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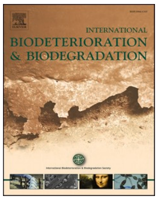

Penicillin and streptomycin in ethanol mist against spore-forming *Bacillus* bacteria isolated from surfaces of historical objects

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ABSTRACT

The objective of the study was to investigate the antimicrobial efficacy of ethanol mist enriched with penicillin and/or streptomycin and to examine its effects on the surface properties of model and historical textile materials from the collections of the Auschwitz-Birkenau State Museum (ABSM) in Oświęcim (Poland). *Bacillus* bacteria, which inhabited historical textile objects in the ABSM, were inoculated onto samples of textiles. Then, penicillin and/or streptomycin suspended in water or ethanol were applied in the form of mist. Sensitivity of the bacterial strains to the antibiotics was tested with disk diffusion (vegetative forms) and agar imprint (spores) methods. After that, surface alterations were analysed using SEM, confocal microscopy and XPS techniques. Even though initial effectiveness of presented disinfection method was observed, both for cells and spores, it resulted only in a temporary inhibition of the growth of tested bacteria. Importantly, subsequent analyses revealed that this treatment did not induce any detectable alterations in the surface morphology or chemistry of the textile materials. The developed method of applying antibiotics together with ethanol mist to increase effectiveness of ethanol against spore-forming bacteria is non-destructive and preserves the original structural and chemical integrity of historical fabric. However, the method has a biostatic effect on spore-forming *Bacillus*, not biocidal, so the addition of tested antibiotics does not allow the desired effect to be achieved. Nevertheless, ethanol in the form of mist without additives is biocidally effective against a wide range of microorganisms.

1. Introduction

The territory of Auschwitz-Birkenau, the former Nazi concentration and extermination camp in Oświęcim, is the most well-known site of martyrdom and genocide in the world. The camp has become a global symbol of the Holocaust, genocide and terror. The Nazis deported to Auschwitz at least 1,100,000 Jews from various European countries, nearly 150,000 Poles - primarily political prisoners - around 23,000 Roma from several European nations, 15,000 Soviet prisoners of war, and 25,000 prisoners of other nationalities, including Czech, French, Yugoslav, Russian, Belarusian, and Ukrainian (Świebocka and Świebocki, 2016). Since 1947, the Memorial Site has housed a museum that preserves the remains of the Auschwitz concentration camp, including numerous movable artifacts, such as: prisoners' personal belongings, camp equipment, items left behind by the SS, archival documents, testimonies of genocide and possessions stolen from the victims. Among these unique objects there are many made of textile materials, such as civilian and prisoner clothing, Jewish tallisim, as well as numerous multi-material objects containing textile components, such as: suitcases, shoes and prostheses (Fig. 1). These unique artifacts are inextricably linked to the lives and tragic fates of the victims of the Auschwitz concentration camp (Pantouvaki, 2014).

The conservators at the Auschwitz-Birkenau State Museum (ABSM) treat all traces of history, including many forms of soiling, as documents

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and evidence to be preserved during the conservation treatments. However, it is essential that such contaminants should not pose a threat to human health or to the historical object itself, nor accelerate the natural degradation processes of the materials (Papis and Jastrzębiowska, 2021).

Textiles, like most organic materials, are considered among the most sensitive to temperature, relative humidity, light and particulate pollution. This high sensitivity is closely related to their particular susceptibility to microbiological degradation (Tímar-Balázsy, 2011; Montegut et al., 1991). Numerous studies and inspections have shown that all types of textile fibres, whether in storage or on display in museums, are vulnerable to biodeterioration (Abdel-Kareem, 2010; Omar et al., 2019; Elmaarefa et al., 2020). Biodeterioration is a complex, multistage process caused by microorganisms, resulting in unwanted alterations in the physical properties of materials. In historical textiles, this may manifest as stains, deposits, structural damage and color changes. The microorganisms responsible for biodeterioration secrete extracellular enzymes (eg. cellulolytic and proteolytic enzymes), pigments and organic acids. This process involves two main mechanisms: assimilation, where the fibres serve as a nutrient source; and degradation, where the fabric is physically damaged by microbial growth and the release of metabolic by-products (Szostak-Kotowa, 2004). The biodeterioration of cotton, linen and jute is based on a degradation of cellulose to glucose by such enzymes as: 1,4-endo-b-D-glucan cellobiohydrolase, endo-1-4-b-D-glucan glucanohydrolase and glucohydrolase of b-D-glucosides. The mechanisms of keratynolysis such as sulfitolysis and proteolysis by peptidases and also deamination (oxidative metabolic processes with the release of ammonia) are responsible for the biodeterioration of wool. On the other hand, the biodeterioration of silk is based on proteolytic decomposition of sericin and fibroin (Gutarowska et al., 2016). Ongoing research is focused on developing disinfection methods that are both effective and safe for historical objects (Favaro, 2020).

Bacteria of the *Bacillus* genus play a significant role in the colonisation of museum collections worldwide (Chaudhary et al., 2019; Noohi and Papizadeh, 2023), including textile collections (Amin, 2018; Lee et al., 2007; Pangallo et al., 2013). They constitute the dominant bacterial genus found on the surfaces of movable objects at the ABSM made of textiles and leather (Wawrzyk et al., 2018; Rybitwa et al., 2020, 2022). Bacteria of this genus may cause biodeterioration of textile objects through their cellulolytic activity (Mazzoli et al., 2018). Moreover, some species may also have a negative impact on human health. For example, *Bacillus cereus* can cause food poisoning and, less commonly, wound or eye infections (Kamar et al., 2013). In immunocompromised individuals, the infections caused by *B. cereus* span a broad spectrum, including severe bacteremia, central nervous system infections such as meningitis and brain abscesses, endophthalmitis, pneumonia and skin infections resembling gas gangrene (Bottone, 2010). *Bacillus subtilis* is not commonly considered a human pathogen, however, it has been isolated on multiple occasions from immunocompromised people suffering from bacteremia, endocarditis, pneumonia and sepsis (United States Environmental Protection Agency, 1997). *Bacillus licheniformis* was associated several times with bacteremia in immunocompromised patients (Blue et al., 1995). There was also a recognised case of reccurent sepsis in immunocompetent patients caused by this species (Haydushka et al., 2012). Rare incidents of soft tissues infections and pulmonary alveolar proteinosis caused by *Bacillus megaterium* were also reported (Bocchi et al., 2020; Guo et al., 2024).

The team at the ABSM laboratory continuously conducts research aimed at identifying methods of disinfection that are safe for historical objects. In one of the initial studies, high effectiveness in disinfecting textile and cardboard objects using vaporised hydrogen peroxide (VHP) was demonstrated. However, this process requires a VHP generator, making it less practical for broader use by other museums (Wawrzyk et al., 2018). In the ABSM laboratory, nearly 100 % decontamination efficiency was achieved using a diode laser, with no damage to the surfaces of leather and cellulosic objects. However, the application of this method is limited to removing the effects of biodeterioration at its early stages (Rybitwa et al., 2020). One of the disinfection methods currently being studied at the ABSM laboratory is 90 % ethanol applied in the form of a mist (EM). So far, this method has demonstrated nearly 100 % effectiveness on textile and leather materials with no observed

Fig. 1. Historical textile objects from the ABSM: A - prisoner blouse, B - Jewish prayer garment (tallit), C - children's dress, D - camp number patch, E – children's slipper.

negative effects on their surfaces (Wawrzyk et al., 2023, 2024). However, as observed during numerous tests in the ABSM laboratory, this method is ineffective in eliminating spore-forming bacteria of the *Bacillus* genus. Therefore, a new approach combining the action of ethanol with antibiotics was proposed.

Penicillin and streptomycin are antibiotics with different mechanisms of action. Due to their synergistic effect, their combination is often used in treating infections or in preventing contamination in cell cultures. Penicillin disrupts the synthesis of the bacterial cell wall, which allows streptomycin to enter the cell and inhibit protein synthesis (Plotz and Davis, 1962). When analysing the susceptibility of *B. cereus* to various antibiotics, streptomycin was shown to be effective in eliminating this species (Yusuf et al., 2018). In the case of *B. subtilis*, its cell wall contains penicillin-binding proteins (PBPs). When these proteins bind penicillin, cell wall synthesis is stopped, ultimately leading to cell death (Mitchell et al., 2024).

Considering the literature data on the susceptibility of the tested bacteria to penicillin and streptomycin, in the ABSM laboratory the biocidal effectiveness of these antibiotics in combination with ethanol mist was tested. Since all substances used in the preservation of heritage objects, including disinfectants, must be safe for the treated objects, the proposed method was also examined for its potential impact on the physical and chemical properties of historical materials. Therefore, the aim of this study was to optimise a disinfection method using ethanol mist enriched with antibiotics, so that it would exhibit biocidal activity against spore-forming *Bacillus* species isolated from the surfaces of textile objects from the ABSM, without negative impact on the surface properties of these materials.

2. Materials

2.1. Textiles

The experimental study was performed on model and historical textile samples. Physicochemical tests were performed on both types of fabrics, while microbiological tests used only the model material due to the limited availability of historical samples. The model material was a contemporary undyed cotton fabric (SDC Enterprises Limited). The historical material was a fragment of cotton fabric, probably from a dress, found inside a historical shoe originating from the ABSM collections.

