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Effect of anaerobic digestion on pathogens and antimicrobial resistance in the sewage sludge

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ABSTRACT

Antimicrobial resistance (AMR) is recognized as a global threat. AMR bacteria accumulate in sewage sludge however, knowledge on the persistence of human pathogens and AMR in the sludge line of the wastewater treatment is limited. Sludge can be used, with or without additional treatment, as fertilizer in agricultural fields. The aim of this study is to obtain knowledge about presence of human pathogens and AMR in the sewage sludge, before and after the anaerobic digestion (AD) applying innovative combinations of methods. Fifty sludge samples were collected. Cultivation methods combined with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and Antibiotic Susceptibility Test (AST) were used obtaining knowledge about the microbial community, pathogens, and antibiotic resistant bacteria while the droplet digital Polymerase Chain Reaction (ddPCR) was performed to detect most common AMR genes. In total, 231 different bacterial species were identified in the samples. The most abundant species were spore-forming facultative anaerobic bacteria belonging to *Bacillus* and *Clostridium* genera. The AD causes a shift in the microbial composition of the sludge ($p = 0.04$). Seven pathogenic bacterial species constituting 188 colonies were isolated and tested for susceptibility to Clindamycin, Meropenem, Norfloxacin, Penicillin G, and Tigecycline. Of the *Clostridium perfringens* and *Bacillus cereus* isolates 67 and 50 %, respectively, were resistant to Clindamycin. Two *B. cereus* and two *C. perfringens* isolates were also resistant to other antibiotics showing multidrug resistance. ARGs (bla_{OXA}, bla_{TEM}, *ermB, qnrB, tet(A)-(W), sulI-II*) were present at 7–8 Log gene copies/kg of sludge. AD is the main driver of a reduction of some ARGs (1 Log) but resistant bacteria were still present. The results showed the usefulness of the integration of the proposed analytical methods and suggest a decrease in the risk of presence of cultivable pathogens including resistant isolates after AD but a persistent risk of ARGs' horizontal transmission.

1. Introduction

Continuous population growth causes an intensification in sewage sludge production (Pellegrini et al., 2016). Sludge is a solid or semi-solid material by-product from wastewater treatment, normally derived from civil or industrial wastewater as well as runoff waters (Campo et al., 2021; Domini et al., 2022). Every year it is estimated that more than 10 million tons of dry matter are produced in the European Union (EU-27) (Campo et al., 2021; Pellegrini et al., 2016)) generating concerns about its disposal. Complying with the EU's "end of waste" policy, since it is rich in organic matter and nutrients, dried sludge is reused in agriculture as a fertilizer or soil conditioner as an alternative to chemical fertilizers (European Commision, 2008). However, sludge could be a repository for organic or inorganic chemical contaminants (heavy metals, pharmaceuticals) as well as biological ones (microorganisms, genes), that can contaminate soil, water, and air (Espinosa et al., 2022). Different studies report some concerns on the reuse of the digestate on agricultural soil, due to the potential modification of the microbial composition it may cause and the Antimicrobial Resistance (AMR) dissemination in the environment (Sanz et al., 2022). A recent real scale WWTP study reported a concentration $6.6x10^3$ to $6.5x10^8$ ARGs copies/g of sludge (Patra et al., 2024). According to the European Directive on Sewage Sludge (86/279/EEC), the sludge from WWTP presents some restrictions for its reuse on agricultural soil, such as prohibiting the shedding of the sludge on land used for certain crops (e.g. vegetables and fruits cultivation, crops for animal grazing) or near water bodies for drinking supplies (Council of European Union, 1986). Raw sludge must be treated to reduce its environmental impact (European Commision,

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2008). Anaerobic digestion (AD) is a green biotechnology in which a microbial community, in the absence of oxygen and high temperatures, decomposes the organic matter present in the sludge producing biogas and, thanks to the extreme conditions, reduces the presence of potential pathogens. AD is well established in a large number of countries with more than 18,000 biogas plants around the EU – thanks also to its beneficial effects on microorganisms (Motola et al., 2023).

Antimicrobial Resistance (AMR) is the ability of microorganisms to survive antimicrobial treatments. The spread of this ability is facilitated by an elevated and uncontrolled use of few antibiotic compounds (European Food Safety Agency, 2023). The World Health Organization (WHO) places the AMR among the ten biggest threats to human health (WHO, 2021) and the EU places it in the top three threats (Health Emergency Preparedness and Response Authority, 2022). AMR is responsible for more than 35,000 deaths in the EU (Directorate-General for Research and Innovation, 2024). In 2015 a disability-adjusted life-years of 874,541 was estimated (Cassini et al., 2019). In 2017 the WHO produced a list of 12 pathogenic bacteria for which new antibiotics are urgently required. Among the critical bacteria, there are carbapenem-resistant Enterobacteriaceae and ESBL-producing Enterobacteriaceae, while Vancomycin-resistant *Enterococcus faecium* is included among the high priority pathogens (World Health Organization, 2017).

Antibiotics are used in human medicine as well as veterinary medicine and as pesticides on crops, easily reaching the soil and near water bodies (U.S. Centre for Disease Control, 2018). WWTP are known reservoirs for antibiotics. Most antibiotics from humans and livestock are excreted, not fully metabolized, and can be found in the wastewater through urine and feces. Tetracyclines have high adsorption capability and are mainly adsorbed in sewage sludge (Guardabassi et al., 2002; Oliver et al., 2020). These antibiotics, together with other compounds such as pharmaceuticals, bactericides, and heavy metals, exert a selective pressure on the bacteria present in the sludge (Amarasiri et al., 2020). Antimicrobial Resistance Genes (ARGs) can be transmitted vertically or horizontally among the microorganisms adsorbed on the sludge (Fouz et al., 2020). AMR bacteria (ARBs) contain mobile genetic material (plasmids or transposons) that confer different resistance mechanisms to antimicrobials. There is limited knowledge on the concentration of ARGs and pathogens in the digestate analyzing the potential effect of the anaerobic digestion for their dissemination after the AD process.

