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THE EFFECT OF SEPARATION TECHNOLOGY ON ANIMAL MANURE ON ANTIBIOTIC RESISTANT BACTERIA AND HORMONAL ACTIVITY

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List of abbreviations

AK	– Amikacin
AR	– Antibiotic resistance
ARB	– Antibiotic resistance bacteria
ARGs	– Antibiotic resistance genes
BRU	– Bedding recovery units
CAZ	– Ceftazidime
CIP	– Ciprofloxacin
CN	– Gentamicin
COL	– Colistin
CRE	– Carbapenem
CS	– Colistin
DHT	– 5a-dihydrotesterone
EC20	– 20% effective concentration
EC50	– 50% effective concentration
ED	– Endocrine disruptor
EMB	– Eosine-Methylene Blue
FEP	– Cefepime
GPA	– Growth promoting antimicrobial
HGT	– Horizontal gene transfer
IC20	– 20% Inhibitory concentration
IMI	– Imipenem
LB	– Luria-Bertani
MCR	– Mobilized colistin resistance
MDR	– Multidrug resistant
MGE	– Mobile genetic elements
MIC	– Minimal inhibitory concentrations
MRP	– Meropenem
OD	– Optical density
PCR	– Polymerase chain reaction
RPM	– Round per minute
SXT	– Trimethoprim sulfamethoxazole
TEQ	– Toxic equivalency quotients
TZP	– Piperacillin tazobactam
WHO	– World Health Organization
YPD	– Yeast Extract-Peptone-Dextrose

ABSTRACT

Composting is considered as one of the possible methods for the management of organic waste such as sewage sludge, or manure due to its capability to reduce the number of microorganisms that are present in the waste products. One of the available systems, that has been introduced as Bedding Recovery Unit (BRU), claimed to have ability to eliminate the presence of pathogenic microorganisms during the intensive composting process. The treated waste products will be used as bedding inside the farm. As this method is environmentally friendly and increase the well-being of animals, it fits in with the circular economy concept and with the animal welfare. However, the activity of the system in the reduction of antibiotic resistant bacteria (ARB), antibiotic resistance genes (ARGs), or hormonal (ED) activity has not been investigated in details. In this study, we collected manure samples from two different dairy farms located in Hungary, prior and after BRU treatment, to evaluate the effectiveness of this system in the removal of AR bacteria (with a special focus on carbapenem and colistin resistant microorganisms), ARGs and ED effect. Samples were analysed in the laboratory of the Institute of Aquaculture and Environmental Safety with traditional cultivation and molecular biological methods; ED effect was determined using a *Saccharomyces* bioreporter assay.

Based on our results, the colistin, carbapenem resistant microorganisms were fully removed in one of the BRU-treated samples where the BRU system was operating properly, however the hormonal activity after BRU treatment trended to be higher. In acetone extracted form, all raw and treated samples shown notable estrogenic activity with the maximum intensification between 374-659%, (where the estrogenic activity of the positive control E2 was measured to be 1175%). In case of BRU-treated samples (extracted with acetone), the androgenic activity was also significant with a maximum intensification of 120 and 101%, respectively (where the positive control DHT's intensification was 269%). At the same time, water extracts of the examined samples did not show significant hormonal activity. Samples with hormonal activity showed as notable difference in the composition of cultivable bacteria with dominance of *Comamonas* species, such as *C. testosteronii* and *C. kerstersii*. Further investigation should be proposed to identify whether the hormonal activity is originating from naturally produced hormones or influenced by the endocrine disruptors (ED). Based on antibiotic resistance profiling, one of the detected species originating from raw manure, *Empedobacter stercoris* was resistant to the examined Aminoglycosides (gentamicin, amikacin) and to colistin, and at the same time, it was only moderately sensitive to imipenem, a Carbapenem agent, which verifies the presence of ARBs in untreated manure and highlight the importance of manure treatment.

1. INTRODUCTION

Agricultural sector contributes with massive profits to every country's economy worldwide. However, the growing demand for livestock breeding and animal-derived products increase the production of livestock manure (Lima et al., 2020). Scientific literature proved that the application of cow manure can effectively improve soil structure and diversity of soil bacterial community (Zhang et al., 2020). However, the extensive use of manure might cause enormous microbial growth and encourage microbes to undergo selective pressure to the environment, resulting the emergence of antimicrobial resistance bacteria (Lima et al., 2020).

The occurrence of antibiotic resistance bacteria is not a new problem to the agriculture sector but it became one of the biggest challenges to this industry since ARBs are threatening the success of antibiotic treatment and may cause adverse effects to the human health. Antibiotic resistance is a condition where bacterial strains are no longer affected by the antibiotic used. Bacteria acquire resistance to antibiotics by horizontal gene transfer (HGT) or genetic mutation due to the selective pressure of antibiotics (Ruuskanen et al., 2016). If these problems left untreated, it may cause the health treatment become more limited and jeopardized. Hence, it is important to monitor antibiotic resistance in the agriculture and environment as well for better control of ARB.

Several methods have been conducted for better management of the animal wastes that may contain ARB and ARGs. One of the possible ways for manure, sewage and sludge management is composting (Marti et al., 2014) as it can prevent the contamination of the environment and reduce the concentration and the risks of antibiotics (Dolliver et al., 2008).
One of the available composting options to control the massive organic waste production by dairy cow farms is the application of Bedding Recovery Unit (BRU) treatment, which is claimed to have the ability to eliminate the presence of pathogenic microorganisms during the intensive composting process.

BRU was examined that was introduced specifically for the management of liquid animal manure. This system uses hydraulic pressure separate liquid and solid wastes: liquid manure from this treatment will be supplied for irrigation of plantation while solid manures are used as bedding inside the farm. The treated waste products will be used as bedding inside the farm as it is environmentally friendly, better for animal well-being, cost-effective. It also enables the reduction of synthetic materials' use such as plastics, straws or rubber mattress that may cause pollution (Gautam et al., 2020, Norring et al., 2008). The ability of BRU treatment to reduce the pathogenic microorganisms also might give another solution for better

management of antibiotic resistant bacteria that otherwise easily proliferate and rapidly evolve inside animal manures.

However, the application of animal composts to the land may have adverse effects and increase the hormonal activity in soils and waters due to the emergence of endocrine-disrupting compounds (EDCs) (Abdellah et al., 2020). The existence of these compounds became a concern as they can mimic the naturally occurring hormones and cause adverse effects to the environment. The application of manure to the farmland is considered as the primary sources of estrogen (Abdellah et al., 2020) and even low levels of estrogen in the water body can affect the hormonal metabolism of aquatic wildlife (Xu et al., 2018). In case of novel systems, such as BRU, the detailed investigation to prove the reduction of antibiotic resistant bacteria (ARB), antibiotic resistance genes (ARGs), or in the reduction of hormonal (ED) activity has not been fully conducted under local circumstances in Hungary.

The purpose of this study was to evaluate the effectiveness of BRU system in the removal of AR bacteria, ARGs and ED effect using the manure samples collected from two different dairy farms located in Hungary, prior and after BRU treatments. The analysis of the samples took place in the laboratory of the Institute of Aquaculture and Environmental Safety with traditional cultivation and molecular biological methods; *Saccharomyces* bioreporter assays were used in this study to determine ED effects.

2. LITERATURE REVIEW

2.1. Antibiotics and antibiotic resistance

2.1.1. Antibiotics in general and the history of antibiotic use

Antibiotics are powerful agents that are used to combat microbial infections with specific modes of action depending on the type of antibiotics used. Bacteriostatic substances have the ability to restrain the bacterial growth, while bactericide agents can kill the infectious germs (Bernatova et al.,2013). Depending on the type of microorganisms they act against, antimicrobials can be antibacterial, antifungal, antiviral and antiparasitic agents (WHO, 2017). For instance, antibacterial drugs are applied against bacterial infections while antifungals are used against fungi. The usage of the antimicrobials must specifically depend on the type of microorganisms as the action of the antimicrobials will not be effective if they are wrongly applied. For example, antibiotics are not effective against fungal, parasite or viral infections and same applied to others. Taking antibiotics while having viral infection will give no effect and can contribute to the emergence of bacterial resistance.

Antibiotics were first discovered in 1929 by Alexander Fleming who accidentally observed that mould supressed the growth of the *Staphylococcus* variants on the culture plates. The mould was then identified as *Penicillium notatum* and named the substance as Penicillin. The discovery of antibiotics benefits the medical sectors and revolutionized in many medicinal aspects. In 1940, the discovery of bacterial penicillinase led to the finding of specific resistance mechanisms to penicillin (Davies & Davies, 2010) and unfortunately, their evolution was followed by a rapid emergence of other antibiotic resistance mechanisms. Until our days, the most serious side effect of antibiotic usage is still antibiotic resistance, causing the bacteria to undergo adaptation to the presence of antibiotics. This naturally occurring phenomenon results in the rise of the novel strains that are resistance towards the present antibiotic regimen (Habboush et.al, 2022).

Antibiotics have different modes of action, for instance to interrupt the genomic structure, or single-carbon metabolism pathways, invade bacteria ribosomes, or inhibit the synthesis of cell wall and the coherent lipid membrane (Crofts et al., 2017). The timelines of antibiotic discoveries and their mechanism of action are summarized in Figure 1 (Madhav et al., 2020).

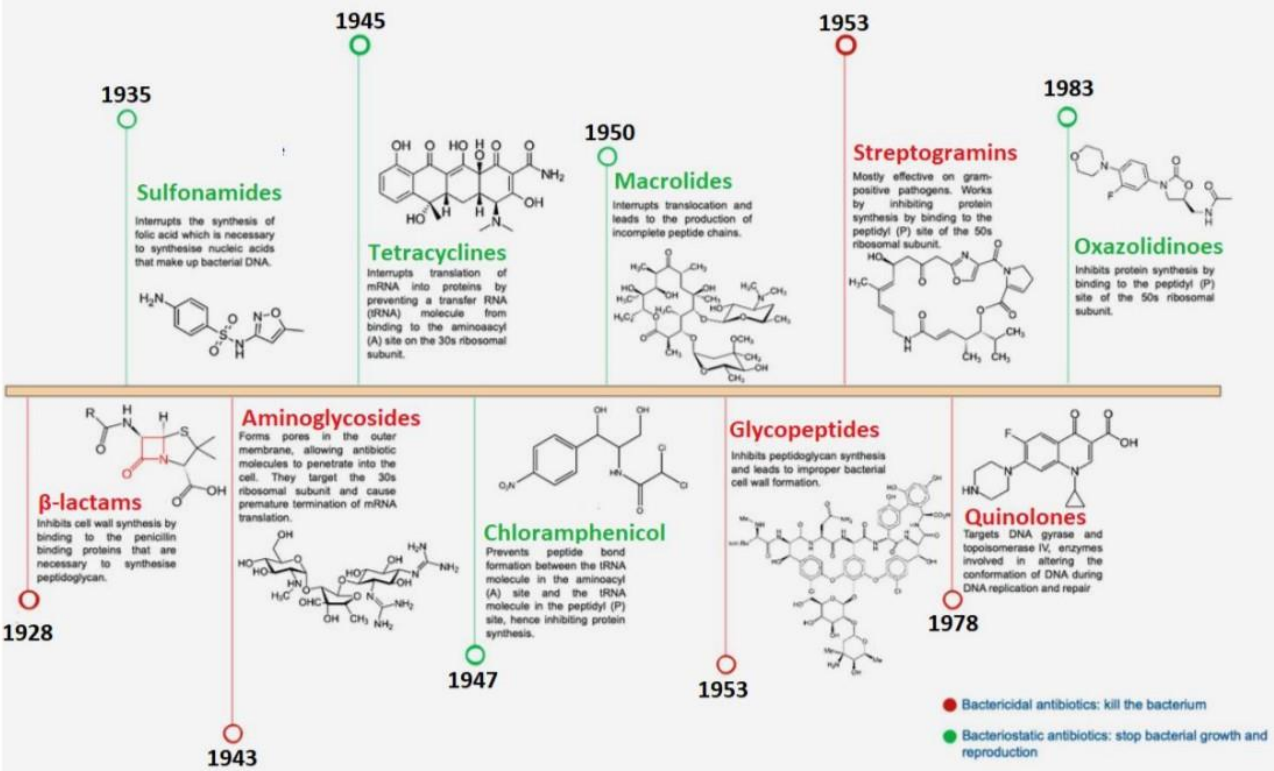

Figure 1. Timeline of antibiotic discoveries and their mechanism of action (Madhav et al., 2020)

2.1.2. Antibiotic resistance

According to the National Foundation for Infectious Diseases, antibiotic resistance (AR) is the condition where the antibiotics can no longer fight off or kill the bacteria causing the infection. Antibiotic resistance is a naturally occurring phenomenon, where vulnerable bacteria become unaffected by the usage of the antibiotics due to mutations, or with acquiring antibiotic resistance genes (ARGs) from other bacteria through the process called horizontal gene transfer (HGT) (Ruuskanen et. al., 2016). Antibiotic resistance inherent from bacterial species is generally referred as ‘intrinsic’ or ‘natural’ resistance and typical for strains of that species, while ‘acquired resistance’ is referring to bacterial strains that belongs to a typically susceptible species, but become resistant to the applied antimicrobial drug (FEEDAP, 2012).

Most of the time, bacterial resistance is a result of the overuse of the antibiotics or the unnecessary intake of the medicine. For example, in the year of 2015, the records showed that about 30% outpatient and 50% of the patients suffered with acute respiratory infections were prescribed with unnecessary antibiotic consumption (Habboush & Guzman, 2022). As a consequence, the treatment of bacterial infection becomes more difficult: the concentration of the antibiotic used for treatment needs to be increased or the agent should be changed to a more effective drug. Over the years, a significant ratio of the bacterial strains (known as superbugs)

became resistant to antibiotics as they undergo this adaptation and evolve (Pepper et al., 2018). These bacteria continue to multiply and trigger infections to the hosts despite the application of the arsenal of available antibiotics.

There are several mechanisms of how bacteria acquire resistance such as increasing the activity of efflux pumps that restrict the accumulation, reducing antibiotic uptake by pumping it out of the cell, enzymatic inactivation by degrading the antibiotic compounds or modified enzymatic reaction, establishing impermeable blockades to prevent the intake of antibiotics into the bacterial cells and altering the cellular structure (Crofts et al., 2017). The main mechanisms of antibiotic actions and the resistance mechanisms are summarized in Figure 2 (Uluseker et al., 2021).

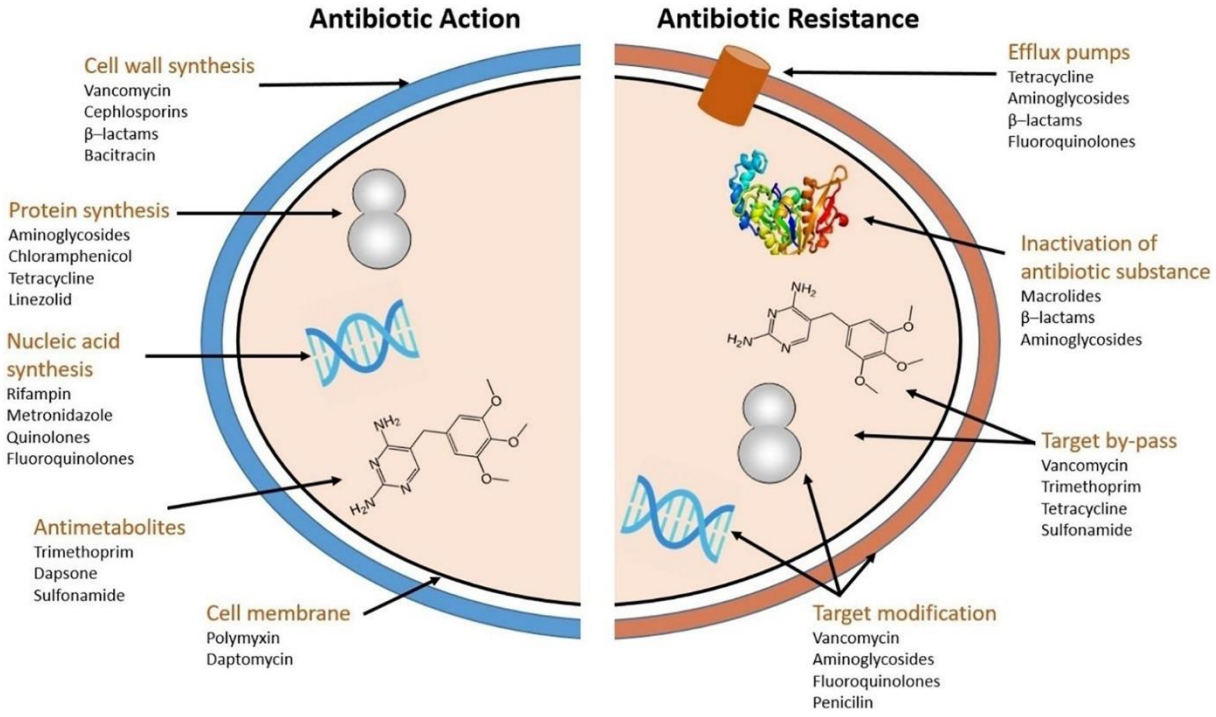

Figure 2 The main mechanisms of antibiotic actions and the resistance mechanisms (Uluseker et al.2021)

The emergence of bacterial resistance required international agreement and standardized terminologies to divide the major types of bacterial resistance for better healthcare strategies. According to the scientific literature, the definition of multi-drug resistant (MDR) is resistant to more than one antimicrobial agent meanwhile extensive drug resistant or XDR means the ability of the bacteria to not only resistance to multiple type of antimicrobial agents but have possibility to become resistant to almost all or all of the approved antimicrobial agents. Pandrug resistant (PDR) came from Greek prefix ‘pan’ refers to the meaning ‘all’, giving the definitions of resistant to entire set of available antimicrobial agents. For bacterial isolates to be categorized

as PDR, it must be tested and proved to be resistant to all approved antimicrobial agents (Magiorakos et al., 2012).

