

Effect of formic acid and heat treatment on *Clostridium sporogenes* spores inactivation in animal by-products

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Summary

The aim of the study was to investigate the effect of acidification and heat on the survival of *Clostridium* spores in third category animal by-products. Poultry minced meat and minced fish waste, contaminated with *Clostridium sporogenes* IW 1306 spore suspension, were subjected to the combined action of formic acid and high temperature of different values.

To assess the efficiency of examined hygienization method, the spore number in biomass samples after acidification and heat treatment was estimated. For this purpose a 10-fold dilution series in peptone water was prepared and heat-treated at 80°C for 10 min. After cooling-down, one milliliter of each dilution was pour-plated onto DRCM medium solidified with agar. Statistical analyses were performed using the Statistica software.

The results of the microbiological analysis show that lowering the pH to about 4 results in increasing the sensitivity of *Clostridium sporogenes* spores to a high – but not exceeding 100°C – temperature. The fastest inactivation of spores occurred after heating the acidified biomass at a temperature of 90°C. In the case of meat the 48-hour exposure to formic acid allowed the thermal inactivation of the spores within 15 minutes.

The investigated method of hygienization may guarantee not only the inhibition of spores germination, but also enables their total elimination, reducing the epidemiological risk related to animal by-products handling.

Keywords: animal by-products, *Clostridium*, acidification, high temperature

For decades animal by-products have been considered valuable sources of easily assimilated proteins, energy and mineral nutrients, which were available for the feeding of livestock. Since 2003 the legal prohibition of using meat and bone meal, resulting from the necessity to reduce the risk of spreading transmissible spongiform encephalopathies (TSEs), considerably reduced these possibilities (8). The implementation of new regulations highlights the importance of effective hygienization measures for all kinds of organic by-products, posing an epidemiological risk for humans and livestock.

Irrespective of the destination this type of biomass, it is characterized by the ability to be subjected to very quick biological decay. As a consequence, even in the case of high risk materials destined for later incineration, it is necessary to stabilize and neutralize biologically the materials for transport and storage.

Processing methods of animal by-products, which should guarantee their microbiologically safety, are precisely described in the relevant regulations of the European Union (7, 21). In most cases, they are based on the lethal effect of elevated temperature, high pressure and exposure time to the individual physical factors, all adjusted to the size of the treated particles.

In the case of animal or aquatic invertebrate by-products a technology is recommended in which, besides the enhanced temperature, a low pH value plays an equal role. Acidification of biomass of plant and animal origin destined for feeding has been used in agriculture for a long time (6, 15, 19, 20). In addition to the natural lactic fermentation processes, the effect of a lowered pH value can also be achieved by addition of an acid, usually an organic one, to the processed material. An example is formic acid, which besides changing the pH, also destroys microorganisms by

forcing their cells to the use of excessive amounts of energy to neutralize the intracellular pH by dissociated acid molecules (16).

In the conducted experiments, the influence of the combined effect of formic acid and high temperature on spores of *Clostridium sporogenes* in animal by-products of the third category was investigated. According to EU regulation, confirmed absence of *C. perfringens* cells in 1 g sample of treated by-products guarantees the efficiency of the method applied. In many scientific studies, however, *C. sporogenes* strains are widely used as a surrogate for pathogenic *C. perfringens* and *C. botulinum* species.

The confirmation of the germicidal efficacy of low pH and heat may constitute the basis for an improvement of existing (or development of new, more efficient) methods for the stabilization and hygienization of animal by-products. The aim of the study was to evaluate the effect of acidification and different values of high temperature on the survival of *Clostridium sporogenes* spores in animal by-products of the third category.

Material and methods

The research was conducted under controlled laboratory conditions. Poultry minced meat (dry matter content ~36%) and minced fish waste (dry matter content ~30%) were placed in glass containers, where they were contaminated with a *Clostridium sporogenes* IW 1306 spore suspension. The strain of *C. sporogenes* was obtained from the Polish Collection of Microorganisms (The Institute of Immunology and Experimental Therapy) in Wrocław, Poland.

