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Effect of sewage sludge liming on drug-resistant bacteria of the Enterobacteriaceae family

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ABSTRACT

Dangerous drug multi-resistant strains of bacteria from the Enterobacteriaceae family are responsible for the deaths of thousands of people every year. The intestinal bacilli are found in large amounts in wastewater and sewage sludge as they are transported with human and animal feces. In the study, microbiological analysis of raw dewatered sewage sludge was performed using Microgen's GNA + B biochemical multi-tests. Isolation of intestinal bacteria was carried out on ENDO agar. Determination of susceptibility of isolated and identified intestinal bacteria to drugs was carried out using ampicillin, co-amoxiclav, cefazolin, cefuroxime, ceftazidime, amikacin, gentamicin, and ciprofloxacin. Antibiograms were performed using Mueller-Hinton II agar. The presence of seven species of intestinal bacteria was found before liming of sewage sludge, including two strains of *Escherichia coli* which demonstrate resistance to ampicillin and amoxicillin with clavulanic acid. Furthermore, the following bacteria from the Enterobacteriaceae family were also present: *Klebsiella pneumoniae*, *Morganella morganii*, *Citrobacter freundii*, *Yersinia aldovae*, *Serratia marcescens* and Enteric Group 68. They showed typical susceptibility to the antibiotics used in the study. The bacteria count in species isolated on ENDO agar ranged from 3.6 to 4.9 × 10^8 CFU/g. Three doses of $Ca(OH)_2$ expressed as CaO weight were used for liming: 0.1, 0.2, and 0.4 kg/kg·d.m. of dewatered sludge. After a three-month process, the following intestinal bacilli were detected in the tested material: *Morganella morganii*, *Serratia odorifera*, *Providencia rettgeri*, *Providencia stuartii*, *Proteus vulgaris*. *Providencia stuartii*, *Proteus vulgaris*, *Morganella morganii* and *Serratia odorifera* proved to be drug-resistant. Bacteria from the *Alcaligenes faecalis*, *Pseudomonas stutzeri*, *Pseudomonas fluorescens* and *Acinetobacter lwoffi* species were also found. Liming process led to a reduction in the number of intestinal bacteria of 6–8 orders of magnitude of the baseline values. None of the doses were able to eliminate Enterobacteriaceae bacteria from the sludge.

Keywords: Bacteria; Enterobacteriaceae; Antibiotics; Sewage sludge; Liming

1. Introduction

The common use of antimicrobial substances in human, veterinary, and industrial animal husbandry treatment is causing an increasing drug resistance among clinically important microorganisms. Individual pathogenic bacteria and fungi have gradually become resistant to the drugs to which they were previously susceptible. Bacteria are capable of developing mechanisms that reduce the effectiveness of each new antibiotic. These can be enzymes that degrade molecules of a substance, pumps that remove it from the cell, or modifications of target proteins. Examples include

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β-lactam antibiotics, where mutations within the gene encoding peptidoglycan transpeptidase can cause complete resistance to this group of compounds, or β-lactamase enzymes that hydrolyze penicillins and cephalosporins. Furthermore, if genes encoding, for example, β-lactamases are localized on plasmids, such resistance can be passed over not only within a specific species with new generations but also through horizontal gene transfer, including interspecies conjugation, or transformation of the naked DNA [1,2].

Along with municipal wastewater, bacteria, both wild strains and those already showing some level of drug resistance, and residues of some antibiotics (some of which are characterized by significant persistence in the aquatic environment and are not always rapidly degraded in wastewater), enter wastewater treatment plants. Next, large amounts of sewage sludge are generated during the processes associated with the removal of pollutants, thus posing a serious problem. This waste is not so easy to deal with mainly for sanitary reasons, and also because heavy metals are sometimes present in amounts that exceed acceptable standards. There are several methods to reduce the count of potentially hazardous pathogenic organisms in the sludge. They show varying degrees of effectiveness in terms of subsequent sanitation parameters. With proper management of the stabilization and treatment, sewage sludge from municipal treatment plants can be used at a later stage for land reclamation or as a fertilizer to improve soil fertility and structure [3]. However, the use of sewage sludge for soil reclamation or fertilization involves the risk of the spread of resistance genes to various antibiotics with the microorganisms present in the sludge. This is a dangerous phenomenon, primarily because of the threat to public health: food products from plants grown on such soils should not be consumed nor should they be used as livestock feed. After soil reclamation with, for example, naturally dried sewage sludge, intestinal bacteria remain present even after a longer period of time (e.g., *Klebsiella*, *Enterobacter* or *Citrobacter*). This seems to be a worrying phenomenon since the strains may also include those showing resistance to one or more antimicrobial substances to which wild strains of these species are naturally susceptible [4].

