Zachodniopomorski Uniwersytet
Technologiczny w Szczecinie

Wydział Biotechnologii
i Hodowli Zwierząt

ZACHODNIOPOMORSKI UNIWERSYTET TECHNOLOGICZNY W SZCZECINIE

Wydział Biotechnologii i Hodowli Zwierząt

Katedra Mikrobiologii i Biotechnologii

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Rozprawa doktorska

Analiza wpływu wirującego pola magnetycznego na efektywność działania substancji przeciwdrobnoustrojowych względem bakterii patogennych

Analysis of the influence of rotating magnetic field on the effectiveness of antimicrobials against pathogenic bacteria

Rozprawa doktorska wykonana w
Katedrze Mikrobiologii
i Biotechnologii pod kierunkiem

dr. hab. inż. Karola Fijałkowskiego,
prof. ZUT

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Publikacje naukowe wchodzące w skład cyklu stanowiącego rozprawę doktorską zatytułowaną:

„Analiza wpływu wirującego pola magnetycznego na efektywność działania substancji przeciwdrobnoustrojowych względem bakterii patogennych”

[D-1] Woroszyło M., Ciecholewska-Juśko D., Junka A., Pruss A., Kwiatkowski P., Wardach M., Fijałkowski K. (2021). The impact of intraspecies variability on growth rate and cellular metabolic activity of bacteria exposed to rotating magnetic field. Pathogens. 10 (11), 1427. DOI: 10.3390/pathogens10111427.

Mój wkład w powstanie tej publikacji polegał na udziale we wszystkich etapach analiz, które obejmowały: wybór i przygotowanie mikroorganizmów, przygotowanie pożywek i reagentów testowych, zorganizowanie stanowiska badawczego, ekspozycję szczepów bakteryjnych na działanie wirującego pola magnetycznego, pomiar gęstości optycznej badanych hodowli oraz przeprowadzenie testów komórkowej aktywności metabolicznej za pomocą spektrofotometrycznych pomiarów fluorescencji, przygotowanie hodowli bakteryjnych do genotypowania, wykonanie posiewów ilościowych, oraz opracowanie wyników i przygotowanie manuskryptu.

Mój udział procentowy szacuję na 65%.

IF – 4,531; 100 pkt. MEiN

[D-2] Woroszyło M., Ciecholewska-Juśko D., Junka A., Wardach M., Chodaczek G., Dudek B., Fijałkowski K. (2021). The effect of rotating magnetic field on susceptibility profile of methicillin-resistant *Staphylococcus aureus* strains exposed to activity of different groups of antibiotics. International Journal of Molecular Sciences. 22 (21), 11551. DOI: 10.3390/ijms222111551.

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Mój udział procentowy szacuję na 65%.

IF – 6,208; 140 pkt. MEiN

[D-3] Woroszyło M., Ciecholewska-Juśko D., Junka A., Drozd R., Wardach M., Migdał P., Szymczyk-Ziółkowska P., Styburski D., Fijałkowski K. (2021). Rotating magnetic

field increases β-lactam antibiotic susceptibility of methicillin-resistant *Staphylococcus aureus* strains. International Journal of Molecular Sciences. 22 (22), 12397. DOI: 10.3390/ijms222212397.

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Mój udział procentowy szacuję na 60%.

IF – 6,208; 140 pkt. MEiN

Wszystkie prace wchodzące w skład cyklu stanowiącego niniejszą rozprawę doktorską zostały opublikowane w otwartym dostępie (Open Access).

Ogólna liczba punktów za cykl prac stanowiących rozprawę doktorską według wykazu czasopism naukowych MEiN z dnia 1 grudnia 2021 r., zgodny z rokiem ukazania się pracy wynosi 380 punktów.

Sumaryczny Impact Factor (IF) za cykl prac stanowiących rozprawę doktorską zgodny z rokiem ukazania się prac wynosi 16,947.

W przypadku wyżej wymienionych prac eksperymentalnych miałam wiodący udział w badaniach, od udziału w opracowaniu koncepcji i metodologii, poprzez udział w wykonaniu wszystkich analiz, po opracowanie, interpretację i opublikowanie wyników. Prowadzone przeze mnie badania miały charakter interdyscyplinarny, dlatego też zostały wykonane we współpracy z ekspertami z różnych obszarów nauk inżynieryjnych, przyrodniczych i medycznych. Większość badań została wykonana w ramach grantu OPUS 14 finansowanego ze źródeł Narodowego Centrum Nauki pn. „Analiza mechanizmów zwiększonej efektywności substancji przeciwdrobnoustrojowych względem biofilmów w obecności wirującego pola magnetycznego”, kierowanego przez dr. hab. inż. Karola Fijałkowskiego, prof. ZUT.

Podczas prowadzenia badań, których wyniki umożliwiły mi przygotowanie publikacji składających się na niniejszą rozprawę doktorską, współpracowałam z naukowcami z Katedry Mikrobiologii i Biotechnologii (KMiB) na Wydziale Biotechnologii i Hodowli Zwierząt (WBiHZ) Zachodniopomorskiego Uniwersytetu Technologicznego (ZUT) w Szczecinie – dr. inż. Radosławem Drozdem i dr inż. Darią Ciecholewską-Juśko. Nawiązałam również współpracę z dr.

Danielem Styburskim z Laboratorium Chromatografii i Spektrometrii Mas na Wydziale Biotechnologii i Hodowli Zwierząt ZUT, dr. hab. inż. Marcinem Wardachem, prof. ZUT z Katedry Maszyn i Napędów Elektrycznych na Wydziale Mechanicznym ZUT, dr. hab. n. med. Adamem Junką, profesorem Uczelni z Katedry i Zakładu Mikrobiologii Farmaceutycznej i Parazytologii (KiZMFiP) na Wydziale Farmaceutycznym Uniwersytetu Medycznego im. Piastów Śląskich (UMW) we Wrocławiu, dr. inż. Pawłem Migdałem z Katedry Higieny Środowiska i Dobrostanu Zwierząt na Wydziale Biologii i Hodowli Zwierząt Uniwersytetu Przyrodniczego we Wrocławiu oraz dr inż. Patrycją Szymczyk-Ziółkowską z Centrum Zaawansowanych Systemów Produkcyjnych na Wydziale Mechanicznym Politechniki Wrocławskiej. Współpracowałam także z dr. hab. Grzegorzem Chodaczkiem z Laboratorium Bioobrazowania Polskiego Ośrodka Rozwoju Technologii PORT we Wrocławiu i dr. Bartłomiejem Dudkiem z Zakładu Mikrobiologii z Instytutu Genetyki i Mikrobiologii na Uniwersytecie Wrocławskim. Ponadto, nawiązałam również współpracę z dr Agatą Pruss z Zakładu Medycyny Laboratoryjnej Wydziału Farmacji, Biotechnologii Medycznej i Medycyny Laboratoryjnej Pomorskiego Uniwersytetu Medycznego w Szczecinie oraz dr. n. med. Pawłem Kwiatkowskim z Samodzielnej Pracowni Diagnostyki Immunologicznej z Wydziału Medycyny i Stomatologii Pomorskiego Uniwersytetu Medycznego w Szczecinie. Współpraca obejmowała wspólne wykonywanie badań i analiz, wykorzystanie sprzętu oraz pomoc w interpretacji otrzymanych wyników, a także przygotowaniu publikacji naukowych.

Załącznik 1. Kopie publikacji naukowych wchodzących w skład cyklu stanowiącego rozprawę doktorską.

Załącznik 2. Kopie suplementów publikacji naukowych wchodzących w skład cyklu stanowiącego rozprawę doktorską.

Załącznik 3. Oświadczenia współautorów publikacji naukowych wchodzących w skład cyklu stanowiącego rozprawę doktorską wraz z określeniem ich indywidualnego udziału.

Załącznik 4. Sumaryczne zestawienie dorobku naukowego.

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Spis treści

Wykaz skrótów

A.D.A.M. – Antibiofilm Dressing's Activity Measurement
ATCC – Amerykańska Kolekcja Hodowli Komórkowych, *ang. American Type Culture Collection*
BHIA – Brain Heart Infusion Agar
CFU – jednostka tworząca kolonię, *ang. colony forming unit*
CLSI – Instytutu Norm Klinicznych i Laboratoryjnych, *ang. Clinical and Laboratory Standards Institute*
ECM – macierz pozakomórkowa, *ang. extracellular matrix*
EPS – zewnątrzkomórkowe związki polimerowe, *ang. extracellular polymeric substances*
EUCAST – Europejski Komitet ds. Badania Wrażliwości Drobnoustrojów, *ang. European Committee on Antimicrobial Susceptibility Testing*
LC-MS/MS – chromatografia cieczowa z tandemową spektrometrią mas, *ang. liquid chromatography–tandem mass spectrometry*
MDR – wielolekooporny, *ang. multidrug resistant*
M-HA – Müller-Hinton Agar
M-HB – Müller-Hinton Broth
MIC – minimalne stężenie inhibitujące, *ang. minimum inhibitory concentration*
MRSA – metycylinooporny *Staphylococcus aureus*, *ang. methicillin resistant Staphylococcus aureus*
MSSA – metycylinowrażliwy *Staphylococcus aureus*, *ang. methicillin susceptible Staphylococcus aureus*
OD – gęstość optyczna, *ang. optical density*
PBP – białka wiążące penicylinę *ang. penicillin binding proteins*
PBP2a – białko wiążące penicylinę 2a, *ang. penicillin-binding protein 2a*
PBS – buforowana fosforanem sól fizjologiczna, *ang. phosphate buffered saline*
PCM – Polska Kolekcja Mikroorganizmów, *ang. Polish Collection of Microorganisms*
PE – pole elektryczne
PEM – pole elektromagnetyczne
PFGE – elektroforeza żelowa w polu pulsacyjnym, *ang. pulsed-field gel electrophoresis*
PM – pole magnetyczne
PPM – pulsujące pole magnetyczne
SCCmec – gronkowcowy chromosom kasetowy *mec*, *ang. staphylococcal chromosomal cassette mec*
SEM – skaningowy mikroskop elektronowy, *ang. scanning electron microscope*
SPM – statyczne pole magnetyczne
TEM – transmisyjny mikroskop elektronowy, *ang. transmission electron microscope*
TSB – Tryptic Soy Broth
WHO – Światowa Organizacja Zdrowia, *ang. World Health Organization*
WPM – wirujące pole magnetyczne

Streszczenie

Globalny problem rosnącej oporności patogenów bakteryjnych na środki przeciwdrobnoustrojowe i wiążące się z nim niepowodzenia w leczeniu infekcji, skłaniają do poszukiwania nowych, alternatywnych metod terapii. Badania dotyczące wpływu pola magnetycznego (PM) na parametry funkcjonalne bakterii są prowadzone przez wiele zespołów badawczych na całym świecie. Jednym ze stosunkowo nowych kierunków badań jest analiza wpływu PM na efektywność działania środków przeciwdrobnoustrojowych wobec bakterii. Jednakże, często badania takie prowadzone są w sposób, który nie obejmuje w analizie części czynników mogących wpływać na obserwowane efekty i tym samym, uzyskane wyniki nie dostarczają satysfakcjonującego wyjaśnienia obserwowanych zjawisk. Dlatego też, wskazane wydaje się prowadzenie dalszych badań w tym zakresie, jak również systematyzacja ich prowadzenia.

Podstawowym celem niniejszej rozprawy doktorskiej była analiza wpływu wirującego pola magnetycznego (WPM) na efektywność działania antybiotyków wobec bakterii z gatunku *Staphylococcus aureus*, w tym szczepów metycylinoopornych (ang. *methicillin-resistant S. aureus,* MRSA) oraz metycylinowrażliwych (ang. *methicillin-susceptible S. aureus,* MSSA). Ponadto, rozprawa doktorska była próbą usystematyzowania sposobu prowadzenia analiz dotyczących wpływu PM na różne parametry funkcjonalne bakterii.

Wszystkie eksperymenty przeprowadzone w ramach opisanego osiągnięcia naukowego wykonano z wykorzystaniem stanowisk badawczych wyposażonych w bioreaktory wspomagane WPM, zbudowane ze stojanu elektrycznego silnika trójfazowego oraz komory procesowej, w której w trakcie ekspozycji umieszczano hodowle bakterii. W badaniach wykorzystano zarówno kliniczne, jak i referencyjne szczepy bakterii należące do 8 gatunków (*Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis, Enterococcus faecalis, Enterobacter cloacae, Moraxella catarrhalis* i *Bacillus cereus*), przy czym eksperymenty związane z efektywnością działania antybiotyków przeprowadzono wyłącznie z użyciem szczepów *S. aureus* (w tym szczepów MRSA i MSSA). W analizach uwzględniono różne parametry związane z ekspozycją bakterii na WPM (np. częstotliwość WPM w zakresie od 5 do 50 Hz i czas ekspozycji w zakresie od 1 do 18 godzin) oraz antybiotyki z różnych grup, charakteryzujące się odmiennym mechanizmem działania, a także różnymi ładunkami elektrycznymi. Przeprowadzone analizy związane były z wpływem WPM na kinetykę wzrostu i komórkową aktywność metaboliczną bakterii oraz efektywność działania antybiotyków.

Uzyskane wyniki wykazały, że oddziaływanie WPM na takie parametry jak kinetyka wzrostu oraz komórkowa aktywność metaboliczna, nie zależą wyłącznie od parametrów opisywanych dotąd w literaturze (np. od gatunku bakterii, kształtu komórki, budowy ściany komórkowej, czy też od rodzaju i parametrów PM i czasu ekspozycji), ale również, w istotnym stopniu od zróżnicowania szczepów bakteryjnych w obrębie gatunku (w tym nawet szczepów należących do jednego typu klonalnego). Ustalono również, że efektywność działania antybiotyków z różnych grup, w tym aminoglikozydów, β-laktamów, fluorochinolonów, glikopeptydów, linkozamidów makrolidów i tetracyklin

wobec *S. aureus* wzrasta pod wpływem WPM. Szczególnie znaczące zmiany obserwowane były w odniesieniu do antybiotyków, których mechanizm działania związany jest hamowaniem syntezy ściany bakteryjnej (β-laktamy i glikopeptydy). Wykazano, że zmiany w efektywności działania β-laktamów były spowodowane osłabieniem komórek bakteryjnych pod względem strukturalnym. Wśród obserwowanych zmian stwierdzono deformacje kształtu i zmiany w wielkości komórek, a także zapadanie się ścian komórkowych i utratę turgoru komórkowego.

W ramach prowadzonych analiz dowiedziono również, że na zmiany w efektywności działania antybiotyków wobec bakterii eksponowanych na WPM wpływ mają takie czynniki jak: częstotliwość WPM, czas ekspozycji oraz stężenie antybiotyku. Wykazano ponadto, że zmiany w efektywności działania antybiotyków pod wpływem WPM są, podobnie jak kinetyka wzrostu oraz komórkowa aktywność metaboliczna, również ściśle związane ze zróżnicowaniem szczepów w obrębie gatunku. Wykazano również, że zmiany w efektywności działania antybiotyków β-laktamowych wobec bakterii manifestują się jedynie w obecności WPM i nie pozostają one utrwalone w komórkach po zaprzestaniu ekspozycji. Udowodniono także, że choć WPM ma wpływ na uwalnianie antybiotyków z nośnika oraz ich dyfuzję w agarze, nie jest to czynnik istotnie determinujący efektywność działania antybiotyków wobec bakterii.

Wyniki uzyskane w trakcie realizacji niniejszej rozprawy doktorskiej istotnie pogłębiły wiedzę dotyczącą zmian w efektywności działania antybiotyków pod wpływem WPM wobec bakterii z gatunku *S. aureus*. Ponadto, po raz pierwszy w tak szczegółowy i kompleksowy sposób przeanalizowano wpływ WPM na komórki bakteryjne. Istotnym osiągnięciem, wynikającym z przeprowadzonych analiz, jest również stworzenie spójnej bazy metodologicznej, która może przyczynić się do systematyzacji badań związanych z wpływem PM na różne parametry funkcjonalne oraz efektywność działania środków przeciwdrobnoustrojowych wobec bakterii.

Abstract

The global problem of increasing resistance of bacterial pathogens to antimicrobials and the associated failures in treating infections are prompting the search for new alternative therapies. Research on the effects of magnetic field (MF) on the functional parameters of bacteria is being conducted by many research teams around the world. Relatively new line of research is the analysis of the effect of MF on the effectiveness of antimicrobials against bacteria. However, often such studies are conducted in a way that does not include in the analysis some of the factors that can affect the observed effects, and thus, the results obtained do not provide a satisfactory explanation of the observed phenomena. Therefore, it seems advisable to conduct further research in this area, as well as to systematize conducting the research.

The main objective of the dissertation was to analyze the effect of rotating magnetic field (RMF) on the effectiveness of antibiotics against *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) strains. In addition, the doctoral dissertation was an attempt to systematize the methodological approaches for analyses on the effects of MF on various functional parameters of bacteria.

All experiments carried out as part of the scientific achievement described herein were performed using RMF bioreactors made of a three-phase electric motor stator and a process chamber in which cultures of bacteria were placed during exposure. Both clinical and reference bacterial strains belonging to 8 species (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Moraxella catarrhalis* and *Bacillus cereus*) were used in the study, with experiments related to antibiotic efficacy carried out exclusively with *S. aureus* strains (including MRSA and MSSA strains). Various parameters related to RMF exposure (e.g. RMF frequency ranging from 5 to 50 Hz and exposure time ranging from 1 to 18 hours) and antibiotics from different groups, characterized by different mechanisms of action, as well as different electrical charges. The analyses carried out were related to the effect of RMF on the growth kinetics and cellular metabolic activity of bacteria and the effectiveness of antibiotics.

The results obtained showed that the effect of RMF on parameters such as growth kinetics and cellular metabolic activity does not depend solely on the parameters described so far in the literature (e.g. bacterial species, cell shape, cell wall structure, or the type and parameters of MF and exposure time), but also, to a significant extent, on the diversity of bacterial strains within a species (including even strains belonging to a single clonal type). It was also found that the efficacy of antibiotics from different groups, including aminoglycosides, β-lactams, fluoroquinolones, glycopeptides, macrolides lincosamides and tetracyclines against *S. aureus* increases under influence of the RMF. Particularly significant changes were observed for antibiotics whose mechanism of action is related to inhibition of bacterial wall synthesis (β-lactams and glycopeptides). Changes in the effectiveness of β-lactams were shown to be due to structural weakening of bacterial cells. Among the changes observed were deformations in cell shape and changes in cell size, as well as cell wall collapse and loss of cell turgor.

The analyses also demonstrated that changes in the effectiveness of antibiotics against bacteria exposed to RMF are influenced by factors such as RMF frequency, exposure time and antibiotic concentration. It was further shown that changes in antibiotic efficacy under RMF are, like growth kinetics and cellular metabolic activity, also closely related to strain variation within species. It has also been shown that changes in the efficacy of β-lactam antibiotics against bacteria are only manifested in the presence of RMF and do not remain fixed in cells after exposure. It has also been shown that, although RMF affects the release of antibiotics from the carrier and their diffusion in the agar, it is not a significant determinant of the effectiveness of antibiotics against bacteria.

The results obtained in the course of this dissertation have significantly advanced the knowledge of changes in the effectiveness of antibiotics under the influence of RMF against *S. aureus* bacteria. In addition, this is the first time when the effect of RMF on bacterial cells has been analyzed in such a detailed and comprehensive manner. An important achievement, resulting from the analyses, is the creation of a consistent methodological basis that can enable the systematization of studies related to the effects of MF on various functional parameters and the effectiveness of antimicrobials against bacteria.

