decimal reduction times at temperature Tx and Ty, and z is the temperature coefficient. With Bigelow's equation, D-values can be estimated for other temperatures than those experimentally examined.

3 Results and discussion

3.1 Composition of fish by-products

The fine ground fish suspension had a high content of fat and a relatively low content of protein and ash (table 1). The main reason is probably that sieving of the homogenized material removed more bone fragments than other constituents. In industrial scale silages, bone sedimentation is frequently encountered. The composition of the fish suspension is therefore probably not very different from industrial silages after bone sedimentation. In spite of a high content of dry matter, the suspension had a low viscosity, allowing effective blending with the spore suspension or acid and rapid temperature equilibration during heating and cooling.

Table 1 Analyses in fine mince of fish by-products before and after gamma irradiation.

		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	cfu per. gram	1.000.000	< 250
Anaerobic sulphite-red. bacteria	cfu per. gram	890	< 10
Anaerobic bacteria on RCA	cfu per. gram		< 10

Cfu: colony forming units. RCA: Reinforced Clostridial Agar

The coarse ground fish suspension was not sieved, and therefore no parts of the mince were removed.

Table 2 Analyses in coarse mince of fish by-products before and after gamma irradiation.

		Results	
Analysis	Unit	Before irradiation	After irradiation
Raw protein Kjeldahl N*6.25	%		11,6
Fat (Ethylacetate extraction)	%		34,0
Total dry matter	%		48,7
Ash	%		1,6
Aerobic microorganisms	cfu per. gram	1.900.000	< 250
Anaerobic sulphite-red. bacteria	cfu per. gram	6.500	< 10
Anaerobic bacteria on RCA	cfu per. gram	1.500.000	< 10

The fish by-products contained a microbial flora that was able to interfere with quantitative analysis of the added model organism. The suspensions were therefore exposed to a low dose of gamma irradiation in order to minimize its inherent microbial flora while still retaining other properties of relevance to our experiments.

Jamdar and Harikumar (2007) demonstrated that 5 and 10 kGy reduced the number of viable bacteria in poultry viscera by 4 and 6 Log_{10} cycles, respectively, while 20 kGy resulted in sterility. Even 20 kGy had little effect on other examined organoleptic and biochemical parameters. Hwang and Hau (1995) showed that 10 kGy had little effect on the activity of proteolytic enzymes and neither resulted in any structural changes of myofibrils. Similar doses are known to be effective against viruses (De Benedictis et al. 2007). The irradiation dose used by us (10 kGy) is the maximum dose allowed in foods, according to The Codex General Standard for Irradiated Foods (CAC/RS 106-1979).

3.2 Acidification of fish by-products

Weak organic acids exist in a pH dependent equilibrium between the undissociated and the dissociated state. Its inhibitory activity is strongest at low pH because this favours the undissociated state of the molecule which is uncharged and freely permeable across the plasma membrane of bacteria. Upon encountering the higher pH inside the cell, the molecule dissociates, resulting in the accumulation of charged anions and protons which disrupt vital cell functions (Brul and Coote, 1999).

Being the strongest organic acid and having a relatively low pKa, formic acid acts both as acidulant and preservative. Even though some of the antimicrobial action of formic acid is based on its pH decreasing effect, inhibition of microbes is mainly associated with the undissociated formic acid molecule (Pölönen, 2000).

To prevent microbial deterioration of formic acid preserved fish mince, the pH of the mixture must be 4,0 or lower which corresponds to an acid expenditure of 1,0 - 3,5 %. Fishery materials with high bone content require the highest acid dosage (Rubin, 1993).

In our experiments (Figure 1), 2-3 times more acid was required to lower pH to 4,0 in coarse mince compared to fine mince, reflecting the higher bone content of the former.

