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SANITARY VALIDATION OF THE PROCESS OF ANAEROBIC DIGESTION OF SLURRY BASED ON THE SURVIVAL OF *Salmonella* SENFTENBERG (W_{775}, H_2S NEGATIVE) AND *Ascaris suum* EGGS

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Abstract. The subject of this study was an assessment of the effectiveness of hygienization of pig slurry during the methane fermentation process. The basic criterion for evaluating the effectiveness of the process was the inactivation rate of *Salmonella* Senftenberg (W_{775}, H_2S negative) and the eggs of *Ascaris suum*. Bacteria suspensions were introduced into slurry inside microbiological carriers of the FILTER-SANDWICH type, while parasite eggs were in special perlon parasitological carriers. The obtained results indicate that during anaerobic digestion of slurry there occurred a rapid elimination of both *Salmonella* Senftenberg (W_{775}, H_2S negative) and *Ascaris suum* eggs. The shape of the regression line shows that the complete inactivation of *Salmonella* Senftenberg (W_{775}, H_2S negative) occurred between the 12^{th} and 13^{th} hour of the process. *Ascaris suum* eggs were completely eliminated after 4 hours. Study results suggest that even in the case of strong contamination of the feed material, the technology of anaerobic digestion guarantees obtaining a microbiologically safe product.

Key words: *Ascaris* spp., digestion, *Salmonella* spp., slurry

INTRODUCTION

Intensification of animal production results in the depositing of large amounts of dangerous faeces into the environment. Biological waste generated in these processes consists of a number of substances that allow for growth of many microorganisms, also pathogens. Apart from the potential health risk, the use of unprocessed animal faeces as a fertilizer also creates the possibility of transmission to the environment of antibiotic

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resistant strains of pathogens, which may pass this trait onto autochthonous soil bacteria [Skowron *et al*. 2015]. Among the microorganisms colonizing slurry there are bacteria, viruses, fungi and eggs of various parasites. Microorganisms introduced into slurry with faeces and posing a serious health risk include: *Salmonella* spp., *Brucella* spp., *Leptospira* spp., *Chlamydia* spp., *Rickettsia* spp., *Bacillus anthracis*, *Mycobacterium* spp., *Escherichia coli* (enteropathogenic strains resistant to antibiotics), *Treponema hyodysenteriae* [Strauch 1991]. Also the presence of parasites and their eggs and oocysts, being the cause of the spread of invasive diseases, in slurry poses a serious sanitary and epidemiological threat [Paluszak 1998]. Elimination of pathogens from slurry is one of the fundamental conditions for its agricultural use. This can be achieved by subjecting the slurry to different processing methods, whose sanitary effectiveness is validated with the use of specific indicator microorganisms.

One of the bioenergy production methods, which at the same time can be used for biological treatment of waste derived from agriculture, is methane fermentation [Szempliński *et al.* 2014]. It results in waste decomposition, generation of a flammable gas that is used for the production of electricity and thermal energy and the formation of post-fermentation residues, the so-called post-ferment or digestate, that have high fertilizer value [Marszałek *et al.* 2011, Kukier *et al.* 2016]. The potential use of fermented slurry for agricultural purposes requires generation and maintainance of appropriate parameters for this process. According to EU regulations 1069/2009 and 142/2011, processing of animal by-products in the process of methane fermentation should result in a reduction of the number of *Salmonella* Senftenberg (W_{775}, H_2S negative) by 5 $\log_{10}$ and a decrease in the number of resistant eggs of parasites *Ascaris* spp. by at least 99.9% (3 $\log_{10}$) [Rozporządzenie Parlamentu Europejskiego... 1069/2009, Rozporządzenie Komisji (UE) 142/2011].

The aim of this study was to assess the sanitary effectiveness of the process of pig slurry digestion based on parameters describing the inactivation kinetics of *Salmonella* Senftenberg (W_{775}, H_2S negative) and *Ascaris suum* eggs exposed to the conditions prevailing inside the fermenter.

MATERIAL AND METHODS

The experiment was carried out in 2015 on the premises of an agricultural biogas plant with a tank capacity of about 4 thousand m^3 , which is located in northern Poland. The biogas plant worked in thermophilic conditions within the temperature range 49-51.5°C. The study was performed on a technical scale with the use of microbiological carriers of the FILTER-SANDWICH type and perlon parasitological carriers that were placed in a tubular capsule constructed specially for this purpose. This allowed for the continuous exposure of the microbiological and parasitological carriers during the validated process of anaerobic digestion.