Intestinal bacteria have been a serious clinical problem for years, as they can cause infections ranging from mild diarrhea or soft tissue infections to very serious pneumonia or even septicemia, characterized by a high mortality rate. Their treatment is difficult in many cases, especially for multidrug-resistant strains, as there is a problem here with the selection of a sufficiently effective antibiotic. In extreme cases, the outcome of therapy depends only on the resilience of the patient's body and his or her immune system [5]. This makes the treatment process difficult and reduces the chances of a patient to recover. An example here is *Klebsiella pneumoniae* New Delhi strain characterized by resistance to most antibiotics to which this species is naturally susceptible. It was first detected in a patient in Sweden who had previously been hospitalized in India. It is able to rapidly degrade both older and many new antimicrobial substances, including almost all β-lactam antibiotics. This is possible by encoding New Delhi metallo-β-lactamase (NDM), which is also sometimes found in pathogenic *Escherichia coli* strains. The microorganism has spread quite rapidly around the world and was also found in Poland as early as 2011. Furthermore, it is most common in the Middle East, the Balkans, and the Indian subcontinent. The genes encoding NDM are plasmid-encoded and can also be transferred between different species of intestinal bacteria. They are most often transmitted by *K. pneumoniae* and *E. coli* [6]. The most important diseases caused in humans by intestinal bacteria found in sludge from municipal wastewater treatment plants are:

- *E. coli* – urinary tract infections, pyelonephritis, inflammations of the gastrointestinal tract, inflammatory diseases of the middle ear, pneumonia;
- *Shigella* sp. – bacterial dysentery;
- *Salmonella* sp. – typhoid fever, paratyphoid fever, self-limiting inflammations of the gastrointestinal tract;
- *Citrobacter* sp. – urinary tract infections, inflammations of the gastrointestinal tract;
- *Yersinia enterocolitica* – inflammations of the gastrointestinal tract, inflammatory bowel diseases;
- *Enterobacter* sp. – neonatal meningitis, septicaemia, infective endocarditis, infected wounds, urinary tract infections;
- *Serratia* sp. – pneumonia (mainly in hospitalized patients), urinary tract infections, septicemia, infective endocarditis;
- *Hafnia alvei* – pneumoperitoneum;
- *Proteus* sp. – urinary tract infections (often complicated), septicaemia, wound infections;
- *Providencia* sp. – urinary tract infections, septicaemia (mostly in hospitalized patients);
- *Morganella* sp. – urinary tract infections, septicaemia (usually in hospitalized patients [5].

During municipal wastewater treatment processes, excess sludge is generated and removed from the bioreactor to keep the process running properly. Bacteria entering the wastewater with fecal matter, rainwater runoff, and numerous microorganisms responsible for the decomposition of organic matter found in wastewater are present in large quantities in excess sludge. Excess sludge is regularly collected and dewatered in wastewater treatment plants. It is then subjected to solar drying or liming to obtain raw material for soil reclamation or as a source of energy [7,8].

Due to the sanitary hazard, sewage sludge after wastewater treatment requires further management. The simplest method is solar drying or composting. However, more radical methods are needed to achieve an adequate reduction of pathogenic and potentially dangerous microorganisms for humans. One of them is liming. Liming can be made with calcium oxide or calcium hydroxide. The usual dose is 0.2–0.4 kg of CaO (calcium oxide) per kg·d.m. of sludge. The process itself takes up to three or four months. A prerequisite for the process is a very thorough mixing of the sludge. Otherwise, the local survival of microorganisms in areas with slightly lower pH can reduce the quality of the final product. The high content of calcium, nitrogen, phosphorus, and potassium makes lime-treated sewage sludge a good quality fertilizer for plants intended and for example, for energy purposes [7,8]. The addition of lime-treated sewage sludge to the soil raises phosphorus and nitrogen concentrations, while the increase in pH is usually not greater

than 0.2. Thus, while meeting biological and chemical standards, it is a valuable fertilizer [9].