Typical examples of bacterial species that can gain multiple resistance to antibiotics are *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *A. baumannii* possesses unusual plasticity that permits the bacteria to undergo adaptation to adverse living environment, making it one of the most important causative agents of nosocomial infections. Some *A. baumannii* strains are considered as multiple drug resistant (MDR) due their capability to resist to beta-lactams (penicillin, cephalosporins, carbapenems) and monobactams (Novovic et al., 2023, Ramadan et al., 2018). *Pseudomonas aeruginosa* have intrinsic, acquired and adaptive resistance mechanisms that enable this opportunistic clinical pathogen to become simultaneously resistant to different antibiotics such as carbapenems (Ramadan et al., 2018).

2.2. Antibiotics in the agriculture

Antibiotics are commonly used worldwide in agriculture sector such as in plantation, aquaculture farm, poultry and livestock production. Antibiotics are applied to the crop yields to increase productivity and function as growth promoters for livestock animals as well as prevention and treatment of diseases (Mann, A. et al., 2021).

2.2.1. Antibiotic usage in animal husbandry

Livestock production is one of the most demanding sectors worldwide as the production not only need to be parallel to the growing number of human population but also influenced by the competition for natural resources which increase the concerns on human wellbeing and the surrounding environment (Lima et al., 2020). As livestock production is believed to be risen in the upcoming years, the breeding methods, nutrition intake and supervision of animal health should be continuously improved to keep its emissions at a socially tolerable level (Thornton, 2010).

Growth promoting antimicrobials (GPA) were widely used in agricultural sector for decades to treat livestock animals. GPA application means that small doses of antibiotics were introduced to animal feed to improve the health of the animals, e.g., veterinary antibiotics were used to improve the meat quality in huge proportions (You & Silbergeld, 2014). Antibiotics also have been used in veterinary medicines for therapeutic procedure to treat the animals with health problems. The treatment was initiated to control or prevent disease metaphylactic or prophylactic purpose (Mann et al., 2021). Metaphylaxis is the application of the medical

products to the animals after clinical disease diagnosis has been applied with the purpose to treat unhealthy animals and prevent the disease from spreading to other animals that are in close contact and risked to be infected by the disease. Prophylaxis is the preventive procedure by applying medical products to the animals before appearance of sign of disease to prevent the occurrence of infection or diseases (Tang et al., 2017). Based on the scientific review, the widely use antibiotics in animal introduction are penicillin, cephalosporins, sulfonamides, quinolones and tetracyclines (Robles-Jimenez et al., 2021). The categorization of veterinary antibiotics per family for food-producing animals are shown in Table 1 (Robles-Jimenez et al., 2022).

Table 1 The categorization of veterinary antibiotics for food-producing animals (Robles-Jimenez et al., 2022)

Antibiotics									
Penicillins 87.1%	**Tetracyclines 87.1%**	**Aminoglycosides 77.1%**	**Macrolides 77.1%**	**Sulfonamides 70%**	**Quinolones 68.6%**	**Polypeptides 64.3%**	**Cephalosporins 58.6%**	**Phenicols 51.4%**	**Lincosamides 51.4%**
Natural Penicillins Benzylpenicillin Penethamate hydroxide Penicillin procaine *Amdinopenicillins* Mecillinam *Aminopenicillins* Amoxicillin Ampicillin Hetacillin *Aminopenicillin plus Betalactamase inhibitor* Amoxicillin_Clavulanic Acid *Carboxypenicillins* Ticarcillin Tobicillin *Ureido Penicillin* Aspoxicillin *Phenoxypenicillins* Phenoxymethylpenicillin Phenethicillin *Antistaphylococcal Penicillins* Cloxacillin Dicloxacillin Nafcillin Oxacillin	Chlortetracycline Doxycycline Oxytetracycline Tetracycline	*Aminocyclitol* Spectinomycin *Aminoglycosides* Streptomycin Dihydrostreptomycin Framycetin Kanamycin Neomycin Paromomycin Apramycin Gentamicin Tobramycin Amikacin	*Azalide* Tulathromycin *Macrolides C14* Erythromycin *Macrolides C16* Josamycin Kitasamycin Spiramycin Tilmicosin Tylosin Mirosamycin Terdecamycin	Sulfachlorpyridazine Sulfadiazine Sulfadimerazin Sulfadimethoxine Sulfadimidine Sulfadoxine Sulfafurazole Sulfaguanidine Sulfamethazine Sulfadimethoxazole Sulfamethoxine Sulfamonomethoxine Sulfanilamide Sulfaquinoxaline *Sulfonamides and Diaminopyrimidines* Sulfamethoxypyridazine Trimethoprim+ Sulfonamide *Diaminopyrimidines* Baquiloprim Trimethoprim	*Quinolones 1G* Flumequin Miloxacin Nalidixic acid Oxolinic acid *Quinolones 2G (Fluoroquinolones)* Ciprofloxacin Danofloxacin Difloxacin Enrofloxacin Marbofloxacin Norfloxacin Ofloxacin Orbifloxacin	Enramycin Gramicidin Bacitracin *Polypeptides cyclic* Colistin Polymixin	*Cephalosporin 1G* Cefacetrile Cefalexin Cefalotin Cefapyrin Cefazolin Cefalonium *Cephalosporin 2G* Cefuroxime *Cephalosporin 3G* Cefoperazone Ceftiofur Ceftriaxone *Cephalosporin 4G* Cefquinome	Florphenicol Thiamphenicol	Pirlimycin Lincomycin
Pleuromutilins 48.6%	**Ionophores 42.9%**	**Novobiocin 31.4%**	**Ansamycin-Rifamycins 30%**	**Fosfomycin 7.1%**	**Streptogramins 5.7%**	**Quinoxalines 4.3%**	**Orthosomycins 4.3%**	**Fusidic Acid 1.4%**	**Bicyclomycin 1.4%**
Tiamulin Valnemulin	Lasalocid Maduramycin Monensin Narasin Salinomycin Semduramicin	Novobiocin	Rifampicin Rifaximin	Fosfomycin	Virginiamycin	Fusidic acid	Lasalocid Maduramycin Monensin Narasin Salinomycin Semduramicin	Fusidic acid	Bicozamycin

Adapted from OIE, List of antimicrobials of veterinary importance [27].

The demand of the livestock production may contribute to the increase of antibiotic resistance due to the selection pressure occurred to the gut microbiome through the animal feeds (You & Silbergeld, 2014). Therefore, researchers agreed that the usage of antibiotic in animal husbandry should be supervised and more controlled (CDC, 2013). On 28th January 2022, (EU) 2019/6 on Veterinary Medicines together with (EU) 2019/4 on Medicated Feeds regulated new order to phase out the practices of antibiotics use for prophylactic purpose and saved these diseases prevention procedures for exceptional conditions (Simjee & Ippolito, 2022). Antimicrobial medicines are restricted to be applied routinely and shall only be used for control (metaphylaxis purpose) when rise the risk of infection might happen and there is no availability of other alternatives (Simjee & Ippolito, 2022).

2.1.1. Antibiotics and Antibiotic Resistance Genes (ARGs) in manures

The massive production of livestock products, such as eggs, meat, milk, and other agricultural goods, left a lot of side products and waste to be taken care of such as the feed residues, waste products, wash water, manure and bedding materials (e.g., sawdust, wheat straw, wood chips, flax straw or even some use rice hulls and peanut) (Shober & Maguire, 2014). Between 2016 and 2019, more than 1.4 billion tonnes of manure were produced annually in the European Union (EU-27) and in the UK (Königer et al., 2021).

The excretion of animal faeces will release a part of the gut microbiota, the microbial community that inhabiting the animal gastrointestinal tract, therefore, gut microbes can appear in manure and the manure-containing waste products. According to Chee-Sanford et al. (2009), the gene pool of microbiome is larger in animal intestines than in human gut and contributes to a variety of antibiotic resistance determinants. Manure is commonly used in the agriculture as soil fertilizer, it may contribute to the occurrence of resistance determinants in the field crops (Ruuskanen et. al., 2016, You & Silbergeld, 2014) and have a greater effect on the diversity and the abundance of acquired resistance genes in soil than chemical fertilizers (Lima et al., 2020). Since antibiotic resistant bacteria (ARB) were found to be abundant without the history of antibiotic intake in composts, we can presume that the microbiome inhabits of the animal gastrointestinal tract might harbour intrinsic antibiotic resistant bacteria (You & Silbergeld, 2014).

The physical, chemical and microbial properties of manure can be dramatically different depending on external factors (Huang et al., 2011). For instance, the consumption of antibiotics by farm animals offers selective pressure for the antibiotic resistance bacteria to grow inside the intestinal tract of the animals and then being excreted out to the environment (Looft et al., 2012; Zhu et al., 2013). Moreover, other substances of manure such as potassium, nitrogen, phosphorus, sulphur and heavy metals such as copper, manganese, zinc, cobalt, selenium, cadmium, nickel and lead continuously give a selective pressure to ARGs through cross-resistance or co-resistance, as ARGs and metal resistance genes are frequently located together in plasmids or other mobile genetic elements (MGEs) (Yuan et al., 2020). Consequently, the usage of manure in the agricultural sector encourages the risk of spread of pathogen and/or antibiotic resistant microorganisms to the environment (Wang et al.,2020).

2.2. Effect of antibiotic resistance on the environment

The extensive antibiotic use in the agriculture causes the establishment antibiotic resistance genes pool in the environment where both pathogenic and non-pathogenic microorganisms gradually acquire resistance genes (Isaacson & Torrence, 2002). It was verified that even small concentrations of antibiotics can increase the number and frequency of naturally occurring resistant strains (Andersson & Hughes, 2012; You & Silbergeld, 2014), since these small amounts can still influence the selective pressure on bacteria under natural or laboratory conditions (Xie et al., 2018). Vice versa, the massive use of antibiotics in animal husbandry consequently affects the soil environment through the produced waste or the application of manure and can cause the upsurge of antibiotic resistance on the land (Xie et al., 2018; You & Silbergeld, 2014).

As a consequence, antibiotic residues can influence the microbial activities in the surrounding environment such as altering degradation procedure of the pollutant and nutrient cycling (Sarmah et al., 2006). The effect of antibiotics, ARGs and co-selective agents on the development of AR in the soil microbial communities is summarized in Figure 4 (Ashbolt et al., 2013).

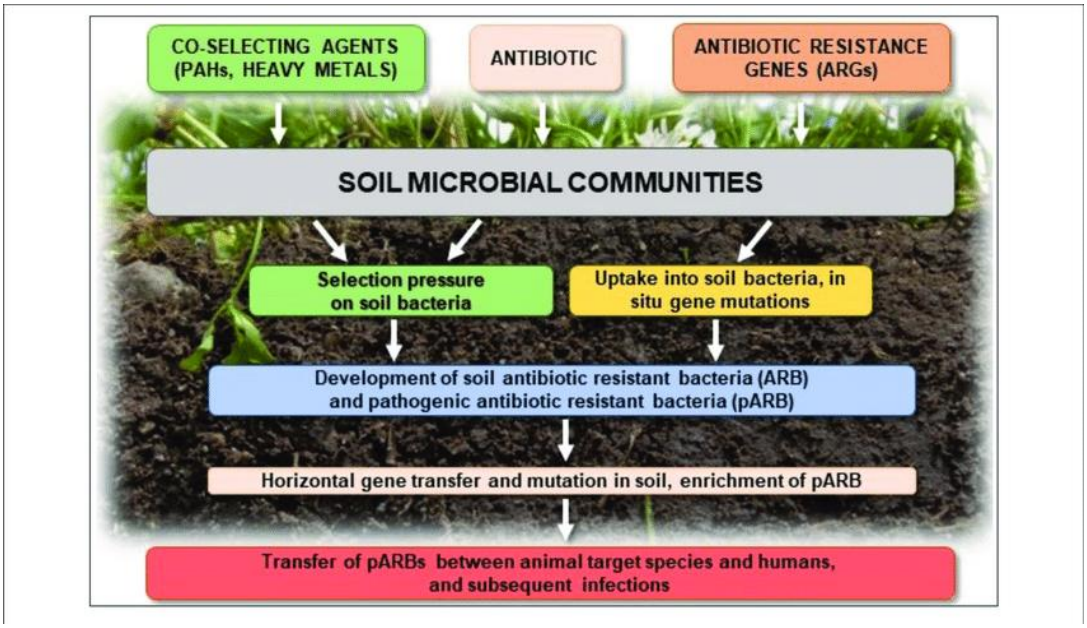

Figure 4 The effect of antibiotics, ARGs and co-selective agents on the development of AR in the soil microbial communities (Ashbolt et al., 2013)

Soil bacteria contributes to the effect of antibiotics and acts as a reservoir of resistance genes (Lang et al., 2010). Antibiotic resistance genes can be distributed in the soil through manure in three ways (Heuer et al., 2011; Xie et al., 2018):

- increase the intrinsic ARGs in soil through the application of manures or organic compounds like composts (Udikovic-Kolic et al., 2014),
- selection pressure on ARGs with the usage of antibiotics in the compost (Gullberg et al., 2011)
- directly introduce manure-derived antibiotic resistance genes to survive together with the host bacteria or to spread among the community members by bacterial horizontal gene transfer

To summarize, the use of organic manure can increase the resistance in soil by adding novel ARGs or increase the already present ARGs (Su et al., 2014; Udikovic-Kolic et al., 2014). The ability of shifting the antibiotic resistance genes from manure to the soil bacteria through HGT mechanisms is indeed the major issue as it can contribute to the distribution of resistance throughout different communities of microbiome (Heuer et al., 2011).

2.3. The effect of soil-originating ARGs and ARBs on human wellbeing

The extensive usage of antibiotics in medical application and agriculture are major sources of AR, as ARB undergo selective pressure during the adaptation process in the environment (Pepper et al., 2018). However, the studies on the transfer of ARB and ARGs from soil of crop field to human are still incomplete and limited (Luby et al., 2016). Mostly foodborne cases transmitted the ARBs and ARGs to human source from agricultural products (Collignon & McEwen, 2019). The possible exposition routes are the direct contact with the contaminated soil through inhalation, ingestion or dermal contact and the transmission through contaminated irrigation water. ARGs can undergo HGT mechanisms with other bacteria including microbes from soil or potential pathogenic bacteria from humans or animals and with the produced crops enter the food chain through consumption of raw fruits or vegetables planted in the treated soil (Marti et al., 2014, Lima et al., 2020). The consumption of the raw agricultural products resulting the ingested resistant bacterial to colonize human gut system and threaten the public human health (Blaak et al., 2014). Irrigation water can spread ARs and ARGs among the microbial communities of natural ecosystems and might affect the animal and human communities across the water streams (Van Overbeek et al., 2014). With the distribution of resistance genes from animals to important natural resources such as soil and water, the human

and animal health can be adversely affected. The resistance of the bacterial can lower the effectiveness of antimicrobial therapy and raising the severity and number of infections in clinical treatment (Collignon & McEwen, 2019).

2.4. One Health Perspective

The massive usage of antimicrobial agents is concerning as it can potentially cause adverse effects on animal and human health (You & Silbergeld, 2014). This is the reason why the efficient handling of antibiotic resistance by holistic approach is a must, by following the guidelines introduced as One Health (McEwen & Collignon, 2018). According to the World Health Organization (WHO), One Health introduces a unified, balanced approach to optimize the health of animals, humans and the environment that is necessary for the prediction, detection, prevention and responsive solution to the global health risks.

One-Health approach creates new ideas or better solutions from multiple disciplines, communities and sectors to search for the root causes of the problems or reducing health threats at the animal-human-ecosystem boundary, and consequently establishes long term solutions (Collignon & McEwen, 2019). Among global health problems, antimicrobial resistance (AMR) illustrates the best the necessity of One Health approach (Velazquez-Meza et al., 2022). AMR closely related to three essential sectors such as agriculture, livestock and human medications. This connection is rather worrying as it can jeopardize epidemiology and public health as the pathogenic strains might develop the ability to become resistant to antimicrobials, allowing them to adapt and proliferate in adverse environment.

One-Health approach joined forces with Food and Agriculture Organization (FAO), WHO and The World Organisation for Animal Health (OIE) to develop Global Action Plan as global effort to address occurrence of AMR problems. Scientific literature listed out some of the key strategies introduces by One-Health approach to combat the AMR issues such as by reducing unnecessary usage of antimicrobials in agricultural sector and restrain antimicrobial dispersal to the surrounding environment. Prophylaxis and the application as growth promoter are strictly prohibited and global surveillance for antimicrobial resistance should be improved for better understanding and monitoring of AMR data and good hygiene practices should be strengthened. To achieve this, investment on the training of human resources should be implement by the countries. Other strategies in combating AMR distribution are by promote sophisticate clinical diagnoses and develop or use vaccines and other alternatives such as probiotics, lysins and phage therapy as treatment strategies (Velazquez-Meza et al., 2022).