The carriers of the FILTER-SANDWICH type (Fig. 1) used in the experiment were made up of a Plexiglass capsule closed on both sides by nitrocellulose filters with a pore size of 0.20 μm placed between plastic perforated trays sealed with a silicone seal.

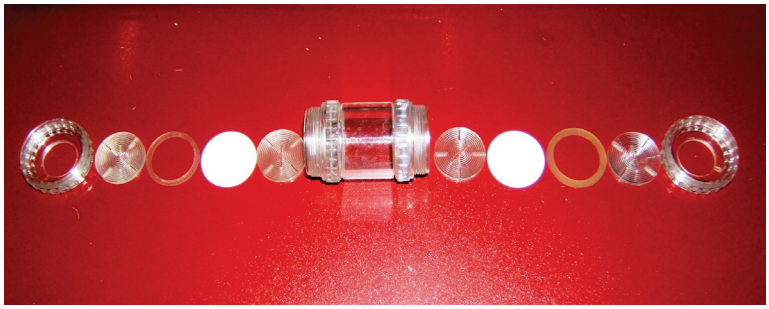

Fig. 1. Carrier of the Filter-Sandwich type

Parasitological carriers (Fig. 2) had the form of perlon sacks having holes with a diameter of 28 μm , which prevented the parasite eggs from getting outside the carrier.

Fig. 2. Parasitological carrier

The protective capsule for the microbiological and parasitological carriers (Fig. 3) was made from a tube with an inside diameter of 70 mm. To ensure that the effects of the internal conditions prevailing in the digester were also felt on the carriers, many perforation holes with a diameter of 8 mm each were drilled in the tube wall. The capsule had 5 separate compartments for carriers. Loading of the compartments occurred through a pivoting flap mounted on hinges.

Prepared sets of carriers were put into raschel sacks and placed in successive capsule sectors. Samples for the study were collected nine times and one sector was emptied at each time of reading.

Fig. 3. Protective capsule for carriers

Material for the study comprised of a typical feed used in biogas plants – 65% pig slurry, 35% maize silage. The assessment of the sanitary effectiveness of the methane fermentation process carried out in the biogas plant was performed in two experimental cycles, based on an estimation of parameters describing the kinetics of quantitative changes in the populations of selected indicator bacteria over time. The following microorganisms and gastrointestinal parasites suggested in the Regulation of the European Parliament and the Council (EC) no. 1069/2009 and the Commission Regulation (EU) no. 142/2011 were used in the experiment: *Salmonella* Senftenberg (W_{775}, H_2S negative) and *Ascaris suum* eggs.

Preparation of suspension of test microorganisms

A suspension of selected test bacteria was prepared based on their 24-hour cultures on Petri dishes with nutritional agar. The medium for making the suspension was sterile physiological saline. Bacterial cell concentration was determined in laboratory conditions with the spectrophotometric method at the wavelength $\lambda = 550$ nm on a level of 4.5×10^9 in 1 ml. The suspension obtained in this way was centrifuged, the supernatant liquid was decanted and bacterial cells were suspended in the pre--fermentation liquid, which was slurry mixed with fragmented maize silage, and introduced into carriers.

Preparation of suspension of *Ascaris suum* eggs

Eggs of *Ascaris suum* were obtained from sexually mature females derived from the casing room of a meat processing plant. After dissection of uteri, the eggs were squeezed with a glass rod into a Petri dish filled with saline solution, in order to obtain a suspension with the proper density. Next, 1 ml of the prepared suspension was introduced into each of the perlon parasitological carriers. After introducing the suspension, the sacks were tightly tied, thanks to which the eggs couldn't get out, although they were exposed to the action of environmental factors prevailing in the

fermenter. At particular times of sampling the sacs were removed, cut open and the contents of the sacs were placed in Petri dishes filled with sterile water. The prepared material was subjected to a 28-day incubation at 28°C, and then the percentage of invasive eggs was determined with the method of triple microscopic counts. In each of the repetitions, 100 eggs were counted and were distinguished between the live – invasive, in which a motile larva was visible and dead, with amorphous dark filling (Fig. 4).

