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AERATION AS AN EFFECTIVE METHOD FOR PATHOGEN ELIMINATION IN PIG SLURRY*

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Abstract

The aim of this study was to investigate the effect of aeration on the survival of indicator and pathogenic microorganisms in pig slurry. After inoculation of the aerated biomass with target bacteria, samples for the microbiological analyses were collected in different time intervals for the period of 12 days. The MPN method was used to determine the number of the investigated microorganisms. The results of statistical analysis showed the lack of significant differences in the theoretical time of survival, elimination rate and the time needed for 90% reduction between different *Salmonella* serotypes. Theoretical survival of *Salmonella* Typhimurium, *Salmonella* Senftenberg $_{W775}$ and enterococci in the aerated slurry ranged from 13 to 25 days. Enterococci were the most resistant to aeration, and their survival time, compared to salmonellas, was significantly higher.

Key words: pig slurry, aeration, *Salmonella*, enterococci

With respect to reports about possible threats to people and animal health as a result of the use of fertilizers of animal origin in agriculture, an extensive action is taken aiming at a reduction of the accompanying epidemiological risk. Bacilli of the genus *Salmonella*, verotoxic strains of *E. coli* (EHEC), *Campylobacter* spp., cysts of protozoans and nematode eggs and a number of viruses were frequently detected in animal feces (Albihn and Vinnerås, 2007; McCarthy et al., 2013; Ottoson et al., 2008). Due to their proved long survival both in feces and in the soil and water environment, the problem of effective elimination of those microorganisms prior to their agricultural use cannot be underestimated.

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Sanitary safety connected with the application of farmyard manure or slurry into the soil is guaranteed by suitably optimized methods of their hygienization. A reliable method for the assessment of slurry sanitization efficiency is based on the influence of the process on the elimination rate of indicator microorganisms, introduced to the slurry prior to its processing. In the present study, strains of *Salmonella* Senftenberg $_{W775}$ and *Enterococcus* spp. were used to this purpose. *Salmonella* Senftenberg $_{W775}$ had already found application in such experiments, due to its increased thermoresistance (Alvarez-Ordóñez et al., 2009; Paluszak et al., 2012; Skowron et al., 2013 b; Velázquez-Estrada et al., 2008). Enterococci are comparatively thermoresistant as well (Salhström et al., 2008). Due to their longer survival in the environment and relatively simple method of determination, they constitute one of the most often determined groups of indicator microorganisms (Wong and Selvam, 2009). In addition, due to its important epidemiological significance, *Salmonella* Typhimurium was integrated into the experiments.

Although slurry produced on individual farms and stock farms is most often stored, the effectiveness of this method from the point of view of reduction of pathogens colonizing slurry is dubious (Albihn and Vinnerås, 2007). It seems that in order to ensure the effective reduction in the number of pathogenic microflora, it is necessary to use more advanced sanitization methods, which in spite of higher amount of work and costs will ensure the satisfying final effect.

The aim of this study was to determine the effectiveness of one biological method for sanitizing slurry – fine-bubble aeration in the mesophilic variant, based on changes in the number of indicator and pathogenic microorganisms introduced to the processed biomass (Plachá et al., 2008; Svoboda, 2003).

Material and methods

Material for the study was fresh pig slurry collected from a pig farm in the Kujawsko-Pomorskie province.

The experiment was carried out on the semi-technical scale in 3 replications, using equipment consisting of a laboratory bioreactor BIOMER 10 (Technostart, Wrocław), allowing aeration of the batch.

Each time, 10 dm^3 of slurry was subjected to aeration with an initial temperature of 35°C. The intensity of aeration ensured maintaining the amount of oxygen dissolved in slurry at a level of 1 mg × dm^{-3}.

Strains of *Salmonella* Senftenberg $_{W775}$ and *S.* Typhimurium used for the study were obtained from The National Salmonella Centre in Gdańsk. Enterococci strains were isolated from environmental samples.

The bacteria were grown in nutrient broth (24-hour incubation at 37°C) obtaining initial suspensions with concentrations from 8.13×10^8 to 9.55×10^8 cfu × cm^{-3}.