1. Wprowadzenie

1.1. Oporność bakterii na środki przeciwdrobnoustrojowe

Rosnąca oporność bakterii na środki przeciwdrobnoustrojowe stanowi jedno z głównych, światowych wyzwań zdrowotnych XXI wieku. Infekcje powodowane przez bakterie wielolekooporne (ang. *multidrug resistant*, MDR) stanowią istotny problem zdrowotny oraz wyzwanie dla medycyny, nie tylko ze względu na zagrożenie zdrowia i życia pacjentów, ale również ze względu na obciążenie ekonomiczne generowane w systemie opieki zdrowotnej (Kern i Rieg, 2020; Serra-Burriel i wsp., 2020). Rosnącą oporność na antybiotyki obserwuje się wśród wielu gatunków bakterii, niezależnie od ich pochodzenia – szpitalnego czy poza-szpitalnego (Dhingra i wsp., 2020; Gerken i wsp., 2021). Większość literatury fachowej opisuje problem infekcji bakteriami MDR na przykładzie ludzi, zwierzęta są natomiast najczęściej wymieniane jedynie w kontekście wektorów przenoszenia (Haag i wsp., 2019). Natomiast zaznaczyć należy, że infekcje powodowane przez MDR są równie istotnym problemem u ludzi, jak i u zwierząt, zarówno gospodarskich, jak i domowych (Morgan, 2008; Vet i wsp., 2014). Dzieje się tak, z uwagi na znaczne zmiany jakim w ostatnich latach uległy realia życia człowieka i towarzyszących mu zwierząt domowych. Obecnie wiele osób żyje ze zwierzętami w znacznej bliskości, np. śpiąc wspólnie z nimi w jednym łóżku. Ponadto, same zwierzęta dzięki postępowi weterynarii dożywają późnych lat życia oraz dotykają je ciężkie schorzenia tj. cukrzyca czy nowotwory, przez co wymagają one zabiegów przeprowadzanych w klinikach weterynaryjnych, w trakcie których mogą ulec zakażeniu (Wieler i in., 2011).

Światowa Organizacja Zdrowia (ang. *World Health Organization*, WHO) zapoczątkowała inicjatywę „One Health" mającą na celu promocję zintegrowanego, systemowego i ujednoliconego podejścia, ujmującego w jedną całość zdrowie publiczne, zwierzęce i środowiska naturalnego w skali lokalnej, krajowej i całej planety. Antybiotykooporność jest problemem, który został określony jako jedno z najważniejszych wyzwań współczesnego świata i objęty wyżej opisaną inicjatywą. Celem powyższej inicjatywy w kontekście antybiotykooporności jest zapobieganie rozprzestrzenianiu się bakterii MDR i zachowanie skuteczności istniejących antybiotyków. Szczególnie podkreślane jest wzajemne powiązanie pomiędzy zdrowiem ludzi a zwierząt (domowych, dzikich oraz hodowlanych) oraz środowiskiem (Abdullahi i wsp., 2021). Jako patogeny modelowe w inicjatywie „One Health" zostały wykorzystane gronkowce, ponieważ są one powszechnie izolowane od ludzi, zwierząt, jak i ze środowiska naturalnego (Tolba i wsp., 2008; Goodwin i wsp., 2012; Turner i wsp., 2019; Algammal i wsp., 2020). Ponadto, wykorzystując analizy genetyczne wykazano, że geny warunkujące oporność na antybiotyki są wymieniane pomiędzy gronkowcami od różnych nosicieli (ludzi i zwierząt), a tymi, które obecne są w środowisku naturalnym (Rossi i wsp., 2020).

1.2. Metycylinooporny *Staphylococcus aureus* w medycynie i weterynarii

Staphylococcus aureus (*S. aureus*) to Gram-dodatni, względnie beztlenowy ziarniak, nie posiadający zdolności ruchu (Madhaiyan i wsp., 2020). *S. aureus* wchodzi

w skład fizjologicznej mikrobioty – około 30% populacji ludzkiej stanowią bezobjawowi nosiciele, przy czym odsetek nosicieli różni się w zależności od wielu czynników np. regionu świata, wieku, przynależności etnicznej czy też płci (Sollid i wsp., 2014; Troeman i wsp., 2018; Abdullahi i wsp., 2021). Istnieje również wiele raportów na temat kolonizacji zwierząt przez *S. aureus* (Nagase i wsp., 2002; Rubin i wsp., 2011; Haag i wsp., 2019). Co jednak najbardziej istotne, *S. aureus* jest jednym z wiodących czynników etiologicznych infekcji w medycynie (Lee i wsp., 2018) oraz w weterynarii (Morgan, 2008; Haag i wsp., 2019; Algammal i wsp., 2020).

Z powodu szerokiego stosowania antybiotyków niektóre szczepy *S. aureus* rozwinęły oporność na metycylinę poprzez wychwyt gronkowcowego chromosomu kasetowego *mec* (ang. *staphylococcal chromosomal cassette mec, SCCmec*), kodującego geny *mecA* lub *mecC*, warunkujące oporność na większość antybiotyków β-laktamowych. Geny te kodują enzym odpowiedzialny za sieciowanie peptydoglikanów w ścianie komórkowej bakterii, tzw. białko PBP2a. Białko to charakteryzuje się niskim powinowactwem do wiązania z β-laktamami, co skutkuje opornością na całą klasę tych antybiotyków (Lee i wsp., 2018). Z biegiem czasu niektóre szczepy MRSA rozwinęły również oporność na antybiotyki z innych grup, w tym na aminoglikozydy, fluorochinolony czy makrolidy, stając się bakteriami MDR (Lee i wsp., 2018; Turner i wsp., 2019).

Szczepy MRSA są najlepszym przykładem bakterii, które początkowo związane wyłącznie ze środowiskiem szpitalnym stały się następnie szeroko rozpowszechnione w społeczeństwie (van Duin i Paterson, 2016). Patogeny te są jedną z głównych przyczyn infekcji u ludzi – począwszy od infekcji skóry, tkanek miękkich i ran po inwazyjne infekcje tj. sepsa, zapalenie opon mózgowo-rdzeniowych, zapalenie płuc oraz zapalenie kości i szpiku (Magill i wsp., 2018; Solis-Velazquez i wsp., 2021). Również u zwierząt MRSA są częstą przyczyną wielu różnych typów infekcji, np. zapalenia wymienia *(*mastitis), infekcji skóry i tkanek miękkich oraz zapalenia stawów i płuc (Morgan, 2008; Haag i wsp., 2019; Algammal i wsp., 2020). W źródłach literaturowych przedstawiono również zakażenia krzyżowe MRSA pomiędzy ludźmi a zwierzętami (Baptiste i in., 2005; Witte i in., 2007). Potencjał chorobotwórczy *S. aureus* opiera się na zdolności tego patogenu do wytwarzania szerokiej gamy czynników wirulencji, w tym polisacharydów otoczkowych, zewnątrzkomórkowych toksyn, enzymów związanych z powierzchnią białek oraz, przede wszystkim, zdolności wytwarzania biofilmu (van Duin i Paterson, 2016; Algammal i wsp., 2020).

1.3. Znaczenie biofilmu *S. aureus* w przebiegu infekcji

Bakterie występują w dwóch formach bytowania – planktonicznych, pojedynczych komórek lub też w formie społeczności bakteryjnej otoczonej macierzą pozakomórkową (ang. *extracelullar matrix*, ECM), nazywanej biofilmem. Szacuje się, że około 40-80% bakterii w naszym otoczeniu funkcjonuje w formie biofilmu, przy czym zmiana formy bytowania pomiędzy komórkami planktonicznymi, a biofilmem może odbywać się płynnie, zależnie od warunków otoczenia (Sauer i wsp., 2002; Flemming i Wuertz, 2019). Biofilm definiowany jest jako ustrukturyzowana, wielowarstwowa społeczność mikroorganizmów otoczona przez ECM. Podobnie

jak w przypadku innych bakterii, ECM w biofilmie gronkowcowym składa się z wody oraz zewnątrzkomórkowych związków polimerowych (ang. *extracellular polymeric substances*, EPS), w skład których wchodzą polisacharydy, białka, kwasy nukleinowe, surfaktanty i fosfolipidy (Becker i wsp., 2014; Czyżewska-Dors i wsp., 2018). ECM chroni komórki bakteryjne przed szkodliwymi dla nich czynnikami otoczenia: metalami ciężkimi, lekami, antyseptykami, dezynfektantami, a także elementami układu odpornościowego gospodarza (Yin i wsp., 2019). Szacuje się, że bakterie w biofilmie mają nawet 1000-krotnie zwiększoną tolerancję na antybiotyki w porównaniu do ich form planktonicznych (Czyżewska-Dors i wsp., 2018).

Infekcje związane z biofilmem można podzielić na dwa główne typy – infekcje biofilmowe związane z kolonizacją urządzeń medycznych oraz przewlekłe infekcje biofilmowe tkanek (Del Pozo, 2018; Sharma i wsp., 2019). *S. aureus* jest zdolny do utworzenia biofilmu na powierzchniach biotycznych (tkankach) i abiotycznych, np. na cewnikach moczowych, cewnikach naczyniowych, implantach zębowych, bądź ortopedycznych (Czyżewska-Dors i wsp., 2018). Najnowsze dane wskazują, że zakażenia, które mają podłoże biofilmowe to około 80% zakażeń występujących u ludzi i zwierząt (Abdullahi i wsp., 2016; Sharma i wsp., 2019). Szacuje się, że około 61% patogenów będących przyczyną infekcji u ludzi jest pochodzenia zwierzęcego (Garcia i Percival, 2011). Powstawanie biofilmów w przebiegu większości infekcji oraz rosnąca oporność bakterii na stosowane rutynowo środki przeciwdrobnoustrojowe zmusza do poszukiwania alternatywnych rozwiązań umożliwiających leczenie infekcji bakteryjnych u ludzi i zwierząt.

1.4. Rodzaje i charakterystyka pól magnetycznych wykorzystywanych w badaniach z organizmami żywymi

Pole magnetyczne (PM) jest obok pola elektrycznego (PE) przejawem pola elektromagnetycznego (PEM). W kontekście badań zaprezentowanych w ramach niniejszej rozprawy doktorskiej można przyjąć, że jest to przestrzeń otaczająca magnes lub przewodnik, przez który płynie prąd. Pole to powstaje poprzez poruszające się ładunki elektryczne i cechuje się tym, że na znajdujące się w jego obszarze naładowane cząsteczki działa siła. Z matematycznego punktu widzenia PM jest polem wektorowym, co oznacza, że każdemu punktowi przestrzeni przypisany jest wektor, który można zilustrować jako wektor o odpowiednim kierunku i długości. Wielkością fizyczną opisującą PM jest indukcja magnetyczna [B]. Indukcja magnetyczna określa intensywność PM (im większa, tym większa jest siła oddziaływania PM). W systemie SI jednostką indukcji magnetycznej jest tesla [T]. Wartość indukcji magnetycznej w teslach zdefiniowana jest przez wielkość siły, jaka działa na ładunek poruszający się w badanym polu.

Zgodnie z dostępną literaturą przedmiotu PM stosowane w badaniach można podzielić na dwa główne rodzaje: PM wywołane przez przepływ prądu stałego lub też PM wywołane przez przepływ prądu przemiennego. PM wywołane przez przepływ prądu stałego, którego przykładem jest statyczne pole magnetyczne (SPM) nie mają częstotliwości (wektor pola jest stały w czasie i przestrzeni) i nie zmieniają się one w czasie, lub też zmieniają się bardzo powoli. Natomiast, PM generowane poprzez przepływ prądu przemiennego zmieniają się w zależności od jego częstotliwości [f]

wyrażanej w hercach [Hz]. Wśród takich rodzajów PM wymienić można pulsujące pole magnetyczne (PPM) i stosowane w niniejszej rozprawie doktorskiej WPM. Wektor PPM pulsuje z częstotliwością prądu płynącego przez uzwojenie. WPM powstaje natomiast w obszarze superpozycji dwóch lub więcej PPM o identycznych częstotliwościach, lecz przestrzennie przemieszczonych w stosunku do siebie. Cechą charakterystyczną WPM jest to, że jego oś wiruje względem układu odniesienia (względem stojana), a zwrot pozostaje stały wzdłuż osi. Opierając się na dostępnej literaturze przedmiotu, można stwierdzić, że badania prowadzone w zakresie wpływu PM na organizmy żywe prowadzone są głównie z wykorzystaniem SPM i PPM, natomiast badania z wykorzystaniem WPM, są stosunkowo ograniczone.

1.5. Wpływ pola magnetycznego na parametry funkcjonalne bakterii

W ostatnich latach prowadzone są liczne badania związane z wpływem PM na różnorodne parametry funkcjonalne bakterii. Analizy prowadzone w przebiegu badań dotyczą m.in. wpływu PM na kinetykę wzrostu i żywotność (Fojt i wsp., 2004; Strašák i wsp., 2005), komórkową aktywność metaboliczną (Konopacki i Rakoczy, 2019), zdolność do tworzenia biofilmu (Raouia i wsp., 2020; Haagensen i wsp., 2021), częstość występowania mutacji (Cellini i wsp., 2008), aktywność enzymów (Wasak i wsp., 2019; Geng i wps., 2020; Li i wsp., 2022), zmiany potencjału błonowego (Oncul i wsp., 2016), morfologię komórki (Sharma i wsp., 2007; Fojt i wsp., 2009), zmiany w przepuszczalności błon komórkowych (Novickij i wsp., 2016) oraz produkcję metabolitów (Gao i wsp., 2011). Z uwagi na złożoność obserwowanych zjawisk nie wyjaśniono jak dotąd wielu mechanizmów związanych z oddziaływaniem PM na bakterie, a część stwierdzeń opiera się jedynie na modelach teoretycznych.

1.5.1. Wpływ pola magnetycznego na kinetykę wzrostu oraz komórkową aktywność metaboliczną bakterii

Na podstawie danych z piśmiennictwa można stwierdzić, że w ramach tematyki związanej z analizami wpływu PM na bakterie, najczęściej analizowane są takie parametry jak kinetyka wzrostu oraz komórkowa aktywność metaboliczna. Dzieje się tak dlatego, że możliwość modyfikacji wyżej wymienionych parametrów komórkowych jest kluczowa w obszarze biotechnologii i medycyny, gdyż ich zmiana może wpłynąć na wydajność przeprowadzanych procesów lub też przebieg infekcji. W literaturze przedmiotu opublikowanych zostało dotychczas wiele badań analizujących wpływ różnych PM (w tym WPM) na wyżej opisane parametry komórkowe bakterii (Tabela 1 w publikacji D-1). Co istotne, analizując dane literaturowe, można zaobserwować brak spójności w wynikach uzyskanych przez różnych autorów – często nawet przy zastosowaniu tego samego rodzaju PM wyniki są przeciwstawne (wskazujące na efekt hamujący, stymulujący lub jego brak) w odniesieniu do tego samego gatunku bakterii (Tabela 2 w publikacji D-1). Niemniej jednak, porównywanie wyników uzyskanych przez różnych autorów sprawia trudność, z uwagi na stosowanie w badaniach m.in. różnych urządzeń generujących PM, odmiennego czasu ekspozycji czy warunków hodowli. Co również istotne, w zdecydowanej większości, badania te zostały przeprowadzone z wykorzystaniem różnych gatunków bakterii, ale nie z wykorzystaniem

różnych szczepów należących do jednego gatunku. W literaturze przedmiotu dostępne są trzy publikacje, w których w analizach ujęty został więcej niż jeden szczep przynależny do tego samego gatunku, jednak autorzy tych prac nie analizowali uzyskanych wyników indywidualnie dla każdego szczepu (Kermanshahi i wsp., 2005; Nawrotek i wsp., 2014; Mousavian-Roshanzamir i Makhdoumi-Kakhki, 2017).

Wyniki uzyskiwane w ramach badań związanych z omawianym zagadnieniem sugerują najczęściej, że wpływ PM na kinetykę wzrostu oraz komórkową aktywność metaboliczną bakterii, zależny jest od rodzaju PM, czasu ekspozycji lub też od takich właściwości bakterii jak ich przynależność gatunkowa, budowa ściany komórkowej czy też kształt komórki bakteryjnej (Fojt i wsp., 2004; Strašák i wsp., 2005; Hu i wsp., 2009; Tessaro i wsp., 2015; Konopacki i Rakoczy, 2019; Masood i wsp., 2020). Niemniej jednak, pomimo, że bakterie można przypisać do określonych gatunków czy grup np. wg budowy ściany komórkowej czy kształtu komórki, to wykazują one znaczną różnorodność, nie tylko w obrębie gatunku, ale nawet szczepu, a do pewnego stopnia nawet w obrębie klonu (Dijkshoorn i wsp., 2000; Nawrotek i wsp., 2012). Dane literaturowe wskazują, że PM działa na bakterie jak czynnik stresowy (Cellini i wsp., 2008; Filipič i wsp., 2012), podobnie jak zmiany np. pH czy temperatury. Dane literaturowe wskazują również, że odpowiedź bakterii na środowiskowe czynniki stresowe jest szczepowo-zależna (Kossakowska i wsp., 2013; Lamret i wsp., 2021; Weiss i wsp., 2021). Można założyć więc, że wpływ PM na różnorodne parametry komórkowe bakterii będzie determinowany charakterystyką danego szczepu, a nawet klonu w obrębie gatunku, a nie tylko cechami bakterii wskazanymi dotychczas w literaturze tj. kształt komórki, budowa ściany komórkowej czy też przynależność gatunkowa.

1.6. Wpływ pola magnetycznego na efektywność działania antybiotyków względem bakterii patogennych

1.6.1. Odziaływanie pola magnetycznego na struktury komórkowe oraz procesy biologiczne w komórkach bakteryjnych

Obecnie wiadomo, że różne rodzaje PM wywierają wpływ na struktury komórkowe bakterii jak również na procesy biologiczne zachodzące w ich komórkach. Wiąże się to przede wszystkim z obecnością w komórkach bakteryjnych cząsteczek paramagnetycznych i diamagnetycznych, np. jonów (m.in. Na^+, K^+ i Ca^{2+}), wolnych rodników oraz makrocząsteczek wchodzących w skład struktur komórkowych, tj. kwasów nukleinowych, białek czy aminokwasów, które mogą mieć charakter dipolów (Zhou i wsp., 2014). W diamagnetykach zewnętrzne PM indukuje powstanie PM o przeciwnym zwrocie, natomiast w paramagnetykach indukowane jest PM o zwrocie zgodnym z kierunkiem zewnętrznego PM – poprzez te oddziaływania, zewnętrze PM wpływa na ruch tych cząsteczek. Ponadto, pod wpływem zewnętrznego PM jony lub dipole generują prąd elektryczny o niskim natężeniu, mogący dodatkowo wpływać na procesy komórkowe (Guo i wsp., 2022). Składniki macierzy biofilmu oraz kwasy tejchojowe obecne w ścianach komórkowych bakterii również są obdarzone ładunkiem elektrycznym i mogą podlegać wpływom zewnętrznego PM (Moryl, 2015).