In the case of calcium oxide, a thermal effect (hydration of this compound is an exoergic reaction) and a significant increase in pH can be achieved. With calcium hydroxide, only the effect of high pH on microorganisms is observed. Almost all bacterial cells, nematode eggs, and viruses are destroyed. Regardless of the choice of calcium oxide or hydroxide for the process, thorough homogenization of the sludge with the reagent is of key importance [10]. An example of the composition of lime-treated sludge is shown in Table 1.

The purpose of this study was to evaluate the effect of the calcium hydroxide liming process on the abundance of Enterobacteriaceae bacteria in raw dewatered sewage sludge from a municipal wastewater treatment plant and the incidence of drug resistance among the intestinal bacteria.

2. Materials and methods

Raw sewage sludge from a municipal wastewater treatment plant in a city with a population of more than 30,000 residents was used for the study. The plant utilizes mechanical and biological processes of wastewater treatment. For the examinations, sewage sludge was sampled after dewatering on a press and used to prepare homogeneous samples. Three test series were performed. The samples were immediately transported to the laboratory and subjected to microbiological analysis. The examinations were performed in triplicate, and the results are presented as means of these replicates. The first stage of microbiological analysis used raw sewage sludge.

Samples for microbiological analysis were prepared by weighing 1 g of sewage sludge and diluted in 9 mL of saline solution. The initial dilution was 10^{-1}. Several decimal dilutions were then performed to obtain a dilution of 10^{-8}. Another stage was to sow 0.1 mL of the above sewage sludge suspensions onto two microbiological plates in triplicate. This was nutrient agar for determining the total bacteria count with lower and medium nutrient requirements, and ENDO agar, used to evaluate bacteria that were supposed to belong to the Enterobacteriaceae family. With selective additives, other bacteria grow much slower or do not develop on this agar (with the exception of Gram-negative non-fermenting bacilli). McConkey's or Levin's agars are most commonly used to culture intestinal bacteria from clinical samples [5], but the authors chose ENDO agar because it is slightly more selective against the Enterobacteriaceae family.

Table 1
Example composition of lime-treated sewage sludge (contents of individual elements expressed as dry matter; in order: nitrogen – total amount, phosphorus, calium, calcium and organic matter) [11]

N-total	3.3%
P_2O_5	3.5%
K_2O	0.3%
CaO	22.3%
MgO	0.6%
Organic matter	43%

After sowing the series of dilutions on these two types of agars, the plates were incubated at the temperature of 37°C for 24 h. Pure Enterobacteriaceae strains were isolated only from ENDO agar at higher sample dilutions: this allows easy collection of single colonies. The next step was streaking (three times), which yielded pure cultures of the tested bacteria.

Preliminary identification included:

- Gram staining,
- Motility test,
- Hugh-Leifson oxidative/fermentation glucose test,
- Cytochrome oxidase test.

Two last trials allowed for the preliminary identification of Gram-negative bacilli other than those from the Enterobacteriaceae family.

Accurate bacterial species identification was performed using Microgen GNA + B biochemical multi-test. These tests are considered as one of the most reliable and have been routinely used for bacteriological diagnostics in microbiological laboratories [12]. Each test consists of two plastic strips with wells (12 wells each) containing dried substrates and indicators. A suspension of the tested bacteria in sterile saline was introduced into these wells. After filling the wells with the bacteria suspension in saline, multi-tests were incubated for 24 h at a temperature of 37°C. The results, in the form of eight-digit codes, were input into Microgen MID 60 software and the species of microorganisms isolated were determined (Fig. 1).

The last stage of the examinations was to prepare antibiograms with the disc diffusion method on Mueller-Hinton II discs. The set of antibiotics was chosen based on the recommendations of the National Reference Center for Microbial Susceptibility [13]. The following substances were used: ampicillin (AM10), amoxicillin with clavulanic acid (co-amoxiclav) (AMC30), amikacin (AK30), gentamicin (CN10), cefuroxime (CXM30), cefazolin (CZ30), ceftazidime (CAZ30), and ciprofloxacin (CIP5). Chloramphenicol was not used due to toxicity for human. The susceptibility of the tested microorganisms to the above-mentioned antibiotics was read using the current indications published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [14].