Using suspensions of such high concentrations allowed observation of the sanitization effectiveness of the selected methods under conditions of the extreme level of slurry contamination. Carriers of the Filter-Sandwich type, containing 10 cm^3 of

slurry, inoculated with the tested *Salmonella* suspensions, were placed in the slurry subjected to aeration (Skowron et al., 2013 b). Enterococci and *S.* Senftenberg $_{W775}$ suspensions were also introduced directly into the sanitized biomass. Samples from the bioreactor were collected 1 hour after inoculation (zero sample), and then after 1, 2, 4, 6, 8, 10 and 12 days.

Prior to biomass inoculation the number of salmonellae and enterococci in raw pig slurry was examined.

In the process of re-isolation of bacteria of the genus *Salmonella*, 1% buffered peptone water was used for initial enrichment (24-hour incubation at 37°C). *Salmonella* were selectively raised on the liquid medium according to Rappaport Vasilliadis (24-hour incubation at 43°C). The BPLS agar was used for growth on the solid medium (24-hour incubation at 37°C). The number of *Salmonella* was determined based on the MPN method in the 3-tube design. The final identification was conducted using the diagnostic sera (Immunolab) according to the scheme of Kauffmann-White.

Enterococci were determined using glucose azide broth (48 h at 37ºC) and kanamycin esculin azide agar (48 h at 37ºC). Final identification was carried out based on the serological Phadabac-D-test (Boule Diagnostics AB, Huddinge, Sweden).

The liquid and solid culture media used in the experiment were supplied by Merck KGaA (Darmstadt, Germany).

Using standard methods of analysis, basic physicochemical properties of the fresh and processed slurry (temperature, pH, redox potential and dry mass content) were estimated.

The results obtained were subjected to statistical analysis involving the determination of regression line equations and calculation of the elimination rate and the theoretical time of survival. The theoretical survival time was calculated from regression line equation: $y = mx + b$, where y is the log MPN of bacteria in a slurry sample at a given time, m is the slope of the curve, x is the time in days and b is the intercept value.

Additionally, the significance of differences of the elimination rate and the theoretical time of survival between different bacterial strains was estimated by means of Tukey's test using the application SAS 9.2 PL.

Results

The results of raw pig slurry examination showed absence of *Salmonella* in the material. The concentration of enterococci did not exceed 10^4 MPN×ml^{-1}.

The number of *Salmonella* Typhimurium, which amounted to 9.55×10^8 MPN×ml^{-1} at the beginning of the experiment, decreased gradually to the level 3.98×10^1 MPN×ml^{-1} (Table 1). The theoretical survival rate calculated on the basis of the regression line equation, amounting to about 13 days, suggests that the complete elimination of those bacteria from slurry would take place in the next two days (Table 2).

Table 1. Number of the studied bacteria and standard deviation (MPN×ml^{-1}) during the mesophilic fine-bubble aeration of slurry

Time of determination	Number of bacteria			
	Salmonella Typhimurium (carrier)	*Salmonella* Senftenberg W_{775} (carrier)	*Salmonella* Senftenberg W_{775}	*Enterococcus*
1 hour	9.55×10^{8} (±0.00)	9.55×10^{8} (±0.00)	9.55×10^{8} (±0.00)	8.13×10^{8} ($\pm1.15\times10^{8}$)
1 day	3.39×10^{8} ($\pm1.44\times10^{8}$)	2.82×10^{8} ($\pm2.14\times10^{8}$)	3.16×10^{8} ($\pm2.47\times10^{8}$)	2.51×10^{8} (±0.00)
2 days	2.63×10^{7} ($\pm5.77\times10^{8}$)	3.09×10^{7} ($\pm3.18\times10^{7}$)	3.89×10^{7} ($\pm8.66\times10^{6}$)	7.24×10^{7} ($\pm9.04\times10^{7}$)
4 days	9.77×10^{5} ($\pm4.31\times10^{5}$)	2.88×10^{6} ($\pm2.65\times10^{6}$)	2.57×10^{6} ($\pm1.53\times10^{6}$)	2.34×10^{7} ($\pm1.69\times10^{7}$)
6 days	6.17×10^{4} ($\pm4.04\times10^{4}$)	3.31×10^{5} ($\pm4.36\times10^{5}$)	1.66×10^{5} ($\pm4.91\times10^{4}$)	5.75×10^{6} ($\pm1.15\times10^{6}$)
8 days	1.55×10^{3} ($\pm7.85\times10^{2}$)	4.07×10^{4} ($\pm5.43\times10^{4}$)	1.66×10^{4} ($\pm3.27\times10^{4}$)	9.33×10^{5} ($\pm2.00\times10^{5}$)
10 days	1.55×10^{2} ($\pm4.86\times10^{2}$)	2.34×10^{3} ($\pm1.78\times10^{3}$)	1.48×10^{3} ($\pm2.11\times10^{3}$)	1.26×10^{5} ($\pm2.02\times10^{5}$)
12 days	3.98×10^{1} ($\pm1.05\times10^{1}$)	2.82×10^{2} ($\pm1.32\times10^{2}$)	1.29×10^{2} ($\pm5.57\times10^{1}$)	3.72×10^{4} ($\pm3.70\times10^{4}$)