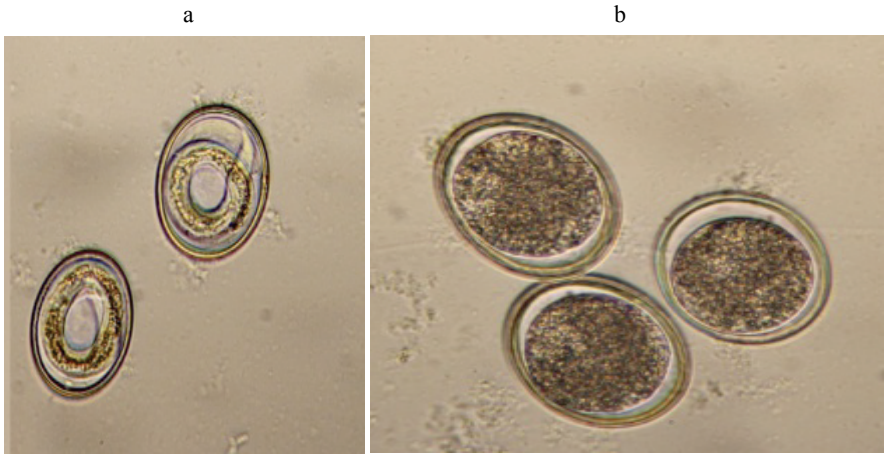

Fig. 4. Live (a) and dead (b) eggs of *Ascaris suum*

Microbiological analyses

Samples for the study were collected after 0, 0.5; 1, 1.5; 2, 3, 4, 8 and 12 hours from the moment of starting the experiment.

In the process of isolation of bacilli from the genus *Salmonella* Senftenberg (W_{775}, H_2S negative) 1% buffered peptone water was used for initial multiplication (24 hours at 37°C). Selective multiplication was performed on the liquid medium according to Rappaport (24 hours at 43°C). The agar medium BPLs was used for growth on a solid medium (24 hours at 37°C). Additionally, serological tests were performed using the suitable set of sera.

The final number of bacteria was determined based on the MPN method in a 3-tube test.

The obtained microbiological and parasitological results were subjected to statistical analysis using the software Statistica 10 PL (StatSoft). Regression line equations were calculated, based on which the theoretical survival times and the decimal elimination rate and time of the studied biological indicators were estimated.

RESULTS AND DISCUSSION

The initial numbers of *Salmonella* Senftenberg (W_{775}, H_2S negative) introduced to slurry during the present study amounted to 4.5×10^9 MPN·ml^{-1}. After 12 hours of fermentation, a decline reaching 8 log was observed (Table 1, Fig. 5, 7). The theoretical time of survival of these microorganisms exceeded 12 hours, at the mean elimination

rate reaching 0.7 log MPN·hour^{-1} (Table 3). The serotype *Salmonella* Senftenberg (W_{775}, H_2S negative) used in the present study is characterized by a large thermoresistance, exceeding that of *Salmonella* Typhimurium by even 30 times [Henry *et al.* 1969]. According to Paluszak *et al.* [1995], it is reasonable to state that estimation of the time needed for the complete elimination of *Salmonella* Senftenberg (W_{775}, H_2S negative) at the same time allows for a determination of the time necessary to eliminate from slurry the other serotypes of bacteria from this genus as these are characterized by a higher susceptibility to environmental factors.

Martens *et al*. [1998] analyzed the survivability of *Salmonella* Senftenberg (W_{775}, H_2S negative), *Salmonella* Enteritidis and *Streptococcus faecium* in bovine slurry subjected to the process of digestion in mesophilic (31°C) and thermophilic (54.5°C and 46°C) conditions. In mesophilic conditions the authors for the last time found the presence of *Salmonella* Senftenberg (W_{775}, H_2S negative) on the 9th day of the process, and at 46°C and 54.5°C, respectively at the 6th and 2nd hour of the process. Burton and Turner [2003], while studying the effect of thermophilic methane fermentation (54°C) on bacilli of the genus *Salmonella*, observed their elimination after less than 7 hours. In the present study the maximal temperature reached was about 47°C, and the fastest inactivation of bacteria occurred between the 4 and 8 hour points of the process. This tendency was particularly noticeable in the first experimental cycle, when their numbers decreased from a value of 10^6 MPN·ml^{-1} to 10^2 MPN·ml^{-1} (Fig. 5) during this time.