Transport składników odżywczych oraz metabolitów bakterii zachodzi z wykorzystaniem kanałów jonowych (Compton i Mindell, 2010). Jony

znajdujące się po obu stronach błony komórkowej są rozmieszczone nierównomiernie, co skutkuje różnicą potencjałów pomiędzy jej częścią wewnętrzną a zewnętrzną. Tym samym, przemieszczenie się jonów pomiędzy warstwami błony przez kanały jonowe powoduje krótkotrwałe zmiany potencjału błonowego komórki. W tym kontekście Hughes i wsp. (2005), Torgomyan i wsp. (2011) oraz Tadevosyan i Trchounian (2009) wykazali, że pod wpływem zewnętrznego PM zmianie ulega rozmieszczenie jonów, dochodzi do zmian potencjałów spoczynkowych błon komórkowych, co prowadzi do zwiększenia intensywności transportu jonowego. Dodatkowo, Qian i wsp. (2016) wykazali, że pod wpływem ekspozycji bakterii na PM dochodzi do uszkodzeń DNA oraz ścian i błon komórkowych bakterii (co skutkuje ich wyższą przepuszczalnością). Z kolei Cellini i wsp. (2008) wykazali, że ekspozycja bakterii na PM indukuje negatywne zmiany w morfologii komórek. Ponadto, Novickij i wsp. (2016) wykazali, że PM może prowadzić do elektroporacji lub trwałego uszkodzenia błon komórkowych. W kontekście wyżej opisanych efektów ekspozycji komórek bakteryjnych na PM wydaje się, że miejsca oddziaływania PM i niektórych antybiotyków na komórki bakterii mogą być zbieżne, przynajmniej w odniesieniu do takich struktur jak kwasy nukleinowe czy ściany i błony komórkowe oraz procesów biologicznych takich jak transport transbłonowy.

1.6.2. Mechanizm działania antybiotyków oraz potencjalny wpływ pola magnetycznego na ich cząsteczki

Aktywność antybiotyków względem bakterii opiera się na ich wybiórczym działaniu na różne struktury komórkowe i procesy biologiczne, skutkiem czego wykazują one działanie bakteriobójcze lub bakteriostatyczne. Zastosowane w niniejszej rozprawie doktorskiej różne grupy antybiotyków charakteryzują się odmiennym mechanizmem działania. Tetracykliny wpływają na wiele procesów w komórce m.in. na oksydatywną fosforylację lub transport przez błonę cytoplazmatyczną, ale ich głównym celem jest hamowanie syntezy białek poprzez wiązanie się z podjednostką 30S rybosomu; makrolidy i linkozamidy wiążą się do podjednostki 50S rybosomu hamując syntezę białek; aminoglikozydy hamują lub zaburzają syntezę białek poprzez wiązanie się z podjednostką 30S rybosomu; β-laktamy hamują syntezę ściany komórkowej bakterii poprzez wiązanie się do białek wiążących penicylinę (ang. *penicillin binding proteins*, PBP); fluorochinolony hamują syntezę DNA poprzez inhibicję aktywności topoizomeraz DNA i gyrazy RNA, a glikopeptydy hamują syntezę ściany komórkowej poprzez wiązanie się do reszt D-alanylo-D-alaniny (Kapoor i wsp., 2017). Co istotne, antybiotyki oprócz różnego mechanizmu działania charakteryzują się również odmiennymi ładunkami elektrycznymi (Zhu i wsp., 2016) i jako cząsteczki posiadające ładunek podlegają oddziaływaniom PM (Rakoczy i wsp., 2017) co potencjalnie może wpłynąć na ich dyfuzję w podłożu hodowlanym czy też stopień penetracji przez struktury biofilmu. Ponadto, niektóre z w/w antybiotyków mają wewnątrzkomórkowe miejsca docelowe, tym samym cząsteczki te muszą przekroczyć barierę ściany i błony komórkowej bakterii. W tym kontekście wykazana przez Novickij i wsp., 2016 zdolność PM do wywołania elektroporacji lub też trwałego uszkodzenia błon komórkowych może potencjalnie umożliwić dyfuzję cząsteczek antybiotyków do wnętrza komórek.

1.6.3. Obecny stan wiedzy dotyczącej wpływu pola magnetycznego na zwiększenie efektywności działania antybiotyków

Jak wynika z dostępnej literatury, zwiększenie efektywności działania antybiotyków wobec bakterii w obecności PM oraz potencjalne mechanizmy leżące u podstaw tego zjawiska zostały zaprezentowane przez kilkunastu autorów (Benson i wsp., 1994; Gaafar i wsp., 2006; Matl i wsp., 2011; Segatore i wsp., 2012; Kamel i wsp., 2013; Kamel i wsp., 2014; Al-Harbi i wsp., 2018; Junka i wsp., 2018; Salmen i wsp., 2018; Wang i wsp., 2021; Juncker i wsp., 2022; Doleib i Saddiq, 2023). Niemniej jednak, analiza opublikowanych wyników badań i ich usystematyzowanie jest trudne m.in. z uwagi na różne rodzaje stosowanych PM, różne czasy ekspozycji hodowli bakteryjnych na PM w obecności antybiotyków, dobór antybiotyków oraz ich stężeń, różnice w systemach generujących PM oraz ilość ujętych w badaniach szczepów bakteryjnych.

Zgodnie z zaleceniami Europejskiego Komitetu ds. Badania Wrażliwości Drobnoustrojów (ang. *European Committee on Antimicrobial Susceptibility Testing*, EUCAST) oraz Instytutu Norm Klinicznych i Laboratoryjnych (ang. *Clinical and Laboratory Standards Institute*, CLSI) oznaczenie wrażliwości na antybiotyki należy przeprowadzić dla każdego wyizolowanego szczepu bakteryjnego. Zalecenia te wynikają z faktu, że stopień wrażliwość wobec antybiotyków jest unikalny dla każdego szczepu. Natomiast, pomimo gruntownego przeglądu dostępnej literatury nie odnaleziono żadnej publikacji, w której efektywność działania antybiotyków względem bakterii pod wpływem PM została przebadana z wykorzystaniem kilku szczepów należących do tego samego gatunku.

Tym samym uzasadnione wydaje się przeanalizowanie wpływu jednego rodzaju PM na efektywność działania różnych antybiotyków wobec różnych szczepów bakterii, biorąc pod uwagę wiele czynników mogących wpłynąć na uzyskane wyniki, w tym mechanizm działania antybiotyku, jego stężenie oraz ładunek elektryczny; czas ekspozycji oraz częstotliwość PM.

2. Hipotezy

Na podstawie dokonanego przeglądu literatury sformułowano następujące hipotezy:

1. Wpływ WPM na parametry komórkowe bakterii, takie jak kinetyka wzrostu oraz komórkowa aktywność metaboliczna jest determinowany zróżnicowaniem szczepów w obrębie gatunku (publikacja D-1).
2. Zwiększenie efektywności działania antybiotyków względem *S. aureus* w obecności WPM jest determinowane mechanizmem ich działania oraz stężeniem, a także częstotliwością zastosowanego WPM i czasem ekspozycji (publikacja D-2, publikacja D-3).
3. Zwiększenie efektywności działania antybiotyków względem *S. aureus* w obecności WPM jest determinowane zróżnicowaniem szczepów w obrębie gatunku (publikacja D-2, publikacja D-3).

4. WPM wpływa na kinetykę uwalniania antybiotyków z krążków bibułowych oraz ich dyfuzję w podłożu agarowym (publikacja D-2, publikacja D-3).
5. Z uwagi na falowy charakter WPM, jego wpływ na integralność ścian komórkowych *S. aureus* nie jest ograniczony czynnikami fizycznymi takimi jak grubość i/lub gęstość komórek w poszczególnych warstwach biofilmu (publikacja D-2).
6. Ekspozycja *S. aureus* na działanie WPM skutkuje zmianami w morfologii komórek tych bakterii, w tym przede wszystkim w strukturze ich ścian komórkowych (publikacja D-2, publikacja D-3).
7. WPM nie wykazuje wpływu cytotoksycznego na komórki *S. aureus* (publikacja D-3).
8. Zwiększona efektywność działania antybiotyków, których mechanizm działania związany jest z hamowaniem syntezy ściany komórkowej, wynika ze zmian strukturalnych w ścianie komórkowej *S. aureus*, indukowanych pod wpływem WPM (publikacja D-2, publikacja D-3).
9. Zmiany w efektywności działania antybiotyków β-laktamowych wobec *S. aureus* zachodzą wyłącznie podczas ich ekspozycji na WPM (brak efektu poekspozycyjnego) (publikacja D-3).

3. Cel

Podstawowym celem publikacji wchodzących w skład osiągnięcia naukowego i stanowiących podstawę ubiegania się o stopień naukowy doktora była analiza wpływu WPM na efektywność działania antybiotyków względem bakterii patogennych.

Cele szczegółowe:

1. Ocena wpływu WPM na kinetykę wzrostu oraz komórkową aktywność metaboliczną bakterii w zależności od czasu ekspozycji, częstotliwości zastosowanego WPM oraz zróżnicowania szczepów w obrębie gatunku (publikacja D-1).
2. Ocena wpływu WPM na efektywność działania antybiotyków wobec *S. aureus* w zależności od ich stężenia oraz mechanizmu działania (publikacja D-2, D-3).
3. Ocena wpływu WPM na efektywność działania antybiotyków wobec *S. aureus* w zależności od częstotliwości WPM i czasu ekspozycji oraz zróżnicowania szczepów w obrębie gatunku (publikacja D-2, D-3).
4. Ocena wpływu WPM na uwalnianie antybiotyków z krążków bibułowych oraz ich dyfuzję w agarze w zależności od częstotliwości zastosowanego WPM oraz ładunku antybiotyku (publikacja D-2, D-3).
5. Ocena wpływu WPM oraz połączonego wpływu WPM i antybiotyku β-laktamowego na komórki bakteryjne w biofilmie (publikacja D-2).
6. Ocena wpływu WPM na morfologię komórek *S. aureus* (publikacja D-2, D-3).
7. Ocena wpływu WPM na liczbę *S. aureus* zdolnych do wzrostu na podłożu stałym oraz na ilość bakterii żywych i martwych (publikacja D-3).

8. Ocena zmian w efektywności działania antybiotyków wobec *S. aureus* preeksponowanych na WPM (efekt poekspozycyjny) (publikacja D-3).
9. Usystematyzowanie sposobu prowadzenia analiz dotyczących wpływu PM na parametry funkcjonalne bakterii oraz efektywność działania substancji przeciwdrobnoustrojowych (publikacja D-1, D-2, D-3).

4. Materiały i metody badawcze

4.1. Bioreaktory wspomagane wirującym polem magnetycznym

Badania przeprowadzono przy użyciu stanowisk badawczych wyposażonych w prototypowe bioreaktory wspomagane WPM (opisane i zaprezentowane w publikacjach D-1, D-2 i D-3) oraz aparaturę kontrolno-pomiarową. Urządzenia zaprojektowano i zbudowano w ramach projektu Lider 5 pt. „Projekt, budowa i optymalizacja działania bioreaktora wspomaganego wirującym polem magnetycznym do produkcji celulozy bakteryjnej", finansowanego przez Narodowe Centrum Badań i Rozwoju, a następnie zautomatyzowano i zoptymalizowano w ramach projektu Opus 14 pt. „Analiza mechanizmów zwiększonej efektywności substancji przeciwdrobnoustrojowych względem biofilmu w obecności wirującego pola magnetycznego" finansowanego przez Narodowe Centrum Nauki. Oba projekty kierowane były przez dr. hab. inż. Karola Fijałkowskiego, prof. ZUT (KMiB, WBiHZ, ZUT).

Urządzenia te składają się z generatora WPM zbudowanego ze stojanu trójfazowego uzyskanego z silnika elektrycznego oraz komory procesowej, w której w trakcie ekspozycji umieszczano hodowle bakterii. Stojan generatora WPM zasilany jest 3-fazowym prądem przemiennym o częstotliwości 50 Hz. W celu zmiany częstotliwości prądu, a tym samym pulsacji WPM wykorzystywany jest przemiennik częstotliwości. Temperatura inkubacji w czasie ekspozycji na WPM kontrolowana jest za pomocą systemu składającego się z termostatów, płaszcza chłodzącego oraz pompy obiegowej. W generatorach w trakcie eksperymentów utrzymywana jest stała wilgotność względna (RH) na poziomie 60%.

W zależności od przeprowadzonej analizy, zastosowano od 1 do 4 różnych częstotliwości WPM (5, 10, 25 lub 50 Hz). Częstotliwość zastosowanego prądu przemiennego determinuje intensywność WPM, wyrażoną jako wartość indukcji magnetycznej oraz odpowiada za kształt fali magnetycznej (determinowanej okresem pomiędzy maksymalną i minimalną indukcją magnetyczną). Wizualizacje obrazujące opisane zależności zaprezentowano w publikacjach D-1, D-2 i D-3. Analizy i wizualizacje związane z charakterystyką WPM wykonano we współpracy z dr. hab. inż. Marcinem Wardachem, prof. ZUT.

4.2. Mikrooorganizmy

W celu oceny wpływu WPM na kinetykę wzrostu oraz komórkową aktywność metaboliczną użyto 8 referencyjnych szczepów bakterii należących do następujących gatunków: *S. aureus* American Type Culture Collection (ATCC) 6538, *P. aeruginosa* ATCC 15442, *P. mirabilis* ATCC 7002, *K. pneumoniae* ATCC 70603, *E. faecalis* ATCC

29212, *E. cloacae* Polish Collection of Microorganisms (PCM) 2569, *M. catarrhalis* PCM 2340 oraz *B. cereus* PCM 449. Dodatkowo, wykorzystano 7 izolatów *S. aureus* (w tym 4 izolaty należące do jednego typu klonalnego, które wyizolowane zostały od krów z mastitis), a także po 3 izolaty z pozostałych, wyżej wymienionych gatunków pochodzące z kolekcji mikroorganizmów KMiB, ZUT oraz z kolekcji mikroorganizmów KiZMFiP, UMW (publikacja D-1).

W celu oceny wpływu WPM na zmiany w efektywności działania antybiotyków wykorzystano 2 referencyjne szczepy *S. aureus* (ATCC 33591 i ATCC 6538) oraz 23 kliniczne izolaty MRSA, a także 5 klinicznych izolatów MSSA pochodzących z kolekcji mikroorganizmów KiZMFiP, UMW (publikacja D-2, D-3).

4.3. Substancje przeciwdrobnoustrojowe

W trakcie badań wykorzystano antybiotyki z następujących grup: glikopeptydy (teikoplanina), makrolidy (erytromycyna), linkozamidy (klindamycyna), aminoglikozydy (gentamycyna), tetracykliny (tetracyklina), fluorochinolony (ciprofloksacyna) oraz β-laktamy (cefalosporyny I generacji – cefazolina, cefradyna, cefaleksyna; cefalosporyna II generacji – cefuroksym; cefalosporyny III generacji – ceftriakson, ceftazydym; cefalosporyna IV generacji – cefepim; cefalosporyna V generacji – ceftarolina; cefamycyny – cefoksytyna, cefotetan; karbapenemy – meropenem, imipenem, ertapenem, doripenem i aminopenicyliny – amoksycylina, amoksycylina z kwasem klawulanowym). Antybiotyki wybrane do analiz charakteryzowały się różnym mechanizmem działania oraz ładunkiem elektrycznym (kationowym, anionowym bądź obojnaczym). W przebiegu analiz zastosowano antybiotyki w postaci krążków bibułowych (Oxoid, UK), pasków Etest (Liofilchem, Włochy) oraz roztworów przygotowanych z proszków (Pol-Aura, Polska; Millipore Sigma, USA) (publikacja D-2, D-3).

4.4. Charakterystyka szczepów *Staphylococcus aureus* wykorzystanych w badaniach

4.4.1. Detekcja genu *mecA*

Obecność genu *mecA* określono techniką PCR. Zastosowano startery opisane przez Oliveira i de Lencastre (2002) i następujące warunki reakcji: 95°C przez 2 min, 30 cykli (95°C przez 30 s, 55°C przez 30 s, 72°C przez 1 min), końcowa elongacja w temperaturze 72°C przez 5 min. Reakcję PCR przeprowadzono w termocyklerze peqSTAR (Peqlab Biotechnologie GmbH, Niemcy), następnie produkty reakcji rozdzielono w 2% żelu agarozowym. Jako kontrolę pozytywną dla genu *mecA* wykorzystano *S. aureus* ATCC 33591, jako negatywną *S. aureus* ATCC 6538 (publikacja D-3).

4.4.2. Analiza pokrewieństwa izolatów *S. aureus*

Pokrewieństwo genetyczne pomiędzy izolatami *S. aureus* określono poprzez trawienie genomowego DNA enzymem restrykcyjnym *Sma*I (Thermo Fisher Scientific, Niemcy), a następnie jego rozdział metodą elektroforezy żelowej w polu pulsacyjnym

(ang. *pulsed-field gel electrophoresis*, PFGE). Rozdział przeprowadzono w zestawie do elektroforezy CHEF-DR III (Bio-Rad Laboratories Inc., Francja) w 1,2% żelu agarozowym. Otrzymane wzory restrykcyjne przeanalizowano przy pomocy programu FPQuest (Bio-Rad Laboratoires, Inc., Francja). Klasyfikacji poszczególnych wzorów restrykcyjnych dokonano z wykorzystaniem metody grupowania UPGMA oraz współczynnika Dice'a. Wyniki przedstawiono w postaci dendrogramu.

Powyższą analizę wykonano we współpracy z dr n. med. Agatą Pruss oraz dr. n. med. Pawłem Kwiatkowskim (publikacja D-1).

4.5. Ekspozycja hodowli bakteryjnych na wirujące pole magnetyczne w celu analizy zmian w kinetyce wzrostu i komórkowej aktywności metabolicznej

Hodowle o gęstości 1,0 (≈ 3 x 10^8 CFU/ml; ang. *colony forming units*, CFU) w skali McFarlanda, rozcieńczono w stosunku 1:10 w podłożu Tryptic Soy Broth (TSB). Otrzymane zawiesiny przeniesiono w objętości 10 ml do probówek i eksponowano na WPM o częstotliwości 5 i 50 Hz przez 9 godzin, w temperaturze 37°C.

Zmiany w kinetyce wzrostu oceniono poprzez pomiary gęstości optycznej hodowli (ang. *optical density*, OD), która pośrednio odzwierciedla ilość komórek bakteryjnych. Pomiary OD wykonano za pomocą czytnika mikropłytek (Tecan Trading AG, Szwajcaria) przy długości fali 600 nm. Zmiany w komórkowej aktywności metabolicznej oceniono z wykorzystaniem odczynnika na bazie resazuryny (AlamarBlue, Thermo Fisher Scientific, USA). Pomiary fluorescencji wykonano za pomocą czytnika mikropłytek (Synergy HTX, BioTek, USA) przy długościach fali wzbudzenia 540 nm i fali emisji 590 nm. Oceny obu parametrów dokonano w 3, 6 i 9 godzinie ekspozycji na WPM.

Uzyskane wyniki porównano do wyników uzyskanych dla nieeksponowanych na WPM hodowli kontrolnych. Hodowle kontrolne inkubowano w takiej samej temperaturze i wilgotności względnej jak hodowle eksponowane na WPM, w bliźniaczym bioreaktorze, z wyłączonym generatorem WPM (publikacja D-1).