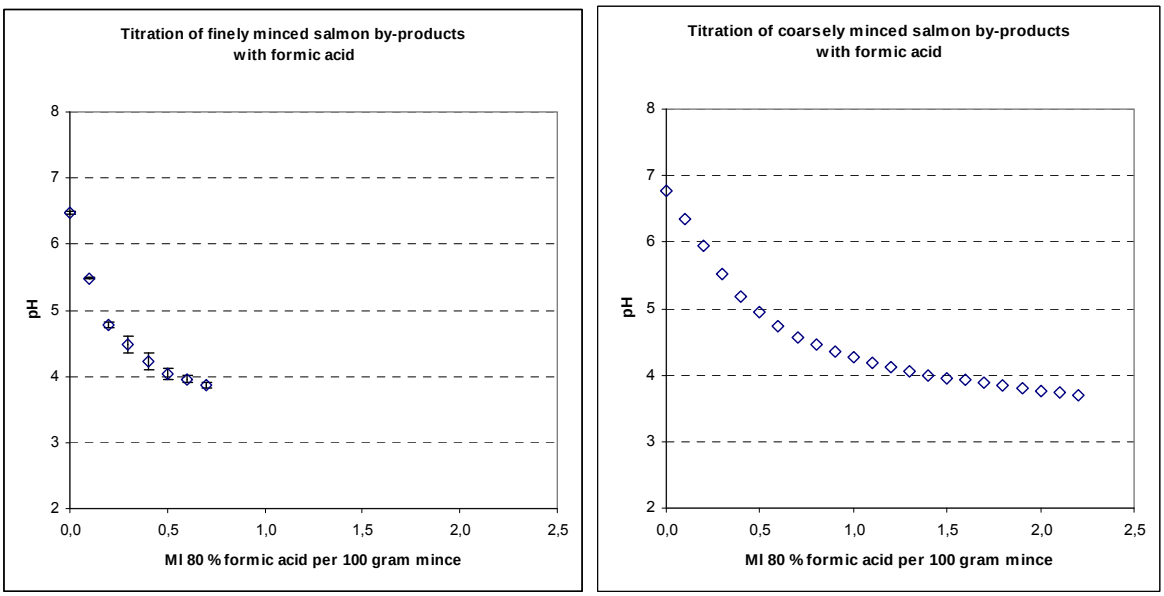

Figure 1 *pH in minced fish by-products versus the amount of added 80 % formic acid. a: fine fish mince (head/backbone : viscera = 1 : 1). b: coarse fish mince (head/ backbone : whole fish : viscera = 1 : 1 : 1).*

3.3 Temperature profiles during heating and cooling

The temperature of the fish suspension exceeded 83 ºC after heating for less than 2 minutes in 85 ºC water bath. Cooling in ice/water reduced the temperature from 85 ºC to 30 ºC in 30 seconds.

With the less viscous phosphate buffer, heating and cooling took approximately half the time.

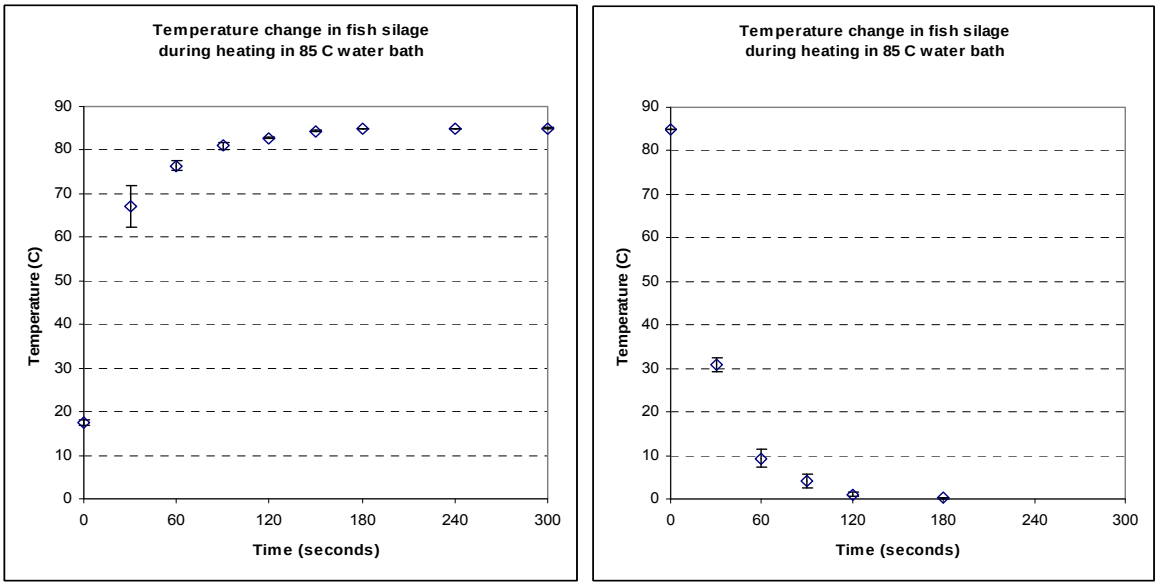

Figure 2 *Temperature equilibration in fine fish mince (4,5 ml in 14x120 mm glass tubes) during heating in water bath (a) or cooling in ice/water (b). Each data point is the average of 3 parallel tests. Vertical bars represent the standard deviation.*

