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Effect of mesophilic fermentation of sewage sludge on drug-resistant bacterial count of the *Enterococcus* genus

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ABSTRACT

Enterococci are one of the major sanitary indices used for determination of water or soil contamination with municipal wastewater. This type of bacteria is present in the alimentary tracts of humans and warm-blooded animals. With the feces, *Enterococcus* bacteria are discharged to wastewater and, eventually, to sewage sludge. This type of waste is sometimes used for soil reclamation, from which it can be transferred to plants used as fodder for farming animals or they can migrate to groundwater, thus posing threat to the environment. One of the methods to limit the pathogenic bacterial count in this type of sludge is to use a fermentation process. This study analyzed dewatered and fermented sewage sludge from one of municipal wastewater treatment plants in Poland that is located near a smaller city in the Silesia Voivodeship. Selective isolation of *Enterococcus* bacteria from sewage sludge was performed and strains were collected for species identification. Total mesophilic bacterial count was also evaluated in the material. The disc method was used to determine drug resistance for strains isolated from sewage sludge and fermented. The antibiograms were used for the following antibiotics: gentamicin, linezolid, streptomycin, ampicillin, erythromycin, imipenem, penicillin, quinupristin, ciprofloxacin, chloramphenicol, vancomycin and tetracycline. Presence of enterococci was not found in the sewage sludge after mesophilic fermentation. Therefore, this process seems to be an efficient method to limit the enterococci content in sewage sludge.

Keywords: Sewage sludges; Pathogenic bacteria; *Enterococcus*; Drug-resistant bacteria

1. Introduction

Bacteria from the *Enterococcus* species are relatively anaerobic Gram-negative cocci that are common organisms in the intestines of humans and animals. Unfortunately, their presence can be also found in oral cavity, on the skin or in the area of the urinary tract. Initially, they were classified as the representatives of the *Streptococcus faecalis* species. However, based on phylogenetic examination that used DNA analysis, they were later separated as an individual genus. Unlike other streptococci, they have lower nutrient requirements. They are also susceptible to smaller antibiotic doses in clinical concentrations. Nowadays, the *Enterococcus* genus includes nearly 20 species, with this number growing constantly. Several of its representatives have been isolated from clinical materials. The most frequent include *E. faecalis* and *E. faecium*. They represent 95% of all isolates that belong to this genus. In the lower part of the intestines, they represent a part of natural microflora (ca. 1% of bacteria). Other, less frequent species are *E. durans*, *E. gallinarum*, *E. hirae*, *E. avium*, *E. raffinosus* and *E. casseliflavus* [1].

The *Enterococcus* bacteria are considered to be opportunistic pathogens which attack a person or animal

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when their immunity is reduced due to various causes. This concerns in particular individuals with congenital or acquired dysfunction of the immunological system. Infection may occur outside the natural environment of enterococci in the form of, for example, infective endocarditis, urinary tract infections or bacteremia. These bacteria are not characterized by many virulence factors. However, in the case of infection they may lead to serious health consequences. Part of them produces adhesin that makes it easier to adhere to tissues. In *E. faecalis*, cytolysins with bacteriocin properties are often detected. An additional problem during treatment is the constantly increasing (also in *Enterococcus* genus) resistance to the most commonly used antibiotics [2]. Some researchers found that plasmids can mediate transfer of genes that cause resistance to certain drugs between Gram-negative bacteria (with phylogenetically distant genomes) and *E. faecalis* and *E. faecium* [3].

These microorganisms are more tolerant to β-lactam antibiotics (cephalosporins, penicillins) than other streptococci. It is sometimes necessary to use a combination of benzylpenicillin with gentamicin or other aminoglycosides. It is not unusual that these bacteria show the presence of mutations concerning genes of proteins in the genome which are the target of gentamicin and streptomycin. This results in a high level of resistance to these drugs without the necessity of synthesis of enzymes which degrade the drugs. Resistance to penicillins is, however, often linked to the presence of specific β-lactamases that cut the β-lactam ring thus inactivating these antibacterial substances [4].