Analyses of density of *Salmonella* Senftenberg $_{W775}$ suspension derived from carriers placed in the reactor proved a slightly slower decrease in the number of their cells than in *Salmonella* Typhimurium (Figure 1). This was confirmed by statistical calculations, according to which at the daily elimination rate of 0.54 log, those bacteria were able to survive in aerated slurry for more than 16 days (Table 2).

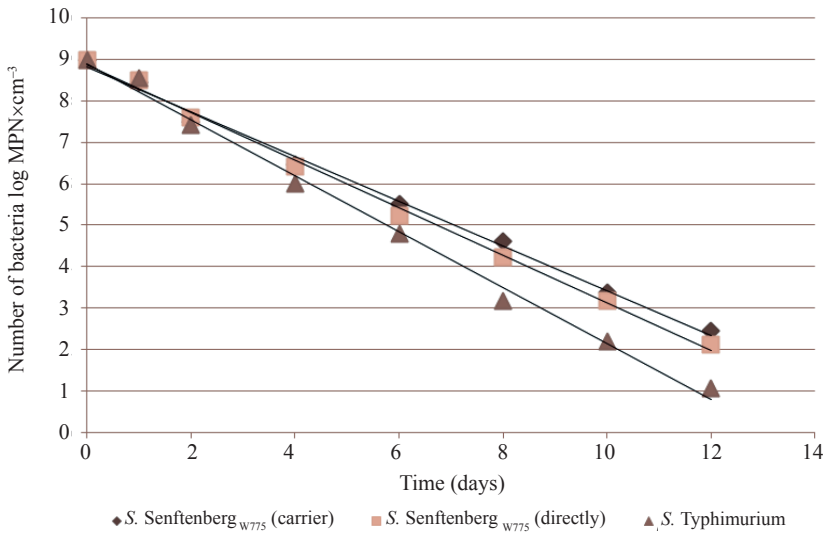

Figure 1. Regression line presenting the kinetics of changes in tested *Salmonella* serotype counts during the mesophilic fine-bubble aeration of slurry

Table 2. Decimal reduction times (DRT), theoretical times of survival (days) and elimination rate of studied bacteria (log MPN×day^{-1}) in slurry

Test bacteria	Way of introduction	Decimal reduction time DRT	Theoretical time of survival	Elimination rate
Salmonella Typhimurium	carrier	1.48 a *(±0.06)**	13.18 a *(±0.84)*	0.68 a *(±0.03)*
Salmonella Senftenberg $_{W775}$	carrier	1.86 a *(±0.44)*	16.37 a *(±1.02)*	0.54 a *(±0.22)*
Salmonella Senftenberg $_{W775}$	directly	1.74 a *(±0.12)*	15.43 a *(±0.89)*	0.57 a *(±0.04)*
Enterococcus	directly	2.82 b *(±0.03)*	24.71 b *(±0.61)*	0.36 b *(±0.004)*

*standard deviation.
a, b – statistically significant differences (P≤0.05) between individual bacteria.

Reduction in *Salmonella* Senftenberg $_{W775}$, introduced directly to the reactor in the form of suspension with a density of 9.55×10^8 MPN×ml^{-1}, in the course of the experiment reached 6 log (Table 1). Similarly to the samples collected from the carrier, their number after 12 days of aeration amounted to 10^2 MPN×ml^{-1} (Table 1).