2.3. Hormonal activity of animal manure

Animal manure is one of the potential hotspots for natural or synthetic hormonal chemicals. The usage of animal manure to the environment may contribute to the pollution of water and soil by endocrine-disrupting chemicals (EDCs) (Abdellah et al., 2020). Hormones regulate a variety of biological functions in animal and human body by binding to specific membrane receptors with specific function for instance metabolism, growth, tissue differentiation and function, development of immune systems and sexual development. The specific hormone-receptor binding on target cells will trigger biochemical reaction with intended effect (Frédéric et al., 2006). The exposure of the hormonal particles to the environment whether by the irrigation process for plantation or for bedding systems of the livestock may alter endocrine function in the affected environment. Hormonal activity may be influenced by the diet and hormonal treatment of the animals (Lorenzen et al., 2004). The hormonal activity commonly being studied in the livestock animals are estrogen and androgen activity while progesterone commonly studied in poultry (Lorenzen et al., 2004). Estrogen particles observed to be present in large quantities in livestock manure for example swine, chicken, cow and duck manure. In the European Union, livestock animals excreted out approximately 33 tons per year of total estrogen while in 2002, United States recorded about 49 tons per year (Xu et al., 2018). Simultaneously, EDCs can be also present and imitate the naturally occurring estrogen causing adverse effects to the environment and to humans (Csenki et al., 2022).

2.4. Possible solutions for the removal of ARB and ARGs from manures

Several techniques have been suggested for the management or elimination of antimicrobial resistance in soil. Some of these techniques aims to reduce or eliminate the concentration of antibiotics, ARGs and microorganisms using anaerobic digestion, sewage treatment, lime stabilization and composting (Collignon & McEwen 2019; Lima et al., 2020; Marti et al., 2014).

2.4.1. Anaerobic digestion

Anaerobic digestion is the procedure that is associated with the reduction of antibiotic residuals, pathogenic microorganisms and organic pollutants (Flores-Orozco et al., 2020). The method also can be used as a renewable energy source as it produces biogas with high methane concentration (Flores-Orozco et al., 2020). However, there is no valid results regarding the state of ARGs during the anaerobic digestion procedure (Zhang et al., 2019).

2.4.2. Composting

Composting is an appropriate procedure to disinfect manure prior to land application (Marti et al., 2014). This process not only transforms the animal waste to a valuable organic matter, but at the same time removes or reduces the number of pathogenic microbes, therefore it secures the safety of the public and the environment and simultaneously gives an economic benefit (Lima, et al., 2020). Composting procedure consists of spontaneous biological reaction using the aerobic digestion that includes the humification and mineralization processes of organic matter (Bernal et al.,2009) under controlled conditions. The temperature, pH, ratio of carbon per nitrogen, oxygen supply, moisture, porosity, nutrient contents, particle size and density of the bulks are the parameters that need to be taken in consideration to supply an optimal condition for microbial growth to degrade the organic material (Bernal et al.,2009). The environment microbes will break down the organic materials, forming a stable composition of final product which is compost (Bernal et al.,2009).

2.4.2.1.Effects of composting strategies on the removal of ARG in the manures

Composting is believed to be an effective method for the management of organic wastes, as it is practical, and helps to reduce the concentration of antibiotics in an economical way. With lowering the risks of antibiotics, composting help to avoid the contamination of the environment before the farmland application of sewage (Dolliver et al., 2008). The elimination of ARBs and ARGs seems to be effective using this technique as high temperature is used in the process that help to kill most of the bacterial species (Qian et al., 2016). However, if some of the host bacteria categorized from thermophile microorganisms, the thermophilic segment of the composting may elevate the number of the bacteria (Duan et al., 2019). An overview of the composting procedure is illustrated in Figure 5 (Zainudin et al., 2022).

Thermophilic composting procedure is verified to decrease the concentration of antibiotics in manure from mg kg−1 to μg kg−1 (Xie et al., 2018). The effectiveness can reach 50-99% in the removal of tetracycline (Dolliver et al., 2008; Kim et al., 2010), >99% in chlortetracycline, and 54 to 76% for monensin and tylosin (Dolliver et al., 2008). At the same time, some antibiotics such as ofloxacin, ciprofloxacin and sulphamethazine are unaffected by thermophilic composting and still detectable at the end of the process (Dolliver et al., 2008; Xie et al., 2016).

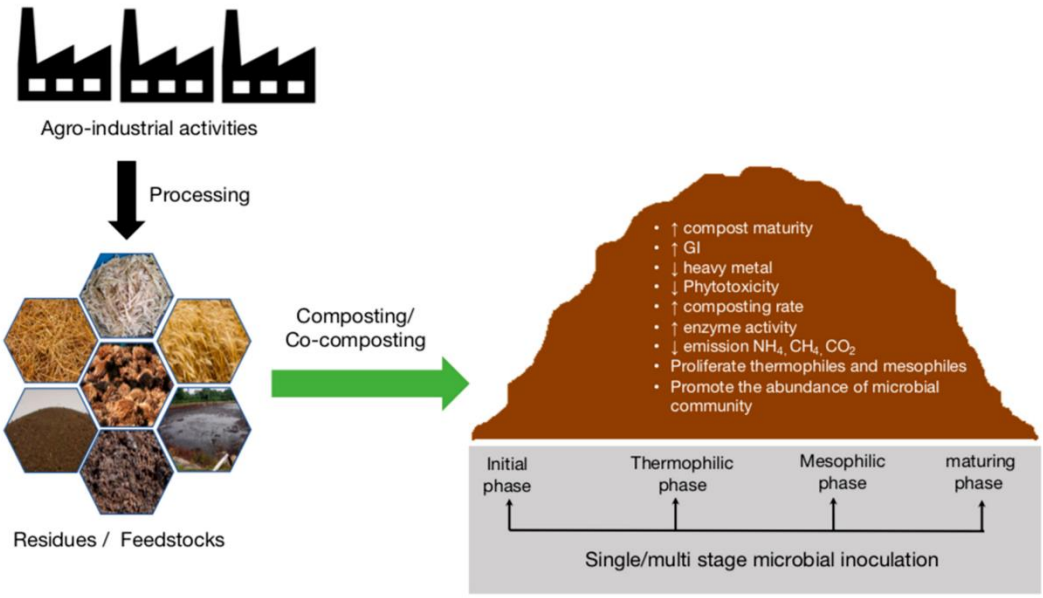

Figure 5 An overview of the composting procedure (Zainudin et al., 2022)

Some study showed that post-manure application gradually getting soil resistance without any addition or changes in the compost materials (Marti et al., 2014; Riber et al., 2014). This might be the period of time where transfer of ARGs from compost to the microbial communities in the associated area takes place (Xie et al., 2018). If the manure application were continuously repeated and run for a long term, it will maintain high levels of antibiotic resistance on soil. Indigenous microbial population in the soil might compete with ARG-harbouring bacteria in the compost materials and gradually supersede from the soil (Marti et al., 2014). Yet, some cases showed the antibiotic resistance genes can be transferred to the indigenous bacterial community inhabiting the soil via horizontal gene transfer (Heuer et al., 2011; You & Silbergeld, 2014). Therefore, the application of compost to the soils can result in a rapid increase and diversity of ARGs and MGEs (Lima et al., 2020). These resistance genes can be directly or indirectly being transmitted through the food chain, manure, sludge-manure soils and water (Lima, et al., 2020, Pepper et al., 2018).

2.5. Bedding Recovery Unit (BRU) as manure management

One of the systems have been introduce as Bedding Recovery Unit (BRU). BRU application is one of the specially developed systems that appears to be a promising method to efficiently remove resistance genes and microorganisms through this mechanism. Through this system, liquid manures are directly used from the farm as the recovery unit of bedding material is safer for the cows. As the bedding material is freshly produced daily, it helps to reduce the

amount of the solid matter and no additional space is needed for the storage. The procedure of producing the bedding material consists of two significant stages which are the separation of solids from the liquid manure continued by the drying process of the compost material. The manure will be pumped into the separator system, and it will give the compression and minimize the moisturization of the solid wastes. The process takes place further in drying drum where the solid materials will experience an intense aerobic drying procedure. The rotatory of the drum will ensure the homogeneity of the waste product. The completion of the treatment process will be monitored by the controlled temperature (see Figure 4).

Figure 6. Schematic figure of the bedding recovery unit (source: http1)

2.5.1. Comparison with conventional bedding materials

Conventional bedding materials are sawdust, straw, wood chips and sand, which is mostly coming outside from the operation system, and in some cases, their usage is not the best solution for the cow. Other than limited availability of the material, the use of the materials might cause injuries to the cow as some of these materials have very tiny and sharp physical characteristics. The used of straw material might increase the unknown bioburden to the cow with the upsurge concentration of solids in the liquid manure. Moreover, the frequent use of the conventional material may associate with higher processing costs of the manure such as for the handling process, storage and physical labour.

Regarding the use of the conventional rubber mats and mattresses as the bedding in some animal farms, the proper maintenance from time to time and the need to replace at least

every 10 years of the usage are the disadvantages. The acquisition costs are already expensive followed by the additional bedding that is required for the coverage of the bearing surface.

2.5.2. Economic benefits of the bedding recovery system

Recycling the liquid manure as the bedding units for the cow not only offers cost savings, but can also lessen the processing and transportation cost of the manure (Dolliver et al., 2008). As the manure available and used up daily, there is no additional space needed for the storage. In the scope of the agricultural benefit, the material is considered as environmentally friendly as there are no additional materials needed for the bedding and only the natural excretion products are used. The solid materials produced by dairy cow consists of all undigested residues from the feed such as the fibre from the hay or silage, hence it can help to reduce the pollution from the usage of the straw or rubber mattress. The bedding material from the manure is highly accepted by the cows, gives comfort to the cow which is good to upsurge the animal well-being.

BRU technology not only cut cost for the handling and the storage of the compost material but also gives a new solution for the farmers to make a good use of the composting compound. Other than the daily availability of the compost material, the bedding material can be gives comfort to the cow and safer to the environment, because the final products is not spread on the crop fields. However, our knowledge about the effect of BRU treatment on the frequency of antibiotic resistance bacteria (ARB), antibiotic resistance genes (ARGs) and hormonal activity is limited.

In this research, we investigated two operating BRU system located in Hungary by comparing the samples before and after the treatment process to see how effective the system in controlling the numbers of ARBs. The evaluation was made by doing the quantification of bacterial growth on Chromatic media for the selection and quantification of colistin and carbapenem resistant microorganisms. Species level identification of the bacterial strains was performed using 16S rDNA PCR and Sanger sequencing. Antibiotic resistance profiles were quantified with MIC determination. Hormonal activity was measured by a *Saccharomyces* bioreporter assay. The objectives of this study were to (1) evaluate the efficiency of the BRU system to the compost material; (2) to analyse and identify ARBs and ARGs in the system; (3) comparing the samples before and after treatment with traditional cultivation and molecular methods.

3. MATERIAL AND METHODS

3.1. Sampling protocol

Sampling was done in two different branch of dairy cow farm situated around Pápa, Hungary using the system introduced as Bedding Recovery Units (BRU) (Figures 7-10). This technology uses the hydraulic system that facilitate the compression of solid waste and hence assist with the separation of solid and liquid waste. The solid wastes continuedly be treated with intense aerobic condition under controlled temperature inside the drying drum. The solid products after the treatment were claimed to be safe for the environment and free from pathogenic bacteria. These treated products were used as the bedding for the cow inside the farm. This bedding not only provides a good solution in the management of manure compost, but it is also more economic and environmentally friendly. During the sampling process, the samples were taken aseptically from the untreated liquid manure and the treated, solid products (coming out from Bedding Recovery Units). The raw samples (before treatment) were labelled as BE1 and BE2 while the samples after BRU treatment were labelled as KI1 and KI2. The sampling was conducted on December 1st, 2022 with the ambient temperature around 3 to 7°C in the morning. We were informed, that the machine from first sampling place (Site 1) was malfunctioning for a few days and have started to operate again on that day. Site 2's BRU system was properly operating in the time of sampling. Samples were temporary stored at 4°C, transported to the laboratory of the MATE – Institute of Aquaculture and Environmental Safety and were processed within 24h.

Figure 7. The system used in the BRU system

Figure 8. The whole system in the farm

Figure 9. The liquid waste before treatment

Figure 10. The solid waste after treatment

3.2. Preparation of samples

The process was initiated by diluting 10 g of samples in 90 ml sterile physiological saline solution containing beads, followed by 30-60 minutes incubation at room temperature on the rotary shaker (round per minute – RPM: 130). This procedure is compulsory as the samples were too dense and inhomogeneous for downstream processing.

3.3. Isolation and identification of emerging bacteria

During traditional cultivation, the isolation of bacteria with critical importance (*Pseudomonas aeruginosa*, carbapenem resistant bacteria, colistin resistant bacteria) was targeted with selective and differential media as follows.

3.1.1. General cultivation

Colony forming units were conducted using LB (Luria-Bertani: Tryptone, 10.0 g; Yeast extract, 5.0 g; NaCl, 9.0 g; Agar, 18.0 g; Distilled water, 1000 mL) and EMB (Eosine-Methylene Blue: Peptone 10.0 g; Lactose, 10.0 g; Dipotassium hydrogen phosphate, 2.0 g; Eosin Y, 0.4 g; Methylene blue, 0.065 g; Agar, 15.0 g) media. LB agar observed to enable a massive bacterial growth as it allows the rapid proliferation and good yields for different species of bacteria (Sezonov et al., 2007) while EMB agar specifically used to distinguish or isolate coliform or faecal coliforms that might be pathogenic microorganisms to the environment. Samples were serially diluted in ten-fold increments and 1 mL of each level of dilution was transferred into sterile Petri-dishes. The given media was gently mixed with the sample, then plates were incubated at 28°C for 96 h. Colony forming units (CFU values) of the original samples were calculated with the arithmetic average of the colonies counted at different levels

of dilutions. Based on visual assessment, colonies morphologies were determined and several bacterial colonies were selected to be streak again to get single colony units of the strains for further analysis and species determination.

3.1.2. *Pseudomonas aeruginosa*

Isolation was performed by inoculating 1 g of undiluted solid samples or 1 ml of liquid samples into asparagine broth containing the mixture of (g/L): L-asparagine, 3; K_2HPO_4, 1; $MgSO_4 7H_2O$, 0.5; glycerol, 10 mL with pH 7.0. Test tubes were incubated for 48 hours at 42°C (Hungarian Standard MSZ 21470-77:1988). In the next step, the bacterial suspensions were spread onto cetrimide agar (MERCK 105284). Plates were incubated at 37°C for 24 hours, then the colonies displaying pyocyanin production and producing trimethylamine odour were further inoculated into acetamide broth containing (g/L): NaCl, 5; acetamide, 1; KH_2PO_4, 2; $MgSO_4.7H_2O$, 1; pH 6.8. Nessler's reagent will verify the decomposition of acetamide by the changes of colour from colourless to yellow or if brown discoloration and/or precipitation takes place. The changes indicating the existence of *Pseudomonas aeruginosa*.

3.1.3. Carbapenem (CRE) and colistin (COL) resistant bacteria

To determine the number and composition of CRE and COL resistant bacteria, the samples were serially diluted into ten-fold increments. 100 µL of dilutions between 10^2 – 10^5 were spread onto the surface of Chromatic CRE/COL agar (Liofilchem) and were incubated for 72h at 35°C. After incubation, colony plates counting was performed. The morphological assessment was done to some bacterial colonies and bacterial colonies were selected to be streak again to get single colony units of the strains for further investigations.

3.1.4. Species level identification

The species level identification of the strains obtained from traditional cultivation on general media and Chromatic COL/CRE cultivation was confirmed by conducting 16S PCR and sequencing using bacterial DNA as detailed below.

3.1.4.1. Isolation of the bacterial DNA

DNA isolation took place using microwave techniques. 1400µL overnight bacterial suspension was transferred into a sterile Eppendorf tube and was centrifugated for 2 minutes with 11000 RPM. The supernatant was then removed and the bacterial pellet was microwaved at maximum power for 2 minutes. The lid of the tubes was opened to prevent evaporation inside

the tube. Next, the tubes were leave at room temperature for 2 minutes before proceed again with microwave for 1 minutes with maximum power. 50 µL of MQ water were then used to suspend the pellet and the samples were vortexed to resuspend the bacterial cells. The tubes were centrifugated again for 2 minutes at 11 000 RPM. After the centrifugation process, the supernatant containing bacterial DNA was transferred into a new and clean Eppendorf tube. Isolated DNA was stored at -20°C until further use.

3.1.4.2. 16S rDNA PCR and sequencing

For 16S rDNA gene sequencing, overnight liquid cultures of the isolated strains were extracted and purified using the MOBIO Ultra Clean Microbial DNA Isolation Kit (MOBIO Laboratories, USA) following the instructions of the manufacturer. For amplification of 16S rDNA genes, bacteria-specific universal primers (27 forward and 1492 reverse) were used (Lane, 1991). The details about 16s rDNA PCR are:

16S rDNA PCR

Mastermix:	
Taq buffer	5µL
27f forward primer	0.5 µL
1492r reverse primer	0.5 µL
dNTP	10.0 µL
Template (DNA)	0.8 µL
Taq polymerase	0.25 µL
MQ water	32.75 µL
Final volume	50.0 µL

Reaction parameters were as follows: 98 °C for 5 s; 32 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 45 s; and final extension at 72 °C for 10 min. The results were validified using agarose gel electrophoresis. 1% agarose gel were prepared using agarose powder in buffer 1X (NaOH, 0.4g; H_3BO_4, 3.044g; distilled water, 1000cm^3). 3.5 µL Eco Safe nucleic acid staining solution (Pacific Image Electronics) was added for staining. Once the agar solidified, 4 µL of DNA of isolates with the addition of 1 µL of DNA loading dye (Thermo Scientific, USA) were loaded onto the gel. The first line of each well was filled with 3 µL GeneRuler DNA Ladder Mix (Fermentas) to verify the approximate size of PCR products. The machine run for 40 minutes in 100V.

