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Management of milking wastewater as a significant control point of microbial pollution in the environment

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Abstract

Introduction and Objective. Milking wastewater (MW) is an under-recognised reservoir of antibiotic resistant bacteria (ARB) and antimicrobial resistance genes (ARGs). The aim of the study is to evaluate MW as a point source pollutant for the presence of ARB and ARGs, to develop a treatment plant, and to address current legislation.

Materials and Method. Milking wastewater was analysed for the total number of bacteria, *Enterococcus* spp., *Staphylococcus* spp., Enterobacterales, and *Clostridium perfringens*. Microbial identification by MALDI-TOF MS was followed by antimicrobial susceptibility testing and detection of ARGs. Wastewater samples were also tested for the antimicrobials presence using immunochromatographic tests. An on-site two-chamber MW treatment plant was designed with consideration of its cost-effectiveness and profitability. The system incorporated filtration through a mixture of fine sand and 10% kaolin, or a fine sand filter bed, yielding hydraulic conductivity coefficients of 5.13 × 10–6 m/s and 5.36 × 10–5 m/s, respectively. The treated effluent was considered suitable for activities not requiring tap water quality.

Results. *Escherichia coli* and *Enterococcus* spp. were identified as ARB. PCR confirmed the presence of ARGs conferring resistance to β-lactams (*cphA, bla*$_{GES1–9,11}$, *bla*$_{TEM1,2}$) and chloramphenicol (*cfr*). Milkling wastewater samples also tested positive for chloramphenicol.

Conclusions. To mitigate environmental contamination, regulations should be established for the release of bioactive substances, with penalties imposed for non-compliance with recommended safety measures.

Key words

bacteria, antibiotics, wastewater treatment, dairy wastewater, legislation, antimicrobial resistance genes.

INTRODUCTION

The European Food Safety Authority (EFSA) has reported an increasing trend in antimicrobial resistant infections and associated mortality in the EU, a pattern observed across all investigated combinations of bacterial species and antimicrobials [1]. From the One Health perspective – a collaborative approach integrating human, animal, and environmental health, and spread of antimicrobial resistance, is a complex issue that necessitates the involvement of experts from multiple disciplines [2]. The widespread use of antimicrobial agents for the treatment of both human and animal infections has led a situation in which antimicrobial resistance adversely affects both groups, imposing a significant economic burden. Given that the majority of antibiotics are used in agriculture, one of the primary objectives of the One Health action plan is to reduce antimicrobial usage and limit the dissemination of antimicrobial resistance genes originating from animal farming [3].

The food industry, including the dairy sector, is considered a major source of wastewater-related environmental pollution. Regulations governing wastewater treatment technologies are constantly evolving and becoming increasingly stringent and complex [4]. It is estimated that the dairy industry generates approximately 4 – 11 million tonnes of waste annually worldwide, posing a significant threat to biodiversity [5]. EU countries are the leading producers of large volumes of wastewater from milk processing; on average, a European dairy facility produces approximately 500 m^3/day. The composition and properties of dairy wastewater (DW), which includes wastewater generated during milking, on-farm cleaning and dairy processing, are shaped by multiple factors, the most significant of which include the scale of production, milk processing methods, process efficiency, cleaning protocols, the type of waste generated, and the economic

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costs associated with treatment. The primary components of DW include proteins, fats, dairy carbohydrates, nutrients, and cleaning agents [6]. Due to their origin, wastewater from the dairy industry harbours aerobic pathogenic microorganisms, such as *Escherichia coli*, *Salmonella* spp., and *Listeria monocytogenes,* as well as anaerobic species, such as *Clostridium perfringens*. *Giardia* and *Cryptosporidium oocysts*, parasites, and viruses may also be present. Some of these microbial species are part of the physiological intestinal microbiota of humans and animals [7].

The livestock sector is associated with the excessive use of antibiotics, contributing to the rise of antibiotic resistant microorganisms. This issue, coupled with the presence of antibiotics and antibiotic resistance genes (ARGs) in the environment, has become a major global public health concern due to the increasing difficulty in treating bacterial infections. The transmission of ARGs between bacterial species significantly exacerbates the spread of antibiotic resistance [8]. The most frequently detected ARGs in livestock waste are those conferring resistance to tetracyclines, sulfonamides, β-lactams, macrolides, lincosamides, streptogramin B, fluoroquinolones, quinolones, florfenicol, chloramphenicol, and amphenicols-categories, representing most antimicrobials used in livestock. Studies reveal significant variability in ARG prevalence, influenced by the geographic location of sampled areas, dosing patterns, and intensity of antimicrobial use, waste type and antibiotic concentrations, which exert distinct selective pressures on microbial communities. Notably, ARG abundance in cattle waste often exceeds that in human waste, likely due to higher residual concentrations of antimicrobials in livestock environments [9].

While various treatment strategies have demonstrated promise in laboratory and pilot-scale studies, there is a lack of evaluations using real-world dairy wastewater. It is important to conduct testing on samples derived from the environment to accurately assess the burden of antibiotic resistant bacteria (ARB) and ARG in dairy wastewater, and to design a treatment plant that performs under operational conditions typical for certain dairy production facilities [10].

Effective wastewater management resulting from dairy product processing therefore appears to be a key aspect of sustainable agricultural practices. The large volumes of dairy wastewater with unique and significantly variable characteristics can have a strong impact on the environment [11]. Consequently, their treatment process should guarantee the effectiveness of load reduction and be cheap and easy to apply on any dairy farm [12]. The dairy wastewater pre-treatment process is usually performed on-site, involving mechanical treatment/screens and water/oil separation [13]. Since, the demand for an environmentally friendly treatment solution is growing [14], the pre-treatment process is often supported with the use of natural coagulants, e.g. rice husk and its ash or activated charcoal [15]. After the pretreatment process, dairy wastewater can be discharged to the sewerage system, re-used (e.g. for land irrigation) after further treatment by using a combination of physicochemical and biological processes or membrane technologies, or finally discharged to the environment aquatic bodies [16]. In Poland, dairy wastewater is usually transferred to municipal wastewater treatment plants after pre-treatment, especially from smaller dairy plants [17].

