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Viability of *Clostridium sporogenes* spores after CaO hygienization of meat waste

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Abstract

The occurrence of the pathogenic species *C. perfringens* and *C. botulinum* spores in animal by-products poses a potential epidemiological hazard. Strong entero- and neurotoxins produced by these bacteria adversely affect human health. To inactivate pathogens present in animal by-products, waste must be subjected to various methods of sanitization. The aim of the presented study was to estimate the effect of different doses of CaO on the viability of spores *Clostridium sporogenes* in meat wastes category 3. During the research, two doses of burnt lime were added to the poultry mince meat and meat mixed with swine blood contaminated with *Clostridium sporogenes* spore suspension. Half of the samples collected for microbiological analyses were buffered to achieve the pH level ~7, the other were examined without pH neutralization. To estimate the spore number, 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 analysis were performed using the Statistica software. Application of 70% CaO caused complete inactivation of *Clostridium* spores in meat wastes after 48 hours. The highest temperature achieved during the experiment was 67 °C. Rapid alkalization of the biomass resulted in increasing pH to values exceeding 12. The effect of liming was not dependent on the meat wastes composition nor CaO dose. The experiment proved the efficiency of liming as a method of animal by-products sanitization. Application of the obtained results may help reduce the epidemiological risk and ensure safety to people handling meat wastes at each stage of their processing and utilization.

Key words

animal by-products, sanitization, liming, spores, *Clostridium*

INTRODUCTION

Anaerobic bacilli of the genus *Clostridium* occur commonly in many environments. Their endospores were isolated also from food products, both of plant and animal origin. Particular danger connected with those microorganisms is connected with contamination of food by pathogenic species [1, 2]. In spite of using effective methods of pathogen elimination during food processing, their presence in different types of wastes generated during those processes still can be expected. Animal by-products pose a potential epidemiological hazard due to a possibility of strains of the species *C. perfringens* and *C. botulinum* appearing in them. They produce strong entero- and neurotoxins which may adversely affect human health [3, 4, 5].

Depending on their epidemiological risk degree, animal by-products are classified into 3 categories. To eliminate pathogenic microorganisms from waste of the highest risk (categories 1 and 2) radical methods of disposal, such as incineration, are applied. Material of lower risk (category 3) may be also subjected to composting or transforming into biogas, with or without prior processing. Properly processed category 3 waste can be used for agricultural purposes and enrich soil environment with a large amount of valuable organic substances.

To guarantee biosafety of the final product, all parameters of meat waste sanitization and disposal are precisely defined in EU legislation.

Most of the methods aiming at animal waste hygienization are based on the lethal effect of high temperature on microbial cells. This requires large financial outlays, connected mainly with creating and maintaining statutory thermal parameters of the process, but also with transport of the wastes to the assigned utilization units. Accordingly, attempts undertaken to develop an alternative technique of meat waste hygienization, resulting in reduction of generated costs, seem to be fully justified. Current regulations of EU Parliament also encourage their Member States to apply for an authorization of alternative methods for animal by-products use or disposal [6, 7].

Objective. The aim of the conducted study was to estimate the effect of different doses of CaO on the viability of spores of bacteria of the genus *Clostridium* in meat wastes category 3. The species applied for this, i.e. *Clostridium sporogenes,* is commonly used in scientific research as a surrogate for pathogenic clostridia [8, 9]. The elimination rate of spores constituted the basic criterion of liming effectiveness as a meat waste sanitization method.

MATERIALS AND METHOD

Microorganism. *Clostridium sporogenes* IW 1306 strain was obtained from The Polish Collection of Microorganisms, located at the Institute of Immunology and Experimental Therapy in Wroclaw.

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Spore production. To obtain spore suspension, 10 ml FTG medium was inoculated with 0.5 ml of *Clostridium sporogenes* cultured on BHI medium (stored at 4 °C). After 24 hours of anaerobic incubation at 37 °C (Anaerobic System, Oxoid), 0.5 ml of the FTG culture was transferred to 10 ml of Duncan-Strong medium. The inoculated tubes were incubated in anaerobic conditions for 7 days at 37 °C.

Spores were harvested by centrifugation at 3,000 × g rpm for 15 minutes, washed twice, suspended in 50 ml of sterile distilled water and stored at 4 °C. Density of the suspension obtained ranged from 10^7–10^8 cfu·ml^{-1}.