2.2. Bacterial strains

The study used a bacterial strain from American Type Culture Collection: *Bacillus subtilis* ATCC 6635, as well as environmental strains isolated from the surfaces of historical objects at ABSM: *Bacillus subtilis* ABSM, *Bacillus cereus* ABSM, *Bacillus licheniformis* ABSM and *Bacillus megaterium* ABSM. Environmental strains were obtained by taking swabs from textile objects showing signs of biodeterioration (Wawrzyk et al., 2023), isolating microorganisms from these samples and preparing suspensions. Suspensions of both the collection and environmental strains were used to inoculate the model and historical fabrics.

2.3. Ethanol and antibiotics

Two types of antibiotic preparations were tested: Penicillinum Procainicum L (TZF), which active substance is benzylpenicillin procaine lecithin (procaine penicillin G) and Crystalline Streptomycin Sulfate IM (Ibrahim Etem). Antibiotics were suspended in sterile distilled water or in 96 % ethyl alcohol (Chempur) depending on the research method (chapters 3.1–3.3).

3. Methods

A diagram showing the research action plan of optimisation of the disinfection process of historical objects using ethanol mist with antibiotics is shown in Fig. 2.

3.1. Antibiotic susceptibility testing by the disk diffusion method

The test was performed based on the EUCAST methodology, Antibiotic Susceptibility Testing by Disk Diffusion*,* Version 10.0 (January 2022). Bacteria were plated on solid Plate Count Agar (PCA) for determination of the total microbial count. Suspensions were prepared from grown cultures in a 0.85 % saline solution with 1 % peptone at a density of 0.5 McFarland standard. The density of all suspensions was verified prior to testing using the plating method on PCA medium and incubated for 24 h at 30 °C. To prepare a uniform suspension of *B. subtilis* bacteria, shaking with glass beads was used, followed by filtration through sterile cotton. Approximately 25 ml of PCA medium was poured into 9 cm diameter Petri dishes and left to solidify. The agar surface was inoculated with a swab dipped in the bacterial suspension and sterile filter paper disks with a diameter of 16 mm (surface area of 2.01 cm^2) were placed on the agar surface. Onto each disk, 0.04 ml of antibiotic water solutions at various concentrations ranging from 0.125 mg/l to 512 mg/l were applied (see Table S1, left part). The plates were incubated for 24 h at 35 °C. After incubation, bacterial growth and the presence of inhibition zones were assessed. The lowest concentration at which an inhibition zone was observed was considered the Minimum Inhibitory Concentration (MIC). Since the aim of this study was not clinical application (as in the EUCAST method), but the protection of museum textiles, more lenient criteria were adopted than those in the document, i.e., even the smallest inhibition zone was considered a positive result.

3.2. Determination of MIC for antibiotics against bacteria inoculated on model fabric using the agar imprint method

The study was conducted using an in-house method that preserves the presence of the antibiotic during the assessment of its effect on bacteria on the material. Bacteria were cultured on a solid medium for preparing *Bacillus* spores according to the PN-EN 1104:2019 standard, with the following composition: beef extract 3.0 g/l; casein peptone 5.0 g/l; sodium chloride 5.0 g/l; agar 12.0 g/l; $MnSO_4$ 10 mg/l. Plates were incubated for 5 days, after which bacterial suspensions were prepared in distilled water at a density of 0.5 McFarland and diluted to achieve a density above 1×10^6 Colony Forming Units per millilitre (CFU/ml). Model fabric samples were sterilised at 180–210 °C for 10 s. Onto a 3×3 cm piece of model fabric, 0.234 ml of bacterial suspensions in water were applied at the following densities: *B. subtilis* ABSM - 1.4×10^7 CFU/ml, *B. cereus* ABSM - 1.3×10^7 CFU/ml, *B. licheniformis* ABSM - 3.1×10^7 CFU/ml, *B. megaterium* ABSM - 9.1×10^6 CFU/ml. The fabrics were dried in a laminar flow cabinet. Next, 0.1791 ml of antibiotic solutions in 90 % ethanol and water, at various concentrations ranging from 0.5 mg/l to 4096 mg/l (see Table S1, right part), were applied to the samples using a pipette. Three variants of antibiotic mixtures in 90 % ethanol were also tested: 64 mg/l penicillin and 64 mg/l streptomycin (Mix1), 512 mg/l penicillin and 128 mg/l streptomycin (Mix2) and 4096 mg/l penicillin and 512 mg/l streptomycin (Mix3). The proportions of the mixtures were selected based on the results obtained using the disk diffusion method for individual antibiotics. Based on this, the lowest effective dose of antibiotics against all tested bacteria was selected separately for penicillin and streptomycin (Mix1 and Mix2). A Mix3 variant was also added, containing the antibiotics at the highest achievable concentrations. The volume of solution applied to the fabric was selected to ensure that the mass of antibiotic in μg/cm^2 was the same as in the disk diffusion method (Table S1). The samples were left to dry. After incubation at room temperature for approximately 24 h, bacteria were transferred from the fabric to agar medium by imprinting onto PCA agar. Cotton samples were placed on well-solidified PCA agar, covered with sterile polypropylene foil and a small Petri dish, and then weighted down. The same time and pressure were applied for each sample: 222 g

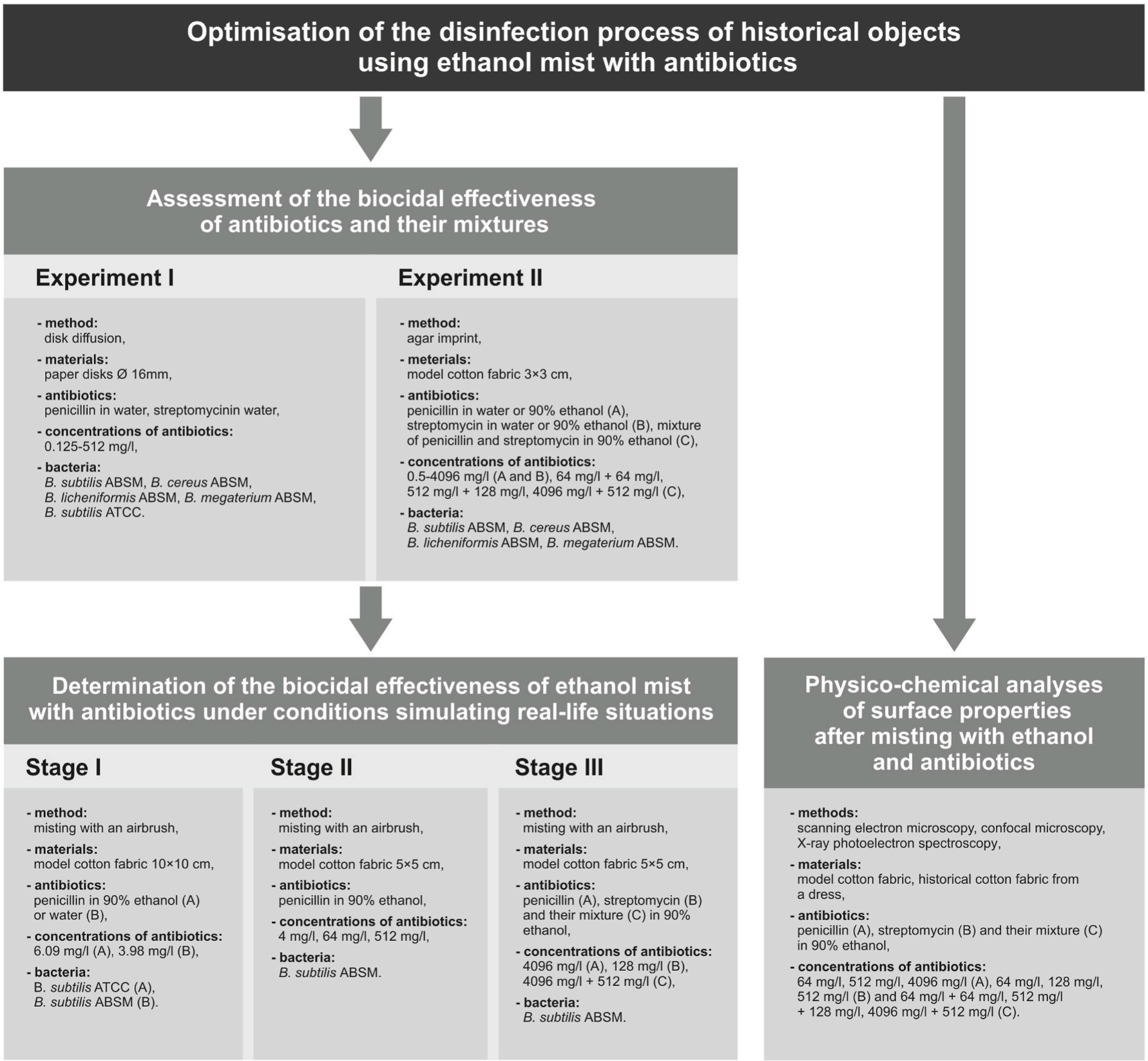

Fig. 2. Diagram of the research action plan.

for 60 s. The plates were then incubated for 24 h at 30 °C. After incubation, bacterial growth was observed on the agar at the imprint site and around it. The MIC was defined as the lowest antibiotic concentration at which no bacterial growth was observed at the fabric imprint site. Fabric samples inoculated with bacteria but without antibiotics were the controls.