The absence of legal regulations governing DW management represents a significant challenge [18]. Studies have shown that a farm-specific wastewater treatment plant can effectively reduce the release of pharmaceutical compounds and their metabolites into the environment, as well as decrease the load of ARB and ARGs originating from agricultural waste [19]. Moreover, the installation of on-site DW treatment plants creates opportunities for at least partial wastewater reuse within farm boundaries, similar to those of rainwater harvesting systems [20]. Several agricultural activities do not require tap water quality, including cleaning livestock buildings, washing and bedding mats for animals, washing vehicles, and cleaning paved surfaces. Thus, implementing such systems could enhance both environmental and economic sustainability by lowering water consumption, reducing costs, and mitigating anthropogenic pressure on the natural environment [21].

The aim of the study is to assess milking wastewater (MW) as a point source pollutant for the presence of ARB and ARGs, propose a treatment plant, and examine relevant legislation, particularly in the field of criminal law.

MATERIALS AND METHOD

Sample collection. The study was conducted on dairy farm located in the Subcarpathian Province in south-eastern Poland. The farm housed a herd of 200 cows, while the building where the samples were taken contained approximately 80 cows, milked using an automated milking system. Milking wastewater samples were collected from the farm sewer at eight time points during milking. The samples were collected in sterile containers under aseptic conditions, and transported under refrigerated conditions (2 – 8 °C) to the Department of Animal Hygiene and Environmental Hazards laboratory at the University of Life Sciences in Lublin, eastern Poland. Initially, each of the eight samples was analysed separately for the presence of antibiotic residues. Subsequently, the samples were pooled for microbiological analysis.

Determination of presence of antibiotics in milking wastewater. The presence of antimicrobials in MW was determined using immunochromatographic tests (4Sensor Test; NUSCANA, Poland), which enable the detection of β-lactams, tetracyclines, chloramphenicol and streptomycin. The test can detect the presence of the following antibiotics at concentrations equal to or below the maximum residue limit (MRL) for milk samples: penicillin G, ampicillin, amoxicillin, oxacillin, cloxacillin, dicloxacillin, nafcillin, cefquinome, cefacetrile, cefalonium, cefazolin, cefoperazone, cefapirin, ceftiofur, tetracycline, doxycycline, oxytetracycline, chlortetracycline, streptomycin, dihydrostreptomycin, and chloramphenicol. In brief, the sample was placed in a sterile container until it reached room temperature and then thoroughly mixed. 200 µL of the sample was added to a test well containing lyophilized reagents, after which the mixture was stirred with an automated pipette and left to stand for 3 minutes. An appropriate test strip was then placed in the well, and after 7 minutes, the results were interpreted according to the manufacturer's instructions. The presence of certain antibiotic was indicated by the appearance of 2 visible coloured lines: one in the appropriate test line region, and one in the control line region.

Microbiological assays. Quantitative analysis of MW was conducted by adding a 20 mL sample to 180 mL of Ringer's solution in a sterile bottle, followed by decimal dilutions. Then, 100 µL of each solution was plated on microbiological media in 2 replicates using the spread-plate technique. The following parameters were determined:

- total number of aerobic mesophilic bacteria on Agar Medium (BTL Ltd., Poland) incubated for 48 hours at 37 °C under aerobic conditions;
- total number of *Enterococcus* spp. on Bile Esculin Agar (BTL Ltd., Poland) incubated under aerobic conditions for 24 hours at 37 °C;
- total number of coagulase-positive *Staphylococcus* spp. on Baird-Parker Agar (BTL Ltd., Poland) incubated for 48 hours at 37 °C under aerobic conditions;
- total number of Gram-negative enteric bacteria on MacConkey Agar (BTL Ltd., Poland) incubated for 24 hours at 37 °C under aerobic conditions;
- total number of coliform bacteria on m-Faekal Coliform (mFC), (BTL Ltd., Poland) incubated under aerobic conditions for 24 hours at 44 °C;
- total number of *Clostridium perfringens* on Tryptose Sulphite Cycloserine Agar (TSC), (Biomerieux Ltd., Poland) incubated for 48 h at 37 °C under anaerobic atmosphere (GENbag anaer; Biomerieux Ltd., Poland).

All parameters were investigated in triplicate. After incubation, colonies were counted by using an automated colony counter (Scan 300; Interscience, France) according to the Polish standard [22] in the authors' modification. The results were expressed as colony forming units per 1 mL of samples (CFU/mL).

For microbial identification, colonies exhibiting distinct morphological characteristics were isolated from selective microbiological media. Colonies were selected if their morphology (e.g., shape, colour, margin) aligned with the bacterial groups targeted by the media. To ensure purity, colonies were subcultured using the streak plate method, followed by incubation under standardized conditions. Single, well-isolated colonies were then subjected to DNA isolation for molecular analysis. Isolates were identified with biochemical methods using a semi-automated Vitek 2 Compact system and dedicated ID cards (Biomerieux, France). Additionally, to confirm biochemical identification, isolates were identified with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), as described previously [23].

After identification, antimicrobial resistance was tested for clinically-relevant bacterial species using the disc diffusion method and/or specific AST cards for the Vitek 2 Compact System (AST-P644, AST-N331, AST-N332; Biomerieux, France). All antimicrobial resistance testing was performed and interpreted according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), [24], or the guidelines of the Clinical and Laboratory Standards Institute (CLSI), [25], if the former were not available.

DNA isolation and detection of antibiotic resistance genes. DNA of Gram-negative bacteria was isolated by the modified boiling method [26] and Gram-positive bacteria by using the spin-column method, according to the manufacturer's instructions (Genomic Mini AX Bacteria Spin; A&A Biotechnology, Poland).

A set of PCR reactions were performed to identify various ARGs encoding resistance to β-lactams (bla_{TEM1-2}, $bla_{CTX-M-1,3,15}$, $bla_{GES-1,9,11}$, $bla_{KPC-1,5}$, $bla_{DHA-1,2}$, $bla_{OXA-1,4,30}$), tetracyclines (*tetA, tetB*), chloramphenicol-florfenicol (*floR, catA1, fexA, cfr*) and streptomycin (*aadA1*, *aadA*, *aadE*) for all bacterial isolates, using the primers and PCR conditions listed in Supplementary Table S1 online. The ARGs analysed in this study were selected for 2 primary reasons: the high clinical significance of β-lactam antibiotics and chloramphenicol, and alignment with the antibiotic profile detectable by the immunochromatographic tests employed in the authors' research. β-lactam resistance genes were prioritized due to their widespread clinical relevance, while chloramphenicol resistance genes were included due to their persistence in agricultural and environmental settings, despite regulatory restrictions.