The One Health approach is defined as the collaborative effort of multiple disciplines to attain optimal health for humans, animals, and the environment −including plants and soil (Hernando-Amado et al., 2019). ARGs can move freely among the three One Health major compartments: humans, animals, and the environment. Environmental microorganisms are, on many occasions, the primary hosts of ARGs that can be transferred to human pathogens. The decrease of animal and plant genetic diversity due to anthropogenic selection favors AMR dissemination homogenizing the hosts (Hernando-Amado et al., 2019). In response to such critical issue, actions against AMR are required from international and national regulatory bodies. The main pillar outlined by the European One Health action plan against Antimicrobial Resistance and the Italian National Plan Against Antimicrobial Resistance 2022–25 (PNCAR) (European Committee, 2017; Ministero della salute, 2017) are prudent use of antibiotics, surveillance, and monitoring in human and veterinary clinical settings as well as in the environmental matrices.

AMR surveillance in the wastewater and sewage sludge is a surrogate of the AMR circulation into the afferent population and can produce relevant epidemiological implications (Daughton, 2012). Such approach is relatively cheap and free from ethical considerations, producing population level data valuable for public health intervention and policy makers decisions (Diamond et al., 2022; Heljanko et al., 2024; O'Keeffe, 2021). WWTP does not remove antibiotics and ARGs during the process; thus, higher concentration of *ermB*, *sulI* and *qnrS* were observed in the effluent of the implant (Rodriguez-Mozaz et al., 2015). Recent studies reported that in Spain WBE data on antibiotics are partially coincident with prescriptions (Bijlsma et al., 2024; Rodriguez-Mozaz et al., 2015). Analyses of the antibiotic resistome of urban WWTP mirror patterns of clinical antibiotic resistance prevalence (Karkman et al., 2020). This also shows how the landscape of the antibiotic compounds mainly administered within the community is associated with the higher persistence in the environment (Mirzaei et al., 2018).

Biomolecular analyses based on the Antimicrobial Resistance Genes (ARGs) amplification present a high sensitivity and specificity and are quantitative high throughput analyses. Droplet digital Polymerase Chain Reaction (ddPCR), is particularlary advantageous because it reduces the effects of the inhibitors present in the environmental matrices, thereby improving the precision of the measurements, which is generally a critical point for such environmental samples. However, drawbacks include the impossibility of discerning live cells from the dead ones (Yamin et al., 2023). MALDI-TOF MS can be used for rapid species identification of microorganisms based on their protein structure, but it has a limitation – it can only identify cultivable microorganisms. Such limit can be overlapped with an additional Antibiotic Susceptibility Test (AST) can be conducted measuring the diameter of the inhibition growth area on plates. However, the MALDI-TOF plus AST and PCR methods give complementary information on AMR. The first can be used to select isolate of interest to test for antibiotic resistance, while PCR-based analyses quantify the ARGs involved in the same mechanisms.

This paper aims to evaluate the impact of the AD in terms of (1) reduction of potential human pathogens and in particular antimicrobial resistant bacteria in the sludge, and (2) persistence of the antimicrobial resistance genes in the sludge during the treatment, including anaerobic digestion. The study was conducted with samples from two full-scale WWTPs, involving different innovative methods for pathogen and AMR detection: MALDI-TOF MS followed by AST and the ddPCR. To our knowledge, this is the first work in which such methods are combined on sludge samples.

2. Material and methods

2.1. Study design

The sampling campaign was carried out from November 2021 to June 2022 in two wastewater treatment plants located in Northern Italy (Table S1). A total of 50 samples were collected: 14 primary sludge, 12 secondary sludge, 13 pre-thickening sludge (influent of the AD), and 11 digestate (effluent of the AD). The physical–chemical properties of the sludge are pH averaged 7.7 ± 0.4, the internal digester temperature 34.9 ± 10.9, Total Suspended Solids% averaged 3.2 ± 2.0 and Volatile Suspended Solids % averaged 68.3 ± 6.2. Each sample was processed at the hygiene laboratory of the Department of Public Health and Pediatrics of the University of Turin. From each sample five aliquots were produced and delivered to the National Research Centre for Working Environment in Copenhagen, Denmark, for subsequent analyses.

2.2. Pilot study

Four WWTP samples were plated at two dilutions on the selected media to identify the correct dilution for counting the colonies. Starting from the thawed samples, 500 μl were added into 4.5 ml of extraction solution for microorganisms (sterile 50 ml flasks 0,85 %NaCl + 0,05 % Tween80). After centrifugation at 500 rpm for 15 min at room temperature, 100 μl of dilution 10^{-1} were added to 900 μl of dilution solution to obtain 10^{-2} dilution, this step was repeated for 10^{-3}.

Each sample was plated on four selective media for bacterial growth ((Staten Serum Institute (SSI): enteric medium for enteric bacteria, Fastidious anaerobic agar (FAA) for anaerobic bacteria, MacConkey agar for the Gram negative bacteria, MRSA agar for methicillin resistant bacteria (SSI Diagnostica, Herredsvejen 2, DK-3400 Hillerød); and three for fungi (Dichloran Glycerol Agar Media (DG18) (Merck KGaA,

Darmstadt, Germany), Malt Extract Agar (MEA37), and Sabouraud glucose agar (Thermo Fisher Scientific Oxoid, Basingstoke, UK)). Four dilutions were produced and plated to identify the suitable ones (Table S2). 100 µl were plated on the selected agar media and incubated overnight at 37 °C to select human pathogens.