3.3. *Determination of the biocidal effectiveness of ethanol mist (EM) with antibiotics applied on fabrics*

Bacteria were plated on solid PCA. Suspensions were prepared from grown cultures in sterile distilled water at a density of 0.5 McFarland. These suspensions were then diluted to achieve a density above 1×10^6 CFU/ml. The density of all suspensions before testing was verified using the plating method on PCA medium and incubated for 24 h at 30 °C. Samples of model fabric with dimensions of 50×50 mm and 100×100 mm were sterilised with UV light for 10 min on each side. Fabric samples were inoculated with microorganisms by applying 2.6 ml of the suspension per 100 cm^2 using a pipette (or proportionally less for smaller samples), then dried in a laminar flow cabinet until constant weight was achieved. Various antibiotic concentrations were used. Antibiotics in aqueous and alcoholic solutions were applied using an airbrush VE 0707, operating at a pressure of 0.2 MPa, equipped with a PA HEAD VLH-5 nozzle (tip diameter 1.05 mm). The mass of the applied solutions was recorded. The distance between the airbrush and the sample was 16 cm, and the application time was approximately 4 s per 100 cm^2. Prepared fabrics were placed in Petri dishes, tightly wrapped in polyethylene foil, and left at room temperature for about 22 h. Subsequently, microorganisms were washed from the fabric by shaking for 5 min at 250 rpm in 100 ml of peptone water containing 0.2 % Tween. Tenfold serial dilutions were performed. The number of bacteria was determined using the agar pour plate method on PCA medium. Incubation was carried out at 30 °C for 24 h. The number of microorganisms and reductions were calculated using the same equations as in previous studies (Guo et al., 2024). For the numbers of bacteria in the untreated and treated with EM with antibiotics samples, arithmetic means and standard deviations were calculated. Differences in the numbers of microorganisms were statistically analysed using one-way analysis of variance (ANOVA) and least significant difference (LSD) tests at a significance level of $p < 0.05$.

In the first test, the biocidal effectiveness of EM with penicillin against *B. subtilis* bacteria was evaluated. Solutions were prepared to match the amount applied in the disk diffusion method at a penicillin concentration of 1 mg/l, what corresponds to the inhibitory dose (MIC). The antibiotic concentration for mist application was determined so that the mass of antibiotic in μg/cm^2, when applied to fabric samples of 100 cm^2, was proportional to the amount applied to filter paper samples of 2.01 cm^2 in the disk diffusion method. The antibiotic solution concentrations were adjusted according to the data in Table S2 so that the volume of solution applied as a mist to the fabric was less than in the disk diffusion method to prevent saturating the sample and only moisten it. For the antibiotic variant with ethanol, the inoculum density was 9.05×10^6 CFU/ml. The appropriate weighed amount of antibiotic was initially dissolved in sterile water and then mixed with 96 % ethanol to achieve a final concentration of 90 % ethyl alcohol, resulting in an antibiotic concentration of 6.09 mg/l. For the antibiotic variant with water, the inoculum density was 3.82×10^6 CFU/ml. The appropriate weighed amount of antibiotic was dissolved in sterile water to reach an antibiotic concentration of 3.98 mg/l (Tables S2 and S7). In all trials, the mass of applied antibiotic was approximately 0.02 μg/cm^2.

In the second test, the effect of penicillin at concentrations of 4 mg/l, 64 mg/l, and 512 mg/l in EM was examined against *B. subtilis* ABSM. The concentrations were selected based on results obtained from the agar imprint method, where the MIC for penicillin on fabric was determined. Three concentrations were tested: the lowest effective concentration (4 mg/l), a medium concentration (64 mg/l), and a high concentration (512 mg/l). The bacterial inoculum density was 9.23×10^6 CFU/ml. The masses of ethanol with penicillin applied on samples were 0.242–0.452 g (for 4 mg/l), 0.296–0.399 g (for 64 mg/l) and 0.415–0.579 g (for 512 mg/l), which correspond to masses of antibiotics shown in Table 2.

The third test involved assessment of the action of high concentrations of penicillin and streptomycin separately and in a mixed solution in 90 % ethanol against *B. subtilis* ABSM. The inoculum density was 7.14×10^6 CFU/ml. Solutions used included penicillin at 4096 mg/l, streptomycin suspension at 128 mg/l, and their mixtures at 4096 mg/l penicillin and 512 mg/l streptomycin (Mix3). The masses of ethanol with antibiotic applied on the samples were 0.12–0.22 g (for streptomycin 128 mg/l), 0.20–0.26 g (for penicillin 4096 mg/l), and 0.19–0.31 g (for Mix3), which correspond to masses of antibiotics shown in Table 3.

3.4. Assessment of the impact of EM with antibiotics on the surface properties of fabrics

Samples of model and historical fabrics were subjected to comparative analyses using several techniques to evaluate the effect of the tested method on the morphology, structure and chemical properties of their surfaces. The study included control samples that were not treated, samples treated with EM alone and samples treated with EM enriched separately and collectively with penicillin and streptomycin dissolved in ethanol.

3.4.1. Scanning electron microscopy

The morphology of the fabric samples was examined using scanning electron microscopy (SEM) with a high-resolution field emission scanning electron microscope JSM-7500F (Jeol), equipped with a Retractable Backscattered Electron detector (RBEI) and an AZtec Live for EDS system (AZtecLiveLite Xplore 30) for characteristic X-ray detection. Before measurement, the samples were mounted on holders and then individually placed on a dedicated holder, introduced into the microscope chamber, and evacuated. The vacuum level during measurement was approximately 9.6×10^{-5} mbar. Elemental composition measurements were performed in micro-areas of the samples prepared in this way. Subsequently, the samples were removed from the microscope, coated with a 50 nm gold layer using an Emitech K575X vacuum sputter coater, then placed back in the microscope and imaged again. For each sample, about 20 images were recorded at various magnifications: 100×, 150×, 250×, 500×, 1000×, 2500×, 5000×, 10,000×, and 25,000×, with an accelerating voltage of 15 kV.

3.4.2. Confocal microscopy

Samples of model and historical fabrics were examined using the LSM 780 fluorescence confocal microscope from Zeiss (Germany), exploiting the autofluorescence phenomenon resulting from cellulose (which is more than 88 % in cotton), proteins, fats, double bonds or aromatic rings being present in the tested material. The fluorescence emission spectra of the test samples were recorded using the 'Lambda mode' function, which detects emission spectra through the 34-channel detector. The samples were illuminated with laser light at 355 nm and 405 nm. Excited light emission was collected from 410 nm to 680 nm in 8.6 nm increments through a 10x/0.45 Plan Achromat objective. The confocal microscope was also used to record 3D images of the materials under study with a 10x/0.45 Plan Achromat objective and 405 nm excitation light.

3.4.3. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) studies were carried out in a multi-chamber UHV system equipped with a SES R4000 hemispheric energy analyzer (Gammadata Scienta) and a non-monochromatic Mg K_α source (12 kV, 10 mA). The base pressure in the analysis chamber was about 3×10^{-10} mbar and about 2×10^{-8} mbar during the experiment. The area of sample analysis was about 5×0.8 mm^2. More details on the recording, calibration and processing of XPS spectra can be found elsewhere (Szota et al., 2023). Since the tested materials were weakly conductive, a calibration was performed on the aliphatic (C-C/C-H) component of C 1s spectrum (set at a binding energy of 285.0 eV).

3.5. Statistical analysis

The arithmetic means and standard deviations for microorganism numbers were calculated. Statistical significance of differences between the results obtained for control samples and samples after misting with ethanol enriched with antibiotics were analysed using one-way Analysis of Variance (ANOVA) and least significant difference (LSD) tests at a level $p < 0.05$. All data were analysed using the statistical software STATISTICA 6.0 (Statsoft, USA).

4. Results

4.1. Assessment of bacterial antibiotic susceptibility based on the disk diffusion method

The measurement of inhibition zone sizes for individual bacterial strains determines their susceptibility or resistance to a given antibiotic. Detailed results of the effects of aqueous solutions of penicillin and streptomycin at various concentrations on four environmental *Bacillus* species and one reference bacterial strain from the ATCC collection are presented in Table S3. Meanwhile, the left part of Table 1 shows the lowest concentrations at which even minimal growth inhibition of the tested bacterial species was observed, designated as MIC (Minimum Inhibitory Concentration).

The MIC results for different bacterial strains indicate varied susceptibility to penicillin and streptomycin. The *B. subtilis* ABSM strain and the reference strain of *B. subtilis* ATCC show the highest susceptibility to penicillin among the tested strains, whereas the environmental ABSM strain exhibits significantly lower susceptibility to streptomycin than the reference strain. *B. cereus* ABSM shows the highest resistance to penicillin among the tested bacterial strains, suggesting low efficacy of this antibiotic against this strain. At the same time, it is susceptible to streptomycin, which may indicate its potential effectiveness in eliminating this microorganism. For *B. licheniformis* ABSM, the MIC for penicillin could not be determined, which may result from very high resistance or technical issues during the test. Conversely, *B. megaterium* ABSM displays the highest susceptibility to streptomycin among the tested strains and moderate susceptibility to penicillin. The results obtained indicate considerable variability in the susceptibility of *Bacillus* species to the tested antibiotics, with the reference strain showing lower resistance compared to the environmental strain (Table 1 and S3).