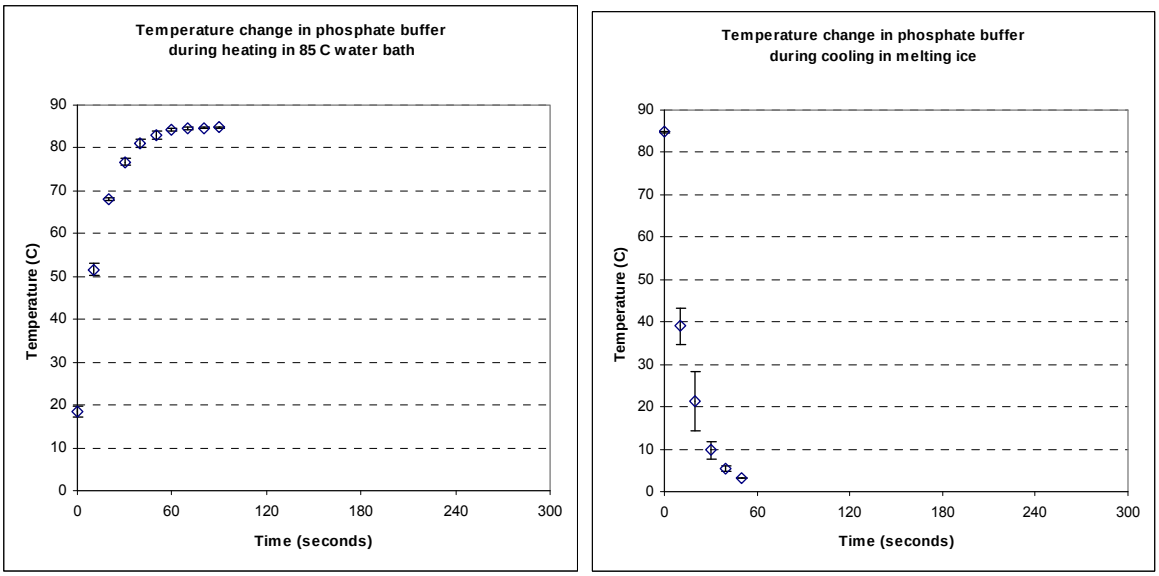

Figure 3 Temperature equilibration in phosphate buffer (2,2 ml in 14x120 mm glass tubes) during heating in water bath (a) or cooling in ice/water (b). Each data point is the average of 3 parallel tests. Vertical bars represent the standard deviation.

3.4 Inactivation of *C.sporogenes* spores at 85 ºC

Endospores are dormant and temporarily non-reproductive structures produced by certain bacteria. Endospores can survive without nutrients. They are resistant to heat and other factors such as ultraviolet radiation, desiccation, freezing and chemical disinfectants.

Transformation of spores to growing, vegetative cells involves activation, germination, and outgrowth. Heat often serves to activate the spores. Generally, heat-resistant strains of *C.perfringens* can be activated by 10–20 min at 75–80 °C.

Only 0,13 - 3,6 % of spores from *C.perfringens* strains associated with food poisoning germinate without heat activation (Doyle, 1989. Walker, 1975). Heat exposure may activate spores but can also injure them. Germination will only occur if damages are repaired, which may require a nutrient-rich recovery medium.

Molin (1992) summarizes D-values for different strains of *C.sporogenes* and *C.perfringens* at 90 °C and 101-103 °C in phosphate buffer or water which indicate that *C.sporogenes* is generally slightly more heat resistant than *C.perfringens*.

C.sporogenes spores replaced *C.perfringens* spores in our D-value determinations. Spores from the two species possess similar heat resistance and *C.sporogenes* is therefore considered as an appropriate surrogate for *C.perfringens* (US FDA, 2001).

3.4.1 Inactivation in phosphate buffer

Inactivation of *C.sporogenes* (ATCC 19404) in a standard medium (0,1 M phosphate buffer, pH 7,0) was examined in order to compare its heat resistance with other strains of the same species.