After the above analysis, the raw sewage sludge was divided into three parts in each test series and calcium hydroxide was added. Three vessels were prepared, with the following concentrations of calcium hydroxide expressed as CaO: 0.1 g·CaO/kg·d.m., 0.2 kg·CaO/kg·d.m., and 0.4 kg·CaO/kg·d.m. of sewage sludge. After thorough homogenization, they were set aside in an airy place at ambient temperature. Samples from the lime-treated sludge from each vessel were taken at monthly intervals and quantitative microbiological analysis on ENDO and A.O. agars was performed each time. Full species identification was performed and antibiograms were prepared for bacteria isolated after the process. At the same time, pH was measured using an electronic pH meter, which made it possible to monitor changes in the pH of the lime-treated sludge. The first research series

continued for six months, while the second and third series continued for three months.

3. Results

The total bacterial count in the raw sewage sludge determined using the nutrient agar was on average 4.6×10^8 CFU in the first test series per 1 g of dewatered sewage sludge. Bacterial count for the bacteria grown on the ENDO agar, that is, those potentially from the Enterobacteriaceae, accounted for around 70% (3.6×10^8 CFU/g) of the total count of microorganisms. These values were converted to 1 g of raw sewage sludge used in the study. In the second series, it was 1.5×10^9 on nutrient agar and 4.5×10^8 on ENDO agar. In the third, it was 2×10^9 on nutrient agar and 4.9×10^8 on ENDO agar. The mean of the three series for the total count of bacteria grown on the nutrient agar was 1.32×10^9, while a count of 4.3×10^8 CFU/g was obtained for bacteria supposed to belong to the Enterobacteriaceae family that grew on ENDO agar. The liming process reduced the bacterial population by 6 to 8 orders of magnitude. For the lowest dose applied, the reduction was of 4 to 5 orders of magnitude.

The pH values ranged from 11 to 12 for the 0.2 and 0.4 kg doses of CaO and about 10 for the lowest lime dose. The results obtained after species identification of pure cultures sampled from ENDO agar and preparing antibiograms are shown in Tables 2–7. For each bacterial species, the results of susceptibility to the antimicrobial substances used in the study are shown as diameters of the growth inhibition zone around the antibiotic-soaked disc in mm (number in parentheses) according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (susceptibility symbols: S – susceptible, R – resistant, I – intermediate). Bold text means drug-resistant species.

4. Discussion

The methodology used in the study allowed for the isolation of the bacteria from the Enterobacteriaceae family from other microbial species present in sewage sludge. Similar to McConkey agar, the capability of lactose decomposition used in ENDO agar as a differentiating factor allows for differentiation between intestinal bacilli which are important from the clinical standpoint. Satisfactory reductions in total bacterial counts were obtained at lime doses of 0.2 and 0.4 kg expressed as kg·CaO/kg·d.m. of sludge. Similar results were obtained in a study by Marcinkowski [10], who reported a reduction in the total count of microorganisms of 7–8 orders of magnitude. Already in the early stages of the process in the first few days, the number of bacteria decreased very quickly as the pH rose rapidly. It seems that reaction values of 11–12 are optimal for conducting this process and allow for effective reduction in the amount of potentially hazardous bacteria in the material. However, this process destroys parasite eggs to a lesser extent. After just 24 h, such sludge meets the standards for bacterial contamination set out, for example, by the US Environmental Protection Agency (EPA) [15]. As shown in the present study,

Table 2
Antibiogram results for individual intestinal bacteria obtained in the first test series before liming