4.6. Ocena wpływu wirującego pola magnetycznego na liczbę komórek *S. aureus* zdolnych do wzrostu na podłożu stałym

Zawiesiny *S. aureus* o gęstości 0,5 (≈ 1-2 x 10^8 CFU/ml) w skali McFarlanda, rozprowadzono za pomocą sterylnej wymazówki na powierzchni podłoża Müller-Hinton Agar (M-HA) i eksponowano na WPM o częstotliwości 5 Hz przez 12 godzin w temperaturze 35 ± 1°C. Następnie, hodowle przeniesiono do probówki z buforowaną fosforanem solą fizjologiczną (PBS) i wykonano rozcieńczenia dziesiętne otrzymanej zawiesiny. W dalszej kolejności rozcieńczone zawiesiny wysiano na podłożach Brain Heart Infusion Agar (BHIA), inkubowano przez 24 godziny w temperaturze 37°C, a następnie zliczono wyrosłe kolonie bakteryjne. Wynik wyrażono jako liczbę CFU/mL.

Uzyskane wyniki porównano do wyników uzyskanych dla nieeksponowanych na WPM hodowli kontrolnych. Hodowle kontrolne inkubowano w cieplarce w takiej samej temperaturze i wilgotności względnej jak w generatorze WPM (publikacja D-3).

4.7. Analiza wpływu wirującego pola magnetycznego na zmiany w ilości żywych i martwych komórek *S. aureus* przy użyciu mikroskopii fluorescencyjnej

Zawiesiny *S. aureus* o gęstości 0,5 (≈ 1-2 x 10^8 CFU/ml) w skali McFarlanda w podłożu Müller-Hinton Broth (M-HB) eksponowano na WPM o częstotliwości 5 Hz przez 12 godzin w temperaturze 35 ± 1°C. Następnie hodowle odwirowano, a uzyskany pellet wybarwiono roztworem SYTO9 i jodku propidyny (Invitrogen, Thermo Fisher Scientific, USA). Zabarwione komórki przeniesiono do płytki 24-dołkowej i analizowano za pomocą mikroskopu fluorescencyjnego Lumascope 620 (Etaluma, USA). Uzyskane obrazy poddano dalszej analizie przy użyciu oprogramowania ImageJ (National Institutes of Health, USA).

Uzyskane wyniki porównano do wyników uzyskanych dla hodowli kontrolnych nieeksponowanych na WPM. Hodowle kontrolne inkubowano w cieplarce w takiej samej temperaturze i wilgotności względnej jak w generatorze WPM.

Powyższą analizę wykonano we współpracy z dr. hab. n. med. Adamem Junką, prof. UMW (publikacja D-3) .

4.8. Ekspozycja *S. aureus* na wirujące pole magnetyczne w celu analizy zmian w efektywności działania antybiotyków

Ocenę wrażliwości na antybiotyki wykonano metodą dyfuzyjno-krążkową zgodnie z wytycznymi EUCAST, oraz metodą gradientowo-dyfuzyjną zgodnie z wytycznymi producenta pasków Etest. Sporządzone zawiesiny o gęstości 0,5 (≈ 1-2 x 10^8 CFU/ml) w skali McFarlanda, rozprowadzono za pomocą sterylnej wymazówki na powierzchni podłoża M-HA. Na tak przygotowanych podłożach umieszczono krążki antybiotykowe lub Etesty. W zależności od wykonywanych analiz hodowle bakteryjne eksponowano na WPM o częstotliwości od 5 do 50 Hz przez okres od 1 do 18 godzin w temperaturze 35 ± 1°C. Po ekspozycji dokonano pomiaru średnicy stref zahamowania wzrostu wokół krążków antybiotykowych lub odczytano wartość minimalnego stężenia inhibitującego (ang. *minimal inhibitory concentration*, MIC) na podstawie stref zahamowania wzrostu wokół Etestu.

Uzyskane wyniki porównano do wyników uzyskanych dla nieeksponowanych na WPM hodowli kontrolnych. Hodowle kontrolne inkubowano w cieplarce w takiej samej temperaturze i wilgotności względnej jak w generatorze WPM (publikacja D-2, D-3).

4.9. Analiza zmian w efektywności działania antybiotyków wobec bakterii preeksponowanych na WPM (efekt poekspozycyjny)

Zawiesiny *S. aureus* o gęstości 0,5 (≈ 1-2 x 10^8 CFU/ml) w skali McFarlanda w podłożu M-HB eksponowano na WPM o częstotliwości 5 Hz przez 12 godzin w temperaturze 35 ± 1°C. Następnie hodowle odwirowano, doprowadzono ponownie do gęstości 0,5 w skali McFarlanda, rozprowadzono za pomocą sterylnej wymazówki na powierzchni podłoża M-HA i wykonano antybiogramy metodą dyfuzyjno-krążkową (z wykorzystaniem cefoksytyny, cefepimu, ceftriaksonu i cefuroksymu)

Uzyskane wyniki (średnice stref zahamowania wzrostu wokół krążków) porównano do nieeksponowanych na WPM hodowli kontrolnych. Hodowle kontrolne inkubowano w cieplarce w takiej samej temperaturze i wilgotności względnej jak w generatorze WPM (publikacja D-3).

4.10. Analiza wpływu wirującego pola magnetycznego na uwalnianie antybiotyków z krążków bibułowych oraz ich dyfuzję w agarze

W pierwszym etapie krążki bibułowe nasycone antybiotykami (cefoksytyna 30 µg, amoksycylina 25 µg, erytromycyna 15 µg, ciprofloksacyna 5 µg, tetracyklina 30 µg, gentamycyna 2 µg publikacja D-2; cefoksytyna 30 µg, cefepim 30 µg, imipenem 10 µg publikacja D-3) umieszczono na powierzchni stałego podłoża agarowego (1,7% agar w wodzie demineralizowanej, w/v) i eksponowano na działanie WPM o częstotliwości 5 i 50 Hz, przez 30 i/lub 120 min (w zależności od przeprowadzonej analizy), w temperaturze 35 ± 1°C. W kolejnym etapie, z powierzchni agaru zdjęto krążki antybiotykowe, a następnie za pomocą korkoboru wycięto krążki agaru o średnicy 6 mm (1 krążek bezpośrednio spod krążka bibułowego oraz 12 krążków rozmieszczonych koncentrycznie w odległości od 1-12 mm od miejsca, w którym umieszczony był krążek antybiotykowy) (Rycina 8 w publikacji D-2). W celu ekstrakcji antybiotyków krążki bibułowe i agarowe przeniesiono do 0,5 ml 50% roztworu metanolu w wodzie dejonizowanej i inkubowano z wytrząsaniem od 2 do 3 godzin. Następnie roztwory z wyekstrahowanymi antybiotykami przefiltrowano i analizowano techniką chromatografii cieczowej z tandemową spektrometrią mas (ang. *liquid chromatography-tandem mass spectrometry*, LC-MS/MS) (1260 Infinity II Series Liquid Chromatograph, Agilent, USA). Analizy ilościowej dokonano odnosząc się do krzywych kalibracyjnych sporządzonych przy użyciu wzorców antybiotyków (Millipore Sigma, USA).

Uzyskane wyniki porównano do nieeksponowanych na WPM układów kontrolnych. Kontrole inkubowano w cieplarce w takiej samej temperaturze i wilgotności względnej jak w generatorze WPM.

Powyższe analizy wykonano we współpracy z dr. inż. Radosławem Drozdem, dr inż. Darią Ciecholewską-Juśko oraz z dr. Danielem Styburskim (publikacja D-2, D-3).

4.11. Analiza zmian w morfologii komórek *S. aureus* po ekspozycji na wirujące pole magnetyczne

Zawiesiny *S. aureus* o gęstości 0,5 (≈ 1-2 x 10^8 CFU/ml) w skali McFarlanda, rozprowadzono za pomocą sterylnej wymazówki na powierzchni podłoża MHA i eksponowano na WPM o częstotliwości 5 Hz przez 12 godzin w temperaturze 35 ± 1°C. Po ekspozycji, z podłoży M-HA wycięto za pomocą korkoboru krążki o średnicy 6 mm. Dodatkowo, do analiz użyto krążków, które wycięto podczas przeprowadzania analiz z wykorzystaniem metody dyfuzyjno-krążkowej (z granicy stref zahamowania wzrostu wokół krążka z cefoksytyną). Następnie, w celu przygotowania do analizy skaningowym mikroskopem elektronowym (ang. *scanning electron microscope*, SEM, Zeiss EVO MA25, Carl Zeiss, Niemcy) krążki utrwalono w 3% aldehydzie glutarowym, odwodniono we wzrastających stężeniach

etanolu i pokryto mieszaniną złota i palladu przy użyciu maszyny Quorum (Quorum International, USA). Natomiast w celu wykonania analizy transmisyjnym mikroskopem elektronowym (ang. *transmission electron microscope*, TEM, JEOL 1200, JEOL, Japonia) krążki utrwalono w 2% aldehydzie glutarowym i odwirowano. Następnie przeprowadzono kontrastowanie z 2% octanem uranylu i 2% czterotlenkiem osmu, po czym hodowle umieszczono we wzrastających stężeniach etanolu i utrwalono w żywicy epoksydowej. W dalszej kolejności przygotowano skrawki z bloków żywicy przy użyciu UltraMicrotome Leica EMUC7 (Leica, Niemcy).

Uzyskane wyniki porównano do nieeksponowanych na WPM hodowli kontrolnych (bez antybiotyku) oraz nieeksponowanych na WPM hodowli wyrosłych wokół krążka z cefoksytyną. Hodowle kontrolne inkubowano w cieplarce w takiej samej temperaturze i wilgotności względnej jak w generatorze WPM.

Powyższe analizy wykonano we współpracy z dr inż. Pawłem Migdałem oraz dr inż. Patrycją Szymczyk-Ziółkowską (publikacja D-3).

4.12. Analiza zmian w integralności ścian komórkowych bakterii w biofilmie traktowanym cefoksytyną i eksponowanym na wirujące pole magnetyczne

Hodowlę bakteryjną o gęstości 1×10^5 CFU/mL w TSB z 1% dodatkiem glukozy, przeniesiono w objętości 1 ml do dołków 24-dołkowej płytki i inkubowano 48 godzin w temperaturze 37°C.

Po inkubacji odciągnięto TSB, a do dołków z biofilmem dodano roztwór cefoksytyny i eksponowano na WPM o częstotliwości 5 Hz przez 12 godzin, w temperaturze 36 ± 1°C. Po ekspozycji roztwór cefoksytyny odciągnięto, biofilm wybarwiono roztworem SYTO9 i jodku propidyny (Thermo Fisher Scientific, USA) i analizowano za pomocą mikroskopu konfokalnego Leica SP8 (Wetzlar, Niemcy). Uzyskane obrazy poddano dalszej analizie przy użyciu oprogramowania Imaris 9 (Abingdon, UK).

Jako kontrole wykorzystano biofilm eksponowany tylko na WPM, biofilm traktowany tylko cefoksytyną i biofilm nietraktowany żadnym z wymienionych czynników. Hodowle kontrolne inkubowano w cieplarce, w takiej samej temperaturze i wilgotności względnej jak w generatorze WPM.

Powyższe analizy wykonano we współpracy z dr. hab. n. med. Adamem Junką, prof. UMW, dr. inż. Grzegorzem Chodaczkiem oraz z dr. Bartłomiejem Dudkiem (publikacja D-2).

4.13. Analiza statystyczna

Dane przedstawiono jako średnie ± odchylenia standardowe średnich (SEM) obliczone z co najmniej trzech różnych pomiarów. Istotność statystyczną różnic między hodowlami eksponowanymi na WPM a kontrolnymi analizowano za pomocą dwukierunkowej analizy wariancji (ANOVA) (publikacja D-1), jednokierunkowej analizy wariancji (ANOVA) (publikacja D-2, D-3) oraz testu post hoc Tukeya (publikacja D-1, D-2, D-3). Różnice uznano za istotne na poziomie $p < 0{,}05$. Analizy statystyczne przeprowadzono przy użyciu programów Statistica 12.5 (StatSoft, Inc., USA) (publikacja D-1, D-3) oraz GraphPad Prism 9.0 (GraphPad Software Inc., USA) (publikacja D-2).

5. Wyniki

Wszystkie przeprowadzone badania zaprezentowane w publikacjach D-1, D-2, D-3 stanowią powiązany ze sobą cykl eksperymentalny. Cykl ten w pierwszej kolejności opisuje ocenę wpływu WPM na kinetykę wzrostu oraz komórkową aktywność metaboliczną 40 szczepów należących do 8 gatunków bakterii. W drugim etapie, przedstawiono analizy związane z wpływem WPM na zmiany w efektywności działania antybiotyków o różnym mechanizmie działania, wobec bakterii z gatunku *S. aureus* (w tym szczepów MRSA i MSSA). Dodatkowo, przeanalizowano wpływ WPM na uwalnianie antybiotyków z krążków antybiotykowych oraz ich dyfuzję w agarze. Ponadto, analizie poddano wpływ WPM na morfologię komórek bakteryjnych *S. aureus*. Oceniono również ilość żywych i martwych komórek oraz zdolność *S. aureus* do wzrostu na podłożu stałym po ekspozycji na WPM. Przenalizowano także połączony wpływ WPM i antybiotyku β-laktamowego (cefoksytyny) na integralność ścian komórkowych *S. aureus*. Ustalono również czy zmiany w efektywności działania antybiotyków β-laktamowych wobec *S. aureus* zachodzą wyłącznie podczas ekspozycji na WPM, czy też pozostają utrwalone po ekspozycji.

Etap 1. Analiza wpływu WPM na kinetykę wzrostu oraz komórkową aktywność metaboliczną różnych gatunków i szczepów bakterii.

Etap ten obejmował:

- Ocenę wpływu WPM na kinetykę wzrostu oraz komórkową aktywność metaboliczną bakterii w zależności od czasu ekspozycji oraz częstotliwości WPM (5 i 50 Hz);
- Ocenę wpływu WPM na kinetykę wzrostu oraz komórkową aktywność metaboliczną w zależności od gatunku bakterii oraz szczepu w obrębie tego samego gatunku bakterii.

Etap 2. Analiza wpływu WPM na zmiany na zmiany w efektywności działania antybiotyków o różnych mechanizmach działania wobec *S. aureus,* w tym szczepów MRSA i MSSA.

Etap ten obejmował:

- Ocenę wpływu WPM na zmiany w efektywności działania antybiotyków wobec różnych szczepów *S. aureus*, w zależności od mechanizmu działania antybiotyku, jego ładunku, stężenia, a także częstotliwości WPM (5-50 Hz) oraz czasu ekspozycji (1-18 godzin);
- Ocenę wpływu WPM na zmiany w efektywności działania antybiotyków wobec różnych szczepów *S. aureus*;
- Ocenę wpływu WPM na uwalnianie antybiotyków z krążków bibułowych oraz ich dyfuzję w agarze w zależności od częstotliwości WPM (5 i 50 Hz) i czasu ekspozycji (30-120 min);

- Ocenę połączonego wpływu WPM i antybiotyku β-laktamowego (cefoksytyny) na integralność ścian komórkowych *S. aureus*;
- Ocenę wpływu preekspozycji na WPM na zdolność *S. aureus* do wzrostu na podłożu stałym;
- Ocenę wpływu WPM na zmiany w ilości żywych i martwych komórek *S. aureus*;
- Ocenę wpływu WPM na morfologię ścian komórkowych *S. aureus*;
- Ocenę zmian w efektywności działania antybiotyków wobec bakterii *S. aureus* preeksponowanych na WPM (efekt poekspozycyjny).

W pierwszej kolejności, analizie poddano wpływ WPM na kinetykę wzrostu oraz komórkową aktywność metaboliczną 40 szczepów należących do 8 gatunków bakterii (*S. aureus, P. aeruginosa, K. pneumoniae, P. mirabilis, E. faecalis, E. cloacae, M. catarrhalis* i *B. cereus*). Uzyskane wyniki, wraz z przeglądem piśmiennictwa przedstawiono w publikacji D-1, a szczegółową statystykę w Suplemencie do publikacji D-1.

W wyniku przeprowadzonych badań ustalono, że WPM wpływa na oba analizowane parametry komórkowe, a obserwowane efekty są zależne od czasu ekspozycji oraz od częstotliwości WPM. Wykazano również, że w zależności od eksponowanego szczepu należącego do tego samego gatunku bakterii wpływ wywierany przez WPM może być stymulujący (tzn. zwiększać tempo wzrostu i/lub komórkową aktywność metaboliczną), hamujący (tzn. działać hamująco na wyżej wymienione parametry) lub może nie występować. Ponadto, ustalono, że nawet w przypadku ekspozycji na WPM 4 izolatów *S. aureus* należących do jednego typu klonalnego, uzyskane wyniki różniły się istotnie. Tym samym, potwierdzono, że wpływ WPM na kinetykę wzrostu oraz komórkową aktywność metaboliczną, oprócz parametrów opisanych dotąd w literaturze (np. kształtu czy budowy komórki bakteryjnej), jest ściśle powiązany ze zróżnicowaniem szczepów w obrębie każdego z analizowanych gatunków bakterii. Jest to obserwacja unikalna, ponieważ po dokonaniu szerokiego przeglądu literatury w zakresie przeprowadzonych badań, nie napotkano publikacji, w której autorzy analizujący wpływ PM na powyższe parametry komórkowe bakterii, zastosowaliby wyżej opisane podejście do doboru materiału badawczego, tzn. ujęli różne szczepy w obrębie jednego gatunku i poddali je analizie indywidualnie.

W kolejnym etapie badań prowadzonych w ramach realizowanej rozprawy doktorskiej, przeanalizowano wpływ WPM na efektywność działania antybiotyków z różnych grup wobec bakterii z gatunku *S. aureus* (w tym MRSA i MSSA). Wyniki analiz uwzględniających wpływ WPM na zmiany w efektywności działania antybiotyków wobec bakterii z gatunku *S. aureus*, wraz z przeglądem piśmiennictwa zostały zawarte w publikacjach D-2 i D-3 oraz w Suplementach publikacji D-2 i D-3.

Stwierdzono, że do największych zmian w efektywności działania antybiotyków pod wpływem WPM doszło, gdy stosowano antybiotyki, których mechanizm działania związany jest z hamowaniem syntezy ściany komórkowej (β-laktamy oraz glikopeptydy). Ponadto, ustalono również, że do zwiększenia efektywności działania antybiotyków pod wpływem WPM wobec *S. aureus* doszło w trakcie stosowania

gentamycyny (6/9 szczepów), erytromycyny (3/9 szczepów), klindamycyny (3/9 szczepów), tetracykliny (3/9 szczepów) i ciprofloksacyny (1/9 szczepów). Wykazano, że wzrost efektywności działania antybiotyków β-laktamowych był obserwowany wobec wszystkich szczepów MRSA ujętych w badaniach, ale nie w przypadku stosowania wszystkich antybiotyków β-laktamowych. Wzrostu efektywności nie odnotowano jedynie dla ceftazydymu w przypadku szczepu ATCC 33591. W odniesieniu do teikoplaniny, której mechanizm działania, podobnie jak β-laktamów, związany jest z hamowaniem syntezy ściany bakteryjnej, zwiększenie efektywność jej działania pod wpływem WPM odnotowano dla 7 z 9 ujętych w badaniach szczepów.

Wyniki uzyskane w tej części doświadczeń wykazały również, że efektywność działania antybiotyków pod wpływem WPM zależy nie tylko od mechanizmu ich działania, ale jest istotnie determinowana zróżnicowaniem szczepów w obrębie gatunku. Ponadto ustalono, że zwiększona efektywność antybiotyków wobec bakterii zachodzi wyłącznie podczas ekspozycji na WPM i efekt ten nie pozostaje utrwalony.