To obtain spore suspensions, FTG medium (10 mL) was inoculated with *C. sporogenes* grown on BHI medium (0.5 mL, stored at 4°C). After 24 hours of anaerobic incubation at 37°C (Anaerobic System, Oxoid) 0.5 mL of the FTG culture were transferred to a Duncan-Strong medium (10 L). The inoculated media were cultured under anaerobic conditions at 37°C for 7 days.

Spores were harvested after centrifugation at 3000 × g rpm for 15 minutes, washed twice, suspended in sterile distilled water (50 mL) and stored at 4°C. The density of the suspension was about 10⁸ cfu·mL⁻¹.

The inoculated biomass, stored for 24 h at 4°C, was acidified with 85% formic acid and the pH level of the acidified mixture was adjusted to about 4.

After 6, 24 and 48 hours of acidification, 3 g samples of the tested material (meat or fish by-product) were introduced into 12 small glass containers and placed in water baths at temperatures of 70, 80 and 90°C (4 samples per each temperature). After 15, 30, 45 and 60 minutes one container with a biomass sample from each bath was removed. The containers were cooled down in an

ice bath and microbiologically examined. The experiments were carried out in triplicate.

To estimate the spore number in the biomass samples, a 10-fold dilution series in 0.1% peptone water was prepared and pour-plated onto DRCM medium solidified with agar. The plates were incubated for 2 days at 37°C under anaerobic conditions.

To analyze the sole effect of acidification the number of *C. sporogenes* spores in not-acidified biomass was determined (Control).

On the basis of the logarithmic values of the obtained results, a statistical analysis was performed. Using the Statistica software, regression line equations were established and the inactivation rate, correlation coefficient (r) and theoretical survival rate of the *Clostridium* spores were estimated. The theoretical survival time was calculated dividing „b” by „a” – the values taken from the regression line equation: $\text{Log}(N) = ax + b$, where N is the number of spores in a biomass at a given time, x is time (hours), a – the slope of the regression line or direction coefficient, accounting for a mean change in the log of the spores number per hour and b is so called “free term”, theoretically corresponding to the log of the spores number at “zero” time. Taking the absolute value of the inverse regression equations slope, D values, defining times to achieve a 90% reduction of the *Clostridium* spores number were also calculated.

Results and discussion

The microbiological studies after 6 h of exposing meat to formic acid revealed a moderate impact of a temperature of 70°C on the number of *C. sporogenes* spores. A considerable higher efficacy, resulting in reduction in the spore number of about two to three orders of magnitude, was observed after heating of the treated biomass at 80°C (Tab. 1). The lowest theoretical survival was measured after 1h of heating at 90°C (Tab. 5).

The number of spores in the acidified minced fish treated with temperatures of 70°C and 80°C, fluctuated only slightly in the range of 5.20 × 10⁴ – 1.05 × 10⁵ cfu·g⁻¹ (Tab. 1). The number of spores declined by not more than 1 order of magnitude, but much more regu-

Tab. 1. Number of spores of *Clostridium sporogenes* in animal by-products subjected to the influence of high temperature after 6 hours of treatment with formic acid (cfu·g⁻¹)

Heating time (min)	Animal by-products type					
	Meat			Fish		
	Temperature					
	70°C	80°C	90°C	70°C	80°C	90°C
Control	3.67 × 10 ⁵			6.67 × 10 ⁴		
0	6.00 × 10 ⁵			1.05 × 10 ⁵		
15	4.63 × 10 ⁴	1.30 × 10 ³	1.00 × 10 ²	7.43 × 10 ⁴	6.90 × 10 ⁴	7.43 × 10 ⁴
30	8.30 × 10 ⁴	8.00 × 10 ²	2.47 × 10 ²	8.07 × 10 ⁴	6.47 × 10 ⁴	3.20 × 10 ⁴
45	4.87 × 10 ⁴	7.00 × 10 ²	1.30 × 10 ²	9.03 × 10 ⁴	5.20 × 10 ⁴	1.11 × 10 ⁴
60	1.02 × 10 ⁵	6.23 × 10 ²	0.30 × 10 ¹	8.20 × 10 ⁴	8.05 × 10 ⁴	1.15 × 10 ⁴

Explanations: Control – not acidified, not heated biomass; 0 – acidified, not heated biomass

larly, when heating the material at 90°C. This made it possible to estimate the theoretical survival at a level of approximately 4.5 h.