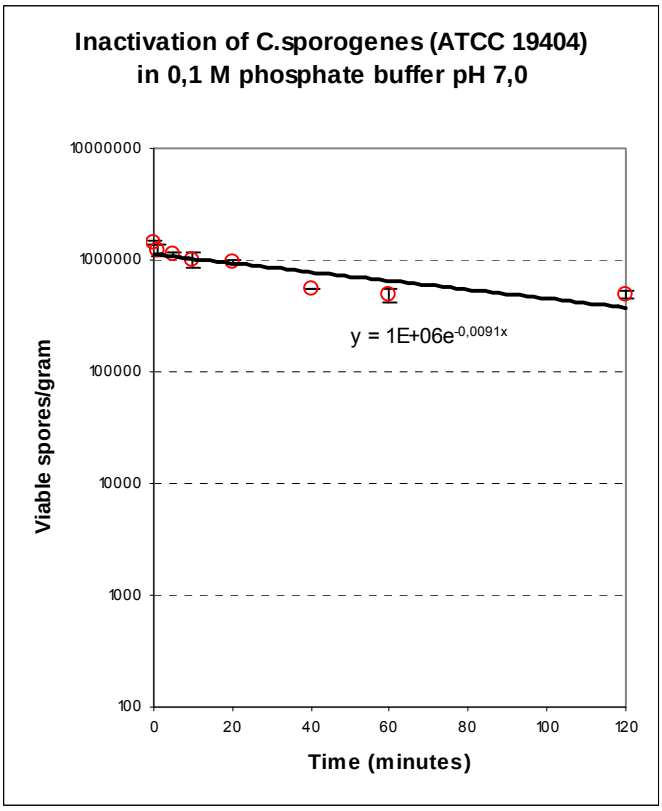

Figure 4 *Inactivation curve for C.sporogenes spores in 0,1 M phosphate buffer pH 7,0 during heat treatment at 85 °C. The straight line is a regression exponential curve. Each data point is average of two parallel analyses. Vertical bars represent the standard deviation.*

Decimal reduction time (D-value) for *C.sporogenes* (ATCC 19404) heated at 85 °C in phosphate buffer pH 7,0 was found to be 253 minutes.

D-values for other strains of *C.sporogenes* in the same medium and at the same temperature were not found in the literature, but could be estimated from D- and z-values obtained in the same medium at different temperatures. Estimated D-value at 85 °C was 348 minutes for strain PA 3679 (D-value at 90 °C = 34 minutes, z = 10,8 °C, Mah et al., 2008) and 82 minutes for strain NCTC 532 (D-value at 115 °C = 0,58 minutes, z = 13 °C, Roberts et al. 1966).

The above data indicate that *C.sporogenes* (ATCC 19404) is not very different from other strains of the same species with regard to heat resistance.

3.4.2 Inactivation in fish by-products

A pre-investigation was carried out in order find appropriate exposure times for the heat treatment at 85 °C of neutral (pH 7,0) and formic acid treated fish mince (pH 4,0). In accordance with the results obtained (Figure 5), it was decided that the heat treatment should last for 30 and 120 minutes at pH 4,0 and pH 7,0, respectively.