In the case of aminoglycosides, the increase in the frequency of resistant species from the *Enterococcus* genus is connected mainly with production of specific enzymes. These include, for example, 2′-phosphotransferase, glycoside 6′-acetyltransferase and 3′-phosphotransferase. For the first time, enterococci with high level of resistance to tobramycin, gentamicin and kanamycin, were isolated and described in 1979. Resistance to aminoglycosides spread very fast all over the world, especially in health care centres [1].

Doctors and researchers are increasingly worried about the instances of resistance of enterococci to glycopeptide antibiotics such as vancomycin and teicoplanin. Vancomycin is now used for hospital treatment of complicated infections caused by Gram-negative bacteria. Fortunately, resistance to these antibiotics remains to be a rear occurrence. However, after isolation of *E. faecalis* resistant to vancomycin first in a hospital in the United States, the enterococci resistant to these drugs have been more and more often recorded in Europe. These species are defined as VRE (vancomycin-resistant enterococci). As demonstrated by the study, the basic reason for the spread of resistance to vancomycin and other glycopeptide antibiotics is the use of avoparcin, which is a homolog of vancomycin. These antibiotics are characterized by total cross-resistance. Contact of enterococci with avoparcin in intestines of farming animals can lead to the development of resistance to vancomycin. Due to WHO activities, the campaigns were started in order to limit the use of antibiotics in animal production. This is supposed to limit the spread of drug resistance in pathogenic microorganisms for humans [5]. Numerous studies have found that antibiotics used in farming animals are transferred through the fertilizers obtained from animal feces [6].

Apart from bacteria from the Enterobacteriaceae family which are always present in the human gastrointestinal tract, enterococci are also transferred to municipal wastewater and sewage sludge with various amounts. This can lead to serious sanitary issues due to waste management. Sometimes the sludge with relatively low heavy metal content is used as a fertilizer for soil reclamation or in the agriculture. However, with presence of potentially dangerous bacteria, including drug-resistant forms, the risk of the spread of resistance in the environment is becoming a real problem [7]. In order to minimize this risk, various methods are currently used in order to reduce the content of potentially pathogenic microorganisms in raw sewage sludge in order for them to be safely managed in the future. This is achieved through stabilization and hygienization, mainly based on the processes of anaerobic fermentation, composting and liming.

Unfortunately, as shown in previous studies, enterococci show a relatively high resistance to unfavorable environmental conditions, for example, elevated temperature that is present during, for example, composting. The process of sewage sludge fermentation does not always sufficiently reduce their content. This depends on the choice of process parameters and its duration. For this reason, the *Enterococcus* bacteria have been often used as an indicator of the effectiveness of the sewage sludge hygienization process. Their lack or a substantial decline in their number indicates that the process is effective [8].

One of the most important processes of stabilization of wastewater and sewage sludge that leads to the reduction in the amount of pathogenic forms of microorganisms is methane fermentation [9,10] which is a complex process that is conducted in anaerobic conditions. With closed fermentation chambers, process temperature can be easily controlled. Furthermore, it is necessary to use the equipment for heating of the fermented sludge in the case of mesophilic or thermophilic fermentation. Temperature is critical to the microbiological composition and biochemical transitions in the substrate [11].

This study analyzed effect of mesophilic fermentation of sewage sludge on drug-resistant bacterial count of the *Enterococcus* genus. Analyzed dewatered and fermented sewage sludge from one of the municipal wastewater treatment plants in Poland that is located near a smaller city in the Silesia Voivodeship. Selective isolation of *Enterococcus* bacteria from sewage sludge was performed and strains were collected for species identification. Total mesophilic bacterial count was also evaluated in the material. The disc method was used to determine drug resistance for strains isolated from sewage sludge and fermented.

2. Materials and methods

The study examined raw sewage sludge after biological processes of activated sludge treatment, directly before the dewatering process. The sludge was sampled from medium-sized municipal wastewater treatment plant in a town in the south of Poland. The sludge represents the waste that should be managed in a way that is safe to both humans and the environment. After taking the sludge samples, they were immediately transported to the laboratory in order to perform analyses.