Reactions were performed in a total volume of 15 µL, including 2.5 µL template DNA, 1 µL of 10 µM each primer, 7.5 µL REDTaq® ReadyMix™ PCR Reaction Mix (SigmaAldrich, USA), and 3 µL of RNase-free water (EURx, Poland). Then, 1.5% agarose gels were used for electrophoresis and Quantum ST5 Xpress v 16.08g (Vilber Lourmat, France) software was used for gel visualisation, documentation, and archiving. Methicillin-resistant *Staphylococcus aureus* ATCC BAA-1707, *Aeromonas veronii* DSM 7386, extended-spectrum β-lactamase producing, and AmpC-positive *Enterobacter cloaceae* were used as positive controls.

Milking wastewater treatment plant. A preliminary conceptual MW treatment system was designed, consisting of an existing septic tank with an active capacity of approx. 5.76 m^3 and 2 concrete settling tanks, each with a diameter of 1.0 m, connected in series. The existing septic tank was intended as a buffer in the event of increased sewage inflow. In the first sedimentation tank, a mixture of sand and kaolin in a mass ratio of 10:1 was used as filling. The sand and kaolin 10% mixture ratio was selected as a result of literature reports and a previous study by the authors concerning the influence of the addition of clay minerals to locally available soils on stabilization, hydraulic characteristics, and pollutants removal abilities [27–31]. Kaolin was selected as an admixture to sand filtration bed due to its frequently reported ability to adsorb pollutants from infiltrated water. Its application would also allow a decrease in the permeability of the sand, resulting in the prolonged duration of wastewater flow through the bed [32]. The height of the filter layer in the first settling tank was assumed to be 0.70 m. Only sand with a filter layer height of 0.50 m was used in the second settling tank as a filling. Additionally, an emergency bypass of the treatment system was designed with a PVC pipe with a diameter of 200 × 5.9 mm and a length of L = 8.0 m. Ozonation of sewage in the first settling tank was proposed as additional equipment. A TRITLEN TR-10 mobile ozonator (GANDEL, Poland) with a 10,000 mg/h capacity was selected. The ozonation of sewage in both sedimentation tanks was assumed once a month for 30 minutes. For this purpose, a connection to the ozonator was designed in the settling tank chamber. The pipe connecting the existing tank with the settling tank was located 2.0 m below ground level. The planned retention time of the sewage in the septic tank is approximately 11.5 days. The existing tank should have a separation partition installed to stop floating fats.

Values of the coefficient of saturated hydraulic conductivity (Ks) of sand, and sand with a 10% kaolin mixture, were

determined under laboratory conditions according to ASTM D5084–24 [33] and ASTM D2434–22 [34] standards, using falling head permeameter HM-5891A (Humboldt Mfg. Co., USA).

Taking into account the determined values of the filtration coefficient for sand (Ks = 5.36E-05 m/s) and the sand-kaolin mixture (Ks = 5.13E-06 m/s), the designed maximum time of sewage flow through the settling tank filled with a mixture of sand and kaolin – approx. 10 days, designed time of sewage flow through the settling tank filled with sand – approx. 12 h (with an assumed hydraulic drop of up to 0.2). Figure 1 presents the scheme of assured design of wastewater treatment plant.

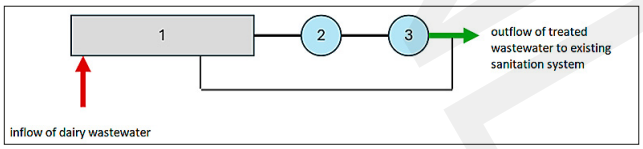

Figure 1. Scheme of assumed wastewater treatment plant. 1 – existing septic tank 3.2 × 1.0 × 2.69 m, Vc = 8.6 m³; 2 – designed concrete tank, cylindrical 1.0 × 2.0 m, Vc = 1.57 m³, filling: sand + kaolin, mass ratio 10:1; 3 – designed concrete tank, cylindrical 1.0 × 2.0 m, Vc = 1.57 m³, filling: sand

As an alternative solution in the technological project MW treatment plant, a system was designed to allow the re-use of treated wastewater for economic purposes instead of tap water in heavy-duty cleaning activities. For this purpose, a concrete tank was designed with a total capacity of V_c = 2.0 m³ and dimensions (length × width × height) of 2.40 m × 1.10 m × 1.25 m, storing treated sewage, equipped with a submersible pump WQ 3-18-0.55 230V (OMNIGENA Michał Kochanowski i Wspólnicy G.P., Poland) for dirty water, with a power consumption of 0.55 kW, efficiency up to 100 dm³/min and a maximum water lifting height of 23 metres, factory-adapted to work with flexible hoses. The tank will be connected to the existing sewage network by a PVC 200 pipe. A flexible, 50 m long pipe was also selected, enabling the use of water at points distant from the reservoir. Treated sewage was assumed to be used for washing bedding mats, barn floors, walls, wash vehicles, and paved surfaces (access roads).

The following components of water demand, allowing to determine the 177.65 m³/year annual value of re-used water, were included in calculations:

- washing bedding mats once every 2 days – 25 dm³/d;
- washing the surface of floors and walls of the cowshed once a year – 1 dm³/m² (area of walls and floors of the cowshed 748 m²);
- cleaning vehicles 4 times a month – 300 dm³/(washing and vehicle), (2 vehicles accepted);
- washing paved surfaces 4 times a month – 2.5 dm³/(washing and m²), (paved surface area 1158 m²).

Considering the presented requirements for the preliminary treatment of wastewater, the proposed solution is characterized by low costs and application of natural materials. It enables the treatment of wastewater with varying load and fluctuating flow rates. Additionally, the solution does not require an electrical power supply, except for the periodic operation of the ozonator. Due to the small space requirements, it can be successfully implemented in most dairy plants, as well as also being flexible for expansion and adaptation to local conditions.