Enterococci proved the highest resistance to the action of stress factors accompanying aeration (Figure 2). At the initial number of cells similar to that of *Salmonella*, its value at the end of the process was 3.72×10^4 MPN×ml^{-1} (Table 1). Theoretically, they could survive in aerated slurry almost 25 days (Table 2).

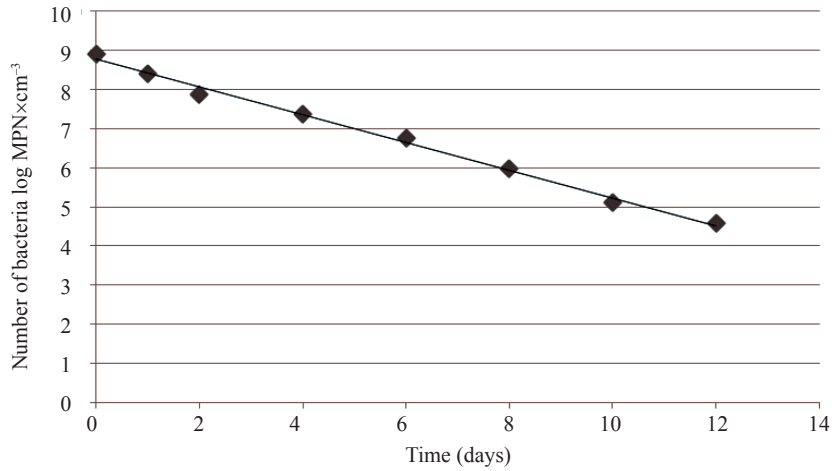

Figure 2. Regression line presenting the kinetics of changes in *Enterococcus* spp. count during the mesophilic fine-bubble aeration of slurry

The results of the statistical analysis showed no significant differences (P≤0.05) between the theoretical time of survival, elimination rate and the time required for the reduction of 90% in the number of the tested serotypes of *Salmonella*. The meth-

od of their introduction to the aerated reactor also did not result in the occurrence of differences. In the case of enterococci, the values of all parameters were significantly different from those obtained for *Salmonella* strains (Table 2).

Changes in the values of physicochemical properties of the aerated pig slurry are presented in Table 3.

After 12 days of the aeration the temperature of the biomass increased from 35°C to 49.3°C. The alkalization of the slurry was observed – the pH ranged from 7.64 at the beginning to 9.20 during final examination. A rise in redox potential was also noted within a range of −271 mV to +174 mV. The biomass content decreased gradually from 5.08% to 1.84% (Table 3).

Table 3. Results of basic physicochemical properties of slurry

Selected physicochemical properties of slurry								
	Time of determination (days)							
	0	1	2	4	6	8	10	12
Temperature (°C)	35.0	37.6	42.0	43.8	45.7	48.1	48.9	49.3
pH	7.64	8.27	8.83	9.05	9.13	9.22	9.27	9.20
Redox (mV)	–78	–271	–212	–14	+62	+117	+151	+174
Dry mass (%)	5.08	4.10	3.35	2.94	2.69	2.33	2.08	1.84

Discussion

The process of aeration allows the diverse utilization of the metabolic activity of autochthonous microorganisms in slurry. On the one hand, their action may result in a considerable reduction in slurry contamination with organic compounds (Plachá et al., 2008). Moreover, the energy generated as a result of this increased biological activity results in an increase in liquid temperature even by 10°C, which can considerably influence the sanitization efficacy of this method (Burton and Turner, 2003; Martens and Böhm, 2009).

Stimulating effect of aeration on the effectiveness of elimination of undesirable microorganisms in animal feces was frequently observed (Venglovski et al., 2009). Hanajijama et al. (2008) reported that aeration results in inactivation of microorganisms of gastrointestinal origin in pig slurry already after 1 day of the process. The number of *E. coli,* which at the initial stage reached 10^7 CFU·ml^{-1}, after 24 hours decreased to the level 10^2 CFU·ml^{-1} and for 6 days of the process did not exceed 10^3 CFU·ml^{-1}. Munch et al. (1987) indicated higher values of T_{90} for indicator and pathogenic bacteria in aerated slurry, than in stored slurry without additional aeration. The results of Plachá et al. (2008) proved that after 48 h of aeration under mesophilic conditions (temperature below 40°C) the number of *S.* Typhimurium decreased below the detection level.