Physical and chemical properties of sewage sludge were examined based on the recommended procedures. The reaction was examined using the potentiometric method, nitrogen content – using the Kjeldahl method, ammonium nitrogen content – using colorometric method (Nessler's reagent), sludge dry mass – using weight method. Furthermore, heavy metal contents, including calcium (Ca), magnesium (Mg) and phosphorus (P), were determined by means of the atomic absorption spectroscopy after previous sludge mineralization of the mixture of strong acids in the microwave mineralizer.

The examinations of the effectiveness of the fermentation process in removing drug-resistant enterococci were conducted in the reaction flasks, where mesophilic fermentation was conducted for consecutive 4 weeks (at the temperature of 37°C) without air access. For control reasons, the same stabilization process was conducted in the experimental flasks with access to air and placed in the room temperature. The experiments were carried out with three repetitions.

Microbiological analyses were performed in raw sludge (by determining the species of isolated bacteria and their susceptibility to selected antibiotics). Next, the total bacterial count and enterococci count was evaluated at week intervals for 1 month during the mesophilic fermentation process. The experiments were performed with three repetitions.

In order to count *Enterococcus* bacteria, we used a standard Koch dilution method with decimal progression used to determine microorganisms count in samples of water, sewage, etc. The first sample of sewage sludge with the amount of 1 g was suspended in a 9 ml solution of sterile saline solution. Next, after mixing 1 mL of this suspension, it was put into another test tube with 9 mL of sterile saline solution. This method was repeated until a range of dilutions from 10^{-1} to 10^{-10} was obtained [2].

Agar plate was used to evaluate total bacterial count in the samples. In order to determine the enterococci count, we used bile esculin agar (BEA) plate with addition of sodium azide, which is substantially selective for the *Enterococcus* genus. This plate is used mainly in the clinical practice. Due to better differentiating properties compared with Slanetz Bartley agar (used routinely in sanitary microbiology), this plate was used for the present study. The examinations were performed using current recommendations concerning the process of sampling and preparation of microbiological samples and bacteriological analyses [2].

After applying of 0.1 mL of the sludge suspension (from various dilutions) on individual dishes with nutrient agar and BEA, they were incubated at the temperature of 37°C for 24 h. The bacterial colonies grown on the BEA with the characteristic blackened area around enterococci (caused by hydrolysis of esculin to esculetin) were sampled for further analyses. The colonies of the discussed bacteria are relatively small, with the size ranging from 0.5 to 1.0 mm with light gray and slightly convex and shiny. Unlike various species of staphylococci, they hydrolyze esculetin after only several hours and do not contain catalase [1,2].

Surface passage is chosen in the case of necessity of further analysis of bacteria, including isolation of a pure strain using the streaking method. Deep passage is in this case impractical as it is much more difficult to sample an individual colony from the agar volume without polluting it with other bacteria and to count the number of colonies with much greater density. Real values in these terms in surface passage are obtained with the highest dilutions. With greater density, bacterial cells tend to connect with or stick to organic particles or internal surfaces of the laboratory glass. With deep passage, part of cells of bacteria more susceptible to higher temperatures can be destroyed after addition to cooled but still liquid agar, which is relatively warm.

Another cause of the choice of surface passage in the case of *Enterococcus* is their behavior on the BEA. With hydrolysis of esculin to esculetin, their colonies and large area around them are blackened. With their greater number, this agar actually changes the color to dark grey or black. If the deep passage had been used, we would not have seen colonies in the agar volume and, therefore, they would be impossible to be sampled for further examinations. Being facultative anaerobic organisms, *Enterococcus* can grow both in anaerobic and aerobic conditions. Faster increase of these bacteria can be ensured by enriching the incubator atmosphere with CO_2. However, it is not necessary.

Pure cultures of enterococci obtained from three-time reduction inoculation were subjected to biochemical analysis by means of Microgen Strep ID commercial biochemical multitests used for identification of streptococci and enterococci. After inoculation with the suspension of bacteria in a special solution, they were incubated for 24 h at temperature of 37°C. Species identification if individual isolates was performed by means of Microgen MID 60.

The period of 24-h incubation is entirely sufficient at temperature of 37°C to obtain growth of *Enterococcus* (colonies with diameter of 0.5–1 mm). Certain strains show a positive response of hydrolysis of esculin to esculetin already in a few hours after inoculation and blacken the agar around colonies which are often not yet visible with the naked eye. The 24-h incubation at this temperature has been routinely used in microbiological laboratories. Therefore, we also used this methodology.