W odniesieniu do zastosowanych częstotliwości WPM (5, 10, 25 i 50 Hz) największy wzrost w efektywności działania antybiotyków, obserwowano najczęściej przy zastosowaniu WPM o częstotliwości 5 Hz. Przy czym, w zdecydowanej większości analizowanych przypadków, efekt zwiększonej efektywności działania antybiotyków pod wpływem WPM obserwowano również przy zastosowaniu wszystkich pozostałych częstotliwości (niemniej jednak był on mniejszy niż w przypadku 5 Hz). Dlatego też, częstotliwość 5 Hz przyjęto w analizach jako optymalną (taką, przy której różnica w strefie zahamowania wzrostu pomiędzy hodowlą eksponowaną i nieeksponowaną na WPM była największa) dla zwiększenia efektywności działania antybiotyków. Dodatkowo, stwierdzono, że różnice w efektywności działania antybiotyków pod wpływem WPM o różnych częstotliwościach są szczepowo-zależne.

Ustalono również, że zmiany w efektywności działania antybiotyków wobec bakterii pod wpływem WPM, nie są zależne od ładunku antybiotyku. Wniosek ten wynikał z obserwacji porównywalnych zmian w efektywności działania amoksycyliny i cefoksytyny wobec szczepów *S. aureus* – antybiotyki te charakteryzują się takim samym mechanizmem działania, ale odmiennym ładunkiem cząsteczki (obojnaczy v. anionowy).

W przeprowadzonych badaniach uwzględniono również ustalenie czasu, w którym różnice w efektywności działania antybiotyków pomiędzy hodowlami eksponowanymi na WPM, a kontrolnymi będą największe. Uzyskane wyniki pozwoliły jednoznacznie stwierdzić, że obserwowany efekt zwiększonej efektywności działania antybiotyków β-laktamowych obserwowany jest od drugiej godziny ekspozycji na WPM i jest on tym większy, im dłuższy czas ekspozycji. Dodatkowo, podobnie jak to miało miejsce w przypadku różnych grup antybiotyków oraz analiz związanych z wytypowaniem optymalnej częstotliwości WPM, ustalono, że czas ekspozycji niezbędny do stwierdzenia zwiększonej efektywności działania antybiotyków jest również istotnie zależny od zróżnicowania szczepów w obrębie gatunku.

W przeprowadzonych analizach zastosowano krążki bibułowe (z jednym stężeniem antybiotyku) oraz Etesty zawierające wyższe niż krążki, a przy tym wykładniczo malejące stężenia antybiotyków. Pozwoliło to ustalić, że niskie stężenia antybiotyków mogą być przyczyną braku różnic w efektywności ich działania pod wpływem WPM, w odniesieniu do hodowli kontrolnych. Analiza wyników wykazała, że brak wpływu WPM na efektywność działania erytromycyny, klindamycyny, tetracykliny, ciprofloksacyny, gentamycyny oraz teikoplaniny był związany ze zbyt niskim stężeniem antybiotyków. Obserwacja ta dotyczyła 4 z 9 szczepów eksponowanych na WPM, u których zaobserwowano obniżenie wartości MIC w porównaniu z hodowlami kontrolnymi (pomimo braku podobnych zmian w średnicach stref zahamowania wzrostu w metodzie dyfuzyjno-krążkowej). W tym kontekście wykazano, że zmiany w efektywności działania antybiotyków pod wpływem WPM mogą nie manifestować się, gdy w badaniach użyte zostanie zbyt niskie stężenie antybiotyku, natomiast podniesienie stężenia może spowodować pojawienie się omawianego efektu. Ponownie wskazuje to na zależność pomiędzy efektem wpływu WPM a zróżnicowaniem szczepów w obrębie gatunku, w odpowiedzi na te same bodźce. Ponadto, podkreśla to również konieczność przetestowania odpowiednio dobranej liczby szczepów w celu wyciągnięcia właściwych wniosków z przeprowadzonych badań.

Przeprowadzono również analizy mające na celu weryfikację czy zmiany obserwowane w efektywności działania antybiotyków wobec bakterii pod wpływem WPM wynikają z bezpośredniego oddziaływania WPM na cząsteczki antybiotyków. Co istotne, antybiotyki użyte w badaniach charakteryzowały się odmiennymi ładunkami elektrycznymi cząsteczek. Wyniki z tej części analiz były odnoszone do wyników testów dyfuzyjno-krążkowych. Na podstawie uzyskanych wyników stwierdzono, że stężenie gentamycyny, ciprofloksacyny i tetracykliny w agarze, w układzie eksponowanym na WPM było wyższe niż w układzie kontrolnym, przy czym w przypadku ciprofloksacyny i tetracykliny wyłącznie w przypadku WPM o częstotliwości 50 Hz, a w przypadku gentamycyny przy obu częstotliwościach. Wzrost stężenia gentamycyny uwolnionej do agaru zaobserwowano niezależnie od zastosowanej częstotliwości WPM, natomiast odnosząc wyniki do wzrostu jej efektywności wobec bakterii, większe różnice (pomiędzy hodowlami eksponowanymi, a kontrolnymi) odnotowano jedynie dla częstotliwości 5 Hz (u 4 z 9 szczepów). W odniesieniu do ciprofloksacyny, wzrost jej stężenia w podłożu nie korelował ze wzrostem efektywności jej działania wobec żadnego ze szczepów ujętych w analizach. Ponadto, jej stężenie w agarze wzrosło jedynie bezpośrednio pod krążkiem, co sugeruje, że WPM wpłynęło na jej uwalnianie, ale nie dalszą dyfuzję w agarze. W odniesieniu do tetracykliny, pomimo wzrostu jej stężenia w układzie eksponowanym na WPM, wzrost efektywności jej działania odnotowano jedynie dla 2 z 9 szczepów ujętych w analizach. W przypadku klindamycyny, w układzie eksponowanym na WPM o częstotliwości 50 Hz jej stężenie było istotnie niższe, niż w układzie kontrolnym, nie mogło zatem przełożyć się na zwiększoną efektywności jej działania. W przypadku erytromycyny nie stwierdzono zmian w uwalnianiu, ani wzrostu jej stężenia w agarze pod wpływem WPM, w odniesieniu do układu kontrolnego (natomiast efektywność jej działania

wzrosła wobec 2 z 9 szczepów). Z kolei w odniesieniu do grupy antybiotyków β-laktamowych ustalono, że w konsekwencji ekspozycji na WPM ich stężenie w agarze jest istotnie mniejsze w porównaniu do układu kontrolnego. Tym samym, odnosząc zmiany w uwalnianiu i dyfuzji antybiotyków do zmian w efektywności ich działania wobec bakterii przyjęto, że bezpośrednie oddziaływanie WPM na cząsteczki antybiotyków nie ma kluczowego znaczenia dla obserwowanego wzrostu ich efektywności. Ponadto, nie udało się wskazać korelacji pomiędzy wpływem WPM na uwalnianie antybiotyków z krążka i ich dyfuzją w agarze, a posiadanym przez nie ładunkiem.

W świetle uzyskanych wyników przyjęto, że zmiany w efektywności działania antybiotyków wobec bakterii nie wynikają z oddziaływania WPM na cząsteczki antybiotyków, są natomiast powodowane bezpośrednim oddziaływaniem WPM na komórki bakteryjne. Dodatkowo, postanowiono zweryfikować hipotezę zakładającą, że cechy biofilmu, takie jak grubość lub gęstość komórkowa w jego poszczególnych warstwach, mają wpływ na aktywność WPM jako czynnika fizycznego o właściwościach falowych (a nie korpuskularnych). W celu weryfikacji powyższego założenia biofilm *S. aureus* traktowany cefoksytyną eksponowano na WPM o częstotliwości 5 Hz. Dodatkowo, przeanalizowano biofilm traktowany wyłącznie cefoksytyną, biofilm eksponowany wyłącznie na działanie WPM oraz biofilm nietraktowany cefoksytyną i nie eksponowany na WPM. Po ekspozycji biofilmy wybarwiono roztworem SYTO9 (barwiącego komórki żywe i martwe) i jodku propidyny (barwiącego komórki martwe, uszkodzone) i wykonano analizę z wykorzystaniem mikroskopu konfokalnego. Stwierdzono, że efektem ekspozycji na WPM są zmiany w ścianie komórkowej bakterii. Wykazano, że liczba komórek z uszkodzonymi ścianami komórkowymi była wyższa w biofilmie eksponowanym na WPM w połączeniu z cefoksytyną, w porównaniu do biofilmu traktowanego wyłącznie cefoksytyną. Co istotne, porównanie komórek w biofilmie kontrolnym (nietraktowanym ani cefoksytyną, ani WPM) z komórkami w biofilmie eksponowanym na WPM, również wykazało wyższy udział komórek ze zmienioną ścianą komórkową w całym pionowym przekroju biofilmu. Tym samym, do pewnego stopnia, potwierdzono również, że falowy charakter WPM powoduje, że jego działanie jako czynnika fizycznego nie jest ograniczone przez wspomniane już cechy biofilmu (grubość czy gęstość komórek). Ponadto, wykazano, że WPM oddziałuje negatywnie na ściany komórek bakteryjnych prowadząc do ich uszkodzenia, tym samym wywierając efekt podobny do antybiotyków β-laktamowych. Założono zatem, że obserwowana, zwiększona efektywność działania antybiotyków β-laktamowych pod wpływem WPM wynika z połączonej aktywności obu tych czynników.

Przeprowadzone badania obejmowały również ocenę wpływu WPM na zmiany liczby komórek *S. aureus* zdolnych do wzrostu na powierzchni podłoża stałego. Eksperyment przeprowadzono w celu oceny potencjału bakteriobójczego samego WPM wobec eksponowanych bakterii. Uzyskane wyniki nie wykazały statystycznie istotnych różnic w liczbie CFU pomiędzy hodowlami eksponowanymi na WPM, a kontrolnymi. Nie stwierdzono również różnic w morfologii wyrosłych kolonii bakteryjnych. Niemniej jednak, eksperyment ten nie dawał pełnego wglądu w stan fizjologiczny komórek bakteryjnych eksponowanych na WPM. Dlatego też, przeprowadzono dodatkowy

eksperyment z wykorzystaniem barwienia komórek bakterii roztworem SYTO9 i jodku propidyny oraz ich dalszej analizie mikroskopem fluorescencyjnym. Eksperyment ten pozwolił ustalić, że u wszystkich badanych szczepów więcej komórek żywych (nieuszkodzonych) znajdowało się w hodowlach kontrolnych, niż w hodowlach eksponowanych na WPM (różnica wynosiła średnio 32,64 ± 6,07%). Co jednak istotne, tylko w przypadku 1 z 3 badanych szczepów korelowało to z istotnie statystycznym wzrostem (w porównaniu do kontroli) ilości komórek martwych (uszkodzonych). W tym wypadku założono, że taka obserwacja mogła wynikać z zastosowanej metodologii, w której podczas płukania i wirowania hodowli dochodzi do dalszych uszkodzeń już naruszonych komórek i w konsekwencji ich usunięcia (wraz ze związanym barwnikiem) z analizowanych prób.

W kolejnym etapie analiz zweryfikowano czy ekspozycja komórek bakteryjnych na działanie WPM wpływa na ich morfologię oraz prowadzi do uszkodzenia ich ścian komórkowych. W tym celu przeprowadzono analizy z wykorzystaniem techniki SEM i TEM, które pozwoliły zobrazować zmiany w komórkach bakteryjnych eksponowanych na WPM. Stwierdzono deformacje kształtu i zmiany w wielkości komórek, ale również zapadanie się ścian komórkowych i utratę turgoru komórkowego (prawdopodobnie na skutek wycieku cytoplazmy). Co istotne, dzięki wynikom tych analiz, wskazujących na powstawanie relatywnie subtelnych zmian zachodzących w komórkach pod wpływem WPM (z wyjątkiem komórek, w których doszło do wycieku cytoplazmy) można przyjąć, że zmiany te mogłyby zostać odwrócone, gdyby komórki zostały wysiane na podłożu hodowlanym i inkubowane bez ekspozycji na WPM. Obserwacje te mogą posłużyć jako potencjalne wyjaśnienie braku istotnych różnic w liczbie CFU pomiędzy hodowlami eksponowanymi na WPM a kontrolnymi oraz spadku w ilości żywych, nieuszkodzonych komórek (w analizie z użyciem mikroskopu fluorescencyjnego) w hodowlach eksponowanych na WPM, w odniesieniu do hodowli kontrolnych. Również brak trwałego efektu poekspozycyjnego, potwierdza subtelność zmian indukowanych w komórkach bakteryjnych przez WPM, co przekłada się na zdolność komórek do stosunkowo szybkiego usuwania uszkodzeń. Wyniki przedstawionych powyżej analiz (dotyczące określenia liczby bakterii zdolnych do wzrostu oraz ilość żywych/martwych komórek w hodowlach eksponowanych na WPM) wsparte dodatkowo obrazowaniem ich morfologii wskazują, że efekt wywierany przez WPM na komórki bakteryjne objawia się poprzez spektrum relatywnie subtelnych zmian, kumulujących się przede wszystkim w skrajnych strukturach komórkowych – ścianach i błonach, nie przekładających się na istotne zmniejszenie poziomu liczby żywych bakterii.

6. Wnioski

Na podstawie wyników uzyskanych podczas badań prowadzonych w ramach rozprawy doktorskiej sformułowano następujące wnioski:

1. Wpływ WPM na kinetykę wzrostu oraz komórkową aktywność metaboliczną jest determinowany częstotliwością WPM oraz czasem ekspozycji, jak również,

w istotnym stopniu, zróżnicowaniem szczepów bakteryjnych w obrębie gatunku (w tym nawet szczepów w obrębie jednego typu klonalnego) (publikacja D-1).

2. Zmiany w efektywności działania antybiotyków pod wpływem WPM wobec *S. aureus* są determinowane stężeniem oraz mechanizmem działania antybiotyku, natomiast nie zależą istotnie od jego ładunku (publikacja D-2, D-3).
3. Intensywność zmian w efektywności działania antybiotyków jest zależna od częstotliwości WPM oraz od zróżnicowania szczepów bakteryjnych w obrębie gatunku (publikacja D-2, D-3).
4. Zwiększona efektywność działania antybiotyków β-laktamowych wobec *S. aureus* jest obserwowana od drugiej godziny działania WPM i zwiększa się wraz z czasem ekspozycji (publikacja D-3).
5. Bezpośrednie oddziaływanie WPM na cząsteczki antybiotyków nie ma istotnego znaczenia dla zwiększonej efektywności ich działania wobec *S. aureus* (publikacja D-2, D-3).
6. WPM zaburza integralność ścian komórkowych *S. aureus* w biofilmie bez względu na jego grubość oraz gęstość komórek w jego poszczególnych warstwach (publikacja D-2).
7. Efekt wywierany przez WPM (bez antybiotyku) manifestuje się poprzez spektrum relatywnie subtelnych zmian w morfologii bakterii (kumulujących się przede wszystkim w skrajnych strukturach komórkowych – ścianach i błonach), nie przekładających się na istotne zmniejszenie liczby żywych, zdolnych do namnażania bakterii (publikacja D-3).
8. Zwiększona efektywność działania antybiotyków β-laktamowych wynika ze zmian strukturalnych w ścianach komórkowych *S. aureus* indukowanych przez WPM, dzięki którym antybiotyki oddziałują na osłabione pod względem funkcjonalnym i strukturalnym komórki (publikacja D-3).
9. Zwiększona efektywność działania antybiotyków β-laktamowych względem *S. aureus* obserwowana jest podczas ekspozycji na WPM, natomiast jeżeli ekspozycja poprzedza traktowanie hodowli bakteryjnych antybiotykami efekt ten nie jest zauważalny – brak długotrwałego efektu poekspozycyjnego (publikacja D-3).

7. Cytowana literatura

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10. Omówienie pozostałych osiągnięć naukowo-badawczych

Działalność naukową rozpoczęłam w 2015 roku w Katedrze Mikrobiologii i Biotechnologii na Wydziale Biotechnologii i Hodowli Zwierząt Zachodniopomorskiego Uniwersytetu Technologicznego w Szczecinie, prowadząc badania w ramach pracy dyplomowej realizowanej podczas studiów magisterskich pt.: „Analiza zdolności wytwarzania biofilmu przez gronkowce koagulazoujemne wyizolowane z przetworów mięsnych". Praca została wykonana pod kierunkiem dr hab. inż. Karola Fijałkowskiego, prof. ZUT i rozszerzona o kolejne wyniki badań w celu przygotowania publikacji naukowej, która ukazała się w 2018 roku (publikacja A-1).

Po obronie pracy magisterskiej, z dniem 1 października 2018 r., rozpoczęłam naukę na studiach doktoranckich. Badania w ramach pracy doktorskiej wykonywałam w Katedrze Mikrobiologii i Biotechnologii Wydziału Biotechnologii i Hodowli Zwierząt Zachodniopomorskiego Uniwersytetu Technologicznego w Szczecinie pod kierunkiem dr hab. inż. Karola Fijałkowskiego, prof. ZUT, czego efektem jest niniejsza rozprawa doktorska, składająca się z cyklu trzech publikacji naukowych. W trakcie mojej działalności badawczej zgromadziłam dorobek naukowy, na który składa się łącznie 7 publikacji naukowych. Podczas studiów doktoranckich uczestniczyłam w badaniach mających na celu opracowanie nowego podejścia do oceny stopnia eradykacji biofilmu z wykorzystaniem obrazów mikroskopowych uzyskanych za pomocą mikroskopii konfokalnej, fluorescencyjnej oraz SEM (publikacja A-2). Dodatkowo, wykonywałam analizy do pracy badawczej mającej na celu analizę mikrobioty gronkowcowej w jamie nosowej owiec prymitywnych ras polskich (publikacja A-3).

Byłam również stypendystką w grancie OPUS 14 finansowanym ze źródeł Narodowego Centrum Nauki pn. „Analiza mechanizmów zwiększonej efektywności substancji przeciwdrobnoustrojowych względem biofilmów". W ramach realizacji grantu byłam wykonawcą zadań badawczych dotyczących analizy wpływu WPM na komórki bakteryjne oraz efektywność działania środków przeciwdrobnoustrojowych względem bakterii z gatunku *S. aureus* oraz *P. auruginosa*.

Dodatkowo, byłam wykonawcą w grancie badawczo-rozwojowym realizowanym w ramach Regionalnego Programu Operacyjnego Województwa Zachodniopomorskiego pn. „Testowanie w warunkach rzeczywistych innowacyjnych maseczek ochronnych (NanoBioCell) z bionanocelulozy". Badania przeprowadzone w realizowanym grancie miały na celu testowanie w ośrodkach ochrony zdrowia biodegradowalnych maseczek ochronnych z przeciwdrobnoustrojowym filtrem na bazie celulozy bakteryjnej. Jako wykonawca analizowałam właściwości przeciwdrobnoustrojowe maseczek oraz oceniałam obecność żywych komórek bakteryjnych na maseczkach po etapie ich testowania.