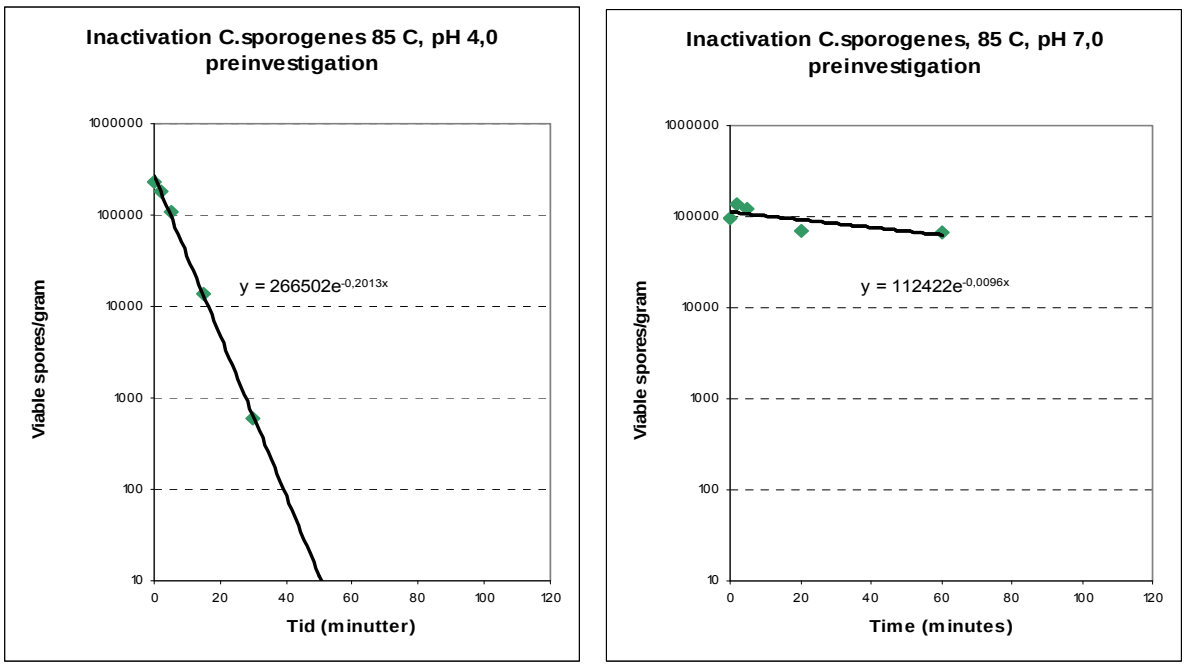

Figure 5 *Inactivation curves for C.sporogenes spores (pre-investigation) in fine fish mince at pH 4,0 (a) and pH 7,0 (b) during heating at 85 °C. The straight lines are regression exponential curves.*

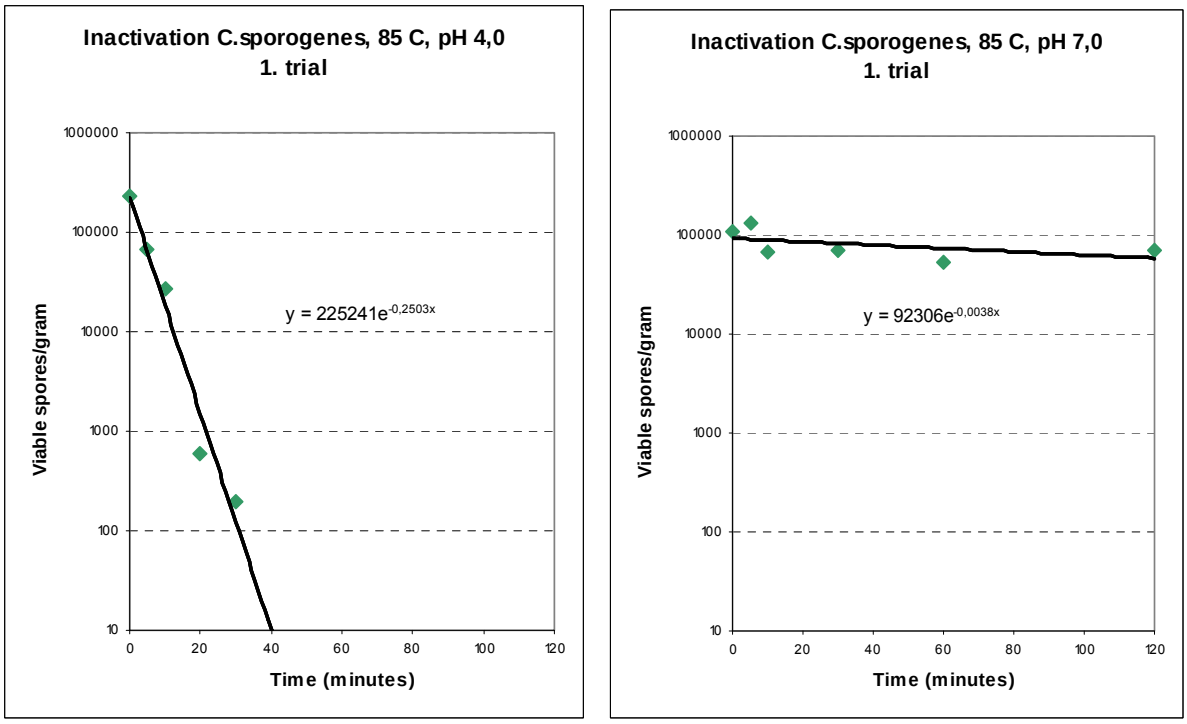

Figure 6 *Inactivation curves for C.sporogenes spores (1.trial) in fine fish mince at pH 4,0 (a) and 7,0 (b) during heating at 85 °C. The straight lines are regression exponential curves.*