Isolated bacteria species	Diameter of the bacterial growth inhibition zone around the disc (mm)							
	Co-amoxiclav (AMC30)	Amikacin (AK30)	Gentamicin (CN10)	Cefazolin (CZ30)	Ciprofloxacin (CIP5)	Ceftazidime (CAZ30)	Ampicillin (AM10)	Cefuroxime (CXM30)
Citrobacter freundii	S (27)	S (25)	S (26)	S (22)	S (30)	S (25)	S (23)	S (26)
Escherichia coli inactive L+	**R (11)**	**S (22)**	**S (25)**	**S (25)**	**S (30)**	**S (25)**	**R**	**S (23)**
Morganella morganii	R (9)	S (25)	S (28)	R	S (26)	S (28)	I (14)	I (18)
Escherichia coli inactive L–	**S (20)**	**S (25)**	**S (26)**	**S (25)**	**S (30)**	**S (28)**	**R**	**S (29)**
Klebsiella pneumoniae	S (30)	S (32)	S (30)	S (28)	S (32)	S (30)	S (20)	S (26)
Enteric Group 68	R (15)	S (25)	S (28)	S (23)	S (30)	S (30)	R	S (28)
Yersinia aldovae	S (25)	S (30)	S (34)	S (27)	S (30)	S (30)	R	S (30)
Serratia marcescens biogp1	S (30)	S (30)	S (30)	S (25)	S (35)	S (35)	S (30)	S (30)

S – susceptible, R – resistant, I – intermediate

Table 3
Antibiogram results for individual intestinal bacteria obtained in the first test series after liming

Isolated bacteria species	Diameter of the bacterial growth inhibition zone around the disc (mm)							
	Co-amoxiclav (AMC30)	Amikacin (AK30)	Gentamicin (CN10)	Cefazolin (CZ30)	Ciprofloxacin (CIP5)	Ceftazidime (CAZ30)	Ampicillin (AM10)	Cefuroxime (CXM30)
Pseudomonas stutzeri	R (25)	S (28)	S (29)	R (16)	S (26)	S (25)	R (20)	R (13)
Alcaligenes faecalis	S (28)	S (26)	S (28)	R (20)	S (30)	S (30)	R (28)	S (25)
Pseudomonas stutzeri	R (20)	S (25)	S (25)	R (15)	I (22)	S (20)	R (15)	R
Providencia rettgeri	S (22)	S (22)	S (25)	S (25)	S (30)	S (28)	R (20)	S (25)

Table 4
Antibiogram results for individual intestinal bacteria obtained in the second test series before liming

Isolated bacteria species	Diameter of the bacterial growth inhibition zone around the disc (mm)							
	Co-amoxiclav (AMC30)	Amikacin (AK30)	Gentamicin (CN10)	Cefazolin (CZ30)	Ciprofloxacin (CIP5)	Ceftazidime (CAZ30)	Ampicillin (AM10)	Cefuroxime (CXM30)
Serratia rubidaea	S (25)	S (32)	S (33)	S (35)	S (40)	S (35)	I (15)	S (30)
Klebsiella pneumoniae	S (26)	S (30)	S (35)	S (35)	S (39)	S (35)	S (29)	S (34)
Klebsiella pneumoniae	S (28)	S (31)	S (35)	S (38)	S (42)	S (45)	S (22)	S (35)
Klebsiella pneumoniae	S (22)	S (25)	S (30)	S (30)	S (33)	S (30)	S (21)	S (30)
Burkholderia pseudomallei	R (10)	S (30)	S (30)	S (26)	S (32)	S (35)	R	S (30)
Klebsiella pneumoniae	S (27)	S (25)	S (25)	S (30)	S (35)	S (35)	R (12)	S (30)
Pseudomonas fluorescens	R	S (35)	S (32)	S (25)	S (30)	S (32)	S (28)	S (35)
Yersinia enterocolitica	**R (16)**	**S (30)**	**R**	**S (28)**	**S (26)**	**R (15)**	**R**	**S (24)**
Klebsiella pneumoniae	S (18)	S (30)	S (30)	S (32)	S (25)	S (38)	S (32)	S (35)
Escherichia coli inactive L+	**R**	**S (30)**	**S (32)**	**S (28)**	**S (30)**	**S (35)**	**R**	**S (33)**

Table 5
Antibiogram results for individual intestinal bacteria obtained in the second test series after liming