The nucleotide sequence determination was performed with the Big Dye Terminator version 3.1. Cycle Sequencing Kit (Applied Biosystems, USA) and sequences were analysed with ABI 3130 Genetic Analyzer (Applied Biosystems, USA). Prior to capillary gel electrophoresis, products were purified by ethanol precipitation. Ethanol precipitation were done by adding the acetate mix (NaAc (3M), 3µL; EtOH (95%), 62.5µL; MQ water,14.5µL) to the sequencing PCR product. Then, the products were incubated for 10 minutes in room temperature before being centrifuged for 30 minutes, 3220g at 4°C. Supernatant was discarded after the process and EtOH (70%) solution were added for 180µL. The centrifugation process was done again for 20 minutes, 3220g at 4°C. The residual supernatant was discarded and the remaining residual liquid were left to evaporate by incubate it at 50°C for 20 minutes. As the pellet were gained, 20µL of Hi-Di formamide (Thermo Fischer, USA) was added. The solution was incubated for 24 hours at 4°C.

The obtained (> 400 bp) sequences were edited and assembled using MEGA5 software® and were searched for homology in the EzBioCloud database (Yoon et al. 2017). Sequence homology over 98.5% was accepted as species-level identification.

3.2. Antibiotic resistance assays/profiling

After species level identification, 5 different strains of bacteria were selected and cultivated on Mueller-Hilton agar (MERCK 105435) following the recommendation of the EUCAST (European Committee on Antimicrobial Susceptibility Testing) and the manufacturer's instruction. Minimal inhibitory concentrations (MICs) of the strains were detected using MIC test strips (MAST Diagnostica) containing amikacin (AK), trimethoprim sulfamethoxazole (SXT), colistin (CS), cefepime (FEP), meropenem (MRP), gentamicin (CN), Ceftazidime (CAZ), imipenem (IMI), piperacillin tazobactam (TZP), ciprofloxacin (CIP), representing different class of antibiotics (listed in Table 2). 20 mL Mueller-Hinton agar was poured into sterile Petri-dishes and let to be solidified. Overnight bacterial cultures were used to prepare a bacterial suspension in 5 mL sterile physiological saline suspension until it reaches 0.5 MacFarland density. Bacterial suspensions were spread onto Mueller-Hinton agar, then MIC test strips were placed onto the surface with sterile forceps. The breakpoint of MICs was visually determined after 24h incubation at 35°C and were interpreted as susceptible (S), or resistant (R) in accordance with the clinical breakpoints of EUCAST (http2). If the strains displayed resistance to two or more antimicrobial class, it will be considered as multidrug resistant (MDR) (Magiorakos et al., 2012).

Table 2. List of the classes of antibiotics used for testing

CLASSES OF ANTIBIOTICS	ANTIBIOTICS	CODE	CONCENTRATION RANGES	**RESISTANCE (ACCORDING TO EUCAST)***
Aminoglycosides	GENTAMICIN	CN	**0.016-256**	**2-18<**
	AMIKACIN	AK	**0.016-256**	**1.0-16<**
Carbapenems	MEROPENEM	MRP	**0.002-32**	**2-8<**
	IMIPENEM	IMI	**0.002-32**	**0.5-4.0<**
Cephalosporins	CEFTAZIDIME	CAZ	**0.016-256**	**1-8<**
	CEFEPIME	FEP	**0.016-256**	**0.25-8.0<**
Fluoroquinolones	CIPROFLOXACIN	CIP	**0.002-32**	**0.6-.0125<**
Penicillins	PIPERACILLIN-TAZOBACTAM	TZP	**0.016-256**	**0.25-16<**
Miscellaneous	COLISTIN	COL	**0.016-256**	**2-4<**
	TRIMETHOPRIM-SULFAMETHOXAZOLE	SXT	**0.02-32.0**	**0.125-0.5<**

3.3. Detection of antibiotic resistance genes (ARGs)

Bacterial DNA was isolated from all samples (BE1, BE2, KI1 and KI2) using the DNeasy® PowerSoil® Pro Kit according to the manufacturer's instructions. 16S rDNA PCR was then conducted to amplify 16S gene followed by agarose gel (1%) electrophoresis with the addition of 3.5µ ECO Safe® nucleic acid staining solution to visualize the results to verify the presence of bacterial DNA.

For ARG testing, our target group was the mobilized colistin resistance gene family (*mcr* genes). Colistin, as a last resort antibiotic against MDR and carbapenem-resistant infections is used in this experiment. Plasmid-mediated colistin-resistance genes means an increasing threat of ARGs to public health (Lin et al., 2022). The experiment was designed to have simultaneous detection of five known transferable resistance genes of colistin (*mcr-1* to *mcr-5*) in the examined four samples using a multiplex PCR method (Rebelo et al., 2017). The results then were visualized by gel electrophoresis. Primer Mix consisted of specific primer pairs from *mcr-1* to *mcr-5* genes. Primers used and reaction parameters for the procedure and their relevant references are detailed in Table 3. Positive control strains were obtained from the Technical University of Denmark (DTU).

Table 3: Primers and reaction parameter used for 16S rDNA and multiplex *mcr-1* to *mcr-5* PCR

*Lane, 1991
**Rebelo et al., 2018

3.4. Hormonal activity screening

The screening of hormonal activity of the samples obtained before and after BRU treatment, was performed using bioluminescent yeast bioreporter assays of *Saccharomyces cerevisiae*. Artificially modified *S. cerevisiae* strains are estrogen/androgen-inducible bacterial lux-based bioreporters constructed by the insertion of tandem estrogen/androgen response elements (see Figure 11 with the example of the estrogen bioreporter), where chemical sensing (bioluminescence) can be used for the assessment of ED activity in the environment (Sanseverino et al., 2005).

Genes	Primer sequence		Amplicon size (bp)	Specification	Reaction parameters
16S rDNA*	F: R:	5'- AGAGTTTGATCMTGGCTCAG - 3' 5'- GGTTACCTTGTTACGACTT- 3'	1400	Species level identification	95°C 2' 25 x (94°C 25''58°C 40''72°C 40'') 72°C 1'
*mcr1***	F: R:	5'- AGTCCGTTTGTTCTTGTGGC - 3' 5'- AGATCCTTGGTCTCGGCTTG - 3'	320	Plasmid-mediated colistin resistance genes	94°C 15' 25 x (94°C 30''58°C 90''72°C 60'') 72°C 10'
*mcr2***	F: R:	5'- CAAGTGTGTTGGTCGCAGTT - 3' 5'- TCTAGCCCGACAAGCATACC - 3'	715	Plasmid-mediated colistin resistance genes	94°C 15' 25 x (94°C 30''58°C 90''72°C 60'') 72°C 10'
*mcr3***	F: R:	5'- AAATAAAAATTGTTCCGCTTATG - 3' 5'- AATGGAGATCCCCGTTTTT - 3'	929	Plasmid-mediated colistin resistance genes	94°C 15' 25 x (94°C 30''58°C 90''72°C 60'') 72°C 10'
*mcr4***	F: R:	5'- TCACTTTCATCACTGCGTTG - 3' 5'- TTGGTCCATGACTACCAATG - 3'	1116	Plasmid-mediated colistin resistance genes	94°C 15' 25 x (94°C 30''58°C 90''72°C 60'') 72°C 10'
*mcr5***	F: R:	5'- ATGCGGTTGTCTGCATTTATC - 3' 5'- TCATTGTGGTTGTCCTTTTCTG - 3'	1644	Plasmid-mediated colistin resistance genes	94°C 15' 25 x (94°C 30''58°C 90''72°C 60'') 72°C 10'

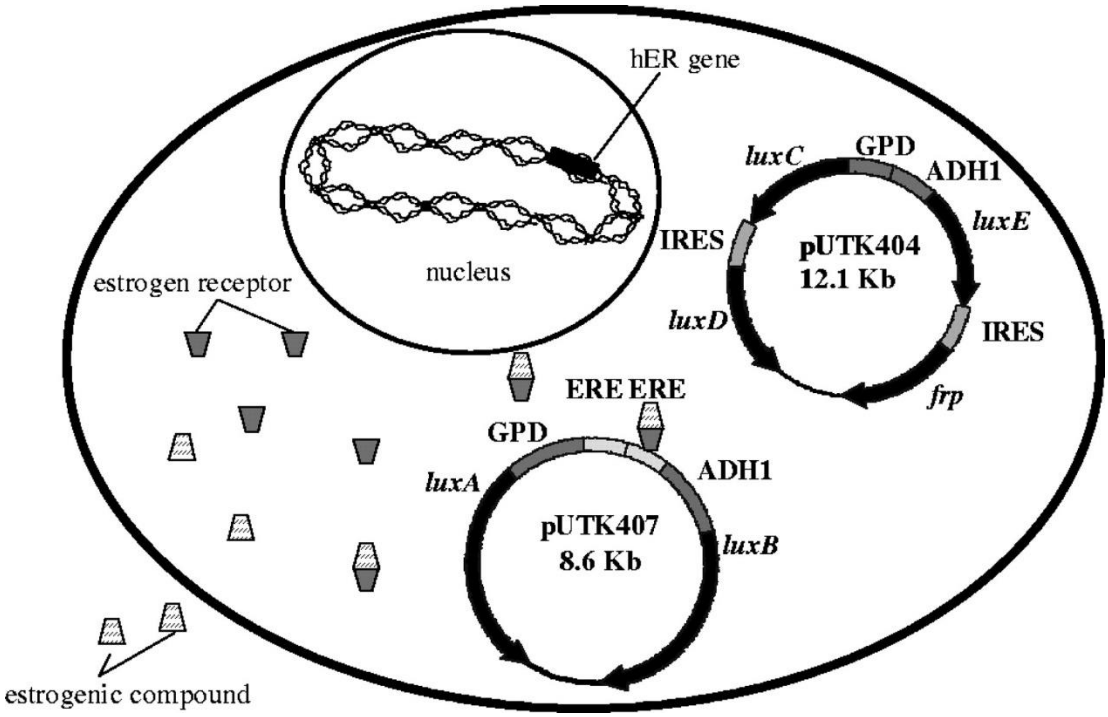

Figure 11. Illustration of artificial Saccharomyces cerevisiae BLYES (Sanseverino et al., 2005)

In this experiment, artificially modified *Saccharomyces cerevisiae* strains were used as yeast-based bioreceptors to analyse the androgenic (BLYAS) and estrogenic (BLYES) activity of the manure samples before (BE1/BE2) and after (KI1/KI2) BRU treatment. Designated BLYES strain was constructed with the insertion of tandem estrogen response elements situated in between of yeast promoters GPD and ADH1 on pUTK401, causing the expression of *luxA* and *luxB* to create pUTK407. Second plasmid or pUTK404 is co-transformation plasmid that contain genes needed for synthesis of aldehyde (*luxCDE*) and FMN reduction (*frp*) that can induce respond to bioluminescent bioreporter when in contact with ED compounds. Results with strain BLYES were compared to the estrogenic assay that uses *lacZ* reporter strain (YES) in yeast to validate the results. BLYR strain was used as a control yeast strain to measure toxicity as it was recommended by the scientific literature (Sanseverino et al., 2005, Sanseverino et al., 2008). This experiment is crucial to detect the hormone-related compounds of the treated/untreated manure. The initial process of the experiment is the extraction of the solid and liquid samples originating from the dairy farms using Soxhlet-extractor (Behr-Labor behrotest ®) as shown in Figure 12. In our case, water and acetone extract were parallelly obtained from the original samples for a more comprehensive analysis.

Figure 12. Extraction method used prior to estrogen/androgen activity measurement

The extracted samples were then diluted by using two separate dilutant: aceton and sterile distilled water with the dilution of 0.25mg/mL, 0.125mg/mL and 0.00781mg/mL. BLYAS, BLYES and BLYR strains were inoculated overnight in YPD medium consists of 1% yeast extract, 2% peptone and 2% of glucose at 30°C with constant shaking at 200 RPM.

On the next day, the optical density of the liquid cultures of the yeast strains were measured by using Genesys 10S UV-Vis (Thermo Scientific) spectrophotometer and set to have an OD_{600} of 1.0. Then 200 µL of each yeast culture were transferred to each test assay using multiple black 96-well Microfluor microtiter plates by Dynex Technologies, Chantilly, VA. Each black 96-well microtiter plate was labelled with the designation of BLYAS, BLYER and BLYR to prevent confusion while checking the hormonal activity. Duplicate plates were created to each test assay using the BLYR toxicity control strain. 17b-estradiol and 5a-dihydrotestosterone (DHT) with concentration ranges between 2.5 x 10^{-12} and 1.0 x 10^{-6}M were used as positive controls for the observation of estrogen and androgen assays while medium with cells and medium with addition of cells and methanol were included in wells as negative controls. The hormonal activity was expressed in bioluminescence that was measured 1 second per well versus the log of chemical concentration (M) by Perkin-Elmer, USA Victor^{TM} X Light 2030 Luminescence Reader. The graph was generated in sigmoidal curve illustrate the activity of the hormonally active compounds. The value of EC50 or 50% effective concentration was measured by the midpoint of sigmoidal dose-response curve linear portion. To determine the

variability among the assays, the mean and standard deviation values were calculated using each standard value of EC50. The value of EC20 were also decided by the measurement of chemical concentration at 20% above bioluminescence background. The concentration of chemical at 20%, lower than the bioluminescence background, was determined as toxic responses (IC20). To measure the toxic equivalency quotients (TEQ), EC50 (or EC20) of 17b-estradiol (estrogen) or DHT (androgen) need to be divided by EC50 (or EC20) of the test chemical. The assays were incubated for 5 to 6 hours to reduce detection limit and get to maximum bioluminescence.

4. RESULTS

4.1. General cultivation

The detectable bacterial cell counts (Colony forming units) on the applied general media (Luria-Bertani and EMB medium) are summarized in Figure 13.

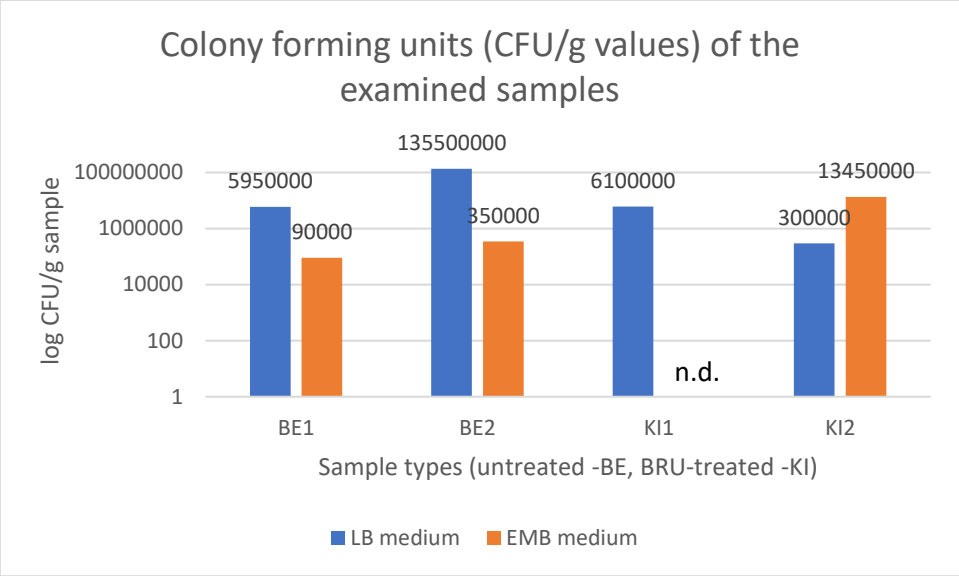

Figure 13. The detectable bacterial cell counts on the applied general media (Luria-Bertani and EMB medium)

Based on this data we can conclude that the difference between the two sampling sites and between the treated and non-treated samples were not significant, the detectable CFU values varied between 10^5-10^7 CFU/g sample. In the case of Site 1, the BRU system was not operating due to technical problems in the last few days, as it can be seen on Figure 13: the microbial cell counts were almost the same before and after treatment on LB agar. Unfortunately, we were not able to measure KI1 sample on EMB medium due to technical errors. Samples of the properly operating BRU system (Site 2, BE2, KI2) showed higher colony forming units (CFU) on LB agar before treatment (BE2) that eventually declined after BRU treatment (KI2) on LB agar, but at the same time, we measured a higher CFU value on EMB medium.

4.2. Isolation and identification of emerging bacteria

4.2.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic pathogen that commonly infecting human body. The emergence of *P. aeruginosa* can contribute to wide array of infections such as otitis

externa, osteomyelitis and pneumonia that can lead to more serious health problem (Wilson MG, Pandey S., 2022). In our study, several observations were made to identify the existence of *Pseudomonas aeruginosa* in the samples. On cetrimide plates, samples prior to BRU treatment (BE1 and BE2) showed growth in all dilution level while KI1 only have bacterial growth on 10^3 dilution level. But during the final verification using acetamide broth, Nessler-reagent displayed all negative results, proving the absence of *Pseudomonas aeruginosa* in all examined samples.

Since the ambient temperature was very low (3-7°C) in the sampling period, the absence of thermotolerant *P. aeruginosa* in the raw samples is not surprising. At the same time, Udikovic-Kolic et al. (2014) stated that *Pseudomonas* spp. can increase in number in manure-amended soil particles. The fact that this species was not present after BRU treatment (KI1, KI2), which, due to the higher temperature, provide more favourable conditions for its growth (Kaszab et al., 2011), confirms the effectiveness of BRU system in the control of an important opportunistic pathogen and prove that BRU system successfully eliminate some emerging bacteria from the waste discharge.

Based on scientific literature, it's still questionable that the occurrence of *Pseudomonas aeruginosa* in organic soil fertilizers is problematic, or not since in study conducted in France, *P. aeruginosa* were rarely detected in agricultural soil treated with manure (Deredjian et al., 2014). It is concluded that the sporadic presence of this bacterial species can be attributed to the soil structure, the physico-chemical characteristics and the climate. Since BRU treated organic material is used for bedding, the absence of *P. aeruginosa* in the treated samples ensures the animal health and the safety of this practice.