Economic analysis method. Cost-efficiency of the designed wastewater treatment plant was based on the calculated Dynamic Generation Cost (DGC) indicator:

$$DGC = p_{EE} = \frac{\sum_0^{t=n} \frac{IC_t + EC_t}{(1+i)^t}}{\sum_0^{t=n} \frac{EE_t}{(1+i)^t}}$$

where: DGC – dynamic generation cost, IC_t – annual investment costs (PLN), EC_t – mean annual operation and maintenance costs (PLN), ^{t} – year of investment operation, from 0 – n (years), i – discount rate (%), p_{EE} – unit cost of ecological effect (PLN/m³), $^{EE}_t$ – annual ecological effect (m³).

The following assumptions were made for DGC calculations:

- investment costs incurred in full in the first year – 13,888.08 PLN;
- average annual operating costs, including cost of electricity, servicing, and replacing pumps, rubber hose, filter bed (every 5 years), and the ozonator (every 10 years) – 317.41 PLN;
- annual ecological effect – 177.65 m³/year;
- investment useful life – 30 years;
- discount rate i – 5%.

Analysis of the economic effectiveness (profitability) of the assessed investment variant was carried out based on the calculated benefit-cost ratio (BCR) indicator value, determining the value of the ratio of investment profits to the costs incurred. The value of the BCR ratio for a profitable investment should be BCR ≥ 1.0. The BCR indicator was determined using the formula:

$$BCR = \frac{PV_b}{PV_c} = \frac{\sum_{t=0}^{n} \frac{CF_{bt}}{(1+i)^t}}{\sum_{t=0}^{n} \frac{CT_{ct}}{(1+i)^t}}$$

where: BCR – benefit-cost ratio, PV_b – value of discounted investment profits (PLN), PV_c – value of discounted investment costs (PLN), CF_{bt} – flow of financial profits for year t (PLN), CF_{ct} – flow of investment costs for year t (PLN).

To determine the possible profits of the investment, it was necessary to determine the value of possible profits/savings in the analysed project. In the analysed case, the possible profits included financial savings resulting from reduced by 177.65 m³/year consumption of tap water for the demands presented above.

The value of the annual investment profits was determined based on:

- estimated water demand – 177.65 m³/year;
- value of the local water tariff GW14 – 4.56 PLN/m³ + VAT [35];
- value of the local sewage tariff G9S – PLN 12.71/m³ + VAT [35].

The determined annual value of the investment profits was determined as equal to 3,313.43 PLN. Additionally, for the same assumptions (calculated investment costs and possible profits) the simple payback period (PP) was determined according to the formula:

$$PP = \frac{IC}{NCF}$$

where: *PP* – payback period (years), *IC* – initial investment costs (PLN), *NCF* – net cash flow (PLN/year).

Analysis of legal regulations. An analysis was conducted of the currently applicable legal regulations relevant to environmental contamination caused by cattle farming. The legal Acts examined included, among others, the Criminal Code (*Journal of Laws* of 2024, Pos. 17), [36], Code of Petty Offences (*Journal of Laws* of 2023, Pos. 2199), [37], Act of 7 December 2023 – Environmental Protection Law (*Journal of Laws* of 2024, Pos. 54), [38], Act of 10 July 2024 – Water Law (*Journal of Laws* of 2024, Pos. 1087), [39] and the Act of 7 June 2001 on Collective Water Supply and Collective Sewage Disposal (*Journal of Laws* of 2001, No. 72, Pos. 747), [40].

RESULTS

Presence of antimicrobials in milking wastewater. The MW samples were analysed for the presence of 4 classes of antimicrobial drugs: β-lactams, tetracyclines, chloramphenicol, and streptomycin. Analysis revealed chloramphenicol in 2 out of 8 samples collected during the milking process, while no traces of other antimicrobials were detected. According to the manufacturer's specifications, the detection limit for chloramphenicol was 0.3 µg/L, indicating that the positive samples contained chloramphenicol at concentrations ≥ 0.3 µg/L.

Presence of antibiotic resistant bacteria and antibiotic resistance genes in milking wastewater. A quantitative microbiological analysis of the MW was carried out as part of the study, including the determination of a total number of aerobic mesophilic bacteria, faecal streptococci, coagulase-positive staphylococci, enterobacteria, coliforms, and *C. perfringens* bacteria. Analysis revealed a significant level of contamination by various microbial groups, ranging from 1.8×10^4 CFU/mL for *C. perfringens* to 1.1×10^7 CFU/mL for aerobic mesophilic bacteria. No growth of *Enterococcus* sp. was observed (Tab. 1).

Table 1. Microbiological contamination of milking wastewater (MW)

Parameter	Result (CFU/mL)
Total number of aerobic mesophilic bacteria	1.1×10^7
Total number of *Enterococcus* spp.	0
Total number of coagulase-positive *Staphylococcus* spp.	3.9×10^4
Total number of Gram-negative enteric bacteria	5.8×10^4
Total number of coliform bacteria	2.3×10^5
Total number of *C. perfringens*	1.8×10^4

CFU/mL – colony forming units per 1 mL of sample.

After enumeration, the characteristic bacterial colonies were isolated and identified. The most relevant species identified are shown in Tables 2 and 3, alongside their phenotypic and genotypic antimicrobial resistance profiles. Gram-negative rods dominated, including genera such as *Serratia*, *Yersinia*, *Aeromonas*, *Citrobacter*, *Raoultella*, *Hafnia,* and *Escherichia*. Among Gram-positive cocci, numerous *Staphylococcus* spp. were isolated, including coagulase-positive *S. pseudintermedius*.

Despite visible growth on Bile Esculin Agar characteristic of *Enterococcus* spp. (dark brown to black medium around bacterial colonies), all investigated colonies were identified by MALDI-TOF-MS and classified as *Lactococcus lactis*. Other identified microorganisms were represented by typically environmental species, such as *Corynebacterium xerosus* and *Aerococcus viridans,* and were excluded from antimicrobial susceptibility testing.

Following identification, microorganisms with potential clinical relevance were assessed for their susceptibility profiles to antimicrobials commonly used in the treating bacterial infections (Tab. 2, Tab. 3). Additionally, *Staphylococcus* spp. and all Gram-negative rods (including Enterobacterales and *Aeromonas* spp.) were screened for the presence of ARGs encoding resistance to beta-lactams, tetracyclines, chloramphenicol-florfenicol and/or streptomycin.