Experimental design. The research was conducted in laboratory conditions. Poultry mince meat (dry matter content ~36%) and meat mixed with swine blood (dry matter content ~25%) were placed in a glass, thermally insulated container. After contamination and mixing the biomass with 10 ml *Clostridium sporogenes* spore suspension, burnt lime was added in 2 doses – 50 and 70% (expressed in dry matter).

Samples for microbiological analyses were collected after 0.5, 1, 2, 3, 6, 9, 12, 24 and 48 hours from the beginning of the experiment. Half of the samples were buffered with $Na_2HPO_4 \times 2H_20$ solution to achieve the pH level ~7, the rest were examined without previous pH neutralization.

Spore enumeration. To estimate the spore number in biomass samples, 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. The plates were incubated for 2 days at 37 °C in anaerobic conditions.

Statistical analysis. Statistical analyses were performed using Statistica software. On the basis of the results obtained, regression lines were drawn. Using regression line equations the inactivation rate and theoretical survival rate of the *Clostridium* spores were calculated.

RESULTS

The results of the initial part of the experiment showed that the lower CaO dose, corresponding to 50% of the meat dry mass, resulted in a not very rapid, but regular decrease in the spore number. After 48 hours their population lost about 3 log units (Tab. 1). Statistically calculated theoretical survival rate of the spores in not neutralised and buffered samples ranged from 73–91 h, respectively (Tab. 5).

Application of a higher CaO dose (70%) caused complete elimination of *Clostridium* spores in meat after 48 hours. Starting from the 2nd hour of the experiment, their number reached the values of the detection limit – 10^{-1} cfu·g d.m.$^{-1}$ (Tab. 2). There were no differences in theoretical survival of the spores in buffered and not buffered meat samples – its value did not exceed 34 h (Tab. 5).

After addition of 50% CaO, *Clostridium* spores introduced into meat and swine blood mixture survived theoretically 61 h in not buffered samples, and 92 h in biomass after pH neutralization (tab. 5). Their initial number, 7.07×10^5 cfu·g d.m.$^{-1}$, declined after 24 h to the level of 10^2–10^3, and after one more day it ranged about 10^1–10^{-2} cfu·g d.m.$^{-1}$ (tab. 3).

As a result of increasing CaO dose to 70%, reduction of spores population in meat-blood mixture reached 4–5 log units after 48 h of the experiment (Tab. 4). In not buffered samples, the theoretical survival rate was lower, compared to samples of pH=7, and oscillated around 59 and 69 h, respectively (Tab. 5).

Enriching the biomass with CaO resulted in rapid alkalization of the environment and increasing pH to values exceeding 12. The effect of liming was not dependent on the meat wastes composition nor CaO dose (Tab. 1, 2, 3, 4).

Subjection of the biomass to the CaO action caused a fast increase in temperature during the first hours of the experiment. The highest temperature of 67 °C was observed in

Table 1. Effect of CaO (dose 50%) on the number of *C. sporogenes* spores and pH values in meat (cfu·g d.m.$^{-1}$)

Time of sampling (h)	Buffered samples	Not buffered samples	pH*
0	6.84×10^5		6.11
0.5	4.21×10^4	3.48×10^4	12.64
1	3.12×10^3	2.76×10^3	12.60
2	8.89×10^2	2.06×10^3	12.59
3	2.76×10^2	6.01×10^2	12.59
6	4.68×10^2	1.32×10^2	12.57
9	3.12×10^2	9.97×10^2	12.57
12	1.35×10^3	3.60×10^2	12.56
24	1.90×10^2	2.40×10^1	12.54
48	1.72×10^2	1.52×10^2	12.56

*pH values before neutralization of the samples

Table 2. Effect of CaO (dose 70%) on the number of *C. sporogenes* spores and pH values in meat (cfu·g d.m.$^{-1}$)

Time of sampling (h)	Buffered samples	Not buffered samples	pH
0	3.64×10^5		6.63
0.5	7.67×10^3	6.23×10^3	12.56
1	1.38×10^2	7.45×10^1	12.54
2	2.76×10^1	0.48×10^1	12.52
3	0.78×10^1	0.66×10^1	12.48
6	0.24×10^1	nd	12.48
9	1.44×10^1	0.12×10^1	12.45
12	0.48×10^1	0.12×10^1	12.43
24	nd	0.06×10^1	12.43
48	nd	nd	12.17

Table 3. Effect of CaO (dose 50%) on the number of *C. sporogenes* spores and pH values in meat mixed with blood (cfu·g d.m.$^{-1}$)