During the pilot study, two sample from the same implant stored with glycerol and two without were tested as described above in order to evaluate the potential effected related to the absence of glycerol during the freezing process.

2.3. MALDI-TOF MS

Serial dilutions were prepared to be plated on the selected media (Table S1); a 100 µl aliquot was plated and incubated at 37 °C for 24–48 h. Identification of bacteria and fungi was performed using matrix-assisted laser desorption–ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) on a Microflex LT mass spectrometer (Bruker Daltonics, Bremen, Germany) using the Bruker Biotyper 3.1 software with the BDAL standard library and filamentous library 4.0 (see, e.g., (Møller et al., 2022) for details). Taxonomic identification was based on the species identification score: score $\geq$ 1.80 corresponds to secure genus identification/probable species identification; 1.70–1.99 probable genus identification (Fedorko et al., 2012); and isolates with score $<$ 1.70 were unidentified. MALDI-TOF target plates for bacteria were prepared using the direct method according to the manufacturer's instructions; some enteric bacterial colonies on SSI were difficult to identify for the presence of polysaccharides; ethanol extraction was required after overnight incubation in Tryptic soy agar (TSA). MALDI-TOF target plates for fungi were prepared after a modified ethanol extraction. Each isolate was tested in duplicate. Spots were covered with 1 µl of matrix (α-cyano-4-hydroxycinnamic acid).

2.4. Antimicrobial resistance (AMR)

2.4.1. Antibiotic Susceptibility test (AST)

Among the species identified with MALDI-TOF MS, seven pathogenic bacteria present in the annex III of the Directive 2000/54/CE (European Parliament, 2000) were selected to test for their susceptibility to the most administered antibiotic classes in Italy (European Centre for Disease Prevention and Control, n.d.): lincosamide (clindamycin), carbapenems (meropenem), quinolones (norfloxacin), penicillin (penicillin G), and tetracyclines (tigecycline) (Thermofisher^{TM} Scientific Inc.). Colonies of the selected species were isolated from the plates and grew overnight on the same medium. Isolates were tested a second time with MALDI-TOF MS to assure the purity of the colony before the Antibiotic Susceptibility Test (AST). The ASTs were conducted measuring the diameter of the inhibitory area following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines for disk diffusion tests (EUCAST, 2024) (Table 1).

2.4.2. DNA extraction and ARGs quantification

Nucleic acid extraction from samples was conducted in duplicate using a DNeasy Powersoil Pro Kit (QIAGEN, Venlo, Netherlands) following the manufacturer's instructions. DNA was eluted in 100 µl of elution solution and quantified using a TECAN Infinite M200 Pro spectrophotometer, a NanoQuant Plate (Tecan Trading AG, Switzerland) and i-Control™ software (version 1.11.10). Quality of the extracts was evaluated with 260/280 and 260/230 ratios at the spectrophotometer and with electrophoretic gels. The 260/280 ratio was 1.65 $\pm$ 0.22. Extracts were stored at −80 °C for subsequent biomolecular analysis. To select extracts on which to conduct further studies, excluding very poor extractions, a cut-off value for DNA of 5 ng µl^{-1} was set (Botes et al., 2013), finally obtaining 50 samples.

Seven ARGs (*bla$_{OXA}$, bla$_{TEM}$, ermB, qnrB, sulI, sulII, tet(A), tet(W)*) were chosen for their capacity to add a resistance ability against the most prescribed groups of antibiotics in Italy (European Centre for Disease Prevention and Control, n.d.) The gene encoding for the 16S rRNA V3- V4 region was added to evaluate the bacterial presence. Primers were selected comparing more than three papers and after Primer BLAST alignment. Antimicrobial resistance was measured using the biomolecular method of the droplet digital PCR (ddPCR, Bio-Rad, Hercules, Cal., USA) on the DNA extracts. Tested genes induce resistance to the same groups of antibiotics mentioned in paragraph 2.4.1, after a screening phase conducted amplifying the ARGs with T100 thermal cycler (Bio-Rad, Hercules, Cal., USA) and gel electrophoresis, *ermB, bla$_{TEM}$, bla$_{OXA}$, qnrB, tet(A)/(W)* respectively and, *sulI-II* for sulfonamides ones were quantified (Table S2) with droplet digital PCR (ddPCR).

2.5. Statistical analyses

Statistical analysis was performed using both IBM SPSS Statistics (version 29.0.0) and R v.4.2.1 (R Core Team, 2022, packages vegan, ampvis2 (Andersen et al., 2018)). ARGs concentrations were expressed as Log of gene copies per kg of sludge and as ratio of the Log of AMR gene copies on the *16S rRNA* copies. These data, adjusted considering the bacterial presence, were then used in all statistical analyses. A descriptive analysis of all the variables was performed. We reported categorical variables as absolute numbers and percentages and as mean and standard deviation for continuous variables. Differences between species identified, gene concentrations, chemical and physical parameters were analyzed using a nonparametric test (Mann-Whitney *U* test and Kruskal-Wallis) for independent samples by ranks, comparing the plant type and the type of sludge. Data collected about species abundance were transformed using the Hellinger method and analyzed through a Redundancy analysis (RDA) using the "vegan" package in R for the anaerobic digestion effect and the implant origin. Statistical significance was evaluated with an ANOVA test. The Spearman rank-order correlation coefficient was determined. A p-value of $p < 0.05$ was considered significant, a value < 0.01 was considered highly significant.

3. Results

3.1. Microbial composition

A total of 67 % bacterial and 87 % fungal isolates were identified at the species level. A fraction of 11 % of the tested isolates had specific spectrum that were not found in the reference library and for 18 % of the

Table 1
Antibiotic Susceptibility Test. The most administered antibiotic classes were selected (included in the work), based on EUCAST breakpoints the antibiotics were selected for each organism.