The analysis demonstrated that most of the Gram-positive and Gram-negative bacteria detected in the MW were susceptible to antimicrobials. None of the *Staphylococcus* spp. isolates exhibited resistance to the tested antimicrobials; however, presence of the *cfr* gene, which encodes a methyltransferase and confers resistance to 5 classes of antibiotics (phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A), was noted for *S. haemolyticus*. The observed phenotype-genotype discrepancy may be explained by the presence of a cryptic ARG harbouring mutation that prevents its expression. Notably, multidrug resistance (MDR) was identified in *L. lactis* isolated from DW. The isolate exhibited resistance to 3 classes of antimicrobials: oxacillin (MIC = 1 µg/ml), rifampicin (MIC>2 µg/ml), and sulfamethoxazole/trimethoprim (MIC = 160 µg/ml). Among Gram-negative bacteria, antibiotic resistance was observed in *A. veronii* and *E. coli*. The *E. coli* isolate was resistant to amoxicillin (MIC = 16 µg/ml), but none of the investigated resistance genes were detected, whereas one *A. veronii* isolate exhibited resistance to tetracycline and harboured multiple ARGs: *cphA*, $bla_{GES\text{-}1,9,11}$, $bla_{TEM\text{-}1,2}$ genes, encoding for TEM-type beta-lactamase and extended-spectrum β-lactamases, including carbapenem-hydrolyzing metallo-beta-lactamase and GES-type carbapenemase. Exemplary gel electrophoresis results are shown in Figure 2. Intrinsic antibiotic resistance was observed in multiple bacterial species, including resistance to amoxicillin in *Hafnia* spp., and to cefuroxim in *Serratia* spp. and *Citrobacter* spp.

Economic analysis of treatment plant. The calculated value of the cost-efficiency indicator DGC for the designed system of treated MW reuse was equal to 4.86 PLN/m^3. Considering the values of the local water and sanitation fees GW 14 (4.56 PLN/m^3 + VAT) and G9S (12.71 PLN/m^3 + VAT) the proposed solution appears to be both attractive and cost-effective. The calculated value of the BCR indicator of economic profitability was equal to 2.843 (-). The calculated value of the simple Payback Period indicator with the same assumptions was determined as equal to 6.74 years.

Analysis of legal regulations. There are currently no specific legal regulations governing the disposal of milk contaminated with antibiotics into the environment. Instead, general provisions on environmental protection and wastewater management are applicable in such cases. The general

Table 2. Results of phenotypic and genotypic antimicrobial resistance for chosen Gram-positive bacteria isolated from milking wastewater (MW)

ID (No. of isolates)	Resistance phenotype	OXA	AMK	GEN	E	CC	LZD	DAP	TEI	VAN	TET	TIG	RIF	SXT	CIP	LEV	C	ARGs
		MIC (µg/ml)													IZ (mm)			
Staphylococcus chromogenes (2)	-	≤0.25	≤2	≤0.5	0.5–1	0.25	2	≤0.12	1–2	≤0.5	≤1	≤0.12	≤0.03	≤10	30	30–31	24–26	
S. pseudintermedius (1)	-	≤0.25	≤2	≤0.5	1	≤0.12	2	≤0.12	2	≤0.5	≤1	≤0.12	≤0.03	≤10	33	31	25	
S. haemolyticus (1)	-	≤0.25	≤2	≤0.5	0.5	≤0.12	1	≤0.12	2	≤0.5	≤1	≤0.12	≤0.03	≤10	30	31	28	*cfr*
S. schleiferi (1)	-	≤0.25	≤2	≤0.5	0.5	≤0.12	1	≤0.12	2	≤0.5	≤1	≤0.12	≤0.03	≤10	30	31	28	
S. sciuri (1)	-	0.5	≤2	≤0.5	0.5	0.5	2	≤0.12	1	≤0.5	≤1	≤0.12	≤0.03	≤10	24	25	27	
Lactococcus lactis (1)	OXA, RIF, SXT	1	≤2	≤0.5	≤0.25	0.25	2	≤0.12	1	≤0.5	≤1	≤0.12	>2	160	nd			

OXA – oxacillin; AMK – amikacin; GEN – gentamycin; E – erythromycin; CC – clindamycin; LZD – linezolid; DAP – daptomycin; TEI – teicoplanin; VAN – vancomycin; TET – tetracycline; TIG – tigecycline; RIF – rifampicin; SXT – sulphamethoxazole/trimethoprim; CIP – ciprofloxacin; LEV – levofloxacin; C – chloramphenicol; ARGs – antibiotic resistance genes; MIC – minimum inhibitory concentration; IZ – inhibition zone; nd – not determined.

Table 3. Results on phenotypic and genotypic antimicrobial resistance for chosen Gram-negative bacteria isolated from milking wastewater (MW)

ID (No. of isolates)	Resistance phenotype	AMC	PIP/TAZ	CRX	CTX	CAZ	FEP	IPM	MER	AMK	GEN	TOB	CIP	TIG	SXT	TET	C	ARGs
		MIC (µg/ml)														IZ (mm)		
Serratia fonticola (4)	-	≤2	≤4	>32	≤0.25	≤0.12	≤0.12	≤0.25	≤0.25	≤2	≤1	≤1	≤0.25	≤0.5	≤20	21–22	25–27	
Yersinia intermedia (3)	-	≤2	≤4	≤1	≤0.25	≤0.12	≤0.12	≤0.25	≤0.25	≤2	≤1	≤1	≤0.25	≤0.5	≤20	26–27	24–26	
Aeromonas veronii (2)	TET	nd														12; 21	24–26	*cphA*, $bla_{GES-1,9,11}$, $bla_{TEM-1,2}$
Citrobacter braakii (1)	-	≤2	≤4	4	≤0.25	≤0.12	≤0.12	≤0.25	≤0.25	≤2	≤1	≤1	≤0.25	≤0.5	≤20	21	24	
Raoultella planticola (1)	-	≤2	≤4	≤1	≤0.25	≤0.12	≤0.12	≤0.25	≤0.25	≤2	≤1	≤1	≤0.25	≤0.5	≤20	26	27	
Hafnia terrigena (1)	-	8	8	≤1	≤0.25	≤0.12	≤0.12	≤0.25	≤0.25	≤2	≤1	≤1	≤0.25	≤0.5	≤20	25	24	
Escherichia coli (1)	AMC	16	≤4	4	≤0.25	≤0.25	≤0.12	≤0.25	≤0.25	≤2	≤1	≤1	≤0.25	≤0.5	≤20	19	22	