After isolation on the BEA (selective agar for growing *Enterococci* from various types of samples), pure strains were obtained using the streaking methods based on the morphological properties. Next, Microgen Strep ID biochemical multitests were used. These multitests are standardized, reliable and used, besides bioMérieux Products, in numerous clinical laboratories all over the world. We used the methodology recommended by test manufacturer. Another stage included performing the extended antibiograms for isolated enterococci that included nearly all important compounds used with respect to these bacteria in medicine. Antibiograms were performed on the Mueller–Hinton agar according to the most recent standards used in clinical microbiology, for example, EUCAST (European Committee on Antimicrobial Susceptibility Testing).

The disc diffusion method was used for the following antibiotics: gentamicin, linezolid, streptomycin, ampicillin, erythromycin, imipenem, penicillin, quinupristin, ciprofloxacin, chloramphenicol, vancomycin and tetracycline. The examinations were based on current recommendations published by the National Reference Centre for Drug Sensitivity of Microorganisms and indications of the EUCAST [12].

The disc diffusion method has been used worldwide for routine evaluation of sensitivity of microorganisms to

selected antibiotics. In this method, special microbiological agar (Mueller–Hinton Agar in various versions) is used to examine the discs saturated with selected antibiotics which are put on the evenly distributed bacterial suspension with specific density (ca. 0.5 in the McFarland standards on the turbidimetric scale). These are standardized discs with specific content of the active substance. During incubation, the antibiotic diffuses to the agar and, if microorganisms are sensitive to its effect, a zone of growth inhibition is formed around the disc. This method is used to determine sensitivity or resistance of this microorganism. The methodology was developed by the EUCAST [12].

3. Results and discussion

Physical and chemical parameters of raw sewage sludge and sewage sludge after the process of mesophilic methane fermentation are included in Table 1.

Total heterotrophic aerobic and relatively anaerobic bacterial count ranged in the raw sewage sludge at the level of 10^8 to 10^9 CFU as calculated per 1 g of sludge. The bacterial count that belongs to the *Enterococcus* genus which was grown in the form of characteristic colonies with the esculin hydrolysis zone around was 3×10^4 CFU per 1 g sludge on average. A gradual decline in the enterococci number was observed during the mesophilic fermentation process. After 4 weeks, no colonies from the genus discussed in the study were found for the BEA plate. In the control samples, the enterococci count was also reducing with time but to a significantly lower degree. In the first 2 weeks of the fermentation process, total count of other bacteria was increased. This was caused by the elevation of temperature to 37°C and the change in their conditions. After this period, total heterotrophic bacterial count was reduced to the level similar to that found in raw sewage sludge. Furthermore, total heterotrophic bacterial count in control tests was relatively stable. Tables 2 and 3 present changes in enterococci bacterial count during the experiment, whereas Table 4 compares the results for resistance of individual species isolated from raw sewage sludge before the fermentation process.

The bacterial count for those supposed to be from the *Enterococcus* genus that was grown on the BEA in the form of characteristic colonies was reducing with time. Five species of enterococci were isolated: four from the *E. faecium* and one from the *E. gallinarum* species. They differed slightly in their biochemical profile and susceptibility to selected antibiotics (Table 4). For this reason, four *E. faecium* species, despite being from the same genus, were analyzed separately. All the enterococci demonstrated reduced sensitivity to imipenem. Furthermore, one of the strains of *E. faecium* was resistant to erythromycin, penicillin and ciprofloxacin. *E. gallinarum*

Table 1
Physical and chemical parameters of raw sewage sludge and sewage sludge after the process of mesophilic methane fermentation

Parameter	Values	
	Raw sewage sludge	Fermented sewage sludge
pH (in H_2O)	6.4	7.2
Dry matter content, %	6.7	7.9
Total nitrogen,% d.m.	6.3	3.6
Total phosphorus, % d.m.	1.0	1.1
Ca, % d.m.	8.96	7.14
Mg, % d.m.	0.37	0.35
Pb, mg/kg d.m.	20.3	20.5
Ni, mg/kg d.m.	8.4	9.8
Hg, mg/kg d.m.	0.19	0.24
Zn, mg/kg d.m.	870	1,543
Cu, mg/kg d.m.	44.6	49.4
Cr, mg/kg d.m.	17.9	31.9