A temperature of 70°C did not lead to a significant reduction in the number of spores of *C. sporogenes* in meat acidified for 24 hours. However, the reduction of spore number observed after the application of higher temperatures reached about three to four log units. Heating at 90°C resulted in the inactivation of the tested microorganisms to a level close to the detection limit (Tab. 2).

The effect of heating the fish waste acidified for 24 h at 90°C was a sharp decline in the number of spores from 3.50×10^4 to 1.02×10^1 cfu·g⁻¹ (Tab. 2). The application of lower temperatures was not as effective and resulted in a reduction in the number of spores of *Clostridium* of about one order of magnitude.

During the acidification of the meat material for 48 h, the time required to completely eliminate *Clostridium* spores at a temperature of 80 and 90°C was 1 h and 15 min, respectively (Tab. 3). The lowest tested temperature of 70°C only slightly affected the tested microorganisms which according to statistical calculations could survive under these conditions longer than 8 h (Tab. 5).

Lowering the pH of fish waste in combination with a temperature of 70°C resulted in a very slow reduction of spores. Their number varied in a narrow range from 4.45×10^4 to 9.36×10^4 cfu·g⁻¹. Although it did not result in a decrease in the number of spores below 10^4 cfu·g⁻¹ heating at a temperature of 80°C significantly reduced the theoretical survival time. The highest efficiency in the inactivation of spores of *Clostridium* in the biomass was observed after the application of a temperature of 90°C, when already after 45 minutes their presence could not be detected in the examined samples (Tab. 3).

Tab. 2. Number of spores of *Clostridium sporogenes* in animal by-products subjected to the influence of high temperature after 24 hours of treatment with formic acid (cfu·g⁻¹)

Heating time (min)	Animal by-products type					
	Meat			Fish		
	Temperature					
	70°C	80°C	90°C	70°C	80°C	90°C
Control	2.33×10^5			1.50×10^5		
0	3.90×10^5			3.50×10^4		
15	7.70×10^4	8.73×10^3	1.31×10^1	5.33×10^4	5.63×10^4	1.90×10^4
30	4.27×10^4	2.90×10^2	0.30×10^1	8.97×10^4	1.67×10^4	3.07×10^3
45	5.57×10^4	7.00×10^2	1.00×10^1	3.23×10^4	3.57×10^4	1.37×10^2
60	5.40×10^4	7.37×10^2	nd	2.67×10^4	6.83×10^3	1.02×10^1

Explanation: nd – not detected

Tab. 3. Number of spores of *Clostridium sporogenes* in animal by-products subjected to the influence of high temperature after 48 hours of treatment with formic acid (cfu·g⁻¹)

Heating time (min)	Animal by-products type					
	Meat			Fish		
	Temperature					
	70°C	80°C	90°C	70°C	80°C	90°C
Control	2.13×10^5			1.13×10^5		
0	1.80×10^5			7.67×10^4		
15	7.43×10^4	7.13×10^2	nd	8.33×10^4	7.01×10^4	6.93×10^3
30	6.47×10^4	3.67×10^1	nd	9.36×10^4	2.97×10^4	1.01×10^3
45	6.57×10^4	1.00×10^1	nd	7.20×10^4	4.03×10^4	nd
60	3.17×10^4	nd	nd	4.45×10^4	1.97×10^4	nd

Explanation: nd – not detected

Tab. 4. Equations of regression lines, correlation coefficient (r) and theoretical survival time (h) of spores of *Clostridium sporogenes* in acidified animal by-products subjected to high temperatures