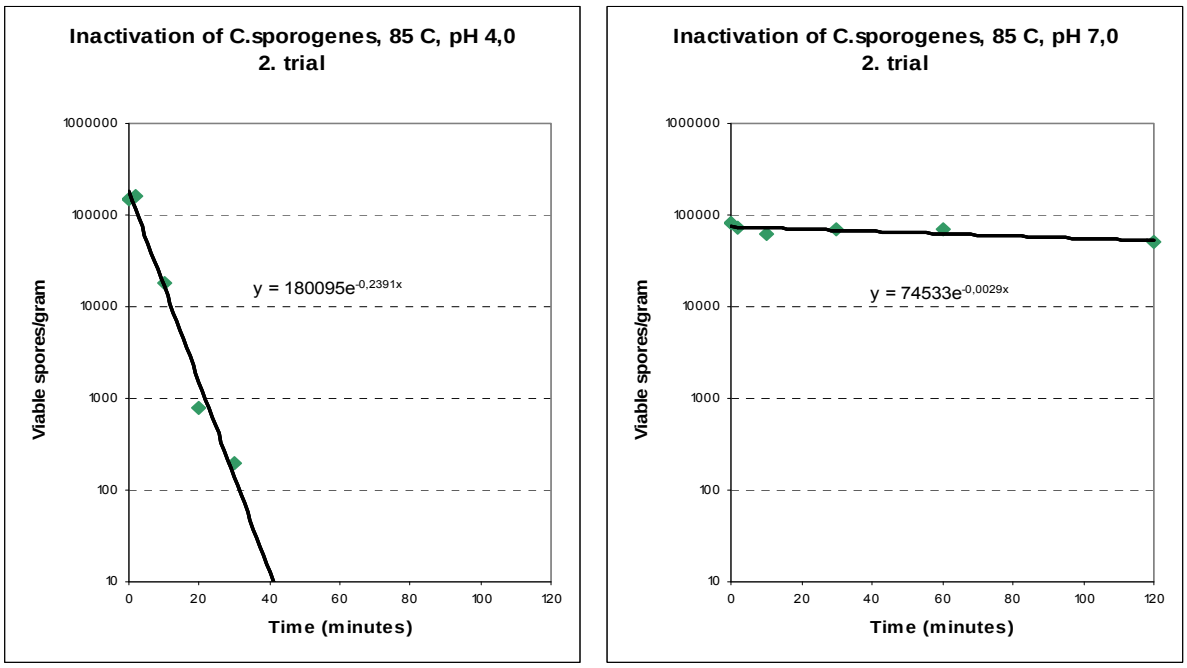

Figure 7 *Inactivation curves for C.sporogenes spores (2.trial) in fine fish mince at pH 4,0 (a) and 7,0 (b) during heating at 85 °C. The straight lines are regression exponential curves.*