Time of sampling (h)	Buffered samples	Not buffered samples	pH
0	7.07×10^5		6.38
0.5	7.07×10^4	1.85×10^5	12.62
1	1.54×10^4	8.43×10^3	12.66
2	9.33×10^3	6.43×10^3	12.66
3	9.17×10^3	7.67×10^3	12.65
6	2.31×10^3	1.24×10^3	12.62
9	3.00×10^3	8.43×10^2	12.62
12	1.16×10^3	7.50×10^2	12.59
24	1.09×10^3	3.00×10^2	12.69
48	1.37×10^2	3.03×10^1	12.64

Table 4. Effect of CaO (dose 70%) on the number of *C. sporogenes* spores and pH values in meat mixed with blood (cfu·g d.m.$^{-1}$)

Time of sampling (h)	Buffered samples	Not buffered samples	pH
0	5.50×10^5		7.04
0.5	7.83×10^4	1.81×10^5	12.65
1	4.07×10^4	5.25×10^4	12.67
2	4.83×10^3	2.57×10^3	12.64
3	8.07×10^2	3.07×10^2	12.65
6	7.50×10^2	6.57×10^2	12.62
9	1.47×10^3	4.83×10^2	12.60
12	1.57×10^3	1.01×10^3	12.60
24	3.50×10^2	6.25×10^2	12.58
48	6.43×10^1	0.75×10^1	12.57

Table 5. The elimination rate [log·h^{-1}] and the theoretical survival rate [h] of *C. sporogenes* spores in meat and meat+blood mixture subjected to the quicklime action

CaO dose	Meat		meat+blood mixture	
	buffered samples	not buffered samples	buffered samples	not buffered samples
50%	y = –0.04x + 3.64 91	y = –0.05x + 3.63 73	y = –0.05x + 4.36 92	y = –0.07x + 4.28 61
70%	y = –0.07x + 2.34 33	y = –0.06x + 1.94 32	y = –0.06x + 4.15 69	y = –0.07x + 4.15 59

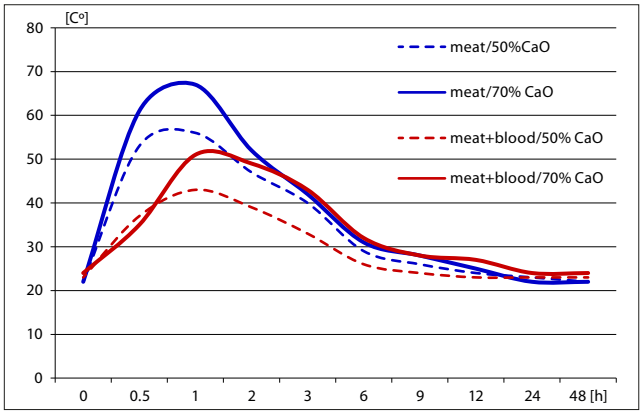

Figure 1. Temperature values in meat and meat+blood mixture subjected to CaO action (C°)

meat treated with 70% CaO. After 24 hours its value declined and remained at the initial level of 22–24 °C (Fig. 1).

DISCUSSION

Specific properties of bacterial spores, resulting mainly from their structure, dehydration degree and the content of specific chemical compounds, provide them with high resistance to the action of unfavourable environmental factors [10, 11]. Therefore, spore- forming bacteria are used as indicators of processes of hygienization of different types of organic wastes. Their inactivation gives a complete guarantee for the effectiveness of the given technology, at the same time confirming the appropriateness of its application.

Category 3 animal by-products, after proper processing, can be introduced into soil in order to supply it with a valuable source of organic substance. The addition of CaO, being a result of using this compound for biomass disinfection, may appear particularly favourable in the case of fertilization soils with low pH [12]. However, due to the potential presence of pathogenic microorganisms, including those forming spores, the utilization technology applied must be characterized by an exceptionally high hygienization effectiveness, ensuring safety for people and animals having contact with this material.

The effectiveness of liming as a method of waste hygienization is based mainly on extreme alkalization of the environment and the action of high temperature generated during exothermic reaction of Ca compounds with water. EU Commission Regulation No. 749/2011 of 29 July 2011 [13], where mixing lime and farmyard manure is regarded as a safe method for inactivation of pathogenic agents, sets the control pH value of the mixture in a pile on a level of at least 12, on condition of keeping it for 60 minutes at the temperature 60 °C, or for 30 minutes at 70 °C.