	Clindamycin (2 µg/mm)	**Meropenem** (10 µg/mm)	**Norfloxacin** (10 µg/mm)	**Penicillin G** (1 unit)	**Tigecycline** (15 µg/mm)
Bacillus cereus	included	included	included		
Clostridium perfringens	included	included		included	
Enterococcus faecalis			included		included
Enterococcus faecium			included		included
Escherichia coli		included	included		included
Klebsiella oxytoca		included	included		
Klebsiella pneumoniae		included	included		

total isolates no spectrum was obtained.

3.1.1. Microbial diversity

The concentration in the sewage sludge varies from 2-4x10^3 to 2.7x10^7 CFU/Kg of sludge. A total of 231 species were identified in the 50 sludge samples (Table S4) that belonged to 88 different genera. Firmicutes and Proteobacteria were the most represented phyla (103 and 84 specie respectively); the most abundant genera were *Clostridium*, *Enterococcus*, *Bacillus,* and *Staphylococcus,* which belongs to the Firmicutes phylum, and *Acinetobacter,* which is a Proteobacterium.

Out of the identified species, 91/231 (39 %) are pathogenic species in the risk 2 or risk 3 groups according to GESTIS database (Institut für Arbeitsschutz der Deutschen Gesetzlichen Unfallversicherung, n.d.). The most abundant bacterial pathogens are *Bacillus cereus, Clostridium perfringens, Enterococcus faecium, Enterococcus hirae, Enterococcus faecalis, Paraclostridium bifermentans, Klebsiella oxytoca, Clostridium butyricum,* and *Acinetobacter lwofii.* In the fungal community, the most abundant are *Candida* and *Saccharomyces* genera. Among the 231 identified species, 7 were selected for resistance testing, including 188 bacterial isolates. The chosen species were firstly selected because they are potential pathogens (European Parliament, 2000). During the pilot study (section 2.2), a difference in the number of colonies growing from samples stored with or without glycerol was evaluated. Of the 231 identified species, 33 grew only in samples containing glycerol. Among the species it is possible to find bacteria, fungi, and yeast which present different levels of tolerance to freezing stress. The absence of a cryoprotectant could have caused severe damages to those microorganisms during the thawing phase.

3.1.2. Biodiversity and wastewater origin

Sludge with different origins had different microbial community compositions. Considering the identified bacterial species, a higher biodiversity is observed in WWTP-S samples from all the stages of the treatment process as compared to WWTP-M (RDA, R^2 = 0.07 and Adjusted R^2 = 0.05; ANOVA test, p = 0.001, (Fig. 1)). Among the pathogens selected, *C. perfringens* concentration is significantly different between the two implants, being higher in the WWTP-M (Mann-Whitney *U* test, p = 0.009).

The fungi isolated are very restricted, limiting statistical analysis. The 43 % and 8 % of samples were positive for *Saccharomyces cerevisiae* respectively for WWTP-S and WWTP-M. Moreover, the 19 % and 8 % of samples were positive for *Candida tropicalis* and 14 % and 62 % for *Candida parapsilosis*. Only a couples of colonies of *Aspergillus niger* were observed.

3.1.3. Anaerobic digestion

The microbial community composition before and after anaerobic digestion differed significantly (RDA, R^2 = 0.063, Adjusted R^2 = 0.043, ANOVA test, p = 0.044) (Fig. 2). Observing the effect on the pathogens of interest, anaerobic digestion does not significantly reduce but increases the *B. cereus* concentration; (Mann-Whitney *U* test, p = 0.029). *Clostriudium perfrigens* seems not to be affected by the extreme conditions of the AD (Mann-Whitney *U* test, p > 0.05). On the contrary, the concentrations of *E. faecalis, E. faecium,* and *K. oxytoca* are significantly reduced (Mann-Whitney *U* test, p < 0.001, p < 0.001 and, p = 0.023 respectively), while *K. pneumoniae* was rarely observed also in the influent (Fig. 3). Analyzing each single phase in the sludge line process before the AD step, no significant difference is observed between the primary and secondary sludges. While, significant difference was generated by AD and it was observed for *C. perfringens* and *E. faecalis* comparing both the primary and the secondary sludge with the effluent of the digester, respectively (Kruskal-Wallis, p = 0.015 and p = 0.042). No bacteria grew on SSI plates from digestate samples.

3.2. Antimicrobial resistance

3.2.1. Antibiotic Susceptibility test on identified pathogens

The seven species were included for their potential pathogenicity according to the European Law on the biological risk in the working