Table 1. Number of *Salmonella* Senftenberg (W_{775}, H_2S negative) in pig slurry subjected to anaerobic digestion, MPN·ml^{-1}

Time of measurement hour	Experimental cycle	
	I	II
0.0	4.5×10^9	4.5×10^9
0.5	2.5×10^9	9.5×10^8
1.0	$2{,}5\times10^8$	4.0×10^8
1.5	4.5×10^7	2.0×10^8
2.0	2.5×10^7	7.5×10^6
3.0	9.5×10^6	2.5×10^5
4.0	2.5×10^6	2.5×10^4
8.0	1.5×10^2	4.5×10^2
12.0	4.5×10^1	2.5×10^1

The effect of fermentation on the invasiveness of eggs of *Ascaris suum* was stronger than that observed for *Salmonella*. This may indicate a significant role being played in the studied process of sanitization by chemical agents, such as pH or the presence of compounds with a potentially toxic effect, to which, in contrast to high temperatures, the eggs of *Ascaris suum* are particularly sensitive [Böhm 2007]. It has been reported that their higher resistance to heat, in comparison with *Salmonella*, may be the cause of the low effectiveness of psychro- and mesophilic processes of processing slurry in respect of the elimination of eggs of the invasive helminths *Ascaris suum* [Gaasenbeek and Borgsteede 1998, Gantzer *et al.* 2001, Kato *et al.* 2003].

In the present study, the inactivation of live eggs of *Ascaris suum* was already found after 4 hours of digestion at a temperature not exceeding 50°C (Table 2). The calculated mean theoretical survivability of eggs was about 8 hours, and the mean time of decimal elimination was about 16%·hour^{-1} (Table 3). Pecson *et al.* [2007] observed a 99%

reduction in *Ascaris suum* eggs in sewage sludge with a temperature of 50°C and pH 7 within about 2 hours. Kato *et al.* [2003] in turn found a similar level of reduction in live *Ascaris suum* eggs at 55°C during 1 hour in sewage sludge under similar conditions.

Table 2. Percentage of invasive eggs of *Ascaris suum* in pig slurry subjected to anaerobic digestion, %

Time of measurement hour	Experimental cycle	
	I	II
0.0	97.66	95.00
0.5	91.00	87.67
1,0	95.33	86.00
1.5	93.67	88.00
2.0	86.67	85.67
3.0	13.00	1.00
4.0	0	0
8.0	0	0
12.0	0	0

Table 3. Regression coefficients characterizing the dynamics of inactivation of *Salmonella* Senftenberg (W_{775}, H_2S negative) and *Ascaris suum* eggs in pig slurry subjected to the process of anaerobic digestion

Microorganism	Regression line equation	Theoretical time of survival hour	Elimination rate log MPN·hour^{-1} % ·hour^{-1}	DRT hour	Coefficient R^2	Correlation coefficient
Experimental cycle I						
S. Senftenberg	y = -0.7035x+9.1357	12.98	0.70	1.42	0.95	0.97
A. suum	y = -16.344x+133.57	8.17	16.34	0.06	0.69	0.83
Experimental cycle II						
S. Senftenberg	y = -0.7018x+8.7465	12.46	0.70	1.42	0.89	0.94
A. suum	y = -16.381x+128.86	7.87	16.38	0.06	0.68	0.82

The crucial moment for the effectiveness of the fermentation process in respect of reduction in the percentage of invasive eggs was the third hour of the process, when the value of this index decreased by several dozen percent (Fig. 6 and 8). Similar results to this study were obtained by Johansen *et al*. [2013], who observed the full inactivation of *Ascaris suum* eggs in slurry at the third hour of their being maintained under anaerobic conditions.