Isolated bacteria species	Diameter of the bacterial growth inhibition zone around the disc (mm)							
	Co-amoxiclav (AMC30)	Amikacin (AK30)	Gentamicin (CN10)	Cefazolin (CZ30)	Ciprofloxacin (CIP5)	Ceftazidime (CAZ30)	Ampicillin (AM10)	Cefuroxime (CXM30)
Alcaligenes faecalis t11	S (23)	S (22)	S (25)	R (14)	S (22)	S (16)	S (18)	R (13)
Providencia stuartii	**R (8)**	**S (18)**	**S (22)**	**R (13)**	**S (28)**	**R**	**R**	**R (11)**
Alcaligenes faecalis	I (17)	S (25)	S (25)	R (15)	S (25)	R (10)	R	R (16)
Proteus vulgaris	**S (23)**	**S (22)**	**S (25)**	**R**	**S (20)**	**R (15)**	**R**	**R (17)**

Table 6
Antibiogram results for individual intestinal bacteria obtained in the third test series before liming

Isolated bacteria species	Diameter of the bacterial growth inhibition zone around the disc (mm)							
	Co-amoxiclav (AMC30)	Amikacin (AK30)	Gentamicin (CN10)	Cefazolin (CZ30)	Ciprofloxacin (CIP5)	Ceftazidime (CAZ30)	Ampicillin (AM10)	Cefuroxime (CXM30)
Escherichia coli	**R (8)**	**S(25)**	**S (24)**	**S(30)**	**S (20)**	**S (27)**	**R**	**S (25)**
Escherichia coli	S (22)	S (26)	S (25)	S (27)	S (40)	S (30)	S (25)	S (28)
Escherichia coli	S (20)	S (24)	S (25)	S (28)	S (40)	S (30)	S (20)	S (25)
Pseudomonas fluorescens	S (28)	S (25)	S (30)	S (20)	S (27)	S (23)	S (26)	S (12)
Morganella morganii	R (10)	S (26)	S (30)	S (25)	S (28)	S (28)	R	S (23)
Pseudomonas fluorescens	S (27)	S (30)	S (33)	S (20)	S (36)	S (27)	S (28)	S (14)
Pseudomonas fluorescens	S (25)	S (26)	S (32)	S (28)	S (35)	S (20)	S (24)	S (15)
Alcaligenes faecalis t11	S (25)	S (28)	R (30)	S (26)	S (36)	S (27)	S (26)	S (16)
Alcaligenes faecalis t11	S (26)	S (30)	S (32)	S (25)	S (35)	S (25)	S (30)	S (15)
Escherichia coli	S (17)	S (24)	S (22)	S (21)	S (33)	S (23)	S (21)	S (25)

a process pH of about 10 is too low for effective hygienization. There are two major limitation of liming sewage sludge: high cost when it used at large scale and necessity of exact mixing sewage sludge with lime. Lack of exact mixing may significantly decrease effectiveness of this process.

E. coli, which is common in humans, was present in the sewage sludge samples tested, represented by three strains. One of them, present on the ENDO agar, showed a characteristic growth attributable to lactose fermentation. The other two did not have abilities to decompose lactose. Both these strains of this species showed lower biochemical activity compared to the species typical of *E. coli*. Therefore, they were classified based on the computer database of the manufacturer of the biochemical multi-tests to the group

Table 7
Antibiogram results for individual intestinal bacteria obtained in the third test series after liming

Isolated bacteria species	Diameter of the bacterial growth inhibition zone around the disc (mm)							
	Co-amoxiclav (AMC30)	Amikacin (AK30)	Gentamicin (CN10)	Cefazolin (CZ30)	Ciprofloxacin (CIP5)	Ceftazidime (CAZ30)	Ampicillin (AM10)	Cefuroxime (CXM30)
Pseudomonas fluorescens	S (27)	S (35)	S (36)	S (15)	S (35)	S (27)	S (17)	S (18)
Morganella morganii	**R**	**S (35)**	**S (35)**	**R**	**S (35)**	**S (30)**	**R**	**S (32)**
Acinetobacter lwoffi	S (30)	S (34)	S (35)	R (30)	S (35)	S (32)	S (28)	S (30)
Pseudomonas fluorescens	S (30)	S (30)	S (33)	R (32)	S (34)	R	S (30)	S (22)
Serratia odorifera biogp 2	**R (15)**	**S (26)**	**S (29)**	**S (18)**	**S (30)**	**S (25)**	**S (22)**	**S (20)**