4.2.2. Carbapenem (CRE) and colistin (COL) resistant bacteria

Colony forming units were observed using Chromatic COL and CRE agar. COL agar observed to have excessive bacterial growth in KI1, where BRU treatment was not operating in the last few days, but less bacterial isolates showed to be grown in BE1 and BE2. Meanwhile CRE agar showed higher CFU values in BE2 but low in BE1 and KI1. Surprisingly, there were no detectable bacterial growth in KI2 (BRU treated sample of the properly operating technology) for both agar types. The detectable bacterial cell counts on the applied general media (Colistin and Carbapenem medium) are summarized in Figure 14.

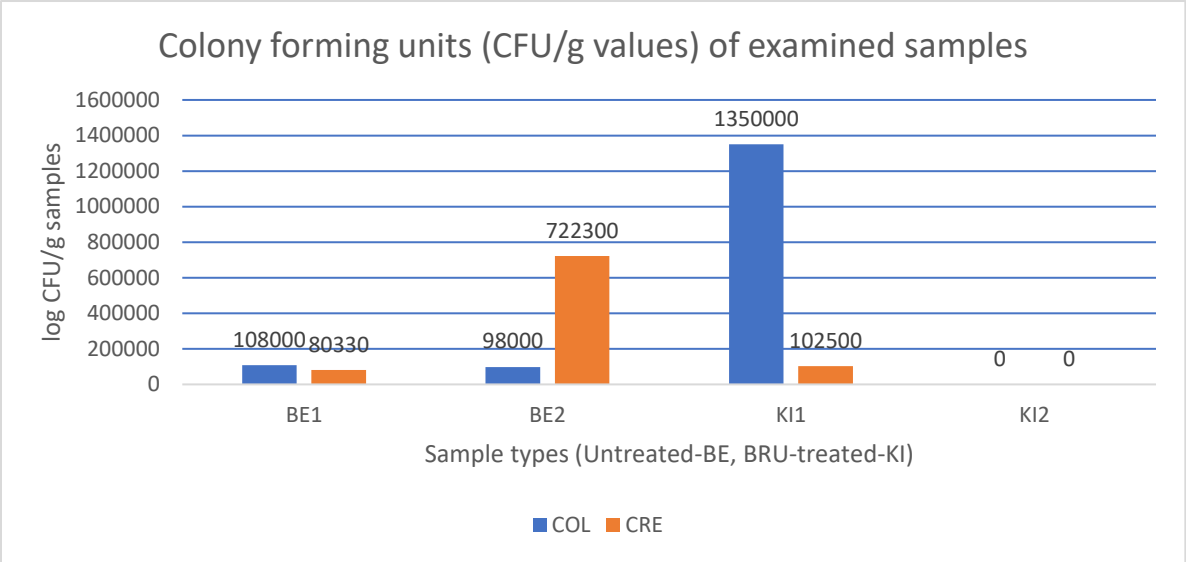

Figure 14. The detectable bacterial cell counts on the applied general media (COL -colistin and CRE - Carbapenem medium)

Based on this measurement, compared to the raw manure, we can conclude that properly operating BRU system can efficiently remove the emergence of COL/CRE resistant bacterial isolates and show no growth of ARBs after BRU treatment procedure. However, there is massive growth of bacteria strains in KI1 on the COL medium and moderate growth on CRE medium. Due to the malfunction of BRU systems occurred on Site 1, this might result on the suitable incubation temperature for certain bacteria strains that were colonizing the organic material of the malfunctioning system.

4.3. Species level identification of bacterial cultures

Based on the morphological characteristics of the bacteria strains, 30 random bacterial colonies of the untreated (BE1/BE2) and BRU-treated (KI1/KI2) liquid manure samples were selected for isolation and identification at species level from all four applied general (LB, EMB) and Chromatic (COL/CRE) media (Figure 15). The details of bacterial strains isolated from untreated (BE) and BRU-treated (KI) samples are presented in Supplementary table.

However, 10 of the isolated strains were stop growing throughout the clean-up process, therefore, we were unable to finish their characterization. The list of the successfully isolated and identified strains and their characteristic features are summarized in Table 4. The risk group classification (according to the German TRBA, http3) is added to Table 4. giving a more comprehensive evaluation of the possible human health concerns of the isolated strains. The composition of the cultivable microorganisms of raw (BE1, BE2) and BRU treated (KI1, KI2) samples are visualized in Figure 16.

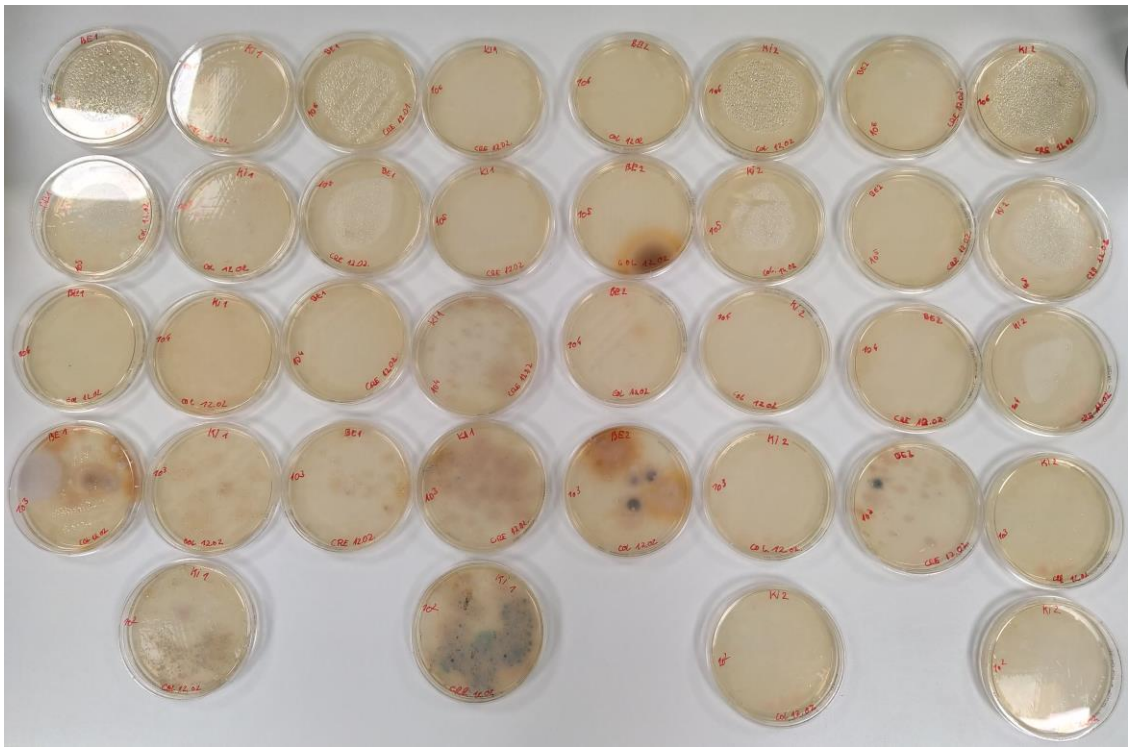

Figure 15. The growth of bacterial isolates on Chromatic COL and CRE plates

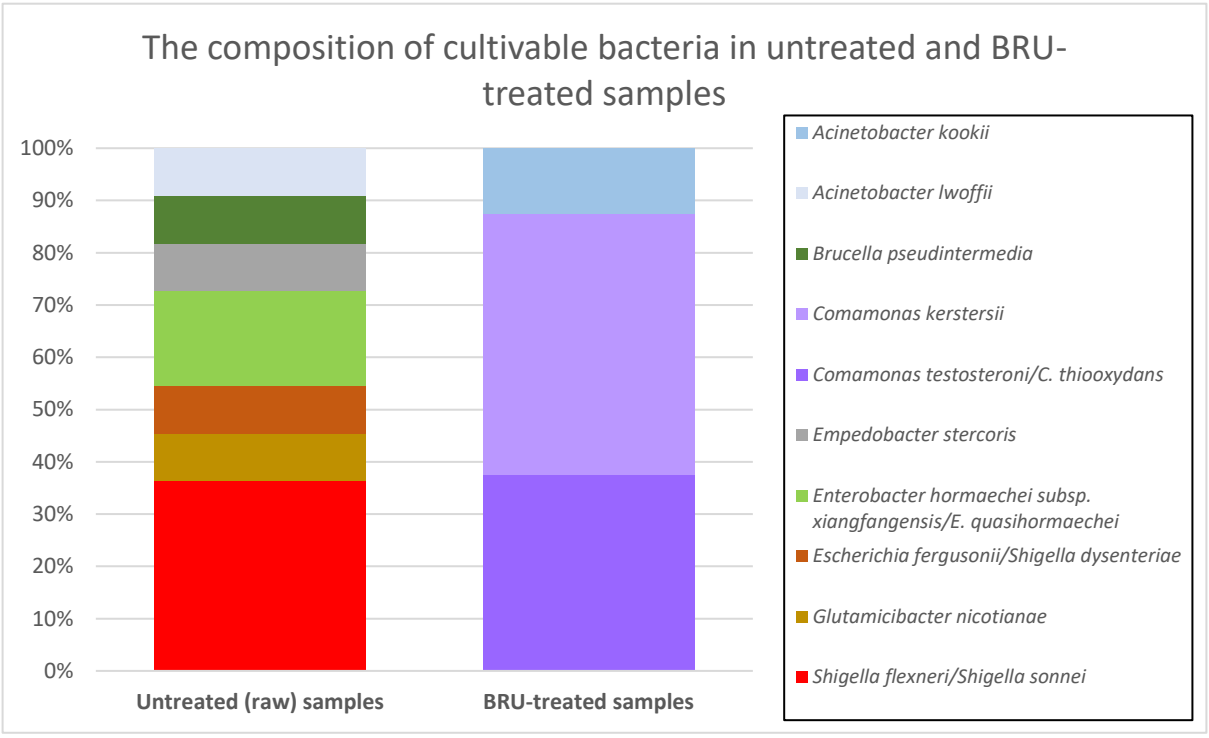

Figure 16. Composition of cultivable microorganisms before and after BRU treatment

Table 4. Bacterial strains isolated from untreated (BE) and BRU-treated (KI) samples and their identification based on EzBiocloud database

Identifier	Source	Medium	Colony morphology	Top hit taxon (16S)	Risk group (http3)	Similarity (%)	Length (bp)
BR5	BE1	LB 10-5	1-2mm, yellow	*Acinetobacter lwoffii*	2 TA	99.47	752
BR11	BE1	EMB 10-3	<1mm, green	*Shigella flexneri/ Shigella sonnei*	2 HT	99.75	793
BR12	BE1	EMB 10-4	2mm, green	*Escherichia fergusonii/ Shigella dysenteriae*	2 HT	99.63	802
BR16	BE1	CRE 10-3	6mm, pink, flat	*Brucella pseudintermedia*	n.d.	99.87	746
BR17	BE1	COL 10-4	1mm, blue, shiny, convex	*Shigella flexneri/ Shigella sonnei*	2 HT	99.87	751
BR6	BE2	LB 10-6	1-2mm, yellow	*Empedobacter stercoris*	n.d.	99.64	840
BR7	BE2	LB 10-6	2-3mm, yellow	*Glutamicibacter nicotianae*	n.d.	99.48	776
BR14	BE2	EMB 10-4	2mm, green	*Shigella flexneri/ Shigella sonnei*	2 HT	99.87	784
BR20	BE2	COL 10-4	1mm, slightly blue, convex, not shiny	*Shigella flexneri/ Shigella sonnei*	2 HT	99.87	742
BR21	BE2	COL 10-4	2mm, pink, flat	*Enterobacter hormaechei subsp. xiangfangensis/ Enterobacter quasihormaechei*	2 HT	99.85	705
BR23	BE2	CRE 10-3	3mm, pink, flat	*Enterobacter hormaechei subsp. xiangfangensis/ Enterobacter quasihormaechei*	2 HT	99.72	725
BR1	KI1	LB 10-6	3-4mm, yellow	*Comamonas testosteroni/ C. thiooxydans*	1	97.9	810
BR2	KI1	LB 10-6	2-3mm, yellow	*Comamonas testosteroni/ C. thiooxydans*	1	97.90	811
BR8	KI1	EMB 10-4	1mm, yellow	*Comamonas testosteroni/ C. thiooxydans*	1	98.01	805
BR9	KI1	EMB 10-5	1-2mm, yellow	*Comamonas kerstersii*	2	99.63	805
BR10	KI1	EMB 10-6	1-2mm, yellow	*Comamonas kerstersii*	2	99.5	796
BR24	KI1	CRE 10-3	2mm, blue, shiny, convex	*Acinetobacter sp.* JFYL_s/ *Acinetobacter baumannii*	2	100/ 97.64	762
BR25	KI1	COL 10-3	1mm, blue, not shiny, convex	*Comamonas kerstersii*	2	99.63	811
BR27	KI1	CRE 10-2	1mm, blue, shiny, convex	*Acinetobacter sp.* JFYL_s/ *Acinetobacter kookii*	2	100/ 98.46	715
BR29	KI1	COL 10-2	1mm, pink, shiny, flat	*Comamonas kerstersii*	2	99.37%	806

Risk Group 1 - Biological agents which are unlikely to cause disease in an individual; Risk Group 2: Biological agents which can cause a disease in an individual, but spreading in the community is unlikely; TA – Some strains have been handled safely over many years in technical applications, therefore can be assigned to the risk group 1; HT - Pathogen for humans and vertebrates, but normally no transmission between the two host groups; n.d. - no data

As it can be seen in Figure 15, the composition of cultivable microorganisms was completely different before and after BRU treatment: in the initial (raw) samples, *Shigella flexneri/Shigella sonnei* group was dominant (35% of the isolated strains) followed by

Enterobacter species (*E. hormaechei* subsp. *xiangfangensis*, or *E. quasihormaechei*). Based on 16S rDNA sequencing the latter two species of *Enterobacter* genus could not be differentiated.

Besides these species, *Empedobacter stercoris* and *Glutamicibacter nicotianae* were detectable in samples before treatment. Other species of bacteria was also found in BE samples, such as *Acinetobacter lwoffii*, *Escherichia fergusonii/Shigella dysenteriae* and *Brucella pseudintermedia*. All strains isolated from raw liquid manure were evaluated as Risk Group 2 microorganisms, which means that these biological agents can cause a disease in an individual and could mean a hazard to employees, but their spreading in the community is not likely. Usually, an effective prevention or treatment is possible for the infections caused by these microorganisms.

According to scientific literature, most of the isolates found before BRU treatment are also commonly found in environment, sewage sludge or manure and some of them discovered to be residing inside the intestinal tract of mammals. *Shigella flexneri/ Shigella sonnei* pathogenic bacteria commonly related to gastrointestinal infection that causing dysentery and foodborne disease (Phiri et al., 2021). *Empedobacter stercoris* is a Gram-negative bacteria belong to *Flavobacteriaceae* group which is commonly discovered in faecal samples (Li et al., 2023) and in manure samples from dairy cattle and breeding sows where these input products were used in a German biogas plant (Schaus et al.,2015). Moreover, *Enterobacter hormaechei subsp. xiangfangensis/ Enterobacter quasihormaechei* are also common pathogenic bacteria found in human gut, causing human infection and hospital-acquired infections (Wu et al., 2020).

In the BRU-treated samples, the observation revealed a completely different microbial composition and identified an outstanding dominance of *Comamonas kerstersii*, followed by *Comamonas testosteroni/C. thiooxydans*, and an unidentified *Acinetobacter* sp., marked as JFYL_s (also known as DSM 11652). The closest relatives of this species are *Acinetobacter kookii* and *Acinetobacter baumannii*.

However, none of these bacteria were detectable before BRU treatment. Of these species, *C. kerstersii* and *Acinetobacter* sp. JSYL_s are known as Risk Group 2 microorganisms, while *C. testosteroni* is Risk Group 1, which is unlikely to cause disease in an individual. All of them were detectable in BRU treated samples originating from Site 1, where the BRU system were stopped working for a few days and switched on again on the day sampling process took place. These usually mesophilic bacteria like Acinetobacters are capable to grow in a temperature ranging from 25 to 45°C, while *C. kerstersii* can tolerate 42°C (Wauters et al., 2003), therefore, BRU machine might took some time to provide a higher temperature for the system to eliminate these pathogenic microorganisms.

Comamonas species are classified in Gram-negative bacteria, commonly residing in wastewater, aquatic and soil environment (Rong K et al., 2022). These bacteria have capability to undergo bioaugmentation and bioremediation process. Bioaugmentation is the supplementary microorganisms that are able to biodegrade intractable molecules found in polluted environment (Nzila et al., 2016). *Comamonas* shown to have resistance to carbapenem antibiotic but the knowledge on how *Comamonas spp.* have the ability to obtain antimicrobial resistance (AMR) genes were still poorly understood (Hem et al., 2022). Unlike other clusters of *Comamonas* species, *Comamonas testosteroni* and *C. thiooxydans* are closely related, causing it to have the top hit taxon with high sequence similarity percentage. C. testosteroni is known as an androgen degrader (Wang et al., 2016) and capable for steroid degradation (Horinouchi et al., 2018).

Acinetobacter spp. are non-motile and aerobic bacteria with the ability to survive under adverse environmental conditions and are commonly related to infections originate from hospitals (Choi et al.,2013). *A. kookii* and *A.lwoffii* are occasionally associated with disease found on human or animal skin, and foods (Schwarz et al., 2020). Due to enormous cases of *Acinetobacter* species detected in cheese, milk and meat, livestock animals could be important reservoir for AMR for *Acinetobacter* spp. that potentially give impact to the public wellbeing (Schwarz et al., 2020).