In the study by Boost and Poon [14], CaO application to sewage sludge resulted in a rapid growth of temperature up to 98 °C. Such a high value, however, retained for a very short time and during next 20 minutes its decrease to only 40 °C was recorded. According to the authors' suggestion, it could not be regarded as a crucial factor in the process of inactivation of spore-forming pathogens.

Maximal temperature observed during the presented experiment amounted to about 67 °C, but it could not be stabilized on that level for longer than one hour (Fig. 1). Taking into consideration the short time of action and exceptional thermoresistance of *Clostridium* spores, obtaining and then retaining high pH for an accordingly long time seems to be most essential for their potential elimination from wastes.

Experiments carried out to hygienize sewage sludge with the use of liming confirm the possibility of fast alkalizing the material up to a pH value exceeding 12 [15, 16]. In the study by Avery *et al.* [17], the addition of CaO to different types of by-products of animal origin also resulted in a rapid growth in pH. The authors observed a definitely faster alkalization rate of cattle blood, compared to a mixture of wastes with a higher content of dry matter.

In the presented study, a similar tendency was not observed, in spite of the different dry matter of analyzed samples. The level of pH increased very fast and exceeded a value of 12 as early as after 30 minutes after mixing the biomass with CaO. It is notable that irrespective of the size of lime dose applied, such high pH remained for next 48 hours (tab.1–4). Stabilizing of increased pH on the same level is of critical importance for preventing the phenomenon of multiplying microorganisms in sanitized material and recontamination of the biomass by microorganisms deriving from the outside environment [14].

The lethal effect of liming on vegetative forms of microorganisms colonizing organic wastes has been scientifically verified, whereas there are few studies evaluating the action of lime on spore forming bacteria [16, 18, 19]. Boost and Ponn [14] examined the survival rates of pathogenic bacteria in limed sewage sludge, determining the minimal pH value (within alkaline reaction) ensuring the inactivation of those microorganisms. The value of 9.5 given for *Clostridium perfringens,* however, must have referred to the sensitivity of the vegetative cells of this species, and it cannot be treated as a point of reference for further comparisons. In the experiment

by Gantzer [20], mixing sewage sludge with CaO caused a decrease in the number of spores of clostridia reducing sulfites by about 0.8–0.6 log MPN. Apart from the natural resistance of spores, the reason for such low hygienization effectiveness could be a relatively low dose of CaO added to sludge, amounting to 25%.

In the presented study, as early as after 30 minutes at least 1 log reduction in the number of *C. sporogenes* spores was observed, irrespective of the kind of material subjected to liming and the amount of the applied dose of CaO (Tab. 1, 2, 3, 4). In meat samples mixed with 70% of this compound, the theoretical survival rate of spores was only 32–33 hours (Tab. 5).

It is notable that in samples subjected to the neutralization of pH, the recovery rate of microorganisms from the tested material was higher. However, those differences decreased along with an increase in CaO dose. A similar tendency was observed earlier when analyzing the effect of calcium compounds on the survival rate of indicator microorganisms not forming endospores [21].

The effect of environmental pH on the survival rate of microorganisms forming spores was previously analyzed, mainly in the context of ability to inhibit their sporulation in acid food products [1, 22]. The action of high pH, resulting from application of Ca compounds, was used in extreme cases, e.g. for sanitization of carcasses of animals which died of a disease.

The liming of animal by-products, using a proper technology, may result in producing soil conditioners making it a source of considerable amounts of valuable and environmentally safe organic matter. The need for setting down detailed parameters of such a method, taking into consideration, among other things, the particle size of sanitized wastes or their dry weight content, justifies the research undertaken on this subject. This refers also to microbiological experiments, aimed at the sanitary risk assessment connected with the production and use of this specific biomass. Application of their results allows minimizing that risk and ensuring the safety of people handling meat wastes at each stage of their processing and utilization.

CONCLUSIONS

1. The results of the research proved that CaO hygienization of meat waste affects the viability of *Clostridium sporogenes* spores.
2. Quicklime doses applied in the experiment (50 and 70%) resulted in a reduction in the number of *Clostridium* spores.
3. The high pH (>12) obtained in limed meat waste was a crucial factor affecting their proper hygienization. The increase in temperature seemed to be too short to guarantee sufficient eradication of bacterial spores.
4. Application of CaO for hygienization of meat waste may effectively decrease health and environmental risk related to their application for agricultural purposes.

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