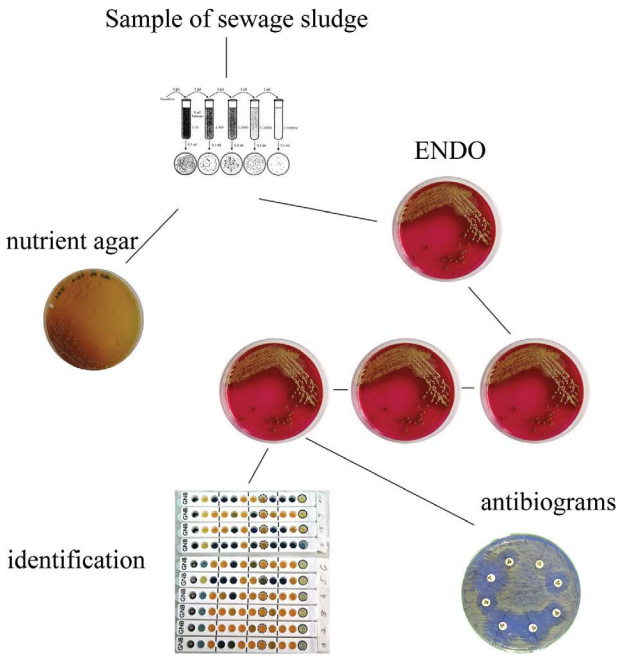

Fig. 1. Diagram of the microbiological examinations conducted in the present study.

of inactive *E. coli* bacilli. Before the use of molecular methodologies in bacteriological practice, the strains of this species without abilities to decompose lactose with reduced general biochemical activity had been sometimes mistakenly classified as *Shigella* species, very similar to the *E. coli* family [5].

Of the eight intestinal bacteria isolated from raw sewage sludge, drug-resistance was found for two strains of *E. coli* and *Yersinia enterocolitica*. *E. coli* inactive L^+ was resistant to ampicillin and to combination of amoxicillin and clavulanic acid, which acted as a β-lactamase inhibitor. Although the resistance to ampicillin is relatively widespread among the intestinal bacilli, it occurs much more rarely in the case of amoxicillin with clavulanic acid. *E. coli* inactive L^- showed resistance only to ampicillin, whereas it was susceptible to co-amoxiclav. However, the wild strains of this species are susceptible to these two drugs in clinical concentrations. In the case of other antibiotics, both strains of *E. coli* showed the standard susceptibility [2]. *Yersinia enterocolitica*, which was characterized by resistance to ceftazidime, gentamicin, and co-amoxiclav, was also isolated from raw sludge in the second test series.

Other intestinal bacteria isolated from sewage sludge were *Klebsiella pneumoniae*, *Citrobacter freundii*, *Morganella morganii*, *Yersinia aldovae*, *Serratia marcescens* biogp1 and a microorganism that has not been named yet, numbered among the Enterobacteriaceae family (Enteric Group 68). *M. morganii* was previously classified as *Proteus morganii*, and more recent studies isolated this species from the *Proteus* genus. *Serratia marcescens* biogp1 did not show the capability of forming the red pigment, typical of the *Serratia marcescens*. Many years ago, it was classified as the *Yersinia enterocolitica biogp 3A*. These types of changes in current classification were possible due to the application of, for example, marker gene analysis or fatty acid profile usage [16].

All the above microorganisms belong to the group of opportunistic bacteria, which, in favorable conditions, can lead to diseases in different body parts, for example, urinary system, respiratory system, soft tissue, joints, etc., including septicaemia. Especially dangerous is pneumonia caused by *Klebsiella pneumoniae*, combined with the local necrosis of the lungs. Infections caused by such microorganisms are more common in hospital environments. The most susceptible people are patients with reduced immune system efficiency, including those after transplantations, older adults, people with AIDS, or with large burn wounds [2].