4.4. Antibiotic resistance assays/profiling

Antibiotic resistant profiles were checked on 5 bacterial isolates that were selected based on their possible human health concerns. According to the German TRBA, *Acinetobacter lwoffii* (BR5), *Escherichia fergusonii/ Shigella dysenteriae* (BR12) and the two *Acinetobacter* JFYL_s strains (BR24 and BR27) were all Risk Group 2 microorganisms (opportunistic human pathogens). The last examined species was *Empedobacter stercoris* (BR6), a Risk Group 1 microorganism (it is unlikely that they can cause disease in healthy individuals), but based on the scientific literature, this species can harbour several antibiotic resistance genes (Cheng et al., 2020).

The results of Minimum Inhibitory concentration (MICs) measurements are summarized in Table 5. Minimum Inhibitory concentration (MIC) indicate the lowest concentration of antibiotic being expressed to fully inhibit visible growth of the isolates under in vitro environment (Kowalska-Krochmal et al., 2021). Antibiotics with lower MIC value consider as effective antimicrobial agents as it displays less drugs are needed to prevent the growth of the microorganisms.

Table 5. The results of Minimum Inhibitory concentration (MICs) measurements

					STRAINS				
Classes of antibiotics	Antibiotics	CODE	CONCENTRATION RANGES	**RESISTANCE (ACCORDING TO EUCAST)***	BR5	BR6	BR12	BR24	BR27
Aminoglycosides	GENTAMICIN	CN	**0.016-256**	**2-18<**	0.023	**4**	0.50	0.125	0.19
	AMIKACIN	AK	**0.016-256**	**1.0-16<**	0.047	**24**	**3**	1.0	1.0
Carbapenems	MEROPENEM	MRP	**0.002-32**	**2-8<**	n.d	0.19	0.016	0.094	0.047
	IMIPENEM	IMI	**0.002-32**	**0.5-4.0<**	n.d.	**1.0**	0.38	**1.0**	0.50
Cephalosporins	CEFTAZIDIME	CAZ	**0.016-256**	**1-8<**	n.d.	1.0	0.125	**4**	**4**
	CEFEPIME	FEP	**0.016-256**	**0.25-8.0<**	n.d.	0.064	0.047	0.75	0.75
Fluoroquinolones	CIPROFLOXACIN	CIP	**0.002-32**	**0.6-.0125<**	n.d.	0.75	0.008	0.047	0.064
Penicillins	PIPERACILLIN-TAZOBACTAM	TZP	**0.016-256**	**0.25-16<**	n.d.	<0.016	0.75	<0.016	<0.016
Miscellaneous	COLISTIN	COL	**0.016-256**	**2-4<**	1.0	**32**	1.0	1.5	1.5
	TRIMETHOPRIM-SULFAMETHOXAZOLE	SXT	**0.02-32.0**	**0.125-0.5<**	0.012	0.032	0.016	0.016	0.008

n.d. – no data (results could not be read)

The table constructed showed several antibiotics effectively inhibit the growth of all chosen isolates such as Trimethoprim Sulfamethoxazole (SXT), Cefepime (FEP), Meropenem (MRP) and Ciprofloxacin (CIP). SXT is an effective combination of drugs consists of Trimethoprim and Sulfamethoxazole commonly used to treat bacterial infection (Kemnic & Coleman, 2022). The mode of action of Sulfamethoxazole is to inhibit folate synthesis of microorganisms, while trimethoprim competes with dihydrofolate reductase enzyme cause it to pause tetrahydrofolate production to the active form of folate and hence resulting inhibition. Single use of these drugs only contributes to bacteriostatic state, but they can act bactericidal when being combined together (Kemnic & Coleman, 2022).

Cefepime (FEP) classified as a fourth-generation cephalosporin which is belong to beta-lactams class of antibiotics. The inhibition method of FEP is by prevent the synthesis of bacterial cell wall by excreting binding enzyme that can cause defects to the cell wall thus leading to autolysis and death to the organism (O'Connor et al., 2022). Their characteristic as zwitterion also benefits FEP for rapid penetration into the cell wall of Gram-negative bacteria,

that is one of the reasons why FEP has better coverage for Gram-negative bacteria than the third-generation cephalosporins (O'Connor et al., 2022).

Next, meropenem is categorized under carbapenem class, has broad spectrum of antibacterial action which incorporates with Gram-negative and Gram-positive, aerobic or anaerobic bacteria, together with strains that resistant to other antibacterials (Blumer, 1997). Ciprofloxacin (CIP) classified in fluoroquinolones group commonly used for the treatment of bacterial infection such as pneumonia and infection occurs in urinary tract. CIP potentially active against various Gram-negatives, especially bacilli such as *Enterobacteriaceae* including *Neisseria*, *Shigella* species, *Salmonella* species and *Escherichia coli* (Thai et al., 2023). The method of CIP to combat bacterial infection is by inhibit topoisomerase of bacterial DNA and DNA-gyrase to prevent the replication of bacterial DNA.

As it can be seen, BR6 (*Empedobacter stercoris*), originating from the raw, untreated liquid manure, showed the highest level of antibiotic resistance: the isolate was resistant to the examined Aminoglycosides (Gentamicin, Amikacin) and to colistin, and at the same time, it was only moderately sensitive to imipenem, a Carbapenem agent. The record of the reading the highest MICs values in amikacin (AK) and colistin (CS) with the record of 24 and 32 mg/L, respectively. Research by Schauss et al. (2015) proved that *Empedobacter stercoris* is a multidrug resistant bacterium as it is found to be resistant to florfenicol and sulfamethaxole but susceptible to oxacillin, enrofloxacin, cefquinome+clavulanic acid, ceftiofur ± clavulanic acid and amoxicillin. In another study, *E. stercosis* also found to be resistant to various antibiotics such as carbapenems, tetracyclines, aminoglycosides, macrolides, phenicols, expanded-spectrum cephalosporins and fluoroquinolenes (Li et al., 2023). The finding that BR6 is resistant to colistin is also in accordance with previous reports about the colistin resistance of *E. stercoris* (Cheng et al., 2020).

The MIC data for *Acinetobacter lwoffii* were incomplete due to the bacterial strains not growing well on the plate and causing it is hard to determine the level of susceptibility of the strains. However, Japoni et al. (2011) concluded that colistin and meropenem are the main antibiotics that effectively inhibit the action of Acinetobacters. This study proved the effectiveness of the drugs towards *Acinetobacter kookii*, *Acinetobacter lwoffii* and *Acinetobacter baumannii* in this study as the measurement of checked antibiotics shown to be less than 1.5 mg/L.

To summarize, based on the research conducted by Udikovic-Kolic et al. (2014), soils with manure application contain higher amount of β-lactam–resistant bacteria than soil with inorganic fertilizer. β-lactam antibiotics commonly used for the treatment of mastitis in dairy

cows. Bacteria might evolve after several application of drugs for the treatment. However, our results still showing that antibiotic resistance is under control, but we should consider that these results are limited due to a smaller number of bacterial isolates tested in the experiments. The repetition of the experiments with high number of bacterial isolates may increase the accuracy of the findings.

4.5. Multiplex PCR for resistance genes of colistin (mcr-1 to mcr-5)

Multiplex PCR were conducted two times to study resistance genes of colistin (mcr-1 to mcr-5) occurred in community DNA (BE1, BE2, KI1, KI2) and in the isolated strains. After screening all 20 strains isolated from raw and treated manure, 2 isolates (BR14 and BR24 belonging to *Shigella flexneri* and *Acinetobacter* sp. JFYL_s, respectively) were candidates for harbouring mcr genes. The other isolates (including colistin resistant BR6) were all negative to the tested *mcr* genes. The results of the second round with the community DNA samples and the BR14, BR24 strains are visualized in Figure 17.

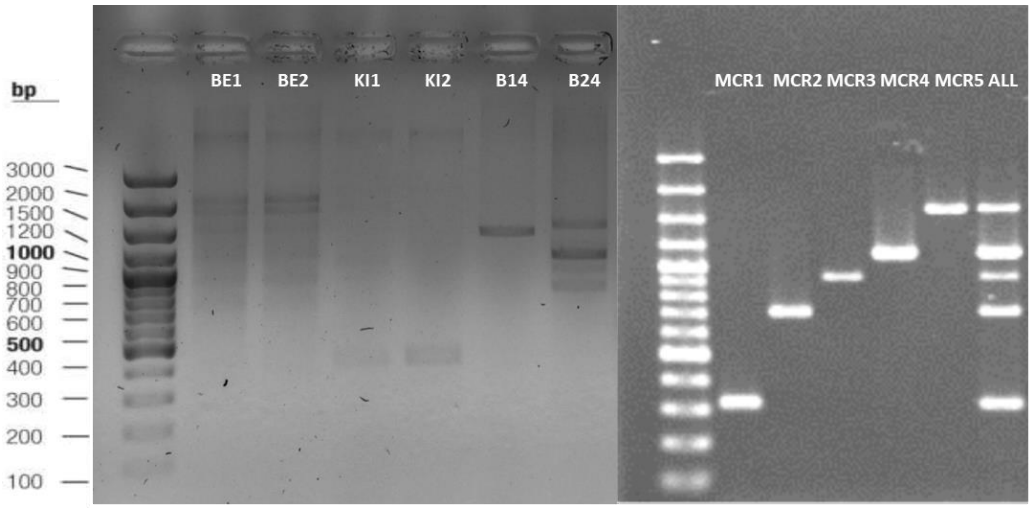

Figure 17. Multiplex PCR for detecting mcr genes (mcr1-5) of the BRU samples (left) and the control strains (right) Source of the control image: Rebelo et al., 2018

Based on our results both community DNA from BE1 and BE2 showed slightly positive results for *mcr-5* gene, while the presence of other types of *mcr* genes are questionable. KI1 and KI2 (BRU treated) samples were negative to all examined *mcr* genes, which emphasize the effectiveness of BRU system in the removal of ARGs. At the same time, both BR strains were positive to mcr-5 and BR24 was positive to mcr-4 and mcr-3, too. Interestingly, BR24 strain did not show phenotypic antibiotic resistance to colistin, which raises the necessity to repeat the colistin resistance testing with broth dilution method and to extend the PCR assay with

additional gene sequencing to verify the presence of the visually observed transferable colistin resistance genes.

Colistin tends to be considered as last antibiotic action to treat the infections triggered by multidrug-resistant of *Acinetobacter baumannii* (Novović & Jovčić, 2023). However, Hameed et al. (2019) states that massive usage of colistin to inhibit infections caused by *Acinetobacter baumannii* and *Pseudomonas aeruginosa* cause the emergence of colistin resistance in both strains. Clinical *Acinetobacter baumannii* found to show approximately 96.7% resistance to antibiotics, and in 2019, *mcr-1* gene was firstly detected in one of the *Acinetobacter baumannii* isolates (Hameed et al., 2019). The fact that we were able to identify and *Acinetobacter* isolate (JFYL_s) that may harbour *mcr* genes is concerning and needs further investigation.

Only 1 out of 4 isolates of *Shigella flexneri* and *S. sonnei* in the samples detected to be resistance to colistin (BR6), but based on multiplex PCR, mcr1-5 genes were not detectable. *Shigella* strains probably undergoes selective pressure and sustain with transferable resistance. Research by Liang et al. (2018) concluded that under selective pressure of colistin, *S. flexneri* obtained transferable and functional *mcr-1* from plasmid-mediated colistin resistance. Colistin resistance transmission occurred during filter mating and might as well transferring mobile elements of host for instance integron and additional resistance genes presence inside the environment. *Shigella* are often multidrug-resistant (MDR) bacteria and reported to be resistant to various types of antibiotics (streptomycin, penicillin, tetracycline, aminoglycosides, ticarcillin, sulphonamides, trimethoprim-sulfamethoxazole, quinolones, sulfafurazole and azithromycin), causing the handling options for the infection become gradually limited (WHO, 2022, Liang B et al., 2018). Since the phenotypic colistin resistance of BR6 is verified, further investigations are necessary to evaluate the presence of additional transferable colistin resistance genes (mcr-6 – mcr 10).

4.6. Hormonal activity screening

Regarding hormonal activity, the result of measurements using acetone and water extracts of BE1/BE2, KI1/KI2 samples (estrogenicity, androgenicity, estrogenic and androgenic activity) with *Saccharomyces cerevisiae* BLYES/BLYAS bioreporter systems are summarized in Figure 16.

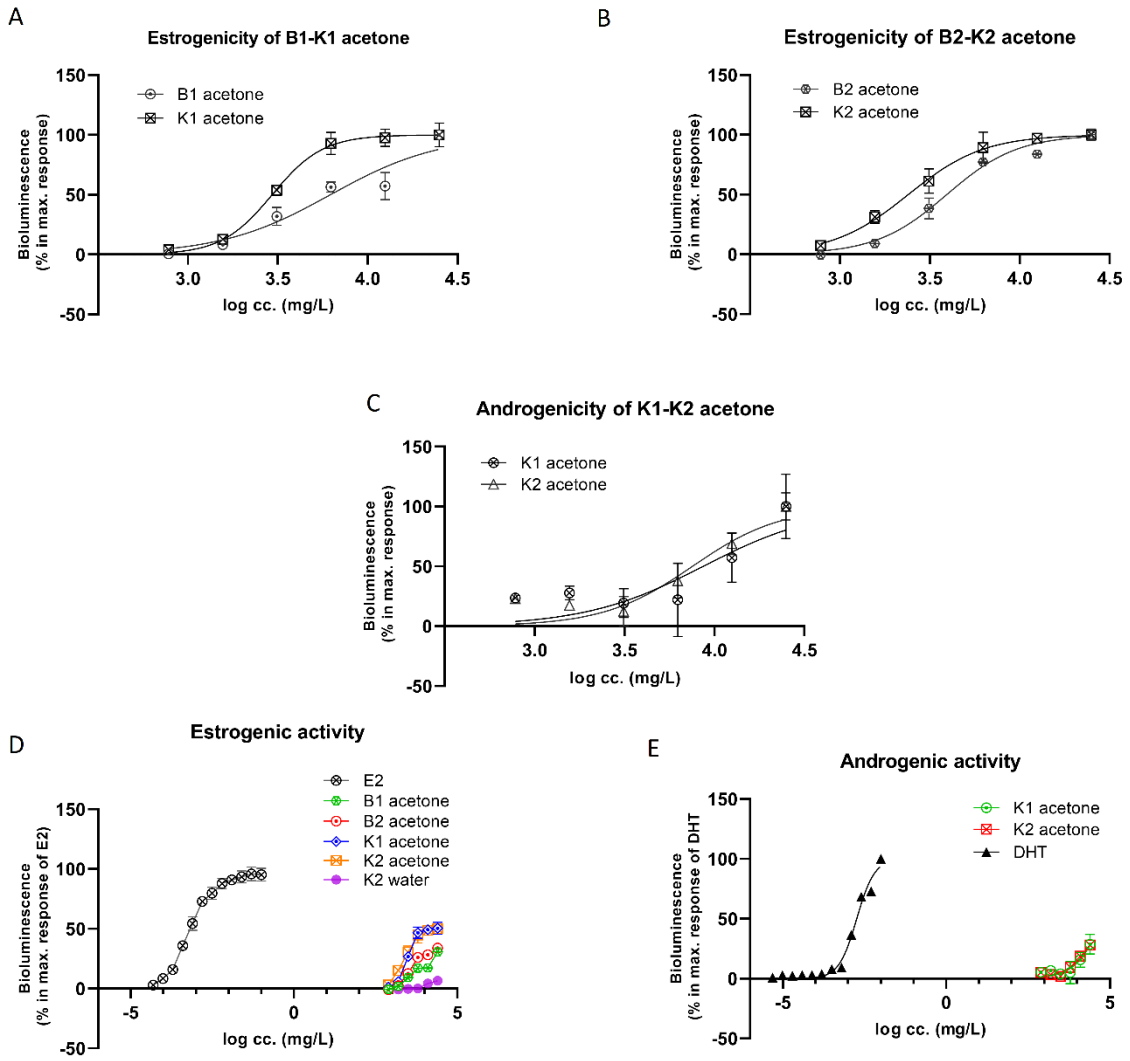

Figure 17. The graph of hormonal activity, the result of measurements using acetone and water extracts of BE1/BE2, KI1/KI2 samples (estrogenicity, androgenicity, estrogenic and androgenic activity) with Saccharomyces cerevisiae BLYES/BLYAS bioreporter systems

As it can be seen on Figure 17, estrogenicity was more pronounced in K1 and K2 (BRU-treated) samples, than in BE1 and BE2 (raw material before treatment) samples (Part A and Part B). Androgenicity was only detectable in K1, and K2 samples (Part C), but there was no significant difference between the level of androgenicity of these two BRU-treated samples.

The hormonal activity was evaluated in comparison with the positive control hormones' (for estrogenic activity, E2, for androgenic activity, DHT, respectively) (Part D and Part E). Based on this comparison, we can conclude that the acetone extract of all four samples (B2, B2, K1, K2) had notable estrogenic activity: the maximum intensification varied between 374-659%, while the estrogenic activity of E2 was measured to be 1175% (Part D). At the same time, the androgenic activity of the acetone extracts K2 and K2 samples was also significant

with a maximum intensification of 120 and 101%, respectively (the positive control DHT intensification was 269%).

Research conducted Yost et al. (2013) shown the same hormonal pattern in swine waste lagoon where the estrogenic activity appeared more abundant than androgenic activity. Based on Haven et al. (2020), the study demonstrated higher and frequent reading of 17α-estradiol, progesterone and 4-androstenedione during winter time. The grazing of overwintered dairy cow and frozen soil have the risk to raised hormones in runoff as frozen soil may retarded degradation process of the hormones. The sampling process that was conducted during winter period may elevated hormonal secretion of dairy cows and as the applied BRU treatment removed the liquid phase to residual hormones were much more concentrated in the solid manures, therefore, a higher hormonal activity was detected after the treatment.