Animal by-products type	Time of acid treatment	Temperature		
		70°C	80°C	90°C
Meat waste	6 h	$y = -0.61x + 5.31$ $r = 0.53$ 8.76 h	$y = -2.49x + 4.73$ $r = 0.77$ 1.90 h	$y = -4.19x + 4.65$ $r = 0.85$ 1.11 h
	24 h	$y = -0.74x + 5.29$ $r = 0.76$ 7.14 h	$y = -2.62x + 4.85$ $r = 0.81$ 1.85 h	$y = -4.52x + 3.89$ $r = 0.79$ 0.86 h
	48 h	$y = -0.63x + 5.16$ $r = 0.92$ 8.27 h	$y = -4.95x + 4.61$ $r = 0.96$ 0.94 h	–
Fish waste	6 h	$y = -0.05x + 4.96$ $r = 0.36$ 95.37 h	$y = -0.14x + 4.93$ $r = 0.49$ 34.84 h	$y = -1.10x + 5.05$ $r = 0.97$ 4.59 h
	24 h	$y = -0.18x + 4.72$ $r = 0.34$ 26.07 h	$y = -0.57x + 4.91$ $r = 0.90$ 8.63 h	$y = -3.69x + 4.94$ $r = 0.97$ 1.33 h
	48 h	$y = -0.21x + 4.96$ $r = 0.68$ 23.14 h	$y = -0.70x + 5.03$ $r = 0.71$ 7.22 h	$y = -5.44x + 5.07$ $r = 0.96$ 0.93 h

The time enabling a 90% reduction in the spore number of *Clostridium*, defined as D value, ranged from 81 to 1200 minutes after heating at 70°C, from

Tab. 5. Effect of acid and heat treatment on the inactivation of *C. sporogenes* spores in animal by-products

Animal by-product type	Time of acid treatment	D-value (min) at:		
		70°C	80°C	90°C
Meat	6 h	98	24	14
	24 h	81	23	13
	48 h	95	12	–
Fish	6 h	1200	428	55
	24 h	333	105	16
	48 h	283	86	11

Tab. 6. The pH value in raw and acid-treated animal by-products

Animal by-products type		Time of acid treatment		
		6 h	24 h	48 h
Meat waste	raw	5.89	5.68	5.66
	acidified	3.90	3.84	3.88
Fish waste	raw	6.69	6.99	6.85
	acidified	4.05	4.18	4.23

12 to 428 minutes at 80°C and from 11 to 55 minutes at 90°C (Tab. 5). After 48 hours of acid treatment of meat, the spores subjected to a temperature of 90°C were inactivated during the first 15 minutes of heating (Tab. 3).

The pH of the meat biomass not treated with formic acid was slightly acidic and ranged from 5.66-5.89. The pH of the acidified material was less than 4 (Tab. 6).

Raw fish material was characterized by a pH close to neutral. The results of pH measurements showed a value of 4.05-4.23 with a slightly increasing trend (Tab. 6).

Animal by-products from the meat and fish industry may be used in different ways, depending on the degree of the potential risk resulting from the possibility of contamination with pathogenic microorganisms. These specific types of biomass, despite the imposed restrictions, may be partially converted into feed for fish or fur animals. It is also a valuable raw material for the production of fertilizers and biofuels (19, 23, 24).

In a situation where the method of waste management may directly or indirectly endanger people and the environment measures shall be taken in order to minimize these risks. Although specifically defined conditions administer the use of animal by-product treatment methods, research is carried out on the effectiveness of alternative technologies that, while maintaining hygiene standards, will be characterized by lower costs and simplicity of implementation (1, 18).

A lot of expectations are connected with methods based on a combined effect of various chemical agents and physical treatments (16, 22). One possibility represents a combined action of low pH and high temperature.

The results of many studies have proven that a reduced pH increases the sensitivity of spores to heat and

demonstrated the synergistic effect of these two factors (9, 10, 16). The application of low pH, often preceding the pasteurization process, is a proven method of food preservation (2, 10). It has been confirmed that pH lower or equal to 4.6 prevents bacterial spore germination, including those of *C. botulinum* (3, 11, 13). Juneja (14) reported that the growth of *C. perfringens* in different types of meat is inhibited by lowering the pH to a value in the range of 4.74-5.17. In our studies, the pH values obtained after acidification of the biomass did not exceed 4.23 (Tab. 6).