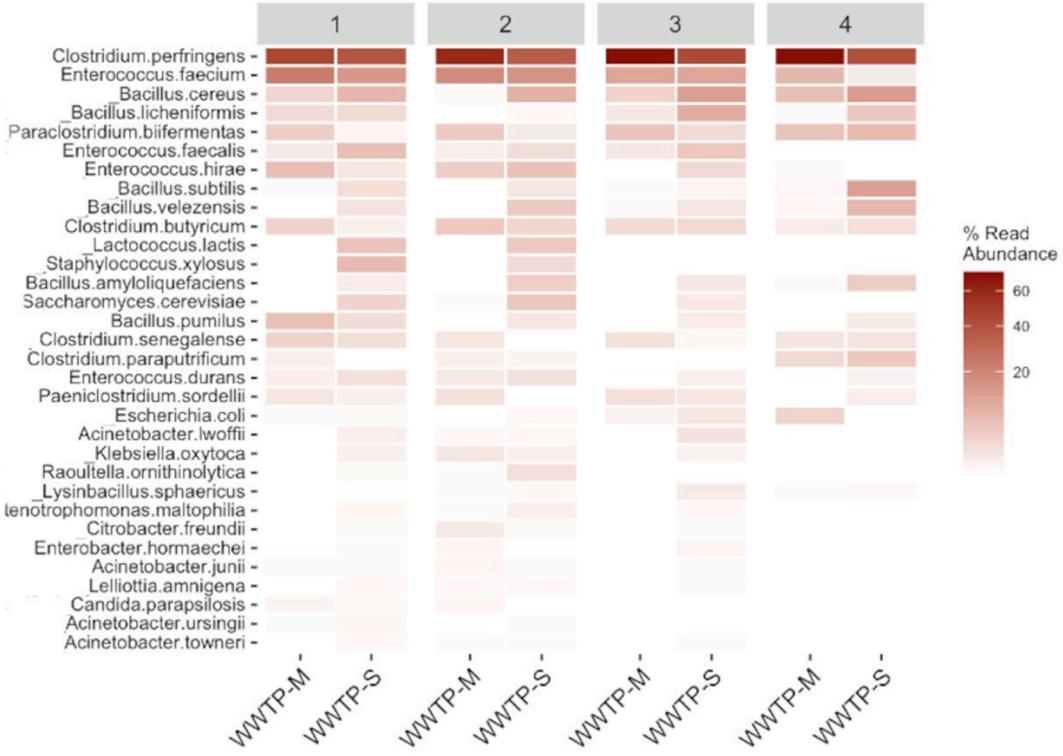

Fig. 1. Relative abundance of bacteria in the two WWTPs, comparing the abundance of the most observed species (present in more than 10 samples) between the two sampled implants (WWTP-M vs WWTP-S) during the sludge treatment. The number of the double column refers to the type of sludge: primary (1), activated (2), pre-AD (3) and post AD (4).

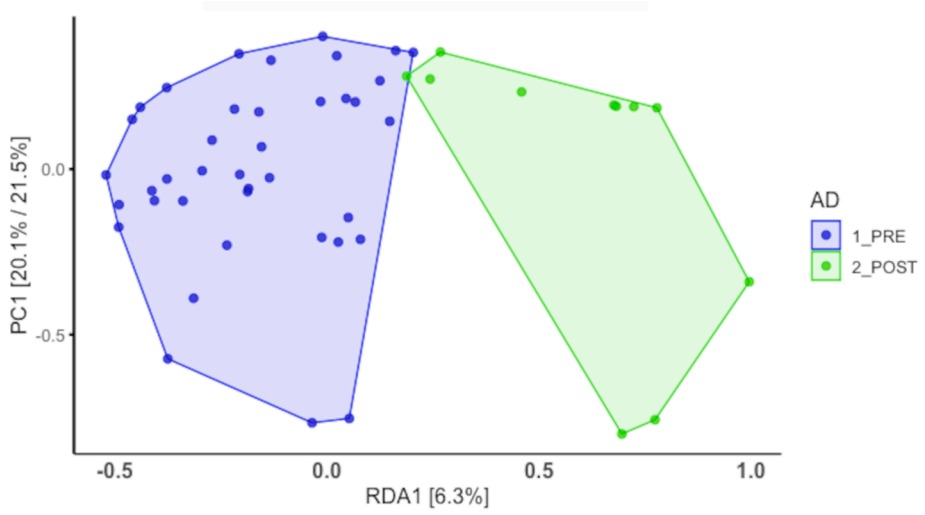

Fig. 2. Effect of anaerobic digestion on the microbial community. Different microorganisms are present in the sludge that enter (blue) and leave (green) the anaerobic digester. The x-axis represents the Redundancy Analysis Axis (RDA), while the y-axis the Principal Component (PC); both axes represent the variation in response to the analyzed variable (Pre vs. post AD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

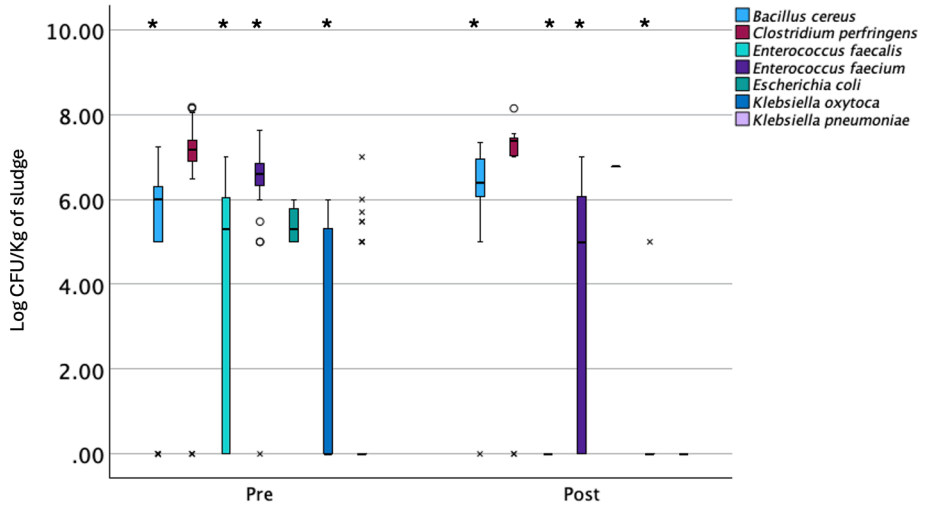

Fig. 3. Concentrations of selected pathogenic bacteria in the influent and effluent of the AD. In the boxplot the concentrations of selected bacteria species before and after the AD are reported. Significant differences in the concentration of the pathogenic bacteria (Mann-Whitney *U* test, $p < 0.05$) during AD are highlighted with *.

environment (European Parliament, 2000). After species identification and selection, the disk diffusion tests were performed for at most three isolates per species per sample for *B. cereus, C. perfringens, E. faecalis, E. faecium, E. coli, K. oxytoca,* and *K. pneumoniae.* Of the *B. cereus* isolates: 50.0 % were resistant to clindamycin, and two of them were also resistant to Norfloxacin, showing multidrug resistance capacity. Of the

Table 2
Antibiotic Susceptibility Test. Resistant isolates out of the total number of isolates tested. Resistant isolates were reported as the isolates from the influent and the (+) effluent of the digester on (/) the total of isolates. Resistance is based on the diameter according to the EUCAST AST disk diffusion test.