AMC – amoxicillin; PIP/TAZ – piperacillin/tazobactam; CRX – cefuroxime; CTX – cefotaxime; CAZ – ceftazidime; FEP – cefepime; IPM – imipenem; MER – meropenem; AMK – amikacin; GEN – gentamycin; TOB – tobramycin; CIP – ciprofloxacin; TIG – tigecycline; SXT – sulphamethoxazole/trimethoprim; TET – tetracycline; C – chloramphenicol; MIC – minimum inhibitory concentration; IZ – inhibition zone; nd – not determined (both investigated isolates did not reach adequate growth in control well of AST card after recommended period)

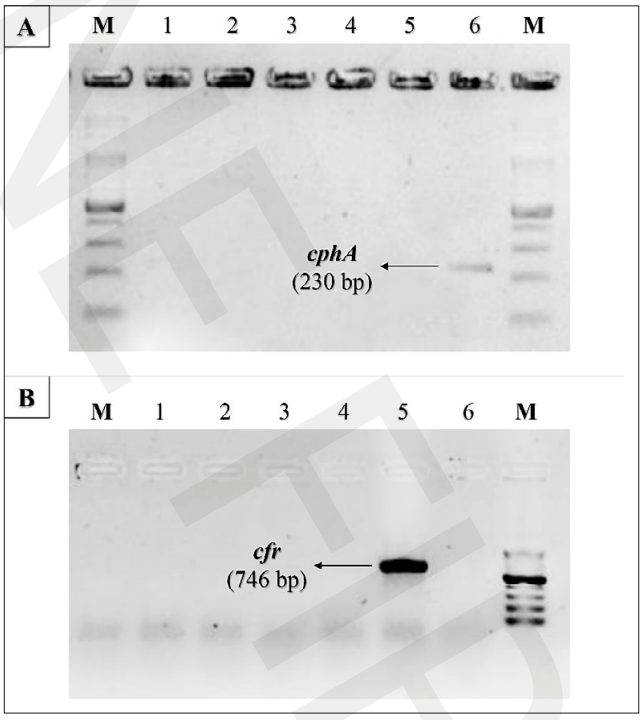

Figure 2. Agarose gel electrophoresis of PCR products. (A) Presence of *cphA* gene (230 bp) in *Aeromonas veronii* isolate (100 bp DNA Ladder Plus, ThermoScientific, USA). (B) Presence of *cfr* gene (746 bp) in *Staphylococcus haemolyticus* isolate (Perfect 100–1000 bp DNA Ladder, EURx, Poland). M – molecular marker

framework of proper practices in this area is established by Directive 2010/75/EU of the European Parliament and of the Council on Industrial Emissions (Integrated Pollution Prevention And Control), [41], which was amended by Directive (EU) 2024/1785 of the European Parliament and of the Council of 24 April 2024, amending Directive 2010/75/EU and Council Directive 1999/31/EC on landfill waste [42]. The amended directive is due to come into force by 1 July 2026.

Regarding the Polish legal framework, key regulations include the aforementioned Act of 7 June 2001 on Collective Water Supply And Collective Wastewater Disposal [40], and the Water Law Act of 20 July 2017 [43], and the Environmental Protection Law Act of 27 April 2001 [44]. Article 6 of the latter introduces 2 fundamental principles for environmental protection:

1) any entity engaging in an activity that may harm the environment is required to prevent such impacts;
2) any entity engaging in an activity whose negative impact on the environment is not yet fully understood must, guided by the principle of precaution, implement all possible preventive measures.

Discharging DW into the environment in violation of general legal regulations can result in liability for several offences outlined in the aforementioned legislation. In particular, Article 28 of the Act on Collective Water Supply and Collective Sewage Disposal [40] is likely to be violated, as it criminalizes non-compliance with the prohibitions in

the Act on sewage management. Additionally, Articles 477 and 478 of the Water Law [39] provide for offences related to the protection of water resources, including unlawful discharge of wastewater into water or soil. Furthermore, in cases of severe environmental contamination, provisions of the Criminal Code [36] may apply. However, in practice, it seems unlikely that a cattle farmer who introduces antibiotic-contaminated milk into the environment would meet the statutory criteria for these offenses. As a rule, the environmental impact of such an action would not be severe enough to constitute an offence under Chapter XXII of the Criminal Code, which generally requires substantial harm to plant or animal life, or significant environmental damage. For more detailed discussion of these legal thresholds, see the work by Sepioło-Jankowska [45]. Additionally, general liability under the Code on Petty Offences may also apply, for instance, Article 109 § 2 of the Code on Petty Offences addresses the contamination of water intended for animal consumption, while Article 162 § 1 of the same Act pertains to soil or water contamination in forested areas [37].

Currently, there are no specific regulations governing the introduction of DW into the environment, despite evidence from studies indicating its potential environmental hazards.

DISCUSSION

In the EU, antibiotic treatment of lactating cows most commonly involves β-lactam antibiotics, such as penicillins and cephalosporins [1]. Compared to other wastewater types discharged from animal farms, DW has been less frequently studied with respect to its microbiological parameters, particularly the presence of ARB and ARGs. Additionally, the prevalence of ARGs in farm waste varies depending on multiple factors, including the extent of antibiotic usage, dosing patterns, and national legislation making it specific to each farm environment [46]. The current study identified the presence of multiple ARGs, including those conferring resistance to chloramphenicol-florfenicol (*cfr*) and β-lactams (*cphA,* $bla_{GES-1,9,11}$, $bla_{TEM-1,2}$) The *cfr* gene was detected in an *S. haemolyticus* isolate, which encodes resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A, including linezolid – a last resort antibiotic used for treating infections caused by multidrug resistant Gram-positive cocci [47].