Table 1

MIC of penicillin and streptomycin in different solvents determined using the disk diffusion method and the agar imprint method.

Bacteria species	MIC of antibiotics [mg/l]					
	disk diffusion method		agar imprint method			
	water solution		90 % ethanol solution		water solution	
	penicillin	streptomycin	penicillin	streptomycin	penicillin	streptomycin
B. subtilis ABSM	1	32	16[a]	256[b]	4	256[b]
B. cereus ABSM	256	8	4096	256[a]	>4096[a]	128
B. licheniformis ABSM	>512	64	64[b]	256	64[b]	512[a]
B. megaterium ABSM	8	4	64[b]	128[a]	64[b]	64
B. subtilis ATCC	1	8	–	–	–	–

- not tested.

[a] Higher MIC, difference between ethanol and water solutions.

[b] Identical MIC, comparison between ethanol and water solutions.

4.2. Determination of MIC for antibiotics on fabric based on the agar imprint method

In the next stage of the study, the ability of bacterial spores to germinate and grow after contact with the antibiotic on the model fabric was tested. MIC on the fabric was determined based on the presence of bacterial growth. MIC was defined as the antibiotic concentration that inhibited germination of spores and growth of bacteria at the fabric imprint site. Detailed results of the effects of penicillin and streptomycin at various concentrations, in 90 % ethanol (Table S4) and in aqueous solution (Table S5), on four *Bacillus* ABSM species using the imprint method are presented in the Supplementary Materials. Right part of Table 1 summarises the MIC concentrations determined for both antibiotics in both solvent variants.

The obtained results indicate a significant influence of the solvent type (90 % ethanol vs. water) on the efficacy of penicillin and streptomycin against different environmental *Bacillus* strains. For *B. subtilis* ABSM spores, the bacteria showed greater sensitivity to penicillin when the antibiotic was dissolved in water than in ethanol. Meanwhile, resistance to streptomycin remained very high regardless of the solvent used, indicating limited effectiveness of this antibiotic against this strain. *B. cereus* ABSM spores exhibited very high resistance to penicillin in alcohol, while in the aqueous antibiotic solution it was not possible to clearly determine an effective dose, which may suggest extremely high resistance or technical difficulties. Sensitivity to streptomycin was slightly higher in water than in ethanol but still limited, suggesting partial resistance. For *B. licheniformis* ABSM spores, the sensitivity to penicillin was similar regardless of the solvent. However, a marked increase in resistance to streptomycin was observed when the antibiotic was applied in water, which may suggest that the chemical environment affects the antibiotic's effectiveness. In the case of *B. megaterium* ABSM, the sensitivity to penicillin remained stable regardless of the solvent. Streptomycin, however, showed better efficacy in aqueous solution than in alcohol, suggesting that the form of solvent may significantly influence the antibiotic's activity against spores of this strain. The differences discussed in MIC depending on the solvent indicate that the antibiotic formulation may play an important role in its effectiveness. In most cases, the aqueous antibiotic solution was more effective than the ethanol solution, especially for penicillin. Streptomycin, on the other hand, was often less effective against the tested strains regardless of the form used.

The study of the effects of antibiotics on model fabrics against *Bacillus* bacteria using the agar imprint method was also conducted for mixtures of both antibiotics in three different variants (Mix1-3) in 90 % ethanol. The results of bacterial growth in the imprint test for the mixtures of penicillin and streptomycin are presented in Table S6. It was shown that Mix2 and Mix3 inhibit the germination and growth of all tested environmental *Bacillus* strains, while Mix1 did not achieve this effect only in the case of *B. cereus* spores. The results obtained for the antibiotic mixtures (Table S6) are consistent with the effects of the individual antibiotics (Table 1 - right part, S4 and S5). For example, penicillin alone at a concentration of 64 mg/l inhibits the bacteria *B. subtilis, B. licheniformis* and *B. megaterium*, but does not inhibit *B. cereus.* Streptomycin inhibits germination of spores and growth of this bacterial species at a concentration of 256 mg/l as well as at concentrations of 128 mg/l and 64 mg/l, where single colonies were observed on the agar after imprinting the fabric. Similar results were obtained for the antibiotic mixture at these concentrations. Fig. S1 shows example images of Petri dishes after imprinting fabric inoculated with *B. cereus* spores and treated with antibiotics in ethanol at various concentrations (P1-P3, S1-S3), as well as a control sample without antibiotics (C). The last row shows plates from the antibiotic mixtures test (Mix1-Mix3). Only at the highest tested penicillin concentration (P3) no bacterial growth was observed at the imprint site, unlike at the lower concentrations (P1 and P2). For streptomycin, this effect was already achieved at a concentration of 256 mg/l (S2). In the photo labeled Mix1, small individual colonies can be seen at the fabric imprint site, indicating incomplete effectiveness of this mixture. Using higher antibiotic concentrations, i.e., Mix2 and Mix3, complete inhibition of *B. cereus* spores' germination and growth at the imprint site may be seen.

4.3. Assessment of the biocidal effectiveness of ethanol enriched with antibiotics applied in the form of a mist

The first test was intended to verify whether the antibiotic dose, which inhibits growth of *B. subtilis,* determined by the disk diffusion method, would show the same effect when applied as an additive to ethanol mist on model fabric, stored in a sealed container, and measured by a quantitative method (Table S7). In the first variant, the effect of penicillin at a concentration of 6.09 mg/l in a 90 % ethanol solution was evaluated on a collection bacterial strain. The applied 90 % ethanol and penicillin dose of 0.018–0.027 μg/cm^2 acting for approximately 22 h did not cause a statistically significant reduction in the number of *B. subtilis* ATCC (16.16 %, 0.08 log). The second variant was performed on the environmental strain of *B. subtilis* with penicillin at a concentration of 3.98 mg/l in an aqueous solution. It was shown that applying 0.018–0.022 μg/cm^2 of this antibiotic using an airbrush did not cause a statistically significant decrease in the number of the environmental strain after 22 h of contact (21.50 %, 0.11 log).

The second test aimed to assess the effect of penicillin at three concentrations in EM (Table 2). None of the tested penicillin concentrations, i.e. 4 mg/l, 64 mg/l and 512 mg/l, caused a statistically significant reduction in bacterial counts. Interestingly, at the highest tested concentration of penicillin in ethanol, corresponding to 2.5–3.6 μg/cm^2 of dry antibiotic (EM + P 512 mg/l), no growth of *B. subtilis* ABSM was observed on agar for the "0″ dilution in 2 out of 3 tested replicates (Table 2, column "dilutions").

The third test aimed at evaluating the effectiveness of penicillin and streptomycin in EM at the highest possible concentrations, as well as the efficacy of their mixture against the *B. subtilis* environmental strain. The

Table 2
Evaluation of the effectiveness of different concentrations of penicillin in 90 % ethyl alcohol against *B. subtilis* ABSM applied as an additive to the ethanol mist.

Type of sample	Mass of antibiotic [µg/cm^2]	Number of bacteria [CFU]						Reduction in the number of bacteria	
		Dilutions [CFU]				Calculated [CFU/sample]	Average [CFU/sample]		
		10^0	10^{-1}	10^{-2}	10^{-3}			[%]	[log]
untreated	0	nc	nc	nc	79	7.90×10^6	7.90×10^6	–	–
treated with EM + P 4 mg/l	0.022	nc	nc	nc	58	5.75×10^6	1.18×10^7	nr	nr
	0.012	nc	nc	nc	64	6.35×10^6	$\pm8.09\times10^6$		
	0.016	nc	nc	nc	232	2.32×10^7			
treated with EM + P 64 mg/l	0.309	nc	nc	nc	71	7.10×10^6	5.82×10^6	26.37	0.13
	0.229	nc	nc	nc	69	6.90×10^6	$\pm1.68\times10^6$		
	0.259	nc	nc	nc	35	3.45×10^6			
treated with EM + P 512 mg/l	3.586	**0**	nc	nc	65	6.45×10^6	5.80×10^6	26.58	0.13
	2.769	nc	nc	nc	57	5.70×10^6	$\pm4.95\times10^5$		
	2.571	**0**	nc	nc	53	5.25×10^6			

Average number of bacteria: mean ± standard deviation.
CFU - colony forming unit.
EM + P - ethanol mist enriched with penicillin.
nc - noncountable.

Table 3
Evaluation of the efficacy of high doses of penicillin and streptomycin and their mixture in 90 % ethyl alcohol against *B. subtilis* ABSM applied as a mist.