Apart from drug resistance with respect to the antibiotics from the penicillin group, it is worrying that both *E. coli* belong to the inactive strains. In light of many analyses, most of them are enteroinvasive *Escherichia coli* (EIEC), which cause diarrhea with different intensities, in both adults and children. In farm animals such as pigs, they are often found as an etiological factor in dangerous diarrheas. In order to verify whether the *E. coli* isolated from the sewage sludge belongs to the EIEC group, serological tests should be performed or the PCR tests should be performed to demonstrate the presence of the *ipaH* gene [17,18]. This gene is found in closely-related and entirely pathogenic bacilli from the *Shigella* genus and it represents a good diagnostic marker in the case of diarrheas caused by these bacteria [19]. Due to the presence of a hospital and health centers in the area where municipal wastewater is collected to the wastewater treatment plant where the sewage sludge was collected for the study, it can be concluded that these health institutions can be the sources of two isolated *E. coli* inactive strains.

A study published by Stańczyk-Mazanek et al. [4] found that intestinal bacteria can survive in sewage sludge and then in soil for a relatively long time. After six months of soil fertilization with sewage sludge containing these bacteria to the soil, relatively hazardous species such as *E. coli*, *Enterobacter kobei*, *Citrobacter freundii* and *Klebsiella oxytoca* can be found in the soil. In some cases, the period where the presence of these bacteria can be found in soil is several years. Therefore, if the drug-resistant forms are present in sewage sludge, the use of such sludge can involve a significant risk of the spread of drug resistance in the environment [4].

In particular, health centers can be a source of drug-resistant microorganisms, as antibacterial substances are used there in high amounts. During one of the analyses of sewage sludge from the wastewater treatment plant coming from the Hsin-Chy General Hospital, Taiwan, in 2005, intestinal bacteria of *E. coli*, *Klebsiella* or *Citrobacter* species were found. The percentage of drug-resistant strains in the general count of these bacteria was 94% for ampicillin, 71% for cefazolin, and below 10% for imipenem. Interestingly, the total number of drug-resistant strains of intestinal bacteria present in sewage sludge from this hospital was higher than in the hospital among the strains isolated from patients. This demonstrates favorable conditions for the selection of resistant strains in raw wastewater [20].

In the case of the bacteria from the *E. coli* species, an increased number of drug-resistant species is observed in the sludge from health centers, especially the bigger ones. Sooner or later, long-term exposure to numerous antibiotics stimulates the selection of less susceptible cells. The capability of fast decomposition of a large amount of β-lactam antibiotics such as penicillin and cephalosporins is more and more often found among intestinal bacteria. This is caused by the formation of enzymes from the group of extended-spectrum β-lactamases (ESBL). In a study on intestinal bacteria in sewage sludge from wastewater treatment plants in Austria, the researchers found the *E. coli* species which formed these enzymes in 44 of 72 samples. Such results are of great concern from a public health perspective. The subsequent treatment of sewage sludge substantially decreased the total number of intestinal bacteria present in the sludge, including the drug-resistant forms. The highest efficiency was found for liming and thermal treatment [21].

5. Conclusions

- The liming process carried out using doses of 0.2 and 0.4 kg expressed in kg·CaO/kg·d.m. of sewage sludge destroyed almost all intestinal bacteria and other microorganisms present in the sludge. A reduction of 6–8 orders of magnitude was achieved.
- The lime dose of 0.1 kg expressed in CaO/kg·d.m. of sewage sludge proved insufficient for effective hygienization.
- In the first test series, only one species of intestinal bacteria (*Providencia rettgeri*) survived 6 months after sludge liming and showed no drug resistance.
- In the second test series, *Providencia stuartii* and *Proteus vulgaris* survived among the intestinal bacteria after 3 months of sludge liming. These species have proven to be drug-resistant.
- In the third test series, *M. morganii* and *Serratia odorifera biogp 2* intestinal bacteria survived after 3 months of liming. These species have proven to be drug-resistant.
- Very low abundance of all bacteria in limed sludge at doses of 0.2 and 0.4 kg·CaO/kg·d.m. persisted until the third month of the liming process.
- drug-resistant strains of intestinal bacteria were mostly less susceptible to older antibiotics, including cefazolin, ampicillin, and co-amoxiclav.
- Resistance of several isolated strains of intestinal bacteria to more recent antibiotics, including ceftazidime and ciprofloxacin, may be of concern.
- After ca. 3 months of the process, there was a significant increase in the count of saprophytic bacteria.
- It was shown that with decreasing pH, intestinal bacteria were replaced by typical saprophytic bacteria commonly found in the environment such as *Pseudomonas* sp. and *Alcaligenes* sp.

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