According to the scientific literature, most of endocrine particles such as estrogen and aryl hydrocarbon were highly found in the solid fraction of the manure. The available information about androgens in sewage treatment systems is still limited, but the hormonal activity in dairy wastewater maybe influenced by biotransformation phenomenal progesterone to testosterone. Testosterone found to be most potent in biotransformation of androgenic particles (Cai et al., 2012). Moreover, androgenic activity may be influenced by lactation phase of the dairy cow as the level of androgenic compounds observed to be declined during pregnancy and lactation period of dairy cow (Cai et al., 2012). The existence of hormones, estrogen and aryl hydrocarbon found to be mainly induced by hormones and other pollutants that still left unidentified and might be due poor removal during storage phase of the manure but the aerobic treatment can effectively reduce the number the particles (Combalbert et al., 2012).

5. CONCLUSION, SUGGESTIONS

In 2022, two actively operating bedding recovery unit systems were sampled and analysed to evaluate the effect of BRU treatment on the frequency and composition of antibiotic resistant and pathogen bacteria, on antibiotic resistance genes and on hormonal activity.

According to my results, the BRU treatment did not cause a significant decrease in the viable cell counts (CFU values), but the number of antibiotic (colistin and carbapenem) resistant bacteria was dramatically decreased in the properly operating BRU system of Site 2. This result proves that the introduced BRU system can contribute to elimination process of some pathogenic microorganisms from raw manure and hence proves safer and environmentally friendly materials to be used as bedding inside the dairy farm. Opportunistic pathogen *P. aeruginosa* was not detectable in any of the examined samples.

Since traditional cultivation was performed only once, in the winter period, it is suggested to repeat the experiment under different weather conditions (e.g., seasonally) to more comprehensively evaluate the effectiveness of the BRU system. The number of the isolated COL and CRE resistant strains should be further increased and characterized with phenotypic MIC determination to get a more detailed picture about the bacterial composition and its antibiotic resistance in raw and treated manure. Since cultivation methods have limitations, the microbial community should be evaluated with next-generation sequencing methods (e.g., 16S rDNA amplicon sequencing) to reveal the complexity of the bacterial community.

Antibiotic resistance genes should be further analysed with PCR, or Real-Time PCR assays to get more information about their frequency in manure and BRU treated samples. The quantification of the detectable ARGs would be a further step to evaluate the removal capacity of the bedding recovery unit. In future investigations, the number of ARGs should be increased and other types of antibiotic resistance genes such as *blaOXA*-like genes, or further *mcr* genes should be identified to reveal their occurrence.

The detected hormonal activity of samples after the BRU treatment needs further attention too. This research can be further investigated in the future with some suggestion to focus on the hormonal activity of the after-treatment products to confirm whether the hormonal activity is naturally produced hormones or influenced by the endocrine disruptors (ED) that can mimics the character of natural hormones. Higher ED particles presence in the final products, might affect the hormonal changes of farm animals and hence causing the raise of unwanted health problem. ln the future, liquid manure that being discharged to the plantation sites for the

irrigation process should also be considered for better control or protection of plantation from pathogenic microorganisms.

Further research can supervise and prevent the increase of antibiotic resistance genes inside the farmland due to direct exposure of liquid manure, therefore, a regular monitoring of dairy farms is suggested. For decision makers, it can be suggested to have more stringent control on the use of antibiotics, especially related to the agricultural sectors as it has direct exposure to the consumer. The surveillance of the usage of antibiotics and antibiotic resistance infection should be improved for better management.

For the residents, the application of the antibiotics should be reduced and avoid depending too much on the antibiotics for disease control. Over intake of the medicines can cause the body to gain antibiotic resistance. Instead, the intake of probiotics or vitamin should be considered as solution for health problems and to improve immune system of the body. Good hygiene practices also can help in prevent diseases and infection

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7. REFERENCES

Abdellah, Y. A. Y., Zang, H., Li, C. (2020): Steroidal Estrogens During Composting of Animal Manure: Persistence, Degradation, and Fate, a Review. Water, Air, & Soil Pollution, 231(11): 547.

Andersson, D.I., Hughes, D. (2012): Evolution of antibiotic resistance at non-lethal drug concentrations. Drug Resistance Updates, 15, 162–172.

Ashbolt, N. J., Amézquita, A., Backhaus, T., Borriello, P., Brandt, K.K., Collignon, P., Coors, A., Finley, R., Gaze, W.H., Heberer, T., Lawrence, J.R., Larsson, D.G., McEwen, S.A., Ryan, J.J., Schönfeld, J., Silley, P., Snape, J.R., Van den Eede, C., Topp, E. (2013): Human Health Risk Assessment (HHRA) for environmental development and transfer of antibiotic resistance. Environmental Health Perspectives, 121(9): 993-1001.25

Bernal, M.P., Alburquerque, J.A., Moral, R. (2009): Composting of animal manures and chemical criteria for compost maturity assessment. A review. Bioresources Technology, 100, 5444–5453.

Bernatova, S., Samek, O., Pilat. Z., Sery, M., Jezek, J., Jakl. P., Siler, M., Krzyzanek, V., Zemanek, P., Hola, V., Dvorackova, M., Ruzicka, F. (2013): Following the Mechanisms of Bacterostatic versus Bactericidal Action Using Raman Spectroscopy. Molecules, 18(11): 13188–13199.

Blaak, H., Van Hoek, A.H., Veenman, C., Docters van Leeuwen, A.E., Lynch, G., van Overbeek, W.M., de Roda Husman, A.M. (2014): Extended spectrum B-lactamase- and constitutively AmpC-producing Enterobacteriaceae on fresh produce and in the agricultural environment. International Journals Food Microbiology, 168–169, 8–16.

Blumer, J.L. (1997): Meropenem: evaluation of a new generation carbapenem. International Journal Antimicrobial Agents. 1997 Mar;8(2):73-92.

Cai, K., Elliott, C. T., Phillips, D. H., Scippo, M.-L., Muller, M., Connolly. (2012): Treatment of estrogens and androgens in dairy wastewater by a constructed wetland system, Water Research, 46(7): 2333-2343.

CDC (Centers for Disease Control and Prevention) (2013): Antibiotic Resistance Threats in the United States.

Chee-Sanford, J. C., Mackie, R. I., Koike, S., Krapac, I. G., Lin, Y. F., Yannarell, A. C., Maxwell, A., Aminov, R. I. (2009): Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. Journal of Environmental Quality, 38: 1086–1108.

Cheng, Y., Chen, Y., Liu, Y., Guo, Y., Zhou, Y., Xiao, T., Zhang, S., Xu, H., Chen, Y., Shan, T., Xiao, Y., Zhou, K. (2020): Identification of novel tetracycline resistance gene *tet*(X14) and its co-occurrence with *tet*(X2) in a tigecycline-resistant and colistin-resistant *Empedobacter stercoris*. Emerging Microbes and Infections, 9(1):1843-1852.

Chen, Y.L., Wang, C.H., Yang, F.C., Ismail, W., Wang, P.H., Shih, C.J., Wu, Y.C., Chiang, Y.R. (2016): Identification of Comamonas testosteroni as an androgen degrader in sewage. Scientific Reports. 6:35386.

Choi, J. Y., Ko, G., Jheong, W., Huys, G., Seifert, H., Dijkshoorn, L.. Ko, K. (2013): Acinetobacter kookii sp. nov., isolated from soil. International Journal of Systematic and Evolutionary Microbiology, 63(pt_12):4402-4406.

Collignon, P.J., McEwen, S.A. (2019): One Health-Its Importance in Helping to Better Control Antimicrobial Resistance. Trop. Med. Infect., 4, 22.

Combalbert, S., Bellet, V., Dabert, P., Bernet, N., Balaguer, P., Hernandez-Raquet, G. (2012): Fate of steroid hormones and endocrine activities in swine manure disposal and treatment facilities. Water Research, 46(3):895-906.

Crofts, T.S., Gasparrini, A.J., Dantas, G. (2017): Next-generation approaches to understand and combat the antibiotic resistome. Nature Reviews Microbiology, 15, 422–434

Csenki, Z., Gubó, E., Garai,, E., Bakos, K., Kánainé, S., Dóra, Vásárhelyi, E., Urbányi, B., Szakál, P., Plutzer, J. (2022): Microinjection Based Zebrafish Embryo Test for the Detection of Estrogenic Substances in Slurry Based Irrigation Water and Its Combined Application with Yeast Estrogen Screen. Agricultural Water Management: An International Journal, 272. 1-11.

Davies, J., Davies, D. (2010): Origins and evolution of antibiotic resistance. Microbiology Molecular Biology Reviews, 74:417–433.

Deredjian, A., Colinon, C., Hien, E., Brothier, E., Youenou, B., Cournoyer, B., Dequiedt, S., Hartmann, A., Jolivet, C., Houot, S., Ranjard, L., Saby. N.P., Nazaret, S. (2014): Low occurrence of Pseudomonas aeruginosa in agricultural soils with and without organic amendment. Front Cell Infect Microbiol. 4:53.

Dolliver, H., Gupta, S., Noll, S. (2008): Antibiotic degradation during manure composting. Journal of Environmental Quality, 37, 1245–1253

Duan, M., Zhang, Y., Zhou, B., Wang, Q., Gu, J., Liu, G., Qin, Z., Li, Z. (2019): Changes in antibiotic resistance genes and mobile genetic elements during cattle manure composting after inoculation with Bacillus subtilis. Bioresources Technology, 292.

European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 10.0, 2020. http://www.eucast.org/clinical_breakpoints/.

European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters.Version 13.0, 2023. http://www.eucast.org.

Federal Institute for Occupational Safety and Health. TRBA 466. Classification of prokaryotes (bacteria and archaea) into risk groups. Edition: December 2010. GMBl 2010, No. 68-80 of 06.12.2010, pp. 1428-1667.

Federal Institute for Occupational Safety and Health. Classification of Biological Agents in Risk Groups. https://www.baua.de/EN/Topics/Work-design/Biological-agents/Classification.html

FEEDAP (EFSA Panel on Additives and Products or Substances used in Animal Feed) (2012): Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal, 10(6), 2740.

Flores-Orozco, D., Patidar, R., Levin, D.B., Sparling, R., Kumar, A., Cicek, N. (2020): Effect of mesophilic anaerobic digestion on the resistome profile of dairy manure. Bioresources Technology, 315.

Frédéric, D.L., Leusch, M., R., van den Heuvel, Heather. F., Chapman, S., Ravi, G., Anna, M.E., Eriksson, L. A., Tremblay. (2006): Development of methods for extraction and in vitro quantification of estrogenic and androgenic activity of wastewater samples, Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 143(1): 117-126.

Gautam. V. N., Shraddha S., Lakhani G.P., Rahul S., Kuldeep N.T. (2020): A Review: Impact of floor type and bedding materials on performance of dairy animals. International Journal Current Microbiology and Applied Sciences 9(5): 1524-1534.

Gullberg, E., Cao, S., Berg, O.G., Ilback, C., Sandegren, L., Hughes, D., Andersson D.I. (2011): Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathogens, 7.

Habboush. Y., Guzman, N. (2022): Antibiotic Resistance. Treasure Island (FL), StatPearls.

Hameed, F., Khan, M.A., Muhammad, H., Sarwar, T., Bilal, H., Rehman, T.U. (2019): Plasmid-mediated mcr-1 gene in Acinetobacter baumannii and Pseudomonas aeruginosa: first report from Pakistan. Revista da Sociedade Brasileira de Medicina Tropical.

Havens, S.M., Hedman, C.J., Hemming, J.D.C., Mieritz, M.G., Shafer, M.M., Schauer, J.J. (2020): Occurrence of estrogens, androgens and progestogens and estrogenic activity in surface water runoff from beef and dairy manure amended crop fields. Science Total Environment.

Hem, S., Wyrsch, E.R., Drigo, B., Baker, D.J., Charles, I.G., Donner, E., Jarocki, V.M., & Djordjevic, S.P. (2022): Genomic Analysis of Carbapenem-Resistant Comamonas in Water Matrices: Implications for Public Health and Wastewater Treatments. Applied and Environmental Microbiology, 88.

Heuer, H., Schmitt, H., Smalla, K. (2011): Antibiotic resistance gene spread due to manure application on agricultural fields. Current opinion in microbiology, 14(3), 236-243.

Horinouchi, M., Koshino, H., Malon, M., Hirota, H., Hayashi, T. (2018). Steroid Degradation in Comamonas 51estosterone TA441: Identification of Metabolites and the Genes Involved in the Reactions Necessary before D-Ring Cleavage. Applied and Environmental Microbiology,84(22):e01324-18.

Huang, P., Li, Y., Sumner, M. (2011): Handbook of Soil Sciences: Resource Management and Environmental Impacts, second edition.

Isaacson, R.E., Torrence, M.E. (2001): The Role of Antibiotics in Agriculture. American Society for Microbiology.

Japoni, S., Farshad, S., Abdi. A. A., Japoni, A. (2011): Antibacterial susceptibility patterns and cross-resistance of acinetobacter, isolated from hospitalized patients, southern iran. Iranian Red Crescent Medical Journal. 11:832-6.

Kaszab, E., Szoboszlay, S., Dobolyi, Cs., Háhn, J., Pék, N., Kriszt, B. (2011): Antibiotic resistance profiles and virulence markers of Pseudomonas aeruginosa strains isolated from composts. Bioresource Technology, 102(2): 1543-1548.

Kemnic, T.R., Coleman, M. (2022): Trimethoprim Sulfamethoxazole. StatPearls Publishing.

Kim, K.R., Owens, G., Kwon, S.I., So, K.H., Lee, D.B., Ok, Y.S. (2010): Occurrence and environmental fate of veterinary antibiotics in the terrestrial environment. Water, Air, & Soil Pollution, 214, 163–174.

Köninger, J., Lugato, E., Panagos, P., Kochupillai, M., Orgiazzi, A., Briones, M. (2021): Manure management and soil biodiversity: Towards more sustainable food systems in the EU. Agricultural Systems.

Kowalska-Krochmal, B., Dudek-Wicher, R. (2021): The Minimum Inhibitory Concentration of Antibiotics: Methods, Interpretation, Clinical Relevance. Pathogens.

Lachmayr, K.L., Kerkhof, L.J., Dirienzo, A.G., Cavanaugh, C.M., Ford, T.E. (2009): Quantifying nonspecific TEM β-lactamase (blaTEM) genes in a wastewater stream. Applied and Environmental Microbiology. 75, 203–211.

Lane, D.J. (1991): 16S/23S rRNA Sequencing. In: Stackebrandt, E. and Goodfellow, M., Eds., Nucleic Acid Techniques in Bacterial Systematic, John Wiley and Sons, New York, 115-175.

Lang, K.S., Anderson, J.M., Schwarz, S., Williamson, L., Handelsman, J., Singer, R.S. (2010): Novel florfenicol and chloramphenicol resistance gene discovered in Alaskan soil by using functional metagenomics. Applied and Environmental Microbiology, 76, 5321–5326.

Li, P., Lei, T., Zhou, Y., Dai, Y., Yang, Z., Luo, H. (2023): EBR-5, a Novel Variant of Metallo-β-Lactamase EBR from Multidrug-Resistant Empedobacter stercoris. Microbiology Spectrum, 11(2).

Liang, B., Roberts, A.P., Xu, X., Yang, C., Yang, X., Wang, J., Yi, S., Li, Y., Ma, Q., Wu, F., Qiu, S., Song, H. (2018): Transferable Plasmid-Borne *mcr-1* in a Colistin-Resistant Shigella flexneri Isolate. Applied Environmental Microbiology. 84(8):e02655-17.

Lima, T., Domingues, S., Da Silva, G. J. (2020): Manure as a potential hotspot for antibiotic resistance dissemination by horizontal gene transfer events. Veterinary sciences, 7(3), 110.

Lin, H., Chen, W., Zhou, R., Yang, J., Wu, Y., Zheng, J., Fei, S., Wu, G., Sun, Z., Li, J., Chen, X. (2022): Characteristics of the plasmid-mediated colistin-resistance gene mcr-1 in Escherichia coli isolated from a veterinary hospital in Shanghai. Front Microbiology.

Looft, T., Johnson, T.A., Allen, H.K., Bayles, D.O., Alt, D.P., Stedtfeld, R.D., Sul, W.J., Stedtfeld, T.M., Chai, B., Cole, J.R., Hashsham, S.A., Tiedje, J.M., Stanton, T.B. (2012): In-feed antibiotic effects on the swine intestinal microbiome. Proceedings of the National Academy of Sciences of United States of America, 109(5):1691-6.

Lorenzen, A., Hendel, J.G., Conn, K.L., Bittman, S., Kwabiah, A.B., Lazarovitz, G., Massé, D., McAllister, T.A., Topp, E. (2004): Survey of hormone activities in municipal biosolids and animal manures. Environmental Toxicology, 3:216-25.

Luby, E., Ibekwe, A.M., Zilles, J., Pruden, A. (2016): Molecular methods for assessment of antibiotic resistance in agricultural ecosystems: prospects and challenges. Journal of Environmental Quality, 45, 441–453.

Madhav, A., Will, R.C., Mutreja, A. (2020): The Evolution of Microbial Defence Systems Against Antimicrobial Agents. In: Thomas, S. (eds) Antimicrobial Resistance. 1-31.

Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G,, Harbarth, S., Hindler, J.F., Kahlmeter, G,. Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T., Monnet, D.L. (2012): Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical Microbiology Infection. 18(3):268-81.