Table 2
Changes in total heterotrophic mesophilic bacterial count and *Enterococcus* genus in the process of mesophilic fermentation (CFU/g)

Agar Microbiological (type of microorganism)	Raw sewage sludge	Week 1	Week 2	Week 3	Week 4
Nutrient agar (total heterotrophic mesophilic bacterial count)	$3{\cdot}10^9$	$1.9{\cdot}10^{10}$	$1.6{\cdot}10^{10}$	$5{\cdot}10^9$	$3{\cdot}10^9$
BEA (total count for *Enterococcus* genus bacteria)	$3{\cdot}10^4$	$3{\cdot}10^3$	$5{\cdot}10^2$	$9{\cdot}10^1$	0

Table 3
Changes in total heterotrophic mesophilic bacterial count and *Enterococcus* genus in control samples (with air access) during the experiment (CFU/g)

Agar Microbiological (type of microorganism)	Raw sewage sludge	Week 1	Week 2	Week 3	Week 4
Nutrient agar (total heterotrophic mesophilic bacterial count)	$3{\cdot}10^9$	$1.8{\cdot}10^9$	$3{\cdot}10^9$	$2.5{\cdot}10^9$	$2.9{\cdot}10^9$
BEA (total count for *Enterococcus* genus bacteria)	$3{\cdot}10^4$	$3.5{\cdot}10^4$	$1.3{\cdot}10^4$	$7{\cdot}10^3$	$9{\cdot}10^2$

Table 4
Results of antibiograms performed for bacteria of *Enterococcus* genus isolated from raw sewage sludge

Bacterial species	Gentamicin (CN120)	Linezolid (LNZ30)	Streptomycin (S300)	Ampicillin (AM10)	Erythromycin (E15)	Imipenem (IMP10)	Penicillin (P10)	Quinupristin (SYN15)	Ciprofloxacin (CIP5)	Chloramphenicol (C30)	Vancomycin (VA30)	Tetracycline (TE30)
E. faecium	S	S	S	S	S	R	R	S	S	S	S	MS
E. gallinarum	S	S	MS	S	S	R	R	R	S	MS	R	MS
E. faecium	S	S	S	S	S	R	R	S	S	S	S	S
E. faecium	S	S	S	S	R	R	R	S	R	S	S	S
E. faecium	S	S	S	S	S	R	S	S	S	S	S	S

Symbols used for susceptibility of bacteria to antibiotics: S – susceptible, MS – medium susceptible, R – resistant.

was resistant to imipenem, quinupristin, vancomycin and penicillin and medium sensitive to streptomycin. Three in four species of *E. faecium* demonstrated resistance to benzylpenicillin. No enterococci were found after 4 weeks of mesophilic fermentation in sewage sludge (Table 2). This led to the elimination of this infection factor from the material using the analyzed hygienization method. A reduction in the count of enterococci in control samples (not subjected to the fermentation process) was also observed (Table 3). This was probably caused by the fact that with time, they are replaced by the typical saprophytic bacteria. The relatively small number of isolated species of these bacteria is likely to be caused by the fact that the samples were taken at the end of November, when the temperature in the sewage treatment plant was relatively low.

However, one large health care institution is in the area of the same town. This is the most probable source of drug-resistant bacteria, especially due to the frequency of the use of individual groups of antibiotics in clinical practice. This phenomenon is also reflected by therapies conducted in patient's homes. For this reason, raw sewage sludge from this treatment plant represents a serious sanitary risk if the decision on their use for soil reclamation or agriculture is made in the future. Due to a relatively low content of heavy metals and optimal physical properties, sludge from this treatment plant can be used in nature. Raw sewage sludge from this plant contains bacteria resistant to the antibiotics of last resort. Similar results have been also documented by other researchers [13]. Everage et al. [13], who examined the effectiveness of wastewater treatment, found that *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Enterobacter cloacae* that show resistance to many antibiotics, were present in raw wastewater. Some of them survived the treatment processes and were transferred to the environment with clean water and sewage sludge.