Type of sample	Mass of antibiotic [µg/cm^2]		Number of bacteria							Reduction in the number of bacteria	
			Dilutions [CFU]					Calculated [CFU/sample]	Average [CFU/sample]		
			10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}			[%]	[log]
untreated	0		nc	nc	nc	47	2	4.45×10^6	5.06×10^6	–	–
			nc	nc	nc	56	3	5.36×10^6	$\pm4.29\times10^5$		
			nc	nc	nc	57	2	5.36×10^6			
treated with EM +S 128 mg/l	0.29		nc	nc	nc	66	6	6.52×10^6	5.89×10^6	nr	nr
	0.15		nc	nc	nc	53	5	5.29×10^6	$\pm5.03\times10^5$		
	0.22		nc	nc	nc	59	6	5.86×10^6			
treated with EM +P 4096 mg/l	10.69		**0**	**0**	26	39	9	4.14×10^6	4.38×10^6	13.38	0.06
	9.18		**0**	**0**	5	50	3	4.95×10^6	$\pm4.05\times10^5$		
	8.27		**0**	**0**	217	40	5	4.05×10^6			
treated with EM +Mix3	S	P	**0**	**0**	128	48	5	4.76×10^6	5.06×10^6	0	0
	0.25	7.91							$\pm4.60\times10^5$		
	0.33	10.49	**0**	**0**	6	57	6	5.71×10^6			
	0.40	12.86	**0**	**0**	nc	47	6	4.71×10^6			

Average number of bacteria - mean ± standard deviation.
CFU - colony forming unit.
EM + S - ethanol mist with streptomycin.
EM + P - ethanol mist with penicillin.
EM + Mix - ethanol mist with streptomycin and penicillium.
nc - noncountable.
nr - no reduction.

results of these studies are summarised in Table 3. None of the tested concentration variants or antibiotic combinations caused statistically significant reduction in bacterial count on the fabric after application in EM in amounts of 0.12–0.31 g per 100 cm^2 of fabric. In Table 3, the “dilutions” column presents partial results from colony counts on plates for all tested tenfold dilutions. For high concentrations of penicillin in ethanol (EM + P 4096 mg/l and EM + Mix3), no bacterial growth was observed on agar for dilutions “0” and “-1” in all tested replicates, similarly to the previous test.

4.4. Assessment of the impact of EM with antibiotics on the surface properties of fabrics

4.4.1. Scanning electron microscopy

Samples of both model and historical fabrics were subjected to comparative analyses to examine the effect of different ethanol misting variants on the properties of surfaces. The study included control samples that were not treated, samples treated with EM alone and samples treated with EM enriched with: penicillin at concentrations of 64 mg/l, 512 mg/l and 4096 mg/l, streptomycin at concentrations of 64 mg/l, 128 mg/l and 512 mg/l and also their combinations: Mix1-3.

In the case of model cotton fabric, each SEM image reveals a regular material structure composed of interwoven cotton threads. Comparing SEM images of model cotton samples suggests that separate or combined applications of ethanol, penicillin and streptomycin do not noticeably affect the structure of the cotton fibres (Fig. S2). Morphological comparisons of samples at higher magnification (5000×) also shows no significant differences on the surfaces of tested samples (Fig. S3).

SEM/BSE morphological studies of historical fabric samples showed that all samples have a woven fibre structure with a loose weave. Comparing the structure of model cotton and historical fabric reveals that historical fabric is less compact and less regular. Fibres of the control sample are covered with a large amount of biological material, fungal spores, plant pollen and substantial amounts of clay, evidenced by the presence of clearly visible, well-crystallised hexagonal grains adhering to the fibres of the fabric (Fig. 3A). Elemental analysis by EDS

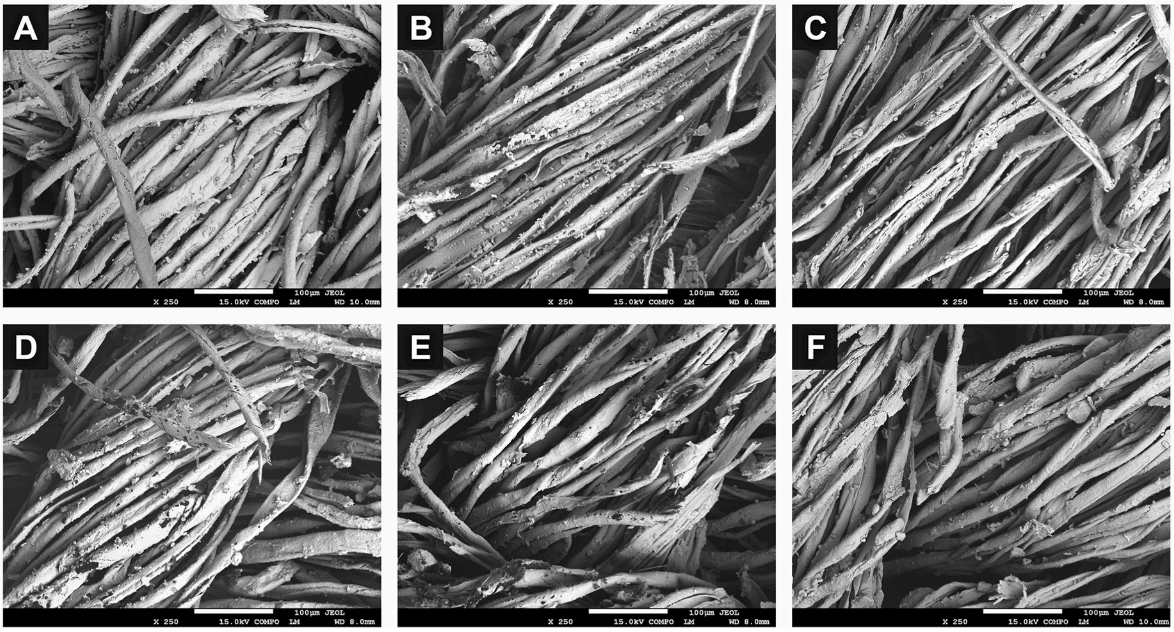

Fig. 3. SEM images of historical cotton samples: A – control; treated with: B - EM, C - EM + P 64 mg/l, D - EM + P 4096 mg/l, E – EM + S512 mg/l, F - EM + Mix3 (magnification 250×).

confirmed the presence of Si, Al, Na, K, Ca, Mg, Cl, Fe in micro-areas of the samples, indicating contamination of the fabrics with commonly occurring aluminosilicates (so-called clays). Treatment with EM alone resulted in the removal of some clay and somewhat reduced the amount of biological material and fungal spores on the fibre surfaces (Fig. 3B). However, these contaminants were still observed in large amounts on the fibres of both samples. The application of penicillin solution did not remove the clay contamination, some fibres were heavily coated or even glued together by clay. Penicillin treatment had a positive effect by reducing biological material and fungal spores on fibre surfaces (Fig. 3C and D). A promising effect was observed after streptomycin treatment. Samples treated with streptomycin solution showed a small amount of clay contamination in the examined micro-areas. Moreover, the fibre surfaces were less covered with biological material and fungal spores compared to the control material (Fig. 3E). A similar effect was observed for fabric samples treated with the antibiotic mixture Mix3. These samples also showed minimal clay contamination in the examined micro-areas and reduced biological material and fungal spores on the fibre surfaces relative to the control (Fig. 3F).

Microscopic observations demonstrated that the use of ethanol, penicillin, streptomycin and their mixtures does not affect the structure of cotton fibres in either model or historical fabrics. At the same time,

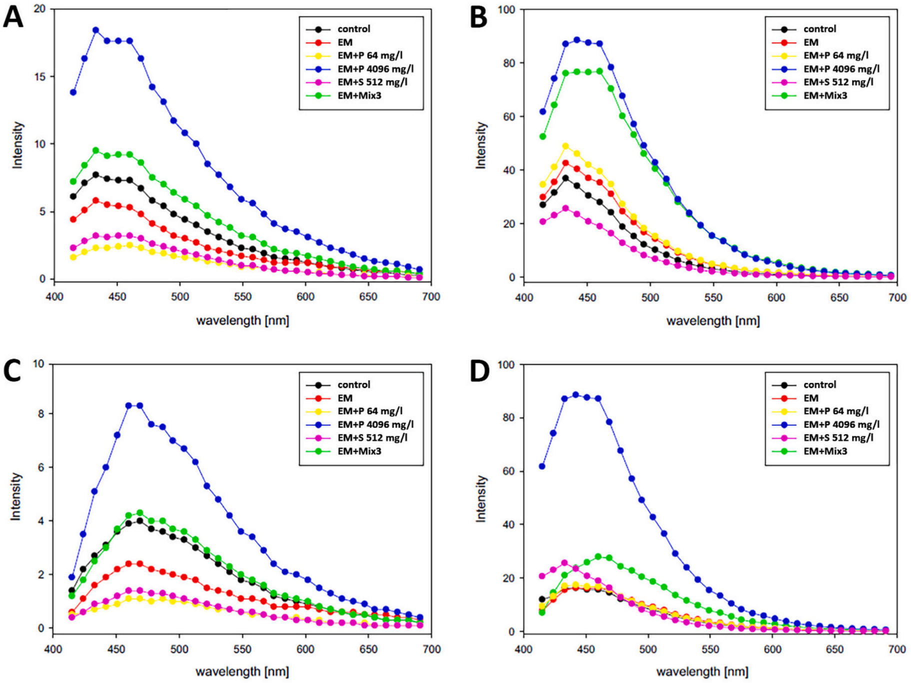

Fig. 4. The fluorescence emission spectra of: A – historical fabric excited with 355 nm, B – model fabric excited with 355 nm, C - historical fabric excited with 405 nm, D – model fabric excited with 405 nm.

these treatments, especially with streptomycin and antibiotic mixture, effectively reduce the presence of biological material and clay contamination on fibre surfaces.