	Clindamycin (2 μg/mm)	**Meropenem** (10 μg/mm)	**Norfloxacin** (10 μg/mm)	**Penicillin G** (1 unit)	**Tigecycline** (15 μg/mm)
Bacillus cereus	5 + 2/14 (50.0 %)	0 + 0/14 (0.0 %)	0 + 2/14 (14.4 %)		
Clostridium perfringens	48 + 6/81 (66.7 %)	0 + 0/81(0.0 %)		1 + 1/81 (2.5 %)	
Enterococcus faecalis			0 + 0/20 (0.0 %)		0 + 0/20 (0.0 %)
Enterococcus faecium			1 + 0/54 (1.9 %)		0 + 0/54 (0.0 %)
Escherichia coli		0 + 0/7 (0.0 %)	0 + 0/7(0.0 %)		0 + 0/7(0.0 %)
Klebsiella oxytoca		0 + 0/10 (0.0 %)	0 + 0/10 (0.0 %)		
Klebsiella pneumoniae		0 + 0/2(0.0 %)	0 + 0/2(0.0 %)		

C. perfringens isolates, 66.7 % were resistant to clindamycin, and two isolates were also resistant to Penicillin G. Of the 54 *E. faecium* isolates, 1 was resistant to Norfloxacin. None of the tested isolates was resistant to Meropenem or Tigecycline (Tab 2). No significant reduction caused by the anaerobic treatment was observed concerning the concentration of the resistant isolates (Mann-Whitney *U* test, $p = 0.118$). In the digestate 6 colonies of *Clostridium perfringes* and 2 colonies of *Bacillus cereus* were still present (Table 2). The resistance tests on fungi were limited to *A. niger* but no positive results were observed.

3.2.2. Antibiotic resistance genes

The mean concentration observed for 16S rRNA was 10.41 ± 0.56 Logs gene copies/kg of sludge, the AR genes were 9.06 ± 0.71 *sulI*, 8.86 ± 0.93 *ermB*, 8.56 ± 0.62 *tet(A)*, 8.32 ± 2.12 *sulII*, 7.44 ± 1.94 bla_{OXA}, 7.20 ± 0.66 bla_{TEM}, 6.81 ± 3.05 *tet(W)*, 6.76 ± 1.24 *qnrB* (Table 3). Anaerobic digestion significantly reduces the concentration of bla_{OXA}, bla_{TEM}, *qnrB, sulII, tet(A)* and *tet(W)* (Table 3). Analyzing by implant, in WWTP-S the reduction is significant for *tet(A), qnrB, sulII* and *tet(W)* ($p = 0.008$, $p = 0.018$, $p = 0.030$, and $p = 0.030$ respectively), while for WWTP-M they are bla_{TEM}, *sulII* and *tet(A)* ($p = 0.06$, $p < 0.001$, and $p = 0.032$ respectively). Still, anaerobic digestion is not able to eliminate such genes and the residual concentration is not negligible.

Normalized concentrations for ARGs are similar to the data obtained per kg of sludge (Fig. 4), the relation between the genes is not affected by the 16S rRNA concentration as it is stable in the influent and the effluent of the digester. Analyzing each implant, the number of samples is reduced, for example bla_{OXA} is no longer significant (Mann Whitney *U* test, for normalized data not divided by implant $p = 0.008$ – divided $p > 0.05$). However, regardless of the unit of measurement chosen to express the ARGs concentrations, the decrease is limited (below 1 Log).

Comparing the concentration obtained for each phase of the sludge line, *tet(A), qnrB,* bla_{TEM}, and *sulII* present significant difference between the influent and effluent of the digester, also when considering the two implants individually (Kruskal-Wallis, $p = 0.061$, $p < 0.001$, $p < 0.001$, and $p = 0.012$, respectively). Significant difference between the primary and the secondary sludge were observed for *qnrB* and bla_{TEM}.

3.2.3. AST and ddPCR integration

Cultural and biomolecular analyses were conducted to evaluate the presence and the persistence of AMR elements. Firstly, the correlation among ARGs concentration and the concentration of the seven evaluated bacteria was calculated; *ermB* correlate with *B. cereus* and *C. perfringens* (Spearman's Rho: 0.33 and 0.29, $p = 0.021$ and $p = 0.038$), *sulI* correlate with *B. cereus* and *K.oxytoca* (Spearman's Rho: 0.30 and 0.29, $p = 0.037$ and $p = 0.043$), *tet(A)* correlate with *C. perfringens* and *E. faecalis* (Spearman's Rho: −0.28 and 0.31, $p = 0.024$ and $p = 0.027$), bla_{OXA} correlate with *E. faecalis* (Spearman's Rho = 0.32, $p = 0.024$), *qnrB* correlate with *E. faecalis* (Spearman's Rho = 0.32, $p = 0.026$), *sulII* correlate with *E. faecalis* (Spearman's Rho = 0.28, $p = 0.046$), *tet(W)* correlate with *E. faecium* (Spearman's Rho = -0.28, $p = 0.046$). On the contrary, no significant correlation was observed among ARGs and the percentage of positive isolates of the previously mentioned bacteria in the AST for each antibiotic (Mann-Whitney *U* test, $p > 0.05$).

Table 3
Effect of the AD on ARGs. Mean copies of ARGs (Log gene copies/kg sludge) before and post AD. Significant differences according to the Mann-Whitney *U* test are indicated in bold.