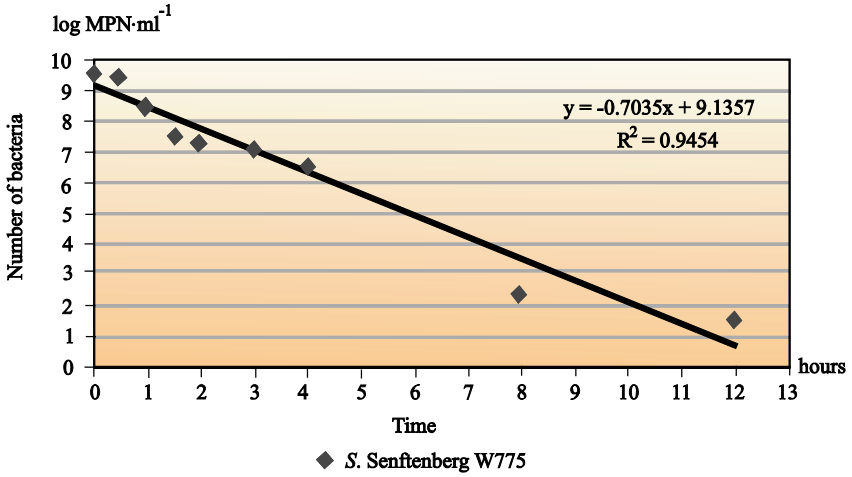

Fig. 5. Regression line characterizing changes in the number of *Salmonella* Senftenberg (W_{775}, H_2S negative) during digestion of pig slurry (cycle I)

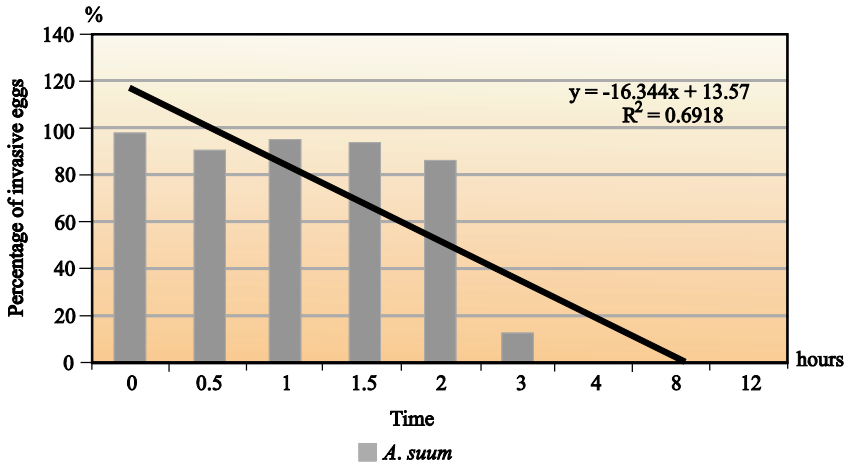

Fig. 6. Regression line characterizing changes in the percentage of invasive eggs of *Ascaris suum* during digestion of pig slurry (cycle I)

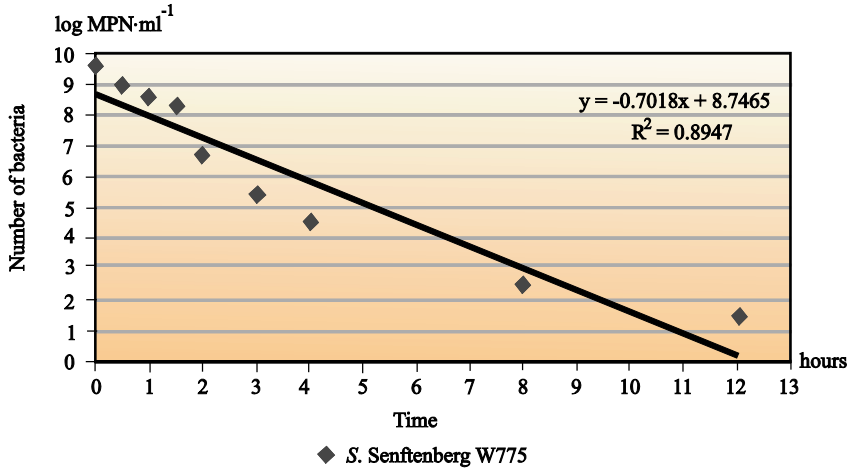

Fig. 7. Regression line characterizing changes in the number of *Salmonella* Senftenberg (W_{775}, H_2S negative) during digestion of pig slurry (cycle II)