Ponadto, wykonałam dwa zlecenia (trzecie jest w trakcie realizacji) na prace badawcze dotyczące oceny wpływu pola magnetycznego o częstotliwości w zakresie od 0 do 2000 Hz, na zmiany w efektywności działania antyseptyków na bazie dichlorowodorku oktenidyny oraz poliheksanidu wobec

bakterii z gatunku *S. aureus* w formie hodowli planktonicznych oraz biofilmu. Zlecenia realizowane są we współpracy z firmą Azyro S.A. oraz Scout Scientific Outsourcing, a ich celem jest opracowanie urządzeń emitujących PM, które w połączeniu z substancjami przeciwdrobnoustrojowymi, byłyby stosowane w celu eradykacji biofilmów z ran przewlekłych. W praktyce klinicznej są już stosowane urządzenia, które emitują PM i są stosowane w leczeniu przewlekłych ran, nie powikłanych infekcją (Reinboldt-Jockenhöfer i wsp., 2022; Piomic). Tym samym, w świetle rosnącego zainteresowania terapiami z wykorzystaniem PM oraz trudnościami w leczeniu infekcji bakteryjnych, obrany kierunek badań uznałam za charakteryzujący się wysokim potencjałem aplikacyjnym.

Ponadto, podczas studiów doktoranckich odbyłam staż krajowy w Katedrze Mikrobiologii Farmaceutycznej i Parazytologii Uniwersytetu Medycznego we Wrocławiu. Brałam także czynny udział w 6 konferencjach o zasięgu krajowym i międzynarodowym (m.in. FEMS, World Microbe Forum, Międzyuczelniane Sympozjum Biotechnologiczne „Symbioza" i inne) oraz aktywnie uczestniczyłam w projekcie popularyzującym treści naukowe wśród uczniów „Licealista w świecie nauki". Jestem również członkiem Polskiego Towarzystwa Mikrobiologów oraz przez wiele lat angażowałam się w działalność Studenckiego Koła Naukowego Mikrobiologów działającego w Katedrze Mikrobiologii i Biotechnologii ZUT.

[**A-1**] Woroszyło M., Pendrak K., Ciecholewska D., Padzik N., Szewczuk M., Karakulska J. (2018). Investigation of biofilm formation ability of coagulase-negative staphylococci isolated from ready-to-eat meat. Acta Scientiarum Polonorum Zootechnica. 17 (4), 27-34. DOI: 10.21005/asp.2018.17.4.04.

[**A-2**] Krasowski G., Migdał P., Woroszyło M., Fijałkowski K., Chodaczek G., Czajkowska J., Dudek B., Nowicka J., Oleksy-Wawrzyniak M., Kwiek B., Paleczny J., Brożyna M., Junka A. (2022). The assessment of activity of antiseptic agents against biofilm of *Staphylococcus aureus* measured with the use of processed microscopic images. International Journal of Molecular Sciences. 23 (21): 13524. DOI: 10.3390/ijms232113524.

[**A-3**] Karakulska J., Woroszyło M., Szewczuk M., Fijałkowski K. (2022). Identification, superantigen toxin gene profile and antimicrobial resistance of staphylococci isolated from Polish primitive sheep breeds. Animals. 12 (16): 2139. DOI: 10.3390/ani12162139.

8.1. Osiągnięcia naukowe związane z wpływem wirującego pola magnetycznego na efektywność działania substancji przeciwdrobnoustrojowych względem biofilmów

Zdolność patogenów do tworzenia biofilmu na powierzchniach biotycznych i abiotycznych jest wymieniana jako jeden z głównych czynników wirulencji wpływających na trudność leczenia infekcji bakteryjnych. W publikacji A-4 brałam udział w badaniach mających na celu przeanalizowanie zjawiska zwiększonej efektywności antyseptyku na bazie dichlorowodorku oktenidyny pod wpływem WPM wobec biofilmów tworzonych przez bakterie z gatunku *S. aureus* i *P. aeruginosa*. W tym

celu ocenie została poddana żywotność i morfologia komórek tworzących biofilm, a także struktura i skład macierzy biofilmu. Biofilmy były eksponowane na WPM o częstotliwości 5 i 50 Hz przez okres od 1 do 3 godzin. Dodatkowym celem prowadzonych badań była ocena uwalniania antyseptyku pod wpływem WPM ze specyficznego nośnika jakim jest celuloza bakteryjna, stosowana obecnie coraz częściej jako innowacyjny opatrunek ran. Analizie poddano również przenikalność dichlorowodorku oktenidyny przez biofilm pod wpływem WPM. Dokonano tego używając wielopoziomowego modelu biofilmu *in vitro*, składającego się z trzech ułożonych na sobie krążków agarowych, z umieszczonym na ich górze krążkiem celulozy bakteryjnej wysyconej dichlorowodorkiem oktenidyny. Przytoczony model biofilmu jest częścią metody A.D.A.M. (ang. *Antibiofilm Dressing's Activity Measurement*) (Junka i wsp., 2017). Skonstruowany w ten sposób model biofilmu *in vitro* umożliwia określenie stopnia przenikalności substancji przeciwdrobnoustrojowej przez warstwy biofilmu.

W wyniku przeprowadzonych analiz wykazano, że zwiększona efektywność działania antyseptyku wobec obu analizowanych szczepów była odnotowana od 1 godziny ekspozycji na WPM. Intensywność obserwowanego efektu zwiększonej efektywność antyseptyku w obecności WPM była determinowana czasem ekspozycji, gatunkiem bakterii oraz warstwą biofilmu (krążkiem agarowym w użytym modelu *in vitro*). Wykazano również, że ekspozycja biofilmów na działanie samego WPM nie spowodowała obniżenia żywotności komórek w biofilmie – tym samym dowiedziono brak efektu cytotoksycznego WPM wobec komórek bakteryjnych w biofilmie. Wykazano ponadto, że WPM intensyfikuje uwalnianie dichlorowodorku oktenidyny z nośnika jakim jest celuloza oraz zwiększa jego dyfuzję przez poszczególne warstwy biofilmu (krążki agarowe w użytym modelu *in vitro*). Udowodniono również, że WPM wpływa na komórki w biofilmie powodując negatywne zmiany w ścianach i błonach komórkowych, tym samym zaburzając ich morfologię. Ponadto, dowiedziono, że ekspozycja biofilmu na WPM zmienia porowatość macierzy biofilmu oraz modyfikuje skład jego części cukrowej.

Co istotne, wyniki prezentowane w publikacji A-4 są zbieżne z wynikami uzyskanymi w ramach trzech publikacji wchodzących w skład niniejszej rozprawy doktorskiej. Stwierdzono zwiększoną efektywność działania dichlorowodorku oktenidyny pod wpływem WPM, którego mechanizm działania, podobnie jak antybiotyków β-laktamowych opiera się na oddziaływaniu na ścianę komórkową bakterii. Dzięki zastosowaniu techniki SEM potwierdzono, że WPM indukuje powstawanie niekorzystnych zmian w ścianach komórkowych *S. aureus* (ale również bakterii z gatunku *P. aeruginosa*). Wykazano również, podobnie jak w badaniach wykonywanych w ramach pracy doktorskiej, że zwiększona efektywność działania substancji przeciwdrobnoustrojowej względem bakterii w obecności WPM jest determinowana czasem ekspozycji.

[**A-4**] Ciecholewska-Juśko D., Żywicka A., Junka A., Woroszyło M., Wardach M., Chodaczek G., Szymczyk-Ziółkowska, P., Migdał, P., Fijałkowski, K. (2022). The effects

of rotating magnetic field and antiseptic on in vitro pathogenic biofilm and its milieu. Scientific Reports. 12 (1), 1-19. DOI: 10.1038/s41598-022-12840-y.

Obecnie zakończyłam wykonywanie badań mających na celu szersze przeanalizowanie efektu poekspozycyjnego wywieranego przez WPM na komórki MRSA.

W ramach badań bakterie w formie hodowli planktonicznych, biofilmu oraz murawki na powierzchni podłoża agarowego eksponowano na działanie WPM o częstotliwości 5 Hz przez 12 godzin. Po ekspozycji hodowle bakteryjne traktowano dwoma antybiotykami (cefoksytyną oraz cefepimem) i dwoma antyseptykami (na bazie dichlorowodorku oktenidyny oraz powidonu jodu) w formie roztworów o trzech różnych stężeniach. Dodatkowo, wykonano również testy dyfuzyjno-krążkowe oraz gradientowo-dyfuzyjne z wykorzystaniem antybiotyków ujętych w analizach.

W badaniach ujęto również szczepy MRSA, które eksponowano na działanie WPM o częstotliwości 5 Hz w kolejnych, 23 pasażach. Analizę tę przeprowadzono w celu oceny, czy powtarzające się narażenie na stres środowiskowy jakim jest zewnętrzne PM spowoduje w komórkach powstanie trwałych zmian w ich wrażliwości względem substancji przeciwdrobnoustrojowych.

W chwili obecnej trwa opracowywanie uzyskanych wyników oraz przygotowywanie publikacji.

Załącznik 1

Kopie publikacji naukowych wchodzących w skład cyklu stanowiącego rozprawę doktorską

Marta Woroszyło

Rozprawa doktorska

ANALIZA WPŁYWU WIRUJĄCEGO POLA MAGNETYCZNEGO NA EFEKTYWNOŚĆ DZIAŁANIA SUBSTANCJI PRZECIWDROBNOUSTROJOWYCH WZGLĘDEM BAKTERII PATOGENNYCH

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Article

The Impact of Intraspecies Variability on Growth Rate and Cellular Metabolic Activity of Bacteria Exposed to Rotating Magnetic Field

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Abstract: Majority of research on the influence of magnetic fields on microorganisms has been carried out with the use of different species or different groups of microorganisms, but not with the use of different strains belonging to one species. The purpose of the present study was to assess the effect of rotating magnetic fields (RMF) of 5 and 50 Hz on the growth and cellular metabolic activity of eight species of bacteria: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Moraxella catarrhalis*, and *Bacillus cereus*. However, contrary to the research conducted so far, each species was represented by at least four different strains. Moreover, an additional group of *S. aureus* belonging to a single clonal type but representing different biotypes was also included in the experiment. The results showed a varied influence of RMF on growth dynamics and cellular metabolic activity, diversified to the greatest extent in dependence on the bacterial strain exposed to the RMF and to a lesser extent in dependence on the frequency of the generated magnetic field. It was found that, with regard to the exposed strain of the same species, the effect exerted by the RMF may be positive (i.e., manifests as the increase in the growth rate or/and cellular metabolic activity) or negative (i.e., manifests as a reduction of both aforementioned features) or none. Even when one clonal type of *S. aureus* was used, the results of RMF exposure also varied (although the degree of differentiation was lower than for strains representing different clones). Therefore, the research has proven that, apart from the previously described factors related primarily to the physical parameters of the magnetic field, one of the key parameters affecting the final result of its influence is the bacterial intraspecies variability.

Keywords: cellular metabolic activity; clone; growth dynamics; rotating magnetic field; species; strain; viability

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1. Introduction

The ability to modify microorganisms' behavior, understood as the level of metabolic activity or rate of cellular division, is pivotal from the point of view of biotechnology and

medicine, as it allows for the increase in the yield of microbiologically derived products or the decrease in the symptoms of infection. The measures applied for such modifications include a vast spectrum of agents/stimuli, which can be chemical or physical. Belonging to the latter category, the electromagnetic field (EMF) can be viewed as a combination of an electric field (EF) and a magnetic field (MF). The first studies on the influence of magnetic fields (MFs) on organisms started at the end of the 19th century. This line of investigation was intensified in subsequent decades, mostly fueled by a growing interest in the impact of fields generated by electric and telecommunication networks on the health and behavior of humans and such animals as bees or birds [1]. In turn, the first reports on the use of MFs to affect microbial growth were released more than 60 years ago [2], while data on the impact of MFs on the viability of microorganisms were presented already in the late 1960s [3,4]. Since that time, numerous studies have been published on the influence of different types of MFs on various parameters associated with microorganisms (Table 1).

Table 1. Review of works showing the influence of magnetic fields on the viability, growth rate, and cellular metabolic activity of various species and strains of bacteria.

Bacterial Species	Intensity	Frequency	Current	Time of Exposure	Field	Biological Effect/Author Conclusions	References
S. aureus *E. coli*	0.5–4 T	-	-	30–120 min	SMF	No influence on growth.	[5]
S. aureus * *S. mutans* * *E. coli* *	30, 60, 80, 100 mT	-	-	Uncertain	SMF	Ferrite magnet caused strength-dependent inhibitory effect on the growth of *S. mutans* and *S. aureus* when cultured under anaerobic conditions. No growth effects were detected on *E. coli* cultures.	[6]
S. aureus FA 812 *E. coli strain* K12 *L. adecarboxylata* 2177	10 mT	50 Hz	-	<30 min	LF-EMF	Decrease in the cell viability and inhibitory effect on the growth rate.	[7]
E. coli *	300 mT	-	-	Up to 50 h	SMF	In the standard medium (LB), no differences between the control and exposed culture were observed. In the modified medium (LB + glutamic acid) after 25 h of cultivation, significant growth stimulation under field exposure occurred in comparison with the control.	[8]
S. aureus (38 strains) *E. coli* (38 strains) Strains were analyzed as a whole group	-	50 Hz	-	30–150 min	SEF	Inhibitory effect on the growth of Gram-negative *E. coli* was greater than Gram-positive *S. aureus.*	[9]
S. aureus FA 812 *R. erythropolis* *E. coli* strain *K12* *L. adecarboxylata* 2177 *P. denitrificans* CCM 982 *S. paucimobilis*	10 mT	50 Hz	-	24 min	MF	The MF caused a decrease in optical densities of bacterial cultures; the effect was higher for rodlike bacteria.	[10]
S. aureus ATCC 49230	5 mT	20 Hz	1.2 A	24 h	LF-EMF	Decreased number of cells by 37.3% for electric field (E = 588 mV·cm^{-1}) was observed.	[11]

Table 1. *Cont.*

Bacterial Species	Intensity	Frequency	Current	Time of Exposure	Field	Biological Effect/Author Conclusions	References
S. aureus 52/03 *B. circulans* B 01115 *M. luteus* B 01072 *P. fluorescens* B 01102 *S. enteritidis* serovar Enteritidis 359/07 *S. marcescens* 15/2/7/2 *E. coli* ATCC 35218	159.2 mT	-	-	Up to 24 h	H-SMF	No influence on growth.	[12]
	477 mT, 12 mT, 2.8 mT	-	-	Up to 24 h	I-SMF		
S. epidermidis ATCC 12228 *S. aureus* ATCC 25923 *E. faecalis* ATCC 29212 *E. coli* ATCC 25922 *K. pneumoniae* ATCC 4352 *P. aeruginosa* ATCC 27853	0.5 mT	50 Hz	-	6 h	ELF-EMF	Inhibitory effect on growth rate.	[13]
S. epidermidis ATCC 35984 *E. coli* ATCC 25922	100 mT	-	-	Up to 4 h	MI-SMF	Inhibitory effect on growth.	[14]
E. coli (MG1655, MG 1655rpoS:kan mutant, DH5α with pRPO22oBTc^r) *P. putida* Dc27	5–50 mT	-	-	Up to 4 h	LD-SMF	Inhibitory effect on growth.	[15]
E. coli ATCC 25922 *P. aeruginosa* ATCC 27853	2 mT	50 Hz	-	4, 6, 8 h	ELF-EMF	No remarkable differences were found in the rate of bacteria growth comparing exposed groups with control groups.	[16]
S. aureus ATCC 43300 *E. coli* ATCC 8739	22–34 mT	1–50 Hz	-	60 min	RMF	Stimulation of the growth dynamics and cell metabolic activity. Higher proliferation rate and cell metabolic activity were found for *E. coli.*	[17]
S. aureus FRI 913 *S. aureus* ATCC 25923 *S. aureus* ATCC 43300 *E. coli* O157:H7 (two strains) *E. coli* E68II/0141	30 mT	50 Hz	-	150 min	RMF	Stimulatory effect on the growth and metabolic activity of *E. coli* and *S. aureus.*	[18]
S. aureus ATCC 6538 *E. coli* O157:H7	2–4 mT	20, 40, 50 Hz	-	1–6 h	ELF-EMF	Inhibitory effect on the growth rate in exposed cultures.	[19]
S. aureus ATCC 43300 *S. mutans* ATCC 35668 *S. xylosus* ATCC 29971 *E. coli* ATCC 8739 *A. baumannii* ATCC 19606 *P. aeruginosa* ATCC 10145 *S. marcescens* ATCC 274 *C. sakazakii* ATCC 29544 *K. oxytoca* PCM 2202	25–34 mT	5-50 Hz	-	60 min	RMF	Increase in the growth and metabolic activity except for *A. baumannii* and *P. aeruginosa.*	[20]

Table 1. *Cont.*

Bacterial Species	Intensity	Frequency	Current	Time of Exposure	Field	Biological Effect/Author Conclusions	References
S. aureus ATCC 25923 *S. epidermidis* ATCC 14990 *S. marcescens* ATCC 264 *E. coli* ATCC 11303	250 µT	6–25 Hz	-	12 h	ELF-EM	Increased growth rate of *S. epidermidis*, *S. aureus*, and *E. coli*, inhibitory effect on the growth rate of *S. marcescens*.	[21]
E. coli (wild strains) *E. coli* ATCC 25922	2–20 mT	-	-	0, 15, 30, 45, 60, 75, 90 min	SMF	Inhibitory effect on the growth rate in exposed cultures at 18 and 20 mT.	[22]
S. aureus ATCC 29213 *S. epidermidis* ATCC 25923 *P. aeruginosa* ATCC 27853		900/1800 MHz	-	12 h	HF-EMF	Exposure of *S. epidermidis* and *S. aureus* to EMF decreased bacterial growth, except for *S. aureus* at 900 MHz at 12 h. Exposure of *P. aeruginosa* to EMF at 900 MHz reduced growth rate, while 1800 MHz had insignificant effect.	[23]
S. aureus ATCC 43300 *E. faecalis* ATCC 29212 *S. mutans* ATCC 35668 *E. coli* ATCC 8739 *S. marcescens* ATCC 274 *K. oxytoca* PCM 2202	up to 18 mT	50 Hz	-	8 h	RMF	Increased growth and metabolic activity of Gram-positive bacteria (up to 25%) and inhibited proliferation of Gram-negative bacteria (up to 17%) (with the exception of *S. marcescens*, no statistical differences were observed).	[24]
S. aureus 155554A *S. epidermidis* 155556A *E. coli* 155065A *P. aeruginosa* 155250A	0.3–0.5 mT; 7.5 mT; 0.5 mT; 0.05–0.5 mT	-	-	24–36 h	B-MF; RM-MF; U-MF; O-MF	Different MFs affect the growth pattern of bacteria differently, depending on the bacterial species.	[25]

B-MF—bar magnetic field, ELF-EMF—extremely low-frequency electromagnetic field, HF-EMF—high-frequency electromagnetic field, H-SMF—homogeneous static magnetic field, I-SMF—inhomogeneous static magnetic field, LD-SMF—low-density static magnetic field, LF-EMF—low-frequency electromagnetic field, MF—magnetic field, MI-SMF—moderate-intensity static magnetic field, O-MF—oscillating magnetic field, SEF—static electric field, SMF—static magnetic field, RMF—rotating magnetic field, RM-MF—round-magnets magnetic field, U-MF—uniform magnetic field. "*"—information regarding the strain was not available. *A. baumannii—Acinetobacter baumannii*, *B. circulans—Bacillus circulans*, *C. sakazakii—Cronobacter sakazakii*, *E. coli—Escherichia coli*, *E. faecalis—Enterococcus faecalis*, *K. oxytoca—Klebsiella oxytoca*, *K. pneumoniae—Klebsiella pneumoniae*, *L. adecarboxylata—Leclercia adecarboxylata*, *M. luteus—Micrococcus luteus*, *P. aeruginosa—Pseudomonas aeruginosa*, *P. denitrificans—Pseudomonas denitrificans*, *P. fluorescens—Pseudomonas fluorescens*, *P. putida—Pseudomonas putida*, *R. erythropolis—Rhodococcus erythropolis*, *S. aureus—Staphylococcus aureus*, *S. enteritidis—Salmonella enteritidis*, *S. epidermidis—Staphylococcus epidermidis*, *S. marcescens—Serratia marcescens*, *S. mutans—Streptococcus mutans*, *S. paucimobilis—Sphingomonas paucimobilis*, *S. xylosus—Staphylococcus xylosus*.