ARG	PRE		POST		DELTA	p-value
	Mean	Dev. Std	Mean	Dev. Std		
16S rRNA	10.41	0.56	10.57	0.22	−0.16	0.504
ermB	8.86	0.93	8.94	0.58	−0.09	0.861
sulI	9.06	0.71	8.55	0.73	0.51	0.055
tet(A)	8.60	0.62	7.89	0.50	**0.71**	0.002
bla_{OXA}	7.65	1.50	7.09	0.71	**0.56**	0.021
qnrB	6.76	1.24	5.88	2.01	**0.88**	0.006
bla_{TEM}	7.20	0.66	6.62	0.36	**0.58**	0.020
sulII	8.32	2.12	6.99	2.43	**1.33**	0.002
tet(W)	6.81	3.05	8.41	1.10	**−1.60**	0.038

4. Discussion

This paper adds new knowledge on the presence of microbial species, including human pathogens and antimicrobial resistant genes, in the sewage sludge and on the impact of the wastewater treatment on their persistence. A combination of cultural method and MALDI-TOF identification were conducted to obtain knowledge about presence of pathogens; ARGs were quantified using the ddPCR. According to literature, no papers used such combination of methods, including the ddPCR, to characterize the microbial community of pre-post AD sewage sludge. The ddPCR presents several advantages, including the higher sensitivity and specificity of the process, the minor influence of the inhibitors of the reaction and other practical positive aspects such as the possibility to process simultaneously a high number of samples in each run plate. Moreover, the validated standards – frequently not available for AMR targets – are not necessary. On the other hand, the instruments and the cost of the reactions are more expensive.

Sampling campaigns conducted for a long period and involving more than one full-scale plant are not so common in literature. Moreover, few studies evaluated the sewage sludge treatment process step by step following the sludge line (primary sludge, secondary sludge, pre-thickening sludge and digestate), to evaluate the effect of the process on the microbial community.

More than half of the number of identified species are not potential pathogens and they are often present in environmental matrices. Firmicutes and Proteobacteria were the most abundant phyla, Firmicutes are widely important in environmental functions, especially in the anaerobic digestion process for their role in organic matter degradation (Liu et al., 2016). Proteobacteria have critical roles in many ecosystems; as member of the Rhizobiaceae family they are relevant in the nitrogen fixation process, contributing to nitrogen availability in the soil (Patel & Sinha, 2011). During the AD, Proteobacteria are also involved in the degradation of the organic matter. The presence of these two phyla in the digestate indicates a healthy community in the digestor, producing a nutrient-rich byproduct ideal for its reuse in agriculture.

The relative abundance differs in the two implants (Fig. 1), with higher biodiversity present at all the stages of the WWTP-S. This may be related to the fact that it receives both urban and industrial wastewater, as well as semi-solid waste from peculiar industries that are not treated in the water treatment, but just added straight in the anaerobic digester (dairy, confectionary, and bio-digester industry). WWTP-M is located near a metropolitan area and the composition of the wastewater is mainly urban and, as expected, a greater presence of human-derived microorganisms was identified. A greater relative abundance of pathogens present in human feces like *Clostridium* and *Bacillus* genera were also present in WWTP-M (Fig. 1), especially *C. perfringens'* concentration is significantly higher in this matrix ($p = 0.009$).

A total of 91 reported species are in risk group 2 and risk group 3 pathogenic microorganisms (Institut für Arbeitsschutz der Deutschen Gesetzlichen Unfallversicherung, n.d.). The anaerobic digestion significantly changes the microbial community of the sludge, reducing the pathogens (Fig. 3).

The selection of the bacterial species for susceptibility testing was based primarily on the potential pathogenicity, and their relevance in a working environment according to the European Directive 2000/54 (European Parliament, 2000).

Among the pathogenic species selected for subsequent susceptibility tests, a reduction of *E. faecalis, E. faecium* and *K. oxytoca,* as well as an increased concentration of *B.* cereus, were observed (Fig. 3). The reduction of *Enterococcus* species is in accordance with what has previously been found after anaerobic digestion (Seruga et al., 2020). Thus, this is also expected to reduce the risk for human health associated with

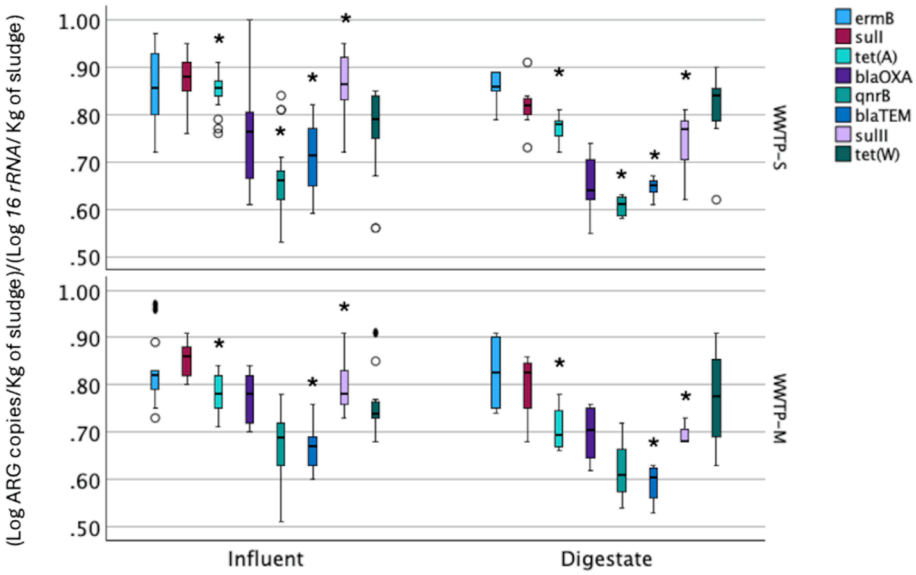

Fig 4. The effect of the AD on ARGs concentrations. Normalized data presented as boxplot. Significant differences (Mann-Whitney *U* test, $p < 0.05$) between the influent (pre) and the effluent (post) of the digester, normalized for *16S rRNA*, are marked with *.

the use or handling of sludge. The AD process seems not to affect the concentration of *B. cereus* and *C. perfringens* as reported in the literature (Flores-Orozco et al., 2024; Lorine et al., 2021). It is worth noticing that *B. cereus* and *C. perfringens* are facultative anaerobic bacteria able to survive in the extreme condition characterizing the anaerobic digestion. Both species belonging to risk group 2 may cause foodborne illness and infections in humans. They persist in digestate, being spread on crops and agricultural soil (Derongs et al., 2020; Govasmark et al., 2011; Lau et al., 2017).