Mann, A., Nehra, K., Rana, J.S., Dahiya, T. (2021): Antibiotic resistance in agriculture: Perspectives on upcoming strategies to overcome upsurge in resistance, Current Research in Microbial Sciences.

Marti, R., Scott, A,, Tien, Y.C., Murray, R., Sabourin, L., Zhang, Y., Topp, E. (2013): Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. Applied Environmental Microbiology. 79(18):5701-9.

McEwen, S.A., Collignon, P.J. (2018): Antimicrobial Resistance: A One Health Perspective. Microbiology Spectrum.

National Foundation for Infectious Diseases. Antibiotic Resistance. Available at: https://www.nfid.org/antibiotic-resistance/

Norring, M., Manninen, E., de Passillé, A.M., Rushen, J., Munksgaard, L., Saloniemi, H. (2008): Effects of sand and straw bedding on the lying behavior, cleanliness, and hoof and hock injuries of dairy cows. Journal of Dairy Science. 91(2):570-6.

Novović, K., Jovčić, B. (2023): Colistin Resistance in Acinetobacter baumannii: Molecular Mechanisms and Epidemiology. Antibiotics. 2023; 12(3):516.

Nzila, A., Razzak, S.A., Zhu, J. (2016): Bioaugmentation: An Emerging Strategy of Industrial Wastewater Treatment for Reuse and Discharge. International Journal of Environmental Research and Public Health, 13(9):846.

O'Connor, A., Lopez, M.J., Eranki, A.P. (2022): Cefepime. StatPearls. Treasure Island (FL): StatPearls Publishing.

Pepper, I.L., Brooks, J.P., Gerba, C.P. (2018): Antibiotic Resistant Bacteria in Municipal Wastes: Is There Reason for Concern? Environmental Sciences Technology, 52, 3949–3959.

Phiri, A.F.N.D., Abia, A.L.K., Amoako, D.G., Mkakosya, R., Sundsfjord, A., Essack, S.Y., Simonsen, G.S. (2021): Burden, Antibiotic Resistance, and Clonality of Shigella spp. Implicated in Community-Acquired Acute Diarrhoea in Lilongwe, Malawi. Tropical Medicine and Infectious Disease,6(2):63.

Qian, X., Sun, W., Gu, J., Wang, X.J., Zhang, Y.J., Duan, M.L., Li, H.C., Zhang, R.R. (2016): Reducing antibiotic resistance genes, integrons, and pathogens in dairy manure by continuous thermophilic composting. Bioresources Technology, 220, 425–432.

Ramadan, R.A., Gebriel, M.G., Kadry, H.M., Mosallam, A. (2018): Carbapenem-resistant Acinetobacter baumannii and Pseudomonas aeruginosa: characterization of carbapenemase genes and E-test evaluation of colistin-based combinations. Infection and Drug Resistance, 11:1261-1269.

Rebelo, A.R., Bortolaia, V., Kjeldgaard, J.S., Pedersen, S.K., Leekitcharoenphon, P., Hansen, I.M., Guerra, B., Malorny, B., Borowiak, M., Hammerl, J.A., Battisti, A., Franco, A., Alba, P., Perrin-Guyomard, A., Granier, S.A., De Frutos Escobar, C., Malhotra-Kumar, S., Villa, L., Carattoli, A., Hendriksen, R.S. (2018): Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5 for surveillance purposes. Euro Surveillance. 23(6):17-00672.

Riber, L., Poulsen, P.H.B., Al-Soud, W.A., Skov Hansen, L.B., Bergmark, L., Brejnrod, A. Norman, A., Hansen, L.H., Magid,J. Sørensen, S.J. (2014): Exploring the immediate and long-term impact on bacterial communities in soil amended with animal and urban organic waste fertilizers using pyrosequencing and screening for horizontal transfer of antibiotic resistance. FEMS Microbiology Ecology, 90, 206–224.

Robles-Jimenez, L.E., Aranda-Aguirre, E., Castelan-Ortega, O.A., Shettino-Bermudez, B.S., Ortiz-Salinas, R., Miranda, M., Li, X., Angeles-Hernandez, J.C., Vargas-Bello-Pérez, E., Gonzalez-Ronquillo, M. (2021): Worldwide Traceability of Antibiotic Residues from Livestock in Wastewater and Soil: A Systematic Review. Animals (Basel). 12(1):60.

Rong, K., Delport, J., AlMutawa, F. (2022): Comamonas kerstersii Bacteremia of Unknown Origin. Case Reports in Infectious Diseases.

Ruuskanen, M., Muurinen, J., Meierjohan, A., Pärnänen, K., Tamminen, M., Lyra, C., Virta, M. (2016): Fertilizing with animal manure disseminates antibiotic resistance genes to the farm environment. Journal of environmental quality, 45(2), 488-493.

Sanseverino, J., Gupta, R.K., Layton, A.C., Patterson, S.S., Ripp, S.A., Saidak, L., Simpson, M.L., Schultz, T.W., Sayler, G.S. (2005): Use of Saccharomyces cerevisiae BLYES expressing bacterial bioluminescence for rapid, sensitive detection of estrogenic compounds. Applied Environmental Microbiology,71(8):4455-60.

Sanseverino, J., Eldridge, M.L., Layton, A.C., Easter, J.P., Yarbrough, J., Schultz, T.W., Sayler, G.S. (2008): Screening of potentially hormonally active chemicals using bioluminescent yeast bioreporters. Toxicological Sciences, 107(1):122-34.

Sarmah, A.K., Meyer, M.T., Boxall, A.B. (2006): A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (Vas) in the environment. Chemosphere, 65, 725–759.

Schwarz, S., Mensing, N., Hörmann, F, Schneider, M., Baumgärtner, W. (2020): Polyarthritis Caused by Acinetobacter kookii in a Rothschild's Giraffe Calf (Giraffa camelopardalis rothschildi). Journal of Comparative Pathology,178:56-60.

Schauss, T., Busse, H.J., Golke, J., Kämpfer, P., Glaeser, S.P. (2015): Empedobacter stercoris sp. nov., isolated from an input sample of a biogas plant. International Journal of Systematic and Evolutionary Microbiology,65(10):3746-3753.

Sezonov, G., Joseleau-Petit, D., D'Ari, R. (2007): Escherichia coli physiology in Luria-Bertani broth. Journal of Bacteriology,189(23):8746-9

Shober, A.L., Maguire, R.O. (2014): Manure Management. In Reference Module in Earth Systems and Environmental Sciences, Elsevier.

Simjee, S., Ippolito, G. (2022): European regulations on prevention use of antimicrobials. Brazilian Journal of Veterinary Medicine, 44.

Su, J.Q., Wei, B., Xu, C.Y., Qiao, M., Zhu, Y.G. (2014): Functional metagenomic characterization of antibiotic resistance genes in agricultural soils from China. Environment International, 65, 9–15.

Tang, K.L., Caffrey, N.P., Nóbrega, D.B., Cork, S. C., Ronksley, P.E., Barkema, H. W., Polachek, A. J., Ganshorn, H., Sharma, n., Kellner, J. D., Ghali, W. A. (2017): Restriction in the use of antibiotics in food animals and antibiotic resistance in food animals and humans. WHO Guidelines on Use of Medically Important Antimicrobials in Food-Producing Animals. Geneva: World Health Organization;

Thai, T., Salisbury, B.H., Zito, P.M. (2023): Ciprofloxacin. StatPearls. Treasure Island (FL): StatPearls Publishing.

Threedeach, S., Chiemchaisri, W., Watanabe, T., Chiemchaisri, C., Honda, R., Yamamoto, K. (2012): Antibiotic resistance of Escherichia coli in leachates from municipal

solid waste landfills: comparison between semi-aerobic and anaerobic operations. Bioresources Technology, 113, 253–258.

Thornton, P.K. (2010): Livestock production: Recent trends, future prospects. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 365, 2853–2867.

Udikovic-Kolic, N., Wichmann, F., Broderick, N.A., Handelsman, J. (2014): Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. Proceedings National Academy of Sciences of the United States of America, 111(42), 15202–15207.

Uluseker, C., Kaster, K.M., Thorsen, K., Basiry, D., Shobana, S., Jain, M., Kumar, G., Kommedal, R., Pala-Ozkok, I. (2021): A Review on Occurrence and Spread of Antibiotic Resistance in Wastewaters and in Wastewater Treatment Plants: Mechanisms and Perspectives. Frontiers Microbiology, 12.

Van Overbeek, L.S., van Doorn, J., Wichers, J.H., van Amerongen, A., van Roermund, H.J., Willemsen, P.T. (2014): The arable ecosystem as battleground for emergence of new human pathogens. Frontiers Microbiology, 5, 104.

Velazquez-Meza, M.E., Galarde-López, M., Carrillo-Quiróz, B., Alpuche-Aranda, C.M. (2022): Antimicrobial resistance: One Health approach. Veterinary World. 15(3):743-749.

Wang, F., Han, W., Chen, S., Dong, W., Qiao, M., Hu, C., Liu, B. (2020): Fifteen-Year Application of Manure and Chemical Fertilizers Differently Impacts Soil ARGs and Microbial Community Structure. Frontiers Microbiology, 11, 62.

Wang, Y., Gong, J., Li, J., Xin, Y., Hao, Z., Chen, C., Li, H., Wang, B., Ding, M., Li, W., Li, W., Zhang, Z., Xu, P., Xu, T., Ding, G.C., Li, J. (2020): Insights into bacterial diversity in compost: Core microbiome and prevalence of potential pathogenic bacteria. Science Total Environment, 718.

Wauters, G., De Baere, T., Willems, A., Falsen, E., Vaneechoutte, M. (2003) Description of Comamonas aquatica comb. nov. and Comamonas kerstersii sp. nov. for two subgroups of Comamonas terrigena and emended description of Comamonas terrigena. International Journal of Systematic and Evolutionary Microbiology, 53(Pt 3):859-862.

Wilson, M.G., Pandey, S. (2022): Pseudomonas Aeruginosa. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing.

World Health Organization (24 March 2022). Disease Outbreak News; Extensively drug-resistant Shigella sonnei infections - Europe.

World Health Organization (17 November 2021). Antimicrobial Resistance.

Wu, W., Feng, Y., Zong, Z. (2020): Precise Species Identification for Enterobacter: a Genome Sequence-Based Study with Reporting of Two Novel Species, Enterobacter quasiroggenkampii sp. nov. and Enterobacter quasimori sp. nov. mSystems., 5(4).

Xie, W. Y., Shen, Q., & Zhao, F. J. (2018). Antibiotics and antibiotic resistance from animal manures to soil: a review. *European journal of soil science*, *69*(1), 181-195.

Xu, P., Zhou, X., Xu, D., Xiang, Y., Ling, W., Chen, M. (2018): Contamination and Risk Assessment of Estrogens in Livestock Manure: A Case Study in Jiangsu Province, China. International Journal Environmental Research and Public Health, 15(1): 125.

You, Y., Silbergeld, E.K. (2014): Learning from agriculture: understanding low-dose antimicrobials as drivers of resistome expansion. Frontiers in Microbiology, 5, 284.

Yost, E.E., Meyer, M.T., Dietze, J.E., Meissner, B.M., Worley-Davis, L., Williams, C.M., Lee, B., Kullman, S.W. (2013): Comprehensive assessment of hormones, phytoestrogens, and estrogenic activity in an anaerobic swine waste lagoon. Environmental Sciences Technology, 47(23):13781-90.

Yuan, W., Tian, T., Yang, Q., Riaz, L. (2020): Transfer potentials of antibiotic resistance genes in Escherichia species strains from different sources. Chemosphere, 246.

Mohd Zainudin, M.H.M., Zulkarnain, A., Azmi, A.S., Muniandy, S., Sakai, K., Shirai, Y., Hassan, M.A. (2022): Enhancement of Agro-Industrial Waste Composting Process via the Microbial Inoculation: A Brief Review. Agronomy, 12, 198.

Zhang, J.; Lu, T., Shen, P., Sui, Q., Zhong, H., Liu, J., Tong, J., Wei, Y. (2019): The role of substrate types and substrate microbial community on the fate of antibiotic resistance genes during anaerobic digestion. Chemosphere, 229, 461.–470.

Zhang, S., Sun, L., Wang, Y., Fan, K., Xu, Q., Li, Y., Ma, Q., Wang, J., Ren, W., Ding, Z. (2020): Cow manure application effectively regulates the soil bacterial community in tea plantation. BMC Microbiology 20(1), 190.

Zhu, Y. G., Johnson, T. A., Su, J. Q., Qiao, M., Guo, G. X., Stedtfeld, R. D., Hashsham S.A., Tiedje, J. M. (2013): Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proceedings of the National Academy of Sciences, 110(9), 3435-3440.

Web references:

http1: https://www.fan-separator.de/en/products/bru-bedding-recovery-unit/bru (date of access: 12-03-2023)

http2: https://www.eucast.org/clinical_breakpoints (date of access: 05-03-2023)

http3: https://www.baua.de/EN/Service/Legislative-texts-and-technical-rules/Rules/TRBA/TRBA.html (date of access: 05-05-2023)

Supplementary table: Bacterial strains isolated from untreated (BE) and BRU-treated (KI) samples

Identifier	Source	Medium/ temperature	Colony morphology	Top hit taxon (16S)	Similarity (%)	length (bp)	Notes
BR4	BE1	LB 10-5	1mm, yellow	n.d.			No growth
BR5	BE1	LB 10-5	1-2mm, yellow	*Acinetobacter lwoffii*	99.47	752	
BR11	BE1	EMB 10-3	<1mm, green	*Shigella flexneri/ shigella sonnei*	99.75	793	
BR12	BE1	EMB 10-4	2mm, green	*Escherichia fergusonii/ Shigella dysenteriae*	99.63	802	
BR15	BE1	CRE 10-3	1mm, blue, shiny	n.d.			No growth
BR16	BE1	CRE 10-3	6mm, pink, flat	*Brucella pseudintermedia*	99.87	746	
BR17	BE1	COL 10-4	1mm, blue, shiny, convex	*Shigella flexneri/ shigella sonnei*	99.87	751	
BR19	BE1	COL 10-3	1mm, slightly blue, convex, not shiny	n.d.			No growth
BR6	BE2	LB 10-6	1-2mm, yellow	*Empedobacter stercoris*	99.64	840	
BR7	BE2	LB 10-6	2-3mm, yellow	*Glutamicibacter nicotianae*	99.48	776	
BR13	BE2	EMB 10-3	1mm, green	n.d.			No growth
BR14	BE2	EMB 10-4	2mm, green	*Shigella flexneri/ shigella sonnei*	99.87	784	
BR18	BE2	CRE 10-4	2mm, slightly blue, not shiny	n.d.			No growth
BR20	BE2	COL 10-4	1mm, slightly blue, convex, not shiny	*Shigella flexneri/ shigella sonnei*	99.87	742	
BR21	BE2	COL 10-4	2mm, pink, flat	*Enterobacter hormaechei subsp. xiangfangensis/ Enterobacter quasihormaechei*	99.85	705	
BR22	BE2	CRE 10-3	10mm, blue flat	n.d.			No growth
BR23	BE2	CRE 10-3	3mm, pink, flat	*Enterobacter hormaechei subsp. xiangfangensis/ Enterobacter quasihormaechei*	99.72	725	
BR1	KI1	LB 10-6	3-4mm, yellow	*Comamonas testosteroni/C. thiooxydans*	97.9	810	
BR2	KI1	LB 10-6	2-3mm, yellow	*Comamonas testosteroni/C. thiooxydans*	97.90%	811	
BR8	KI1	EMB 10-4	1mm, yellow	*Comamonas testosteroni/C. thiooxydans*	98.01	805	

BR9	KI1	EMB 10-5	1-2mm, yellow	*Comamonas kerstersii*	99.63	805	
BR10	KI1	EMB 10-6	1-2mm, yellow	*Comamonas kerstersii*	99.5	796	
BR24	KI1	CRE 10-3	2mm, blue, shiny, convex	*JFYL_s/Acinetobacter baumannii*	100/97.64	762	
BR25	KI1	COL 10-3	1mm, blue, not shiny, convex	*Comamonas kerstersii*	99.63	811	
BR26	KI1	CRE 10-2	2mm, blue, shiny, convex	n.d.			No growth
BR27	KI1	CRE 10-2	1mm, blue, shiny, convex	*JFYL_s/Acinetobacter kookii*	100/98.46	715	
BR28	KI1	COL 10-2	1mm, blue, convex	n.d.			No growth
BR29	KI1	COL 10-2	1mm, pink, shiny, flat	*Comamonas kerstersii33*	99.37%	806	
BR30	KI1	COL 10-2	2mm, pink, convex, not shiny	n.d.			No growth
BR3	KI2	LB 10-5	2-3mm, yellow	n.d.			No growth

DECLARATION

on authenticity and public assess of master's thesis

Student's name:	Nur Hafizah Binti Mohd Yusoff
Student's Neptun ID:	DDXJSA
Title of the document:	The effect of separation technology of animal manure on antibiotic resistant bacteria and hormonal activity
Year of publication:	2023
Department:	MATE, Institute of Aquaculture and Environmental Safety, Department of Environmental Safety

I declare that the submitted master's thesis is my own, original individual creation. Any parts taken from an another author's work are clearly marked, and listed in the table of contents.

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STATEMENT ON CONSULTATION PRACTICES

As a supervisor of **Nur Hafizah Binti Mohd Yusoff** (Student's name) **DDXJSA** (Student's NEPTUN ID), I here declare that the master's thesis has been reviewed by me, the student was informed about the requirements of literary sources management and its legal and ethical rules.

I recommend/don't recommend[1] the master's thesis to be defended in a final exam.

The document contains state secrets or professional secrets: yes no*[2]

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Internal supervisor

[1] Please underline applicable.
[2] Please underline applicable.