For a long time, the use of bacteria, as models of low complexity, to examine cellular replies to MFs was thought to enable reduction of errors associated with the interpretation of experimental results. To make the mechanisms exerted by MFs even more understandable, single-model reference microorganisms, well characterized by genetic markers, are usually used. In spite of this approach, the data presented in the literature on the subject in question are often conflicting, and the mechanisms of MF's biological activity are still not elucidated (Table 1). As an example, some authors reported the antibacterial effect of MFs [7,10,26,27], while others suggested a lack of any significant impact of MFs on microbial growth [12,28], biochemical activity [5], or bacterial adhesion [29]. In turn, other research teams demonstrated a stimulating effect of MFs on microbial cell growth and cell viability [30–32]. Such contradictory results have led to the recognition of the fact that MFs may exert a whole spectrum of biological effects (from none/absence of significant effects through inhibitory/negative to stimulatory/positive ones), depending on the bacterial species analyzed, the nature of the emitted magnetic signals, and the time of magnetic exposure [7,26,33]. Moreover, although research related to the influence of MFs on microor-

ganisms has been globally conducted for several decades, the mechanisms standing behind MF-induced bacterial stimulation/inhibition are not understood. Therefore, it is still not possible to predict, with absolute certainty, how a particular microorganism would behave when exposed to an MF of specific parameters.

As regards the dependence of effects on the physical parameters of MF, in particular its intensity or frequency and the duration of exposure, most of the relationships have been well described and explained. Nevertheless, apart from the physics point of view, the possible effects of magnetic exposure should also be considered from a biological perspective, and in the context of the present study, primarily from the microbiological point of view. In this case, a number of theories suggesting what the observed effect of exposure may depend on can be found in the relevant literature. As an example, according to several authors [7,26,33], biological effects of MFs on the biological function of bacterial cells can be distinguished (inhibitory or stimulatory), depending on the nature of emitted signals and the time of exposure, the microorganism species [18,20,21], the structure and composition of the cell wall [24,25], the bacterial cell shape [7,10,34], or both the shape of the bacterial cell and the structure of the cell wall [25]. However, a thorough review of the available literature shows a lack of consistency of the results obtained concerning the above cellular parameters. Therefore, even when the same type of MF is applied, the results indicating positive and negative effects of exposure for the same species of microorganism are reported (Table 2).

Admittedly, different researchers use different MF-generating systems and conduct their research using different MF parameters, which certainly decreases the possibility of a direct comparison of the obtained results. It should also be noted that, so far, there have been only a few research projects presenting the impact of MF on different bacterial strains belonging to the same species. However, even if such studies were performed, the strains' answer to the exposure was not analyzed individually, or the number of scrutinized strains was too low to draw any significant conclusions regarding their different sensitivities to the magnetic exposure [9,22].

Therefore, in the present work, we attempted to take a step towards systematization of the knowledge in this area, focusing on the impact of only one type of MF (i.e., rotating magnetic field (RMF)) on several species of different bacteria, each represented by several strains. The agenda behind this approach was to investigate how a particular type of MF affects various strains belonging to the same bacterial species.

The purpose of the present study was to assess the effect of the RMF (of two distinguished frequencies, 5 and 50 Hz) on the growth and cell viability/cellular metabolic activity (frequently studied cell parameters of bacteria exposed to MFs) of different bacterial strains (including wild and reference strains) belonging to eight species: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Moraxella catarrhalis*, and *Bacillus cereus*. Moreover, an additional group of *S. aureus* belonging to a single clone (clonal type) was also included in the experiment. The selected bacterial species differed from each other in terms of cell shape (rods/cocci), cell wall structure (Gram-negative and Gram-positive), metabolism (aerobic/facultatively anaerobic), motility, and ability to form spores. We hypothesized that the effect of the RMF is not related (or at least not always) to the above characteristics of a given microorganism but depends on the specifics of an individual strain, and therefore, the determination of the impact of MFs on microorganisms should be performed much more precisely than it has been performed to date.

Table 2. The effects exerted by different types of magnetic fields on bacteria.

Bacterial Species	Positive Effect (Stimulating Effect)	Negative Effect (Inhibitory Effect)	No Influence
		Gram-positive cocci	
E. faecalis	Konopacki and Rakoczy, 2019 [24] RMF	Inhan-Garip et al., 2011 [13] ELF-EMF	
S. aureus	Fijałkowski et al., 2013 [17] RMF Nawrotek et al., 2014 [18] RMF Fijałkowski et al., 2015 [20] RMF Tessaro et al., 2015 [21] ELF-EMF Konopacki and Rakoczy, 2019 [24] RMF	Kohno et al., 2000 [6] SMF Fojt et al., 2004 [7] LF-EMF Kermanshahi et al., 2005 [9] SEF Strašák et al., 2005 [10] MF Inhan-Garip et al., 2011 [13] ELF-EMF Bayir et al., 2015 [19] ELF-EMF Masood et al., 2020 [25] SMF/O-MF Obeimeier et al., 2009 [11] LF-EMF	Grosman et al., 1992 [5] SMF Lászlo and Kutasi, 2010 [12] SMF Salmen et al., 2018 [23] HF-EMF
S. epidermidis	Tessaro et al., 2015 [21] ELF-EMF	Inhan-Garip et al., 2011 [13] ELF-EMF Masood et al., 2020 [25] SMF/O-MF Bajpai et al., 2012 [14] MI-SMF	Salmen et al., 2018 [23] HF-EMF
S. mutans	Fijałkowski et al., 2015 [20] RMF Konopacki and Rakoczy, 2019 [24] RMF	Kohno et al., 2000 [6] SMF	
S. xylosus	Fijałkowski et al., 2015 [20] RMF		
		Gram-negative cocci	
P. denitrificans		Strašák et al., 2005 [10] MF	
		Gram-positive rod	
B. circulans			Lászlo and Kutasi, 2010 [12] SMF
		Gram-negative rod	
A. baumannii		Fijałkowski et al., 2015 [20] RMF	
E. coli	Fijałkowski et al., 2013 [17] RMF Fijałkowski et al., 2015 [20] RMF Nawrotek et al., 2014 [18] RMF	Fojt et al., 2004 [7] LF-EMF Kermanshahi et al., 2005 [9] SEF Strašák et al., 2005 [10] MF Inhan-Garip et al., 2011 [13] ELF-EMF Bayir et al., 2015 [19] ELF-EMF Mousavian-Roshanzamir and Makhdoumi-Kakhki, 2017 [22] SMF Konopacki and Rakoczy, 2019 [24] RMF Masood et al., 2020 [25] SMF/O-MF Bajpai et al., 2012 [14] MI-SMF Filipič et al., 2012 [15] LD-SMF Tessaro et al., 2015 [21] ELF-EMF	Grosman et al., 1992 [5] SMF Kohno et al., 2000 [6] SMF Lászlo and Kutasi, 2010 [12] SMF Segatore et al., 2012 [16] ELF-EMF
K. oxytoca	Fijałkowski et al., 2015 [20] RMF	Konopacki and Rakoczy, 2019 [24] RMF	
K. pneumoniae		Inhan-Garip et al., 2011 [13] ELF-EMF	
P. aeruginosa		Inhan-Garip et al., 2011 [13] ELF-EMF Fijałkowski et al., 2015 [20] RMF Salmen et al., 2018 [23] HF-EMF Masood et al., 2020 [25] SMF/O-MG	Segatore et al., 2012 [16] ELF-EMF
P. fluorescens			Lászlo and Kutasi, 2010 [12] SMF
P. putida		Filipič et al., 2012 [15] LD-SMF	
S. enteritidis			Lászlo and Kutasi, 2010 [12] SMF
S. marcescens	Fijałkowski et al., 2015 [20] RMF	Tessaro et al., 2015 [21] ELF-EMF	Lászlo and Kutasi, 2010 [12] SMF Konopacki and Rakoczy, 2019 [24] RMF

ELF-EMF—extremely low-frequency electromagnetic field, HF-EMF—high-frequency electromagnetic field, LD-SMF—low-density static magnetic field, LF-EMF—low-frequency electromagnetic field, MF—magnetic field, MI-SMF—moderate intensity static magnetic field, O-MF—oscillating magnetic field, SEF—static electric field, SMF—static magnetic field, RMF—rotating magnetic field.

2. Results

2.1. Analysis of the Molecular Diversity between Strains of S. aureus

Macrorestrictive DNA analysis of the investigated staphylococci using PFGE showed that all isolates belonged to different PFGE types and, according to the established criteria (genetic similarity coefficient (Sab) was 65.7%), constituted different clones (Figure 1). Each

S. aureus strain revealed also a distinctive, individual phenotypic pattern and, therefore, according to the established criteria (distinctive features of the strains considered for establishing a biotype), constituted a different biotype (Table 3 and Supplementary Table S1).

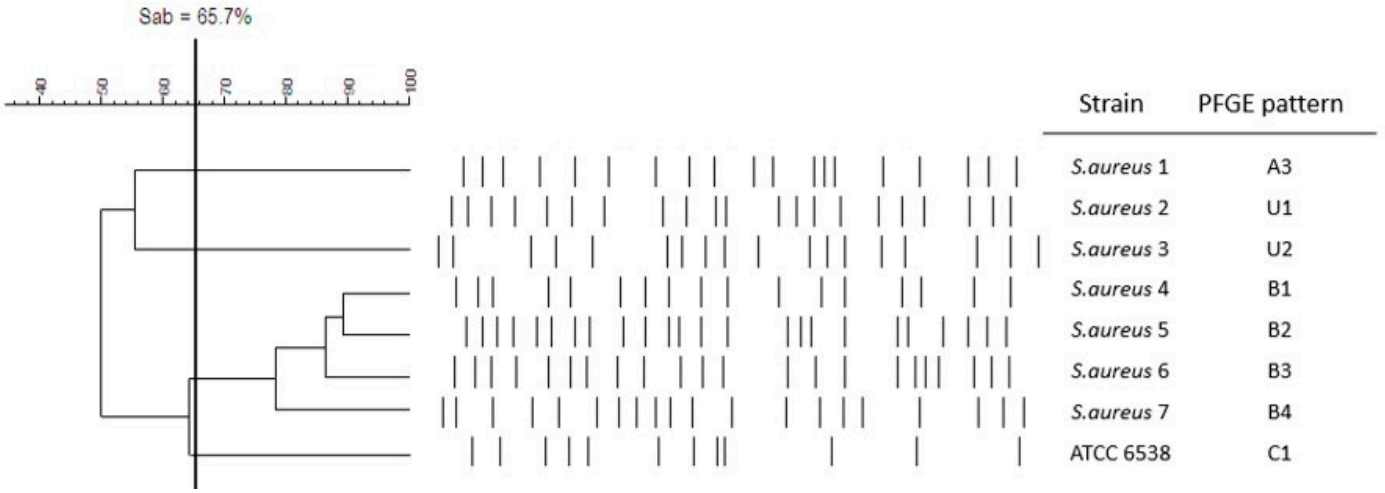

Figure 1. Dendrogram of PFGE clusters and genotypic relationships of *S. aureus* isolates. Cut-off point is equal to 65.7 (Sab = 65.7%).

Table 3. Phenotypic patterns of *S. aureus* strains.

Phenotypic Pattern	Strain Number							
	1	2	3	4	5	6	7	ATCC 6538
dRIB	−	−	−	−	−	−	+	−
NOVO	−	+	−	−	−	−	+	−
ILATk	+	−	+	−	−	+	+	+
O129R	+	+	+	+	+	+	+	−
LAC	−	−	+	−	−	−	−	−
NAG	+	+	+	−	+	+	+	+
BGAL	+	−	+	−	+	−	−	−
AMAN	−	+	−	−	−	−	−	−
MBdG	+	−	−	+	+	+	+	−
AGLU	+	+	−	+	+	+	+	−
dGAL	+	+	+	−	+	−	−	+
ADH2s	+	−	−	−	−	−	−	+

dRIB—D-ribose, NOVO—novobiocin resistance, ILATk—L-lactate alkalinization, O129R—O/129 resistance (comp.vibrio.), LAC—lactose, NAG—*N*-acetyl-D-glucosamine, BGAL—β-galactosidase, AMAN—α-mannosidase, MBdG—methyl-B-D-glucopyranoside, AGLU—α-glucosidase, dGAL—D-galactose, ADH2s—arginine dihydrolase 2. The most differentiating features are summarized in the above table; the complete biochemical characteristics are summarized in Supplementary Table S1.

2.2. The Study of Growth Dynamics and Cellular Metabolic Activity

Because in the preliminary study we confirmed that the results obtained in AlamarBlue were determined to a greater extent by the changes in cellular metabolic activity than cell viability, we assumed that the results obtained in this assay would be presented as changes in cellular metabolic activity (Figure 2).

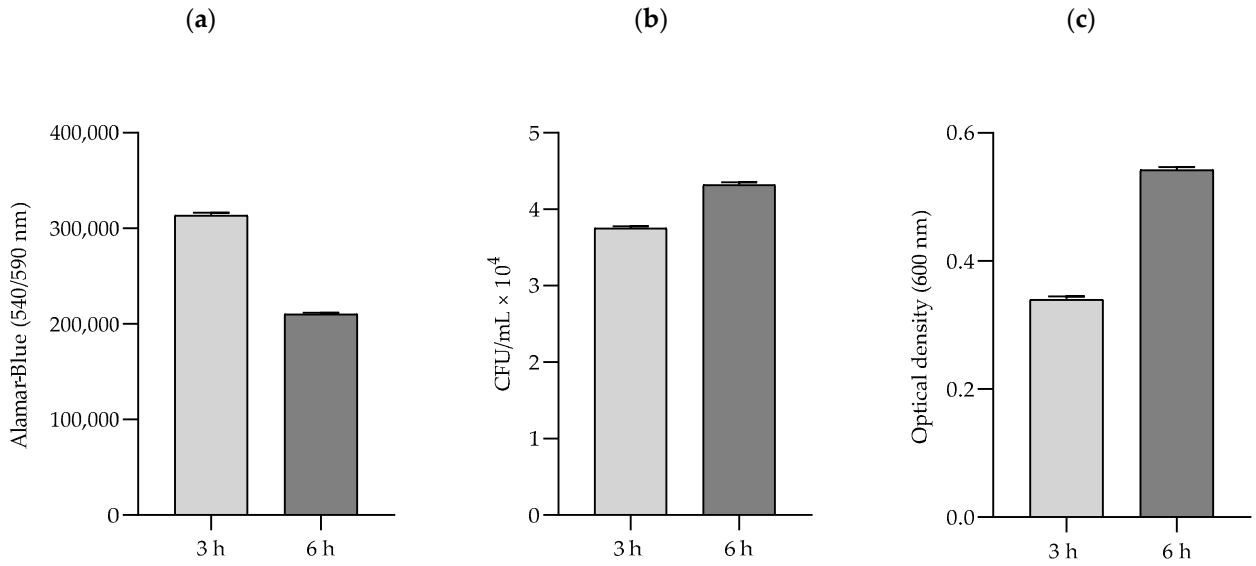

Figure 2. Representative results obtained in different tests: (**a**) Alamar-Blue (cellular metabolic activity/viability), (**b**) CFU number (viability), and (**c**) optical density (growth rate) for the MRSA no. 3 strain exposed to an RMF of 50 Hz. Alamar-Blue shows the changes in cellular metabolic activity and viability (viable cells = metabolically active cells); CFU number shows changes in the number of viable cells (able to form colonies); and optical density shows the growth rate of bacteria, although it does not distinguish between live and dead cells.

Staphylococcus aureus. The results of the effect of the RMF on the growth dynamics and metabolic activity of *S. aureus* cells are presented in Figure 3a (the results of individual statistical analyses are shown in Supplementary Material). In general, the results showed a varied influence of the RMF on the growth dynamics and cellular metabolic activity of *S. aureus*, diversified to the greatest extent depending on the bacterial isolate/strain that was exposed to the RMF, as well as depending on the exposure time, and to a lesser extent depending on the frequency of the generated magnetic field (5 vs. 50 Hz). The reference strain (ATCC 6538) was characterized by a continuous decrease in growth rate. Moreover, the results obtained after 6 and 9 h of exposure to a RMF of 5 Hz and after 9 h of exposure to an RMF of 50 Hz were statistically significantly lower than the values obtained for the control cultures not exposed to an RMF. A progressive decrease in the growth rate, although not as substantial as in the case of the reference strain, was also noted in the cultures of wild strain nos. 4 and 5, and this trend was clearly more pronounced when an RMF with a frequency of 5 Hz was applied. The growth rate of isolate no. 1, after 3 and 6 h of exposure to the RMF, regardless of its frequency, was not statistically significantly different as compared with the control, although during exposure to an RMF of 50 Hz, the frequency of the growth rate markedly increased. Nevertheless, after 9 h of exposure to the RMF (both frequencies) of cultures with this staphylococcal strain, a significant decrease in the growth rate was found. Strain no. 2, in turn, was characterized by a continuous increase in growth rate when exposed to an RMF of 5 and 50 Hz, and strain nos. 3 and 7 did not show any significant changes in this parameter as a result of the magnetic exposure, regardless of its frequency or duration. Therefore, it can be summarized that most of the growth profiles differed so that if only one strain was selected for the purposes of the study, the final conclusion from the obtained results could be different—depending on which strain was included in the analysis.

Similar to the results of growth dynamics, also the results presenting changes in the cellular metabolic activity, which is the second parameter analyzed in the current study, varied between individual strains. In the cultures of the reference strain (ATCC 6538) exposed to an RMF of 5 Hz, no significant changes were found, while a significant increase in cellular metabolic activity was observed after 3 h of exposure to an RMF of 50 Hz. In the case of strain nos. 1 and 4, a continuous decrease in cellular metabolic activity was

observed. For strain no. 1, significant differences as compared with the unexposed control were found after 9 h of exposure to an RMF of 5 Hz (decrease in cellular metabolic activity as compared with the control) and after 3 h of exposure to an RMF of 50 Hz (the results were higher than in the control), whereas in the case of isolate no. 4, significant differences were obtained after 3 and 9 h of exposure regardless of the RMF frequencies (the values were higher and lower as compared with the control, respectively). The cellular metabolic activity of strain nos. 2 and 3 remained below the values obtained for the unexposed controls, except for strain no. 3, whose cellular metabolic activity after 9 h of exposure to an RMF of 50 Hz increased above the values recorded for the control. Nevertheless, the only significant difference (value significantly lower than for the unexposed control) was found for isolate no. 3 after 3 h of exposure to an RMF of 5 Hz. In turn, in the case of strain no. 5, there was a significant increase in cellular metabolic activity as compared with the unexposed control in all time points of measurements, but only during exposure to an RMF of 5 Hz. Whereas when the culture of this strain was exposed to an RMF of 50 Hz, the same tendency was observed except for the last time point, where the value of cellular metabolic activity was slightly lower than for the unexposed control. In the cultures of strain nos. 6 and 7, cellular metabolic activity was above the values obtained for the control, regardless the time point of measurements and the RMF frequencies, while in the case of strain no. 7 and exposure to an RMF of 5 Hz, the highest values of cellular metabolic activity were obtained after 9 h of the experiment, and when the frequency of the RMF was 50 Hz, after 3 h.