The antimicrobial resistance is a public health threat, and the present study shows that it is not fully eliminated by the anaerobic digestion process. The species *B. cereus* is primarily resistant to penicillin and beta-lactams (bla_{OXA}, …) (Fiedler et al., 2019), *C. perfringens* to tetracyclines and beta-lactams (*tet(A), tet(W),* bla_{OXA}, …) (Dan et al., 2022), *E. faecalis* and *E. faecium* are mainly resistant to Vancomycin followed by beta-lactams, tetracyclines and macrolides (Hollenbeck & Rice, 2012), *E. coli, K. oxytoca,* and *pneumoniae* are resistant to beta-lactams (bla_{TEM}) and carbapenems (bla_{OXA}) (Poirel et al., 2018).

It is worth noticing that AD affects the presence and persistence of ARBs and ARGs (Flores-Orozco et al., 2024; Oliver et al., 2020). The temperature of the digester is a key factor in the inactivation of bacteria. ARGs encoding for antibiotic inactivation mechanisms were affected more during mesophilic conditions, while the ARGs encoding for target alteration were reduced more in the thermophilic conditions (Flores-Orozco et al., 2024). In accordance with previous studies, this paper found living resistant spore-forming bacteria in the digestate (Table 2) and a limited reduction of the ARGs concentration was found (<1 Log unit) (Shukla et al., 2024). A limited but significant reduction was observed for *tet(A),* bla_{OXA}, *qnrB,* bla_{TEM}, and *sulII,* while two others of the selected genes, *ermB* and *tet(W)*, seem not to be affected by the AD. In line with the literature, in mesophilic conditions *tet(W)* –which protects the bacterium encoding for an efflux pump- increases (Ma et al., 2011). In the European Union there is a lack of regulation on the concentration of ARGs permitted in the sludge destined to agricultural use, as well as a safety reduction in Log ARGs copies to be obtained by the AD. The limited reduction of the selected genes in the digestate indicates a persistent risk of horizontal transmission in the environmental matrices if the contaminated sludge is spread.

Evaluating the trend of the concentration during the whole sludge treatment, the main factor that alters the trends is the AD.

The absence of correlation between the cultural and biomolecular methods for AMR evaluation highlights the necessity of implementing the two methods, as the first one can lead to an underestimation of the risk AMR dissemination; it is known that only 1.5 % of the species can grow on cultural media (Fröhlich-Nowoisky et al., 2016). The biomolecular analyses by themselves can lead to an overestimation, as they are based on the DNA amplification, and thus they cannot discern the viability of the microorganisms.

The persistence of elevated concentrations of ARGs in the digestate suggests the possibility of horizontal transmission to the bacteria present in the soil. Additionally, a potential underestimation of the microbial community composition and microbial quantification may have occurred due to the storing conditions of the samples without glycerol. Furthermore, unidentified species were not tested for resistance.

The digestate from the two implants follow two different paths. At WWTP-S –after the dehydration- the digestate is collected and send to a composting plant. At WWTP-M, the sludge is dehydrated and then directly stored to be spread on agricultural soil as a soil conditioner. Anaerobic digestion partially reduces the risk, a decrease of culturable microbes and ARGs is observed but it is limited (<1 Log ARGs copies). The presence of a further treatment step, like composting – that can be conducted by the implant itself or by others – reduces the presence of pathogens and ARGs in the digestate as also the concentration of relevant pollutants such as heavy metals, reducing the potential impact on the environment (Gurmessa et al., 2021) and encouraging its reuse in agriculture.

5. Conclusion

This study highlights the usefulness of the combination of ddPCR, MALDI-TOF and AST methods to obtain knowledge about the persistence of harmful biological agents in the effluent of the anaerobic digestion. While both biomolecular and cultural methods have been previously employed in WWTPs for AMR detection, the innovative combination of ddPCR and MALDI-TOF offers several distinct advantages.

Notably, ddPCR exhibits higher sensitivity compared to other PCR-based methods and reduces inhibition effects potentially caused by the chemical composition of sludge. This allows a quantitative assessment of the potential horizontal transfer of ARGs. Furthermore, MALDI-TOF analysis provides data on viable human pathogens and, when combined with AST, knowledge on presence of AMR in pathogenic species.

Together these methods constitute an effective diagnostic tool for the evaluating AMR risk in anaerobic digestion effluent sludge.

A significant reduction in the number of pathogenic species in the sludge was observed supporting the efficiency of the anaerobic treatment. However, spore-forming bacteria (including antimicrobial resistance bacteria), as well as ARGs, are weakly affected by the extreme conditions of the process. Therefore, digestate land application is a suitable option to mitigate the impact of the most common pathogens although a thorough risk assessment is required for spore-forming microbes.

Sentence on AI used:

No use of the any AI support.

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CRediT authorship contribution statement

Elena Franchitti: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Matilde Pedullà:** Formal analysis. **Anne Mette Madsen:** Writing – review & editing, Validation, Supervision, Methodology, Data curation. **Deborah Traversi:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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