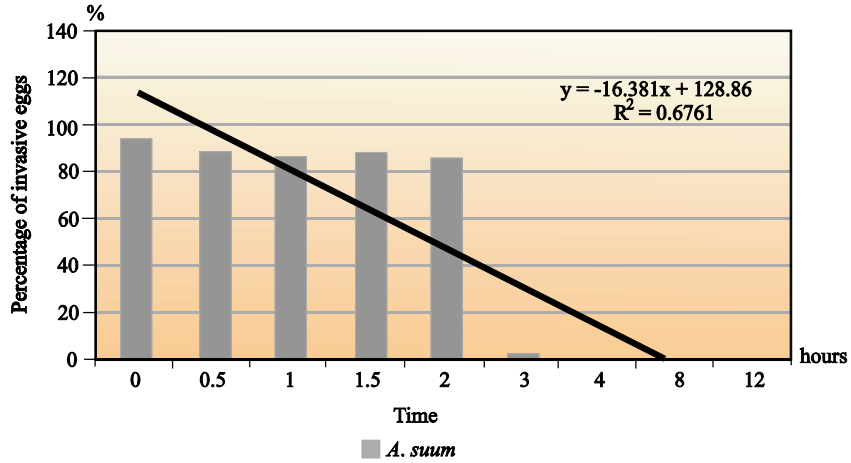

Fig. 8. Regression line characterizing changes in the percentage of invasive eggs of *Ascaris suum* during digestion of pig slurry (cycle II)

CONCLUSIONS

1. Conducted study shows that the process of anaerobic digestion of slurry results in the fast elimination of both *Salmonella* Senftenberg (W_{775}, H_2S negative) and *Ascaris suum* eggs introduced to the bioreactor.

2. Parasite eggs were less resistant to conditions prevailing during the process than would have been expected from the literature data. Their complete elimination took about 4 hours, whereas the theoretical time of full inactivation of *Salmonella* exceeds 12 hours.

3. Results of the study suggest that even in the case of very heavy contamination of the feed material, the process of anaerobic digestion guarantees obtaining a high quality product, microbiologically safe for the environment.

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Rozporządzenie Parlamentu Europejskiego i Rady (WE) nr 1069/2009 z dnia 21 października 2009 r. określające przepisy sanitarne dotyczące produktów ubocznych pochodzenia zwierzęcego, nieprzeznaczonych do spożycia przez ludzi, i uchylające rozporządzenie (WE) nr 1774/2002 (rozporządzenie o produktach ubocznych pochodzenia zwierzęcego).

Rozporządzenie Komisji (UE) nr 142/2011 z dnia 25 lutego w sprawie wykonania rozporządzenia Parlamentu Europejskiego i Rady (WE) nr 1069/2009 określającego przepisy sanitarne dotyczące produktów ubocznych pochodzenia zwierzęcego, nieprzeznaczonych do spożycia przez ludzi, oraz w sprawie wykonania dyrektywy Rady 97/78/WE w odniesieniu do niektórych próbek i przedmiotów zwolnionych z kontroli weterynaryjnych na granicach w myśl tej dyrektywy.

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WALIDACJA SANITARNO-HIGIENICZNA PROCESU FERMENTACJI BEZTLENOWEJ GNOJOWICY W OPARCIU O PRZEŻYWALNOŚĆ PAŁECZEK *Salmonella* SENFTENBERG (W_{775}, H_2S UJEMNE) ORAZ JAJ *Ascaris suum*

Streszczenie. Przedmiotem badań była ocena skuteczności higienizacji gnojowicy świńskiej w procesie fermentacji metanowej. Podstawowym kryterium efektywności procesu było tempo inaktywacji pałeczek *Salmonella* Senftenberg (W_{775}, H_2S ujemne) oraz jaj *Ascaris suum.* Zawiesinę bakterii wprowadzano do gnojowicy wewnątrz mikrobiologicznych nośników typu FILTER-SANDWICH, natomiast jaja pasożytów – w specjalnych perlonowych nośnikach parazytologicznych. Uzyskane rezultaty wskazują, że w trakcie fermentacji beztlenowej gnojowicy nastąpiła szybka eliminacja zarówno pałeczek *Salmonella* Senftenberg (W_{775}, H_2S ujemne), jak i jaj *Ascaris suum*. Z przebiegu prostej regresji wynika, że pełna inaktywacja pałeczek *Salmonella* Senftenberg (W_{775}, H_2S ujemne) miała miejsce między 12. a 13. godziną procesu. Jaja *Ascaris suum* były całkowicie eliminowane po 4 godz. Wyniki badań pozwalają przypuszczać, że nawet w przypadku silnego skażenia materiału wsadowego technologia fermentacji beztlenowej gwarantuje uzyskanie produktu bezpiecznego pod względem mikrobiologicznym.

Słowa kluczowe: *Ascaris* spp., fermentacja, gnojowica, *Salmonella* spp.

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