Practice shows that in the case of naturally acidic materials their earlier acidification is not necessary, whereas products with a higher pH, such as meat and fish, must be acidified. The pH of raw fish waste is almost neutral due to the low carbohydrate content in the muscle tissue and lower *post-mortem* productivity of lactic acid (12). In our own studies, the pH of the non-formic acid treated fish material is also close to 7, while in the case of the meat it was lower (Tab. 6).

In order to lower the pH in organic materials mainly organic acids that have a good ability to penetrate the cell membranes of microorganisms are used (22). Their activity against microorganisms is based on the disturbance of intracellular mechanisms that control energy production and the accumulation of toxic levels of anions derived from dissociated acids (10, 16). An acid-treatment of biomass, despite the proven preservative effect, however, cannot ascertain a sufficient level of hygienization. According to Brown et al. (4) there are confirmed cases of botulism caused by *C. botulinum* that retained its vitality in foods at a pH of 3.5. The risk of alkalization increases when the waste material is mixed with material with a higher pH or when putrefactive microorganisms start to develop. Their metabolism reduces the acidification of the environment. An improperly conducted acidification process may also result in the adaptation of part of the microorganisms to the unfavorable life conditions, or even to the gradual development of resistant strains (16).

In our studies, the number of spores of *C. sporogenes* in fresh and acidified meat and fish by-products was similar, and the differences did not exceed a value of 1 log unit (Tab. 1-3). This proves the moderate effectiveness of the hygienization based solely on the action of formic acid. This is also indicated by the results obtained by Vinnerås et al. (24), who confirmed the presence of *Bacillus* spores and pig parvovirus even after 147 and 168 days, respectively, in acidified animal by-products.

In addition to factors relating directly to the acid used (acid type, concentration, dose and duration of the treatment) the effectiveness of hygienization methods is based entirely or partially on a lowered pH. The properties of the material subjected to this process – its natural pH, tissue structure, buffering capacity, and particle size – also determine the effectiveness (10).

The results of our studies indicate differences in the inactivation rate of *C. sporogenes* spores as affected by heating of the acidified material, depending on its type. In meat waste the inactivation rate was higher than in fish by-products, irrespective of the temperature used in the subsequent thermal treatment (Tab. 4).

Due to the high heat resistance of spores to their rapid destruction, depending on the strain and the conditions prevailing in a given environment, a temperature exceeding 100°C is required. D_{121} values reported in literature for *C. sporogenes* PA 2679 in the phosphate buffer range from 0.19 to 3.50 min (3). Byun et al. (5) confirm this information, at the same time giving predicted D values for temperatures below 100°C. According to their calculations, the time allowing a 90% reduction in spore number at a temperature of 70°C would be more than 100,000 minutes. In our studies the highest D value was 1,200 min. and was observed in acidified fish by-products after 6 hours of heating at 70°C (Tab. 5). Naim et al. (17) indicate that the D_{70} value for *C. sporogenes* spores in carrot-alginate particles being characterized by a pH of 4.5, ranged from 87.7 to 111 minutes. Comparable results were obtained in the present studies, when heating acidic meat waste at 70°C (Tab. 5).

As expected, the fastest inactivation of spores occurred after heating the acidified material at a temperature of 90°C. Particularly satisfactory results were achieved in the case of meat biomass, where the 48-hour exposure to formic acid allowed the thermal inactivation of the spores within 15 minutes (Tab. 3).

The results of the microbiological analysis of hygienization methods based on heating of acidified animal by-products show that lowering the pH to about 4 increases the sensitivity of *Clostridium sporogenes* spores to a high – but not exceeding 100°C – temperature. The obtained results indicate that the presented methods ensure not only the inhibition of spores germination, but also enable their elimination. After optimization of the process parameters and adaptation to individual requirements this will have a number of practical applications that could improve organic waste management and at the same time maintain hygiene standards.

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