Presence of drug-resistant bacteria in sewage sludge, including enterococci, impacts on the activity of big institutions of health care in this area. The awareness of the medical staffs concerning the rational use of antibiotics is also important. It has been improving due to the comprehensive campaigns aimed at limitation of their use only to justified cases. Therefore, fewer doctors prescribe ampicillin or doxycycline to treat common colds. Discharge of municipal waste from health care institutions to the sewerage system can result in a substantial increase in the count of drug-resistant bacteria from sewage sludge after the process of municipal sludge treatment [7]. These bacteria are transferred with feces of people who use antibiotics or those infected with drug-resistant strains. Their presence in municipal waste can be also caused by contact with antibiotics already present in wastewater, where mutants with lower sensitivity to a specific antibacterial compounds are selected. This is conducive to their relatively low concentration in this environment, which is substantially below the minimal inhibitory concentration. The least frequent is resistance of bacteria to vancomycin, which represents the drug of choice in the case of serious infections with Gram-positive cocci. Serious concerns are also raised about animal farming, where substantial amounts of antibacterial drugs are discharged to sewage sludge [14].

Raw sewage sludge can represent the source of enterococci resistant to various types of antibacterial compounds.

Similar situation is also observed in the case of intestinal bacteria which are constantly present in municipal waste. Therefore, this raw material has been gradually eliminated from soil reclamation and subjected to disposal through incineration. One of the simplest forms of hygienization of communal sewage sludge is solar treatment. This does not involve substantial expenditures and is easy to be performed. A reduction of total changes in total heterotrophic mesophilic bacterial count and *Enterococcus* genus in control samples (with air access) during the experiment (CFU/g) bacterial count can be achieved, including those from *Enterococcus* genus, with the fourth to sixth order of magnitude. However, the efficiency of this process largely depends on the time of the year and insolation. Therefore, it cannot be used routinely in the process of sewage sludge management [15]. The liming process can be much more effective in limitation of potentially hazardous microorganisms as due to a high pH level, it allows for a greater reduction in the number of pathogens. However, if the amount of lime is too high, the final product can be unsuitable for further use in the agriculture due to the adverse physicochemical properties.

Whatever the case of the use in nature, sewage sludge should be previously subjected to the stabilization process (e.g., anaerobic fermentation) or hygienization. Therefore, the amount of potentially hazardous bacteria (also those drug-resistant ones) and their spread in soil can be limited. This is especially important since it has been demonstrated that these bacteria can survive in soil for even several years [7].

Sewage sludge can be the source of many intestinal bacteria resistant to antibiotics, which can transfer resistance genes to the bacteria in the environment. The examinations were conducted by the authors demonstrated that mesophilic fermentation reduces the count of intestinal bacteria (enterococci) in the sewage sludge. In the experiment, the process turned out to be efficient and entirely eliminated the microorganisms studied from sewage sludge. It is likely that the products generated during fermentation (e.g., mixture of organic acids) are the factor responsible for this process rather than fermentation temperature. Unlike other streptococci, enterococci can survive 30 min of heating, even at temperature of 60°C. The changing sludge reaction, content of heavy metal and other toxic compounds can also be critical. The authors observed, for example, an increased content of certain heavy metals (zinc, copper and chromium) following the fermentation process compared with their content in sewage sludge (Table 1). Reaction of sewage sludge increased from 6.4 to 7.2.

4. Conclusions

- The present study found presence of drug-resistant forms of *Enterococcus* bacteria in raw sewage sludge.
- Insignificant differences between species of the analyzed heterotrophic bacteria genus were also demonstrated. Four *E. faecium* strains and one *E. gallinarum* strain were isolated.
- The biggest range of drug resistance was found for *E. gallinarum* (determined in sewage sludge), which was resistant to imipenem, quinupristin, vancomycin and penicillin and medium sensitive to streptomycin.
- In its raw form, sewage sludge can represent a sanitary risk in their future applications, for example, soil reclamation.
- 4 weeks of anaerobic fermentation at temperatures of 37°C led to entire elimination of enterococci from the analyzed sewage sludge.
- Therefore, mesophilic fermentation seems to be sufficiently effective in limitation of the enterococci count in sewage sludge.

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