4.4.2. Confocal microscopy

The highest fluorescence intensities were exhibited by samples of both the model and historical fabrics treated with penicillin at a concentration of 4096 mg/l. The fluorescence maximum occurred at 433 nm for the historical fabric and 442 nm for the model cotton when an excitation light of 355 nm was used (Fig. 4A and B). The presence of penicillin at a concentration of 4096 mg/l, applied at a dose of 0.022 g, appears to induce an increase in maximum fluorescence intensity of 140 % in both the historical fabric and the model one compared to the control samples. In contrast, the presence of streptomycin at a concentration of 512 mg/l, applied at a dose of 0.018 g, induces an extinction of the fluorescence maximum by 30 % for model cotton and 58 % for historical fabric. Samples treated with a mixture of 4096 mg/l penicillin and 512 mg/l streptomycin (Mix3, 0.022 g) show an increase in maximum fluorescence intensity of 38 % for historical fabric and 33 % for model cotton. Penicillin at a concentration of 64 mg/l (0.031 g) decreases the maximum fluorescence intensity of the historical fabric by 67 % compared to the control sample. Meanwhile, an increase in the maximum fluorescence intensity of 32 % is observed for the model cotton. For the model cotton, 0.123 g of penicillin was administered at a concentration of 64 mg/l, while 0.031 g was applied to the red fragment of the historical fabric due to the absence of a black fragment in this sample.

Similar emission spectra were obtained for excitation at $\lambda = 405$ nm (Fig. 4C and D). The greatest increase in fluorescence maximum (469 nm) for the historical fabric sample treated with 4096 mg/l of penicillin at 0.016 g was observed. For Mix3, an increase of 7.5 % was observed, as well as a 72 % decrease in penicillin intensity maximum and a 65 % decrease in streptomycin intensity maximum (512 mg/l). For the ethanol-treated sample, the maximum fluorescence intensity decreased by 40 %. For model cotton, excitation at $\lambda = 405$ nm showed the greatest increase in maximum fluorescence intensity at $\lambda = 469$ nm for the penicillin-treated sample (4069 mg/l), at 150 %. An increase in the fluorescence maximum of 72 % and 7.4 % was observed for the samples treated with penicillin at concentrations of 64 mg/l and a mixture of penicillin and streptomycin (Mix3), respectively, while a decrease in the fluorescence maximum of 50 % was observed for the sample treated with streptomycin at a concentration of 512 mg/l. The sample treated with ethanol showed a slight increase in fluorescence intensity of 0.6 % compared to the control sample.

Using a fluorescence confocal microscope, 3D images of the studied materials were also obtained. Analysis of these images showed no significant changes in the morphology of the model cotton fabric or the historical fabric after treatment with ethanol or antibiotics (Figs. S4 and S5). A great deal of impurities, most likely of mineral origin, are observed on the surface of the historical fabric, in both the control sample and the sample treated with antibiotics. Treating the model and historical cotton fabrics with ethanol without or with antibiotics does not affect the structure of their fibres, despite the observed change in fluorescence intensity after treating the fabric surface with ethanol, penicillin or streptomycin, compared to the control samples.

4.4.3. X-ray photoelectron spectroscopy

To demonstrate whether the use of biocidal aerosols affects the chemical structure of historical fabrics, comparative XPS studies were performed on model and historical fabrics for several different EM and antibiotics compositions and procedures. The analysis provided information not only on the chemical composition but also gave a quantitative picture of the chemical bonds present on the surface.

Based on XPS survey spectra, the surface chemical composition of the fabrics studied was obtained (Tables S8 and S9). The summary shows the total atomic concentration of the elements from the surface to a depth of several nanometers. Unfortunately, it is not possible to estimate this parameter more accurately without knowing the density of the material and its structure/texture. For example, for a solid SiO_2 material with density of 2.18 g/cm^3, the penetration depth is about 11.2 nm (Cumpson and Seah, 1997). The calculations take into account the signal from 95 % of the photoelectrons leaving the surface.

As expected for fabric samples stored for a long time in an air atmosphere, the dominant elements on the surface were carbon and oxygen (over 90 %). All samples of the tested materials also contained a small amount of calcium (less than 1 % at.) in the form of sulfates and/or carbonates, most likely originating from water. In addition to the above-mentioned elements, historical fabrics also showed the presence of nitrogen, sulfur, sodium, aluminum and silicon (Table S8). Silicon and aluminum originated from sand (silica) and clay (aluminosilicates) contaminations. Nitrogen and sulfur, in addition to their natural origin (skin, hair, hormones, amino acids), may come from the applied penicillin (N and S) and streptomycin (N). The binding energy of the Na 1s line of 1072 eV suggests the origin of this element from trace amounts of NaCl or Na_2CO_3 (NIST XPS database.).

Considering the chemical composition of the surface, treatment with EM and antibiotics did not cause significant changes on the model fabric (the same amounts of carbon, oxygen and calcium), and only minor changes for the historical fabric. With the same amounts of carbon, oxygen, calcium and nitrogen, changes in the amounts of silicon, aluminum, sodium and sulfur are observed (Tables S8 and S9). This is most likely related to the physical removal (leaching) of dirt of natural origin. It is worth mentioning, however, that the above slight differences in surface chemistry may also be due to the fact that samples taken from different areas of the fabric may have differed slightly in the amount of inorganic soiling already before anyprocesses.

A detailed analysis of the C 1s spectra (Fig. 5, Tables S10 and S11) showed that the amounts of carbon functional groups within the pairs of historical and model samples change very little, indicating that there is no effect of the ethanol and antibiotics used on the surface condition. In the case of the O1s line, no effect of the reagents used can be seen in the model fabric group, while an increase in the amount of hydroxyl groups is observed only in the case of the EM-treated historical fabric (Fig. 6, Tables S12 and S13). A change in the amount of metal-oxygen bonds can also be seen. The effect of EM with antibiotics on the N 1s nitrogen line is negligible and is observed only for the amine groups in the historical fabrics (Tables S14 and S15). No differences were observed for the Ca $2p_{3/2}$ line (not shown here).

No increase in the amount of nitrogen and sulfur is observed in the samples tested, what could indicate surface modifications associated with the application of penicillin and/or streptomycin (naturally containing nitrogen and sulfur, or nitrogen, respectively). Tested processes do not cause changes in the carbon and nitrogen functional groups. Small changes are observed within the oxygen functional groups which may indicate leaching of inorganic soils.

5. Discussion

Before the conservation works at the ABSM, it is essential to ensure the microbiological cleanliness of the objects. Considering that previous studies detected microorganisms on textile objects, whose presence may negatively affect the condition of museum artifacts as well as the health of people working with them (Wawrzyk et al., 2018), developing an effective method for their elimination is crucial. Moreover, the disinfection method used must not damage the properties of the objects' surfaces.

Antibiotics have been studied several times in the context of eliminating microorganisms to prevent biodeterioration of cultural heritage objects, but with varying effectiveness. In one study, 13 different antibiotics were tested for the elimination of 8 species of *Streptomyces* bacteria out of 46 species of this genus found on the paintings of ancient Egyptian tombs in Tell Basta and Tanis tombs. The tested species showed