Currently, the main concern relates to the possible transmission of *cfr*-positive, LA-MRSA (livestock-associated methicillin-resistant *S. aureus*) strains to humans; however, there are increasing reports of *cfr*-positive, coagulase-negative staphylococci in animals [48]. There are only a few reports on the isolation of *S. haemolyticus* harbouring *cfr* gene since the first report in 2009; nevertheless, these strains have gained more clinical importance as in 2021 they were already isolated as an etiological factor of human laryngological infections in Poland [49]. Analysis in the current study also identified the presence of amoxicillin resistant *E. coli,* as well as genes conferring β-lactam resistance in *A. veronii.* The presence of antibiotic resistant *E. coli* in DW has been previously investigated. For instance, Liu et al. found that *E. coli* isolated from wastewater on a dairy farm in China were mostly resistant to β-lactams and tetracyclines. Moreover, the authors concluded that the over-use of cephalosporins and tetracyclines in the study area was the main cause of the development of bacterial resistance [8]. *Aeromonas* spp. are Gram-negative rods commonly isolated from environmental sources, including wastewater, and are capable of withstanding a wide range of environmental stressors. These bacteria are widespread in both human and animal habitats and readily acquire antibiotic resistance, particularly to β-lactams, thus serving as a significant ecological reservoir of ARGs [50]. Notably, the only microorganism isolated in the current study that was classified as MDR, was *Lactococcus lactis*, which exhibited resistance to oxacillin, rifampicin, and trimethoprim-sulfamethoxazole. Previous studies demonstrated that in *L. lactis,* resistance is most often caused by the presence of multidrug transporters, such as the LmrCD transporter, which enhances the ability of the bacterial cell to extrude antimicrobial agents [51].

Despite the limited research on the resistance profile of *L. lactis,* resistance to rifampicin and trimethoprim-sulfamethoxazole has been previously documented in isolates derived from the human oral cavity and sheep's milk, respectively [52]. The level of microbial contamination in wastewater generated during milking is influenced by numerous factors, such as the content of organic matter – including fats, proteins, and lactose – which provides nutrients for microbial growth, as well as the introduction of pathogens from milk or manure, and the types of cleaning processes applied on a farm. Another important factor is the disposal of antimicrobials which, when present in the wastewater, facilitates the selection of ARB. Additionally, variations in production methods, wastewater temperature, and pH fluctuations, create conditions that can either suppress or promote microbial growth [53–55].

The presence of ARGs from dairy wastewater poses a threat to public health. ARB or free ARGs can spread across aquatic systems, terrestrial environments, and as airborne particles, reaching resident bacterial communities through horizontal gene transfer (HGT) mechanisms, such as conjugation, transduction, and transformation. Once acquired by environmental bacteria, ARB can enter human populations through multiple exposure routes, including contaminated water, food, and aerosols, which can result in either immediate clinical infections or undetected colonization that persist asymptomatically and spreads within the human population [9].

According to the current legal regulations, cow's milk must be discarded during antibiotic treatment and throughout a specified withdrawal period. However, antibiotic residues persisting in wastewater may drive the selection and propagation of antibiotic resistance in microorganisms [56]. In the current study, trace amounts of chloramphenicol were detected in MW samples (≥0.3 μg/L). Despite its high toxicity, residues of chloramphenicol have still been detected in milk, most likely due to its unauthorized use or lack of adherence to withdrawal periods after treatment [57]. Under proposed environmental regulatory limits for antimicrobials, the predicted no-effect concentrations (PNECs) for chloramphenicol is 8 μg/L, representing the threshold concentration in wastewater, below which selection of resistant microorganisms is unlikely [58].

Compliance with appropriate withdrawal periods, routine cleaning of milking equipment, and the implementation of educational programmes for farmers, represent key preventive measures aimed at limiting the release of antibiotic

Supplementary Table S1. Specific primers and PCR conditions used for the assays

Genes	Primers sequences (5′ – 3′)	Amplicon size (bp)	Temperature-time conditions	Ref.
		Aeromonas spp.		
cphA	TCTATTTCGGGGCCAAGGG TCTCGGCCCAGTCGCTCTTCA	230	95 °C for 5 min, followed by 35 cycles of: 95 °C for 1 min 55 °C for 1 min 72 °C for 1 min a final extension step at 72 °C for 5 min.	[64]
$bla_{TEM-1,2}$	CATTTCCGTGTCGCCCTTATTC CGTTCATCCATAGTTGCCTGAC	800	94 °C for 10 min, followed by 35 cycles of: 94 °C for 1 min 60 °C for 1 min 72 °C for 1 min a final extension step at 72 °C for 7 min.	[65]
$bla_{CTX-M-1,3,15}$	TTAGGAARTGTGCCGCTGYA CGATATCGTTGGTGGTRCCAT	688		
$bla_{GES-1,9,11}$	AGTCGGCTAGACCGGAAAG TTTGTCCGTGCTCAGGAT	399	94 °C for 10 min, followed by 35 cycles of: 94 °C for 40s 55 °C for 45 s 72 °C for 1 min a final extension step at 72 °C for 7 min.	
$bla_{KPC-1,5}$	CATTCAAGGGCTTTCTTGCTGC ACGACGGCATAGTCATTTGC	538		
$bla_{DHA-1,2}$	GCTTTGACTCTTTCGGTATTCG CGGTAAAGCCGATGTTGCG	997	94 °C for 10 min, followed by 35 cycles of: 94 °C for 40s 60 °C for 40 s 72 °C for 1 min a final extension step at 72 °C for 7 min.	
floR	ATTGTCTTCACGGTGTCCGTTA CCGCGATGTCGTCGAACT	60	95 °C for 5 min, followed by 42 cycles of: 95 °C for 10 s 60 °C for 30 s 72 °C for 30 s a final extension step at 72 °C for 7 min	[66]
catA1	GGGTGAGTTTCACCAGTTTTGATT CACCTTGTCGCCTTGCGTATA	100		
tetA	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576	94 °C for 5 min followed by 35 cycles of: 94 °C for 30 s 50 °C for 40 s 72 °C for 45 s a final extension step at 72 °C for 7 min.	
tetB	AGTGCGCTTTGGATGCTGTA AGCCCCAGTAGCTCCTGTGA	62		
aadA1	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCAT	484	94 °C for 5 min followed by 35 cycles of: 94 °C for 30 s 54 °C for 40 s 72 °C for 45 s a final extension step at 72 °C for 7 min.	[66]
		Enterobacterales		
$bla_{TEM-1,2}$ and its variants	CATTTCCGTGTCGCCCTTATTC CGTTCATCCATAGTTGCCTGAC	800	98 °C for 2 min followed by 32 cycles of: 98 °C for 10 s 56 °C for 30 s 72 °C for 75 s a final extension step at 72 °C for 6 min.	[67]
$bla_{OXA-1,4,30}$	GGCACCAGATTCAACTTTCAAG GACCCCAAGTTTCCTGTAAGTG	564		
$bla_{CTX-M-1,3,15}$	TTAGGAARTGTGCCGCTGYA CGATATCGTTGGTGGTRCCAT	688	94 °C for 10 min followed by 32 cycles of: 94 °C for 40 s 60 °C for 40 s 72 °C for 75 s a final extension step at 72 °C for 6 min.	
catA1	AATAAGATCACTACCGGGCGT GCAACTGACTGAAATGCCTCA	150	95 °C for 5 min followed by 30 cycles of: 95 °C for 10 s 62 °C for 30 s 72 °C for 20 s a final extension step at 72 °C for 6 min.	[68]
floR	ATGGCTCCTTTCGACATCCT CAAGTAGAATTGGCCGTCGC	196		
		staphylococci		
fexA	GTACTTGTAGGTGCAATTACGGCTGA CGCATCTGAGTAGGACATAGCGTC	1272	94 °C for 1 min followed by 34 cycles of: 94 °C for 1 min 58/48 °C for 2 min 72 °C for 3 min a final extension step at 72 °C for 7 min.	[69]
cfr	TGAAGTATAAAGCAGGTTGGGAGTCA ACCATATAATTGACCACAAGCAGC	746		