High temperature is usually the key factor in processes of pathogen elimination from biomass. In the study by Zhu et al. (2002) growing aeration temperature from 15 to 25°C had a substantial effect on survival of aerobic bacteria colonizing slurry and caused their reduction by 75%. Mohammed (2011) observed the increased elimi-

nation rate of enteric pathogens (from 56 to 99.9%) in aerobically treated slurry between 40 and 50°C. Temperature values achieved during the present study exceeded 49°C in the final stage, so it may be assumed that thermal effect of the aeration process was crucial for the elimination of the studied microorganisms (Table 3).

Heinonen-Tanski et al. (2006) reports that aeration of slurry may result in reduction of 90–99.9% of gastrointestinal pathogens, even when it is carried out in the temperature range of 0–30°C. Benefits resulting from the use of the biologically stable fertilizer, safe in respect of hygiene, balance the financial outlays incurred on the aeration system, which makes this method relatively cheap. Also in the study by Hanajijama et al. (2008) aeration resulted in a fast and marked decrease in the number of microorganisms of gastrointestinal origin in slurry, although the temperature of the process, amounting to about 40°C, was similar to the thermal conditions established commonly during the cultivation of this group of microorganisms. This phenomenon may result from the action of many chemical and biological agents accompanying aeration, such as changes in concentration of different substances having inhibitory effect on pathogens, fluctuations of pH and the competitive or antagonistic effect of autochthonous microflora of slurry (Ottoson et al., 2008; Viancelli et al., 2013). According to Ravva and Sarreal (2014) the rapid killing of salmonellae in aerated wastewaters was related to pH increase (from 7.5 to 8.0). A significant growth of pH value observed during the present experiment (Table 3) could be an important factor affecting the survival of the studied bacteria.

In the present study, the number of microorganisms introduced into aerated slurry was reduced during 12 days by 4–7 log (Table 1). Their theoretical survival rate calculated based on regression line equations ranged from 13 to more than 25 days (Table 2). In comparison with data obtained by other authors these inactivation rates are less satisfactory – in most cases a rapid inactivation was observed already within 24–48 hours of aeration (Plachá et al., 2008; Svoboda, 2003).

When comparing the sensitivity of the particular species and serotypes of the studied bacteria, considerably longer survival rate of enterococci was observed as compared with *Salmonella*. Differences between inactivation rate of *S.* Typhimurium and *S.* Senftenberg $_{W775}$, proved to be statistically non-significant (Table 2). Similar observations concerning increased resistance of enterococci to hygienization were made in the study of Salhström et al. (2008). According to Skowron et al. (2013 a), microorganisms also survive longer than *E. coli* and *Salmonella* Dublin in bovine slurry stored at 4 and 20°C.

The results of the present study confirmed high usefulness of Filter-Sandwich carriers in experiments concerning microbiological quality of slurry, carried out in semi-technical scale. The number of *S.* Senftenberg $_{W775}$ isolated both from the contaminated slurry obtained from the Filter-Sandwich carrier and directly from the reactor underwent similar changes, and the differences between them were not statistically significant (Table 2). This confirms the results of the earlier studies concerning this relationship, carried out using bovine and pig slurry stored and subjected to mesophilic fermentation. They proved that introducing bacteria into slurry inside isolated carriers does not modify significantly the results of performed cultures (Skowron et al., 2013 a). This opens wide prospects of application of Filter-Sandwich carriers

as useful devices of study on the effectiveness of different methods for hygienization of liquid animal feces.

In many countries subjecting slurry to treatments reducing the content of pathogenic microorganisms a condition is necessary that enables its use for fertilization. As the results of the present study show, aeration in the mesophilic variant leads to the effective elimination of those microorganisms in a longer time than it is possible in thermophilic processes. However, bearing in mind that economic considerations play an essential role in making decisions concerning the choice of hygienization technology, the relatively low costs generated to obtain proper parameters of aeration process make this method an interesting alternative to high-temperature technologies.

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