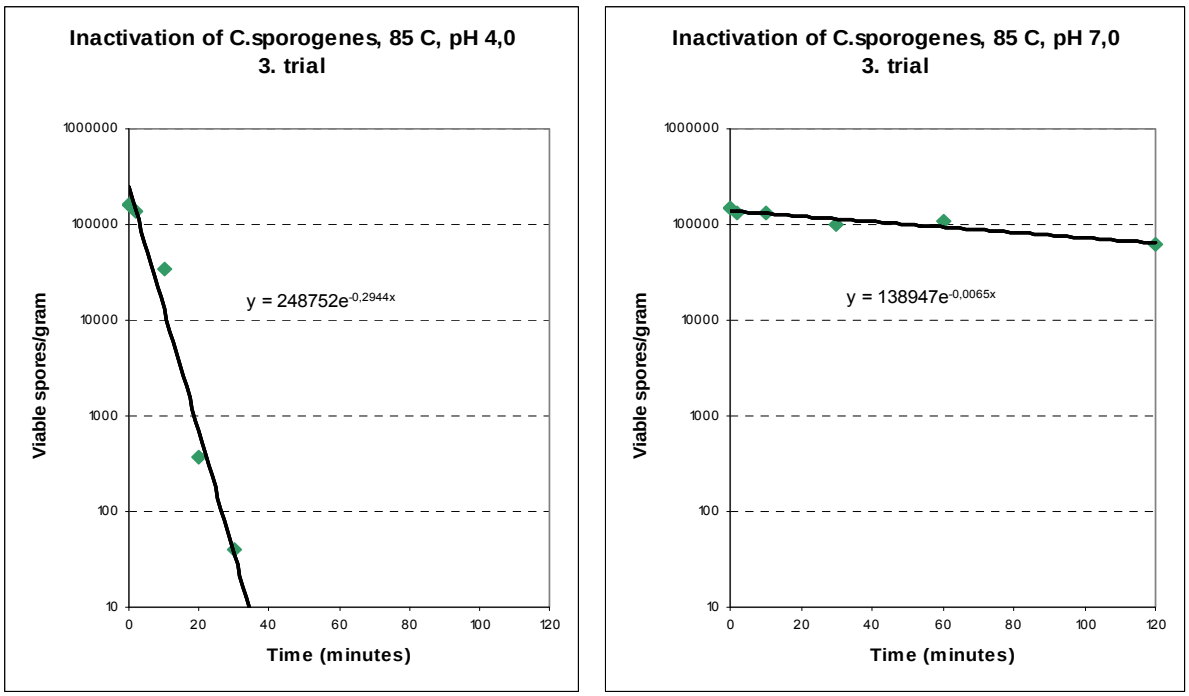

Figure 8 *Inactivation curves for C.sporogenes spores (3.trial) infine fish mince at pH 4,0 (a) and 7,0 (b) during heating at 85 °C. The straight lines are regression exponential curves.*

3.5 Calculated D-values

Table 3 D-values for C.sporogenes spores heat treated at 85 ºC in phosphate buffer pH 7,0 and in fine fish mince at pH 4,0 (formic acid) and pH 7,0. D-values were calculated from the slope of the inactivation curves in figure 4, 6, 7 and 8).

		D-value (minutes)	
MATRIX		pH 4,0	pH 7,0
Phosphate buffer			253
Fine fish mince.	Trial 1	9,2	606
Fine fish mince.	Trial 2	9,6	794
Fine fish mince.	Trial 3	7,8	354

Average D-value at 85 ºC in fine fish mince at neutral pH was 580 minutes. This correspond to 0,04 Log_{10} reductions in 25 minutes. In the presence of formic acid (pH 4,0), average D-value was 8,9 minutes, corresponding to 2,8 Log_{10} reduction in 25 minutes.

3.6 Effect of new processing method

Theoretical concentration of *C.sporogenes* spores after spiking was 200.000 per gram which was also the measured average concentration in the fine mince after 24 hours cold storage (Table 4). Spore concentration in the coarse mince at the same stage of the process (Table 5) could not be measured due to the presence of a large competing flora (approximately 10E8/gram). This flora was absent after the acid treatment, thus allowing quantification of the target organism before and after heat treatment.

In both fine and coarse fish mince the number of viable spores was reduced from approximately 200.000 per gram to less than 200 per gram during the complete process. The total inactivation effect from the new processing method ranged from 3,0 – 4,6 log_{10} reductions which is some more than encountered the first 25 minutes of heat treatment during D-value determination in acidified fine fish mince. This may be due to longer duration of acid treatment in the processing method.

Table 4 *Concentration of viable Clostridium sporogenes (ATCC 19404) spores in fine fish mince stored at 5 ºC for 24 hours after spiking, acid treated (formic acid, pH 4,0) at 20 ºC for 24 hours and heat treated at 85 ºC for 25 minutes. Theoretical spore concentration after spiking was 200.000 per gram. The experiment was repeated four times on different days.*

	Spiked fish mince Stored 5 ºC/24 h	Spiked fish mince Stored 5 ºC/24 h Acid treated 20 ºC/24 h	Spiked fish mince Stored 5 ºC/24 h Acid treated 20 ºC/24 h Heat treated 85 ºC/25 min
Trial 1	210.000	230.000	190
Trial 2	210.000	190.000	20
Trial 3	180.000	230.000	< 5
Trial 4	190.000	190.000	< 5

Table 5 *Concentration of viable Clostridium sporogenes (ATCC 19404) spores in coarse fish mince stored at 5 ºC for 24 hours after spiking, acid treated (formic acid, pH 4,0) at 20 ºC for 24 hours and heat treated at 85 ºC for 25 minutes Theoretical spore concentration after spiking was 200.000 per gram. The experiment was repeated four times on different days.*

	Spiked fish mince Stored 5 ºC/24 h	Spiked fish mince Stored 5 ºC/24 h Acid treated 20 ºC/24 h	Spiked fish mince Stored 5 ºC/24 h Acid treated 20 ºC/24 h Heat treated 85 ºC/25 min
Trial 1	n.d.	86.000	20
Trial 2	n.d.	110.000	5
Trial 3	n.d.	94.000	20
Trial 4	n.d.	120.000	< 5

n.d.: not determined

The results of our study show that heat treatment in the presence of formic acid (pH 4,0) has a much stronger effect than the sum of each separate treatment.