residues into the environment. Additionally, MW treatment plants can be designed not only to reduce the release of ARB and ARGs, but also address additional objectives, including water reuse [57]. In the proposed DW treatment plant project, an ozonation step was included. Ozonation, as previously demonstrated, effectively reduces microbial load [59]. However, for ARB, lysed microbial cells can release ARGs located on mobile genetic elements (e.g., plasmids), posing a risk of post-disinfection 'rebound' resistance – a phenomenon that requires further investigation. Preliminary research on the efficacy of ozonation in reducing ARBs and ARGs loads indicates that 1) ARGs inactivation occurs

subsequent to bacterial cells lysis, and 2) the process may exhibit efficacy in complex wastewater matrices compared to controlled laboratory conditions [60]. Nevertheless, wastewater filtration systems employing sand filters combined with disinfection methods (e.g. ozonation), have been demonstrated to significantly reduce ARBs and ARGs loads, offering a viable solution for MW treatment on dairy farms [61]. Furthermore, economic analysis of the proposed treatment plant, suggest that the investment is economically viable, particularly when treated wastewater is re-used for non-potable applications. The relatively quick return on the incurred costs (PP = 6.74 years) and possible profits resulting from the implementation of the proposed solution may be an encouragement for other milk production companies wishing to modernize their farms. The calculated value of the BCR indicator equals 2.834 for the proposed sewage pretreatment system means that the assumed discounted savings in the assumed investment operation period constitute over 280% of the costs incurred in the same period, which means that the profits/savings outweigh the costs and the proposed solution is profitable. Due to the relatively small building area, the proposed sewage pre-treatment solution is easy to implement on virtually every farm that has a separate dairy sewage system.

Potential challenges related to the implementation of the proposed wastewater treatment solution include the possible high groundwater level in various locations, and the high fluctuation of wastewater inflow. A high groundwater level can cause uplift of the treatment plant elements, in which case the use concrete elements is recommended. In the case of a significant reduction in inflow, there is also a risk of the filter bed being exposed and drying out. Over time, a decrease in the permeability of the filter bed and the possibility of biofilm development are expected. Therefore, typical operational activities that must be carried out periodically are the replacement of the filter material and its disposal to a landfill. Additionally, it is recommended to periodically clean the ozonator nozzle outlets to avoid clogging.

If widely implemented, the proposed solution for treatment on dairy farms would substantially reduce environmental pressure and the occurrence of ARB. At this point, it would be advisable to advocate for the development and dissemination of a 'code of best practices' in this area. If the proposed solutions are broadly adopted, a further step could be to mandate the post-treatment handling of milk in this manner and, moreover, to classify it as a petty offence. Such measures would also fulfill the environmental protection obligations outlined in Directive 2010/75/EU of the European Parliament and of the Council of 24 November 2010 on industrial emissions (OJ L 334, 17.12.2010, pp. 17–119), [41].

Limitations of the study. The study presented a multidisciplinary analysis of MW as a point source of microbial pollution emission; however, several limitations that should be addressed. First, the detection of antimicrobial residues in the analysed samples relied on rapid immunochromatographic tests, which yield solely qualitative data and their use is accompanied with multiple limitations, including false negative results due to low antimicrobial concentration, the presence of interfering substances, such as cleaning agents and cross-reactivity, as well as false positive results arising from hydrophobic interactions of proteins with antibodies, and the reaction of antibodies with non-target compounds [62,63]. Second, the ARGs were detected in cultured bacterial species isolates, whereas direct detection of ARGs in the studied MW samples would likely have increased the probability of detecting ARGs.

CONCLUSIONS

The analysis confirmed that MW constitutes a significant source of ARB and ARGs, representing a notable vector of microbial environmental pollution. The preliminary conceptual framework proposing the re-use of treated MW for heavy-duty cleaning applications, demonstrated both cost-effectiveness and economic viability. Subsequent phases will involve laboratory-scale validation of MW treatment efficiency and the installation of pilot-scale treatment system. Future work should also focus on verifying the efficiency of treatment plants under operational conditions, and establishing a standardized panel of ARGs that can be directly detected in milking wastewater samples, bypassing culture-based methods and microbial identification. In addition, further work should be carried out to improve the operation of the proposed dairy wastewater pre-treatment system. Further activities should primarily include research on hydraulic characteristics and efficiency of purification of various mixtures (other mass fractions and other absorbents). The laboratory tests (model scale), allowing determination of the period of operation without the need for ozonation of the bed and the service life duration of the filter cartridge, are also required.

Extensive use of antimicrobials in livestock significantly contributes to the rise of antimicrobial resistance, leading to the spread of antibiotic-resistant pathogens in animal and human populations. Implementing stricter regulations on antibiotic use, especially in intensive farming, along with improved farming practices and enhanced wastewater treatment, can form an essential part of public health measures that help protect the efficacy of life-saving medicines for future generations.

Data availability. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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