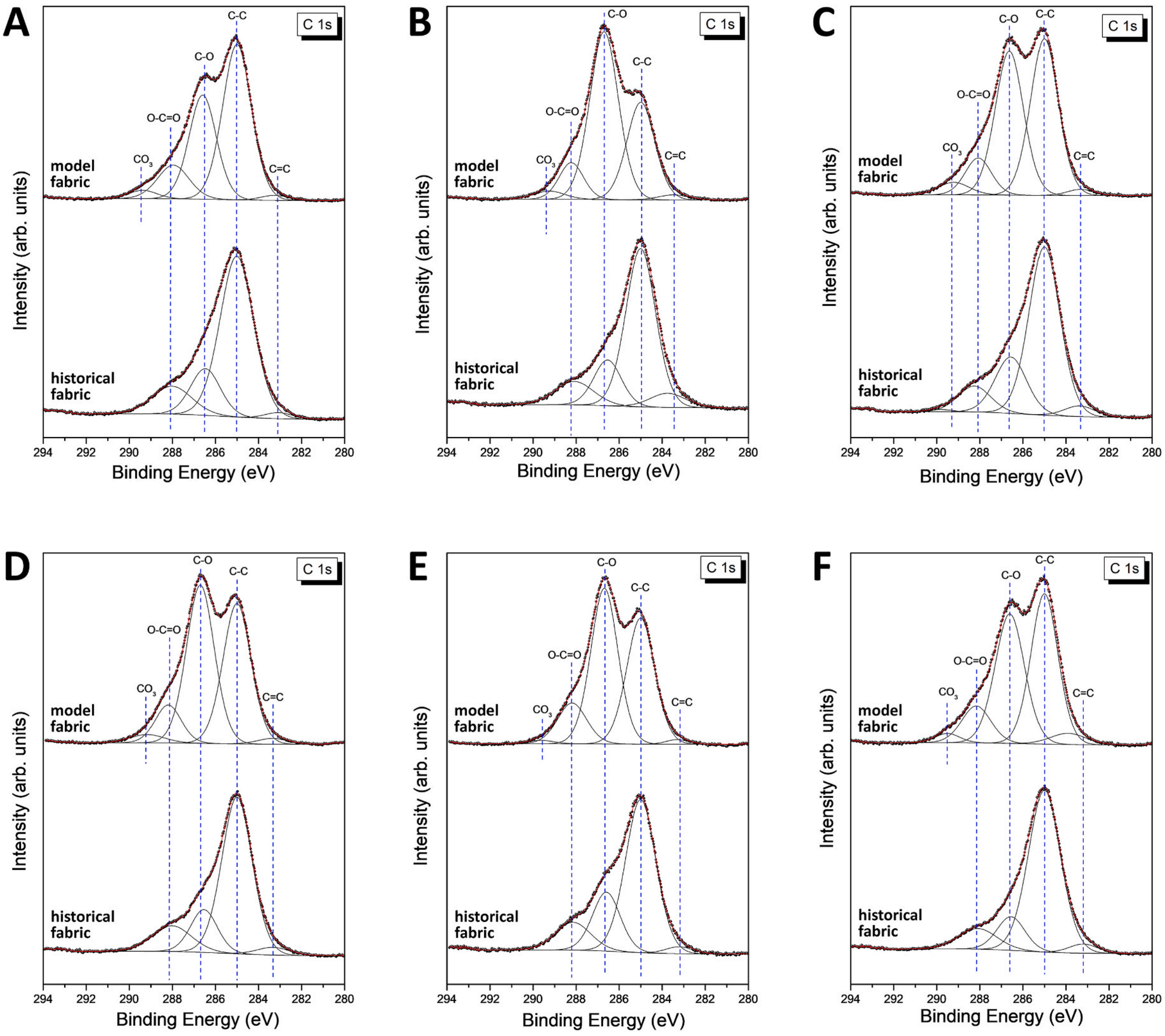

Fig. 5. XPS spectra of C 1s line for model and historical cotton samples: A - control; treated with: B - EM, C - EM + P 64 mg/l, D - EM + P 4096 mg/l, E – EM + S 512 mg/l, F - EM + Mix3.

a high level of resistance to the antibiotics used, which is why a different method, gamma radiation, was ultimately applied for their elimination (Abdel-Haliem et al., 2013). In another study, the antibiotic 6-pentyl α-pyronephenol produced by fungi of the genus *Trichoderma* effectively eliminated *Aspergillus niger* and *Aspergillus flavus* fungi found on ancient Egyptian wall paintings in the Nfer Bau Ptah Tomb, Giza, Egypt (Helmi et al., 2011). Meanwhile, the use of mitomycin C proved effective in eliminating *Blastococcus saxobsidens* bacteria but not the species *Geodermatophilus obscurus* and *Modestobacter multiseptatus,* which cause biodeterioration of stone objects (Gtari et al., 2012). Additionally, in 2015, conservators from the Laboratory of Pompeii Archaeological Site and the company Atramentum eliminated *Streptococcus* bacteria causing biodeterioration of ancient frescoes in the Villa of the Mysteries in Pompeii using amoxicillin (theartnewspaper, 2015).

Considering these literature data, the ABSM laboratory team decided to evaluate the effectiveness of disinfection of textile objects using antibiotics applied as an additive to ethanol in the form of mist, as well as their impact on the surface properties of the fabrics. In this study, 90 % ethanol was deliberately used instead of 70 %, as it contains less water and therefore disinfection does not cause undesirable moisturisation of historical objects. Our previous research has shown that applying a 90 % ethanol mist and then storing the object for 22 h in a sealed bag gives comparable biocidal effect as 70 % ethanol mist (Wawrzyk et al., 2023). Previous studies has also demonstrated high efficacy against filamentous fungi and non-spore-forming bacteria of 90 % ethanol mist itself (Wawrzyk et al., 2023). It was anticipated that the addition of one or two antibiotics might improve biocidal activity against the most difficult to eliminate bacteria, i.e., spore-forming *Bacillus* species.

One of the oldest and most widely used methods for assessing microbial drugs susceptibility in laboratories is the EUCAST disk diffusion method. It is suitable for testing most of the bacterial pathogens and a wide range of antibiotics. This method allows for determination of the minimum inhibitory concentration (MIC), which is the lowest concentration of a biocidal agent that inhibits microbial growth. The MIC is a parameter that characterises, among others, bacteriostatic and bactericidal drugs (Andrews, 2001; Matuschek et al., 2014). Using this method, in the first stage of the study, preliminary antibiotic concentrations were established that could at least minimally inhibit the growth of the vegetative forms of individual bacteria. Additionally, differences in the resistance of various *Bacillus* bacterial strains isolated from historical textile objects were assessed.

In the second stage, a different research methodology was applied, which allowed for the assessment of the MIC with prolonged contact time between the antibiotic and spores of bacteria on the fabric. Samples were stamped onto agar medium, where both bacterial spores and antibiotics were transferred to the agar, enabling the evaluation of the antibiotic's effect on the fabric. This test made it possible to check whether, during storage of objects with antibiotics at the appropriate

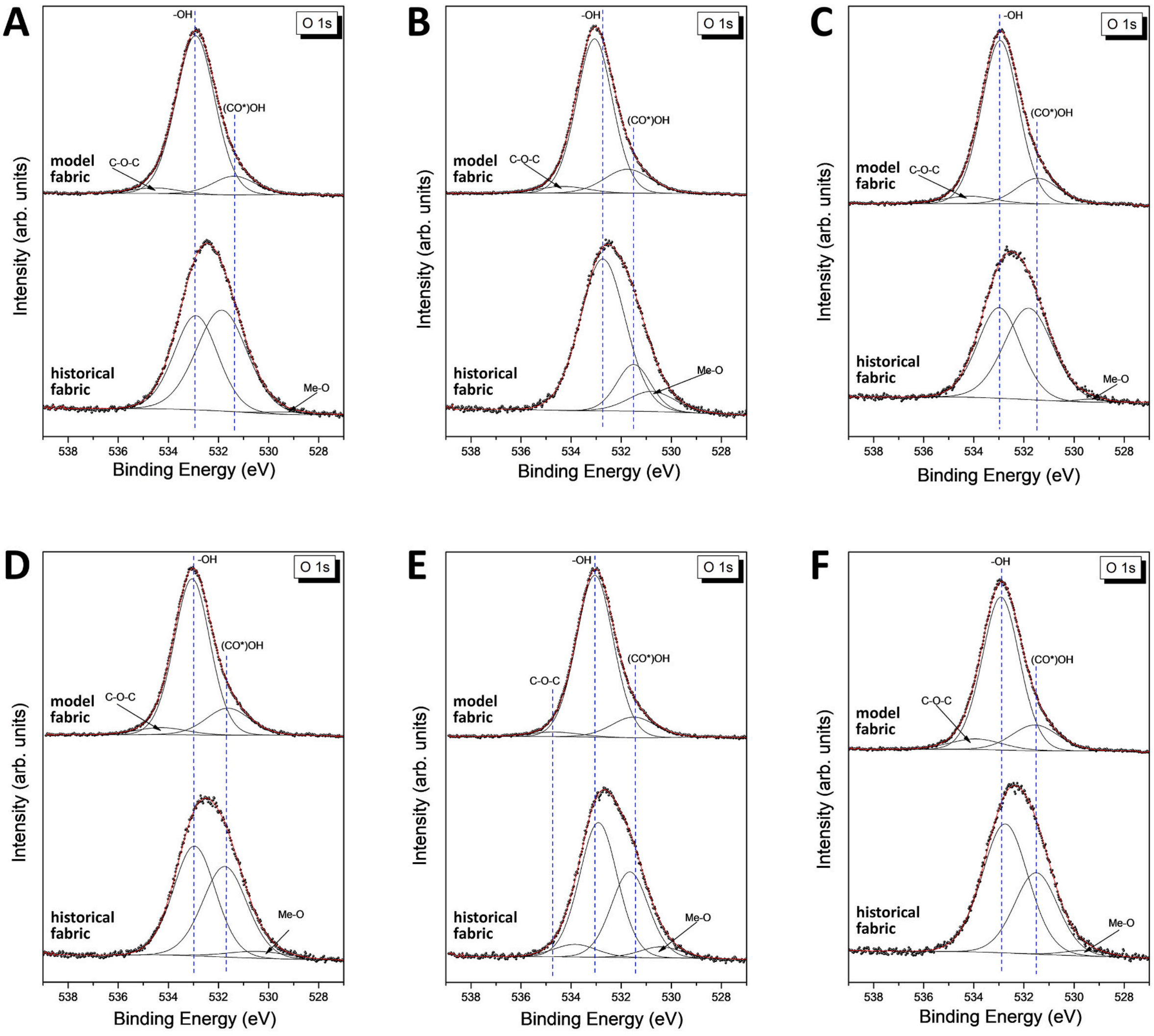

Fig. 6. XPS spectra of O 1s line for model and historical cotton samples: A - control; treated with: B - EM, C - EM + P 64 mg/l, D - EM + P 4096 mg/l, E – EM + S 512 mg/l, F - EM + Mix3.

concentration and bacteria originating from the environment, under museum conditions, bacterial germination and cell multiplication could occur in case of increased environmental humidity and/or contamination with compounds that could serve as nutrients for the bacteria. To perform the test using this method, antibiotic concentrations for each bacterial strain were selected based on the results obtained from the disk diffusion method. For all tested strains, growth inhibition was observed for at least one of the applied antibiotic concentrations. Furthermore, MIC values determined by the agar stamping method for aqueous antibiotic solutions were significantly higher than MICs determined by the disk diffusion method, what is understandable, because in the disk diffusion method vegetative bacterial cells were used, while the imprint method used spores of the same bacterial species. These results confirm that bacterial spores have a much higher resistance to antibiotics than vegetative forms.