Naim et al., (2008) demonstrated significant synergistic effects of temperature and pH on inactivation of *C.sporogenes* (ATCC 11437) spores in both phosphate buffer and carrot-alginate particles. Also, Cameron et al. (1980) found that increasing acidity is accompanied by decreasing heat resistance of *C.sporogenes* (PA 3679) spores.

It has long been recognized that low pH reduces the heat resistance of spores. The relationship between D-values and pH of the heating medium has been described by Mafart and Leguerinel (1998) and Mafart et al. (2001), but the predictive value of those models is uncertain. The effect of pH on spore heat resistance is in one model described by z_{pH} which is the pH change needed to give a ten-fold reduction of D-value. All z_{pH} values reported by Mafart and Leguerinel (1998) lied between 3 and 5. According to this model, pH reduction from 7 to 4 (3 units) in our experiments should result in maximum a ten-fold reduction of D-

value. The actual effect was a 65-fold reduction of D-value, showing that the antimicrobial effect of formic acids is not only due to pH (cfr. Chapter 3.2).

The inactivation effect encountered during the complete new processing method was at least 3 Log_{10} reductions in both fine and coarse (≤ 10 mm particle size) fish mince. This is considered sufficient to ensure the absence of *C.perfringens* after the treatment when taking into account its low abundance in the raw fish silage.

3.7 Incidence of C.perfringens in fish silage

Nygaard (2009) summarized microbiological analysis data representing raw fish silage and processed silage products. The data were provided by the Norwegian fish silage industry.

Of 31 lots raw fish silage (24 lots category 2) and 43 lots fish protein concentrate (16 lots category 2) all contained < 10 *C.perfringens* per gram. This indicates that the prevalence of *C.perfringens* in fish silage and its products is very low.

4 References

Bigelow,W.D. (1921). The logarithmic nature of thermal death time curves. J. Infect. Diseases, 29: 528-536

Brul,S. and Coote,P. (1999). Review: Preservative agents in foods. Mode of action and microbial resistance mechanisms.International Journal of Food Microbiology. 50, 1-17.

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.

Doyle,M.P. (1989). Foodborne Bacterial Pathogens. Marcel Dekker, Inc., New York.

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

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 7218. Microbiology of food and feeding stuffs - General rules for microbiological examinations.

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

Mafart, P. and Leguerinel, I. (1998). Modeling combined effects of temperature and pH on heat resistance of spores by a linear-Bigelow equation. Journal of food science, 63 (1),

Mafart, P., Couvert, O. and Leguerinel, I. (2001) Effect of pH on the heat resistance of spores. Comparison of two models. International Journal of Food Microbiology, 63, 51-56

Mah,J.H., Kang,D.H. and Tang,J. (2008). Effects of minerals on sporulation and heat resistance of *Clostridium sporogenes* International Journal of Food Microbiology. Volume 128 (2), 385-389.

Molin,G. (1992). Heat sterilization. B. Destruction of bacterial spores by thermal methods. In *Disinfection, Preservation and Sterilization*, eds. A. D. Russell, W. B. Hugo and G. A. J. Ayliffe, 499-511. Oxford: Blackwell Scientific Publications.

Naim,F., Zareifard,M.R., Zhu,S., Huizing,R.H., Grabowski,S. and Marcotte,M. (2008). Combined effects of heat, nisin and acidification on the inactivation of *Clostridium sporogenes* spores in carrot-alginate particles: From kinetics to process validation Food Microbiology 25, 936– 941

Nygaard, H. (2009). Thermal inactivation of Clostridium perfringens spores in fish silage. Nofima Report K-343.

Pölönen, I. (2000). Silage for Fur Animals: Preservation Efficiency of Formic Acid and Benzoic Acid in the Ensiling of Slaughterhouse By-Products and their Subsequent Metabolism in Farmed Fur Animals. Academic Dissertation. University of Helsinki. ISBN 951-45-9561-0.

Roberts,T.A., Gilbert,R.J., Ingram,M. (1966). The effect of sodium chloride on heat resistance and recovery of heated spores on Clostridium sporogenes. Journal of Applied Bacteriology, 29, 549-555.

Rubin, 1993. Håndbok i ensilering. ISBN: 82-993089-0-9

Walker,H.W. (1975). Foodborne illness from *Clostridium perfringens*. CRC-Crit Rev Food Sci Nutr 7(1):71–104.

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