Next, it was checked whether the antibiotic concentrations determined by the disk diffusion method would be equally effective under real conditions (when applied on fabrics as an additive to ethanol mist). An aqueous solution was used to maintain the antibiotic dissolution conditions employed in the EUCAST method and an alcoholic solution was used to check whether the antibiotic retains the same activity in the two solvents used, i.e., water (as in the disk diffusion method) and 90 % ethanol (as in the ethanol mist). The results showed that penicillin applied both as a water solution and as an ethanol solution did not have a bactericidal effect against both tested *B. subtilis* strains. This indicates that bacterial growth is only inhibited in the presence of the antibiotic in the disk diffusion method. However, in the above method, fabric samples inoculated with bacteria and treated with antibiotic are washed off after 22 h of incubation. The resulting suspension is diluted for plating and used for determining the number of cells that survived on the sample. During this process, the antibiotic is washed out of the samples and diluted in subsequent tubes. When applied together with bacteria onto agar medium plates, its concentration is too low to inhibit cell growth, that is why colonies form. This may mean that bacterial cells were not eliminated during contact with the antibiotic in the EUCAST method, but only their growth was inhibited. Then, the biocidal efficacy of the ethanol mist with penicillin at three significantly different concentrations, some even exceeding the MICs determined by previous methods, was also tested under conditions simulating real-life situations. Given that none of the tested penicillin concentrations produced even a minimal bactericidal effect, in the final stage, very high concentrations of both antibiotics in ethanol mist were tested. It was shown that achieving a significant disinfecting effect against spore-forming *B. subtilis* is not possible, regardless of how high the antibiotic doses are applied.

Analysis of the literature data indicates that numerous clinical tests have achieved bactericidal efficacy with the combined use of penicillin and streptomycin (Moellering et al., 1972; Miller et al., 1986; Bayer et al., 1980; Le Bouguenec and Buu-Hoï, 1984). This antibiotic mix has also been used prophylactically to reduce the risk of postpartum infections (Kampikaho and Irwig, 1993). It is also a standard treatment in veterinary medicine to prevent and treat bacterial infections in animals (Tufa et al., 2023). Studies have also demonstrated a synergistic effect between ethanol and streptomycin. It was shown that ethanol induces bacterial cell stress, resulting in conformational changes within the ribosomes at the binding site of streptomycin, disrupting the translation process. Consequently, the combined use of ethanol and streptomycin leads to a fourfold increase in bacterial cell toxicity compared to what is predicted by the multiplicative model for unrelated stressors (Haft et al., 2014). Considering the synergistic action of streptomycin and penicillin (Plotz and Davis, 1962; Moellering et al., 1972), a mixture of these antibiotics was prepared and tested to potentially achieve higher biocidal effectiveness. Since the tested strains exhibited different sensitivities to penicillin and streptomycin, three antibiotic mixtures with varying concentrations were prepared to select the mixture most effective against all tested environmental strains. Although results obtained with the agar imprint method were promising, where the mixture of penicillin 4096 mg/l and streptomycin 512 mg/l resulted in no colony growth at the imprint site, tests under real conditions did not confirm this, showing a 0 % reduction.

Nevertheless, analyses of the partial results of colony-forming units (CFUs) counts on individual Petri dishes inoculated from a series of tenfold dilutions of samples after application of EM with antibiotics revealed an interesting pattern. Specifically, for penicillin at 512 mg/l and 4096 mg/l, as well as the mixture of penicillin 4096 mg/l and streptomycin 512 mg/l, no growth was observed from the undiluted suspension after rinsing the samples and in the latter two cases, also from the tenfold diluted suspension. However, growth appeared at higher tenfold dilutions, which may indicate a lack of bactericidal effect and only an inhibitory effect on bacterial cell multiplication. This result is surprising, as penicillin and streptomycin are classified as bactericidal, not bacteriostatic antibiotics (Wright, 1999; Waters and Tadi, 2025). The ability of a small subpopulation of bacteria to survive high doses of antibiotics to which the species is sensitive (according to the EUCAST test) is referred to as antibiotic persistence (Huemer et al., 2020). Following the cessation of antibiotic treatment, under favorable growth conditions, surviving persister cells can repopulate and reestablish the bacterial community (Balaban et al., 2019). The results obtained above indicate possible antibiotic persistence of the studied bacterial species, and perhaps this is why their complete elimination using antibiotics in ethanol applied as a mist proved to be impossible. The duration of the lag phase between the transfer of persister cells to fresh medium and the onset of regrowth is influenced by both the antibiotic concentration and the length of exposure (Himeoka and Mitarai, 2021). Perhaps this is why, in the case of antibiotics applied in ethanol mist, no bacterial growth was observed after plating the undiluted or 10-fold diluted suspensions, as the antibiotic concentration there was higher than in the higher dilutions, where bacterial growth was observed. It is possible that the use of the other kinds of antibiotics in combination with ethanol in the form of a mist could give better results. This may also be because the *Bacillus* bacteria selected for this study are generally very resistant and difficult to eliminate (Setlow, 2006). Therefore, the disinfection method using antibiotics may be applicable to other bacterial species.

To assess the impact of the applied antibiotics on the morphology, structure and chemical composition of historical and model fabrics, physicochemical analyses such as SEM, confocal microscopy and XPS were employed. These methods have been repeatedly used by the ABSM laboratory team in previous studies to evaluate the effects of VHP, diode laser and ethanol mist on the surfaces of historical materials (Wawrzyk et al., 2018, 2023, 2024; Rybitwa et al., 2020, 2022). These techniques have also been successfully used to assess the structure of various other surfaces, including 25-year-old corroded metal and composite materials (Wawrzyk et al., 2022a, 2022b). XPS has also been effectively applied to evaluate the impact of VHP on cotton fabric in the medical field (Wawrzyk et al., 2020).

SEM observations showed that the applied treatments with ethanol mist alone or enriched with antibiotics did not negatively affect the surface morphology or the structure of cotton fibres. All samples, both control and those treated with ethanol and/or antibiotics, exhibited a woven fibre structure with a loose weave. Contamination of the historical fabrics with large amounts of biological material, fungal spores, plant pollen, and substantial amounts of clay, was observed, which decreased after treatment with ethanol and antibiotics.

Changes in the fluorescence intensity of model or historical fabrics may be due to the autofluorescent properties of antibiotics binding to the surface of the cotton material or to the dyes and bleaches used to color the fabrics. While obvious changes in fluorescence intensity occurred after the application of antibiotics to fabric surfaces, no significant changes were observed in the surface morphology of the tested samples.

Historical artifacts often possess protective coatings that differ from the original materials, making it crucial to determine whether disinfectants interact with the underlying historical substance or merely affect these superficial, non-original layers. XPS serves as an effective analytical technique for this purpose. By examining chemical changes within the few nanometers of the surface, XPS can reveal whether a disinfectant has penetrated beyond the protective coating to alter the original material underneath. This level of surface sensitivity is particularly valuable in conservation science, where preserving the integrity of historical objects is paramount. To investigate the chemical changes on the cotton surface caused by bleaching, Topalovic employed XPS analysis. This method allowed comparison of surface chemical changes between a sample of used fabric and a model fabric that had been precleaned to remove readily removable contaminants (Topalovic et al., 2007). In our study, XPS analysis showed no surface modifications associated with the application of ethanol mist itself or together with penicillin and/or streptomycin, so the tested methods used are safe for historical fabrics. Only slight changes in oxygen functional groups were observed, which may indicate leaching of inorganic soiling.

6. Conclusions

The novel method of applying antibiotics in ethanol mist has a biostatic effect on spore-forming *Bacillus* bacteria isolated from the surfaces of historical textile objects from the Auschwitz-Birkenau State Museum. Penicillin and streptomycin alone limit spore germination and cell multiplication of *B. subtilis, B. cereus, B. licheniformis* and *B. megaterium*, but misting with ethanol enriched with these antibiotics does not have a biocidal effect. Penicillin at concentrations of 512 mg/l and 4096 mg/l added to ethanol mist causes inhibition of bacterial cells growth, while no such effect was obtained for streptomycin at any of the tested concentrations.

However, the method is non-destructive and preserves the original structural and chemical integrity of historical fabric, even with high doses of antibiotics. Moreover, the application of ethanol in the form of a mist with antibiotics gently cleans the surface of materials from contaminants of natural origin (e.g. dust, clay and biological materials). Thus, it might be further tested against less resistant bacterial species commonly present on historical artifacts within the ABSM collection, in order to assess its wider applicability and effectiveness.

CRediT authorship contribution statement

Anna Wawrzyk: Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Natalia Pydyn:** Writing – original draft, Visualization, Formal analysis, Data curation. **Dorota Rybitwa:** Writing – review & editing, Visualization, Validation. **Nel Jastrzębiowska:** Writing – original draft, Resources, Funding acquisition. **Lilianna Szyk-Warszyńska:** Methodology, Investigation. **Małgorzata Zimowska:** Methodology,

Investigation. **Jacek Gurgul:** Methodology, Investigation. **Dagmara Zeljaś:** Software. **Filip Bielec:** Methodology, Formal analysis.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2025.106246.

Data availability

Data will be made available on request.

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