For the group of staphylococci belonging to one clonal type, the results were not as noticeably differentiated as for the group of *S. aureus* representing different clones (Figure 3b). Nevertheless, also in this group, different trends in the changes of the analyzed cellular parameters under the influence of the RMF were found. As in the case of the above-discussed group of genetically diverse staphylococci, also in this group, the RMF characteristics were the least significant determinant of the differentiation of the results. When analyzing the results obtained after 3 h of exposure to the RMF, it can be noticed that strain no. 1 was the only one that was characterized by a lower growth rate as compared with the unexposed control (however, the difference was not statistically significant). In contrast, strain no. 2 was the only one that was characterized by a statistically significantly higher growth rate as compared with the unexposed control. After 6 h of RMF exposure, the only significant difference (an increase) in growth rate in comparison with the control was recorded for strain no. 3 exposed to an RMF of 5 Hz, whereas no significant differences in comparison with the control were found for cultures exposed to the RMF for 9 h.

Although they did not show a recurring trend of changes, the results of cellular metabolic activity were clearly less differentiated as compared with the results of growth dynamics. Apart from strain no. 3, exposed to an RMF of 5 Hz, in all other cultures, the lowest values of cellular metabolic activity were obtained after 6 h of exposure, and additionally for strain no. 3, equally low values were also obtained after 3 h of exposure. After 9 h of exposure to an RMF of 5 Hz, a significant increase in cellular metabolic activity was found for all strains except for strain no. 4. In contrast, this strain (no. 4) was the only one whose metabolic activity increased due to exposure to an RMF of 50 Hz (after 9 h).

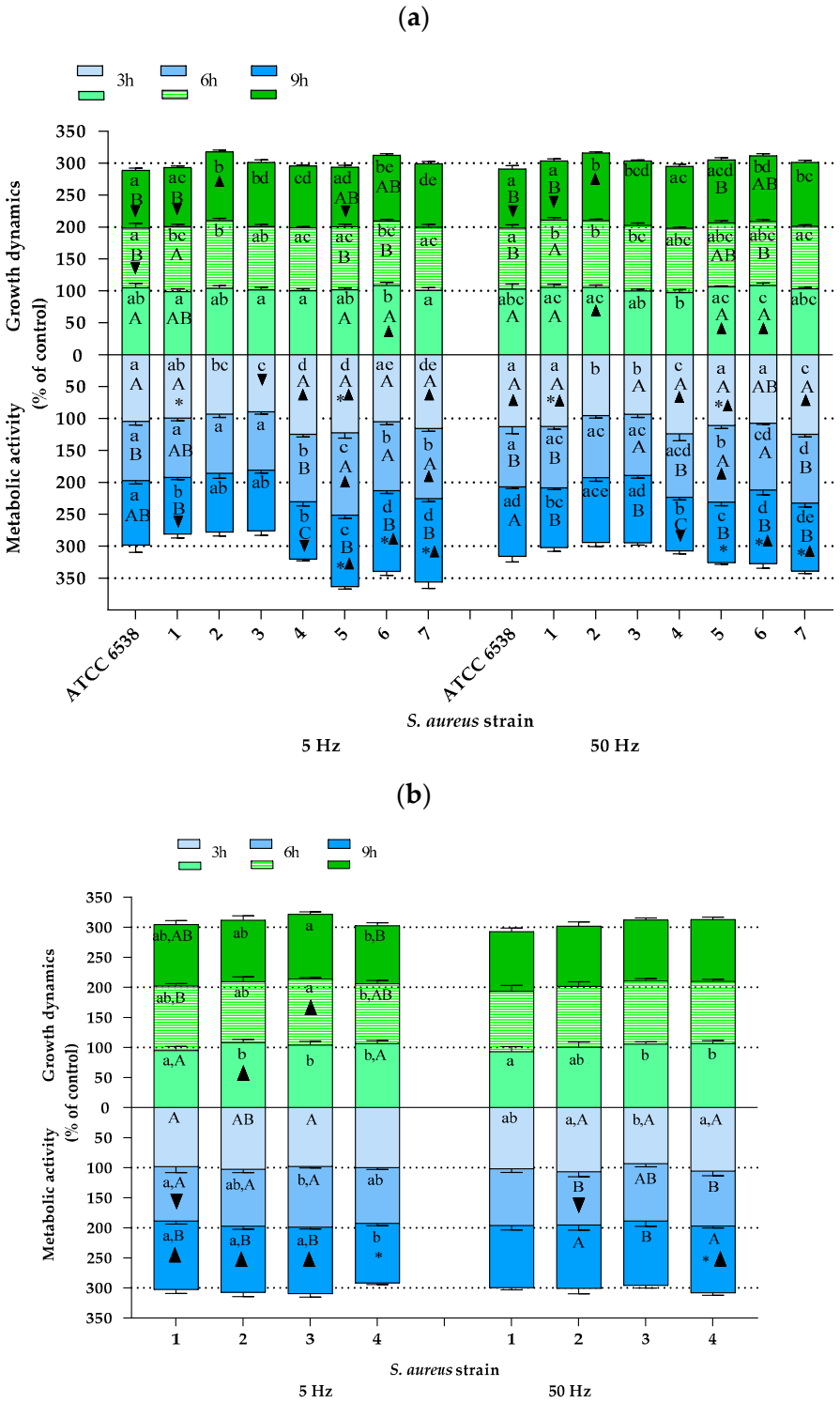

Figure 3. Growth dynamics and cellular metabolic activity of *S. aureus* cultures exposed to a rotating magnetic field (5 and 50 Hz). (**a**) *S. aureus* strains representing different clonal types. (**b**) *S. aureus* strains representing one clonal type. Data are expressed as % of control. The results are presented as a mean ± SEM calculated using six values (three from each biological replicate). Lowercase letters indicate significant differences between strains at a specific time; capital letters indicate significant differences between time points of measurements for a particular strain; the asterisk symbol (*) indicates significant differences between RMF frequencies (5 vs. 50 Hz); ▲▼—significant differences compared with the unexposed control, taking into account the trend (stimulation and inhibition, respectively) ($p < 0.005$).

Other species of bacteria. As shown in Figure 4, also in the case of the remaining bacterial species included in the analyses, the results indicating both changes in growth rate and in cellular metabolic activity due to RMF exposure showed a significant differentiation depending on the bacterial strain analyzed. Similarly, as was found for *S. aureus* strains, the effect of the RMF was mainly determined by the duration of RMF exposure, with the observed trend being in most cases different for various strains of the same species. However, a certain kind of a reproducible trend between strains was found for *P. aeruginosa* strains in the 3rd h of the experiment, where the results of growth rate, except for the reference strain (ATCC 15442) exposed to an RMF of 50 Hz, were not statistically significantly different as compared with the control (Figure 4a). Furthermore, also except for the reference strain, but exposed to an RMF of 5 Hz, the growth rate of other strains decreased (all the values were also significantly lower as compared with the unexposed control) after 6 h of RMF exposure regardless of RMF frequency. Only the results obtained for the reference strain after 9 h of exposure to an RMF of 5 Hz and after 3 and 9 h of exposure to an RMF of 50 Hz were higher as compared with the unexposed controls. On the other hand, in the remaining strains, the growth rate measured after 9 h of magnetic exposure was always below the values obtained for the controls (although the results were not always statistically significant). A repeatable trend was also observed in the analysis of cellular metabolic activity—all the values of this parameter obtained after 3 h and, except for strain no. 1, also after 6 h of exposure (both RMF frequencies) were higher as compared with the control. In contrast, no repeatable trend was observed after 9 h of RMF exposure.

In contrast to the results obtained for *P. aeruginosa*, there were no recurring trends in the changes of growth dynamics in cultures of *E. faecalis*, although in most cases, the results obtained in RMF-exposed cultures were not statistically different as compared with the unexposed controls (Figure 4b). The only exception was found for strain no. 2—the cultures of this strain after 3 h of exposure to an RMF of 5 Hz were characterized by a statistically significantly higher growth rate as compared with the unexposed controls. Moreover, the same trend was also found for this strain when it was exposed for 3 and 6 h to an RMF of 50 Hz. Similarly, also in the case of cellular metabolic activity, strain no. 2 was the most distinct in terms of the observed changes in this parameter as a result of RMF exposure compared with the other strains of *Enterococcus*. In turn, three distinct profiles of changes in growth dynamics under the influence of the RMF were observed in *E. cloacae* cultures (Figure 4c). The first of them was "none" (absence of significant effects) or only slight effect of the RMF on the changes in growth rate, the second one can be described as an increased growth rate after 6 h, and the third one showed a decreased growth rate after 3 and 9 h of RMF exposure. All *E. cloacae* strains showed the highest cellular metabolic activity after 3 h of RMF exposure. In the case of strain nos. 1 and 2, a continuous decrease in this parameter was observed (below the values obtained for the unexposed control after 9 h of exposure). In the case of *P. mirabilis*, no recurring trend in the observed changes was noticed, in the case of both growth dynamics and cellular metabolic activity (Figure 4d). However, it can be seen that most of the statistically significant differences between the RMF-exposed and unexposed cultures indicated a reduced growth rate and, at the same time, increased cellular metabolic activity under the influence of the RMF. The growth rate of *K. pneumoniae*, regardless of the strain, at the first time point of measurement was slightly lower than the one obtained for the control (Figure 4e). In contrast, after 6 h of RMF exposure, the upward trend dominated, and finally, after 9 h, there was no statistically significant difference between RMF-exposed and unexposed cultures. The analysis of *B. cereus* allowed for selecting three different profiles of changes in the growth rate due to RMF exposure, one of which (gradual decrease) was repeated in cultures of two strains (PCM 449 and strain no. 1) (Figure 4f). In the analyses of cellular metabolic activity, it was found that three strains were characterized by the highest values of this parameter after 3 h, followed by its decrease after 6 h of exposure to the RMF (PCM 497 and strain nos. 1 and 3). After 9 h of exposure to the RMF, except for strain no. 1 exposed to an RMF of 5 Hz, the growth rate values for the remaining strains, regardless of RMF frequency, did not differ

from the unexposed control. In the cultures of *M. catarrhalis*, all strains showed the highest growth rate after 6 h of magnetic exposure, although the results differed between strains (e.g., only strain no. 1, when exposed to the RMF, proliferated more rapidly in comparison with the unexposed control (Figure 4g)). Furthermore, all values of this parameter obtained after 3 and 9 h of exposure were lower than in the control (except for strain no. 2 exposed to an RMF of 5 Hz for 3 h and the strains PCM 2340 and no. 1 exposed to an RMF of 5 Hz for 9 h, the rest of the results were statistically significantly different in comparison with the control). In turn, in the case of cellular metabolic activity, the trends of changes in this parameter were not repeated between the analyzed strains.

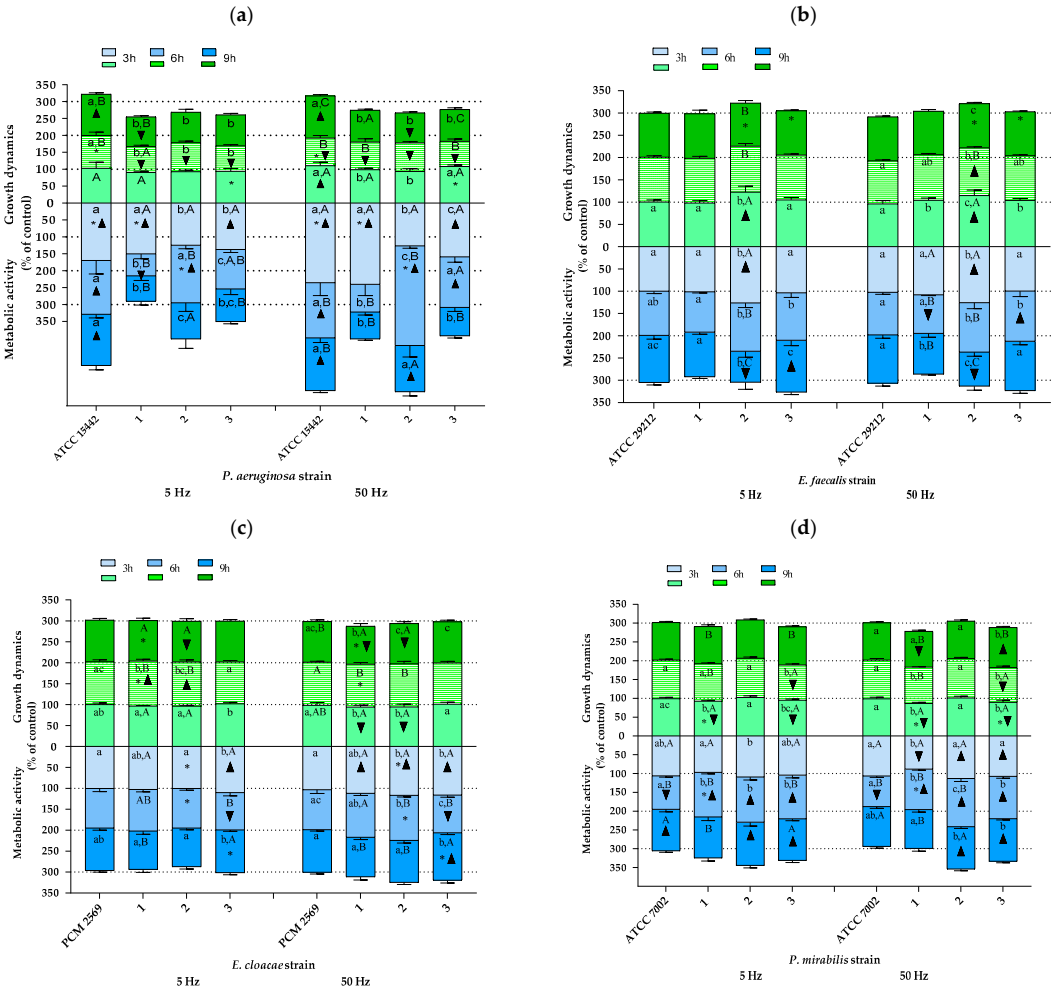

Figure 4. *Cont.*

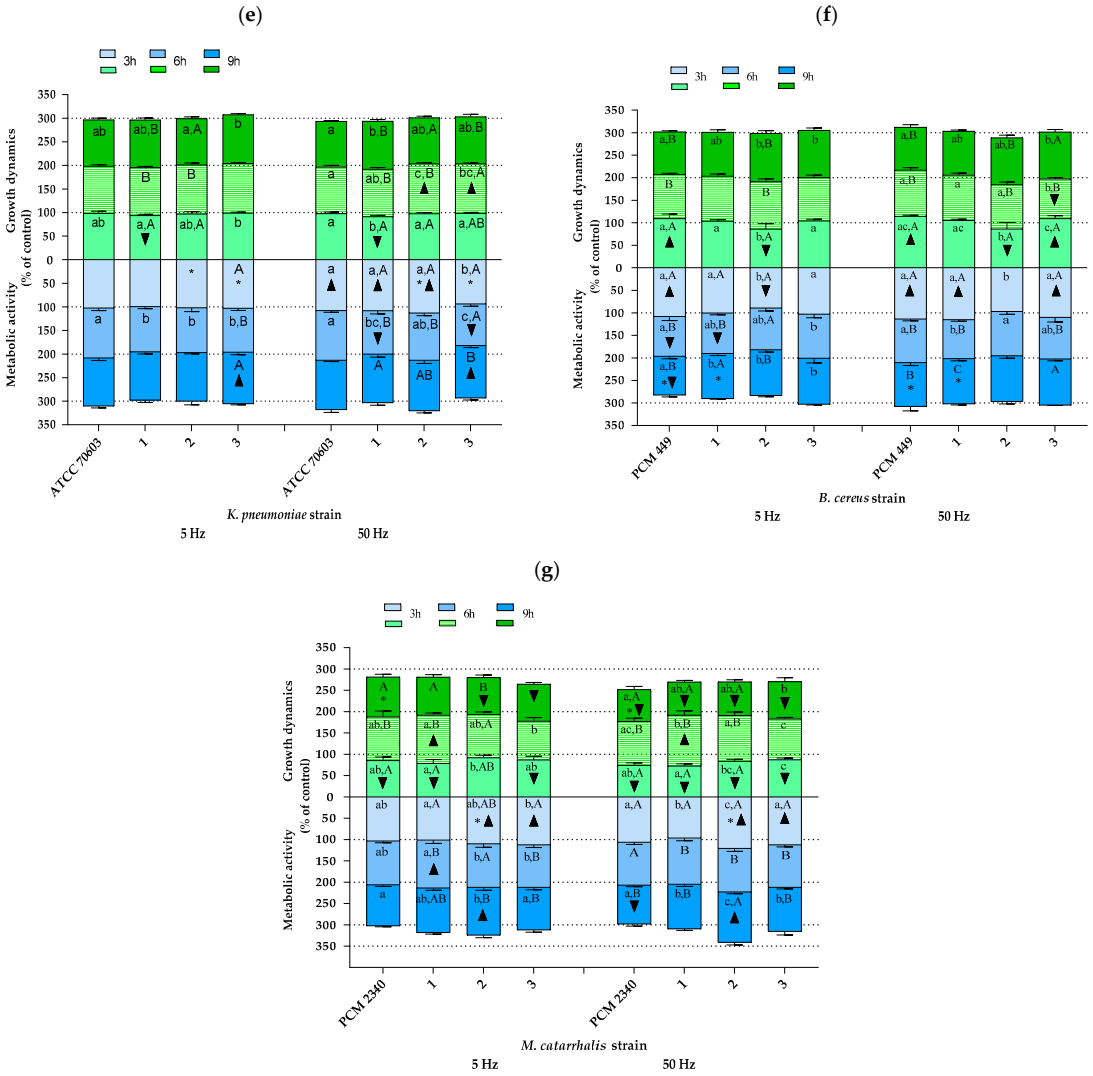

Figure 4. Growth dynamics and cellular metabolic activity of different bacterial strains and species exposed to a rotating magnetic field (5 and 50 Hz): (**a**) *P. aeruginosa*, (**b**) *E. faecalis*, (**c**) *E. cloacae*, (**d**) *P. mirabilis*, (**e**) *K. pneumoniae*, (**f**) *B. cereus*, (**g**) *M. catarrhalis.* Data are expressed as % of the control. The results are presented as a mean ± SEM calculated using six values (three from each biological replicate). Lowercase letters indicate significant differences between strains at a specific time; capital letters indicate significant differences between time points of measurements for a particular strain; the asterisk symbol (*) indicates significant differences between the RMF frequencies (5 v. 50 Hz); ▲▼—significant differences compared with the unexposed control, taking into account the trend (stimulation and inhibition, respectively) ($p < 0.005$).

3. Discussion

Our research has proven that, apart from the previously described factors related primarily to the characteristics of the MF, one of the key parameters affecting the final result of MF influence is the specificity of a given microorganism. By the specificity of a microorganism, we mean not only the species, cell shape, or cell wall structure, as widely considered in the literature [7,10,24,25], but also the individual set of features characteristic for a given strain of a microorganism within a species, referred to as the intraspecies variability [35]. We showed that, depending on a given strain, the effect exerted by the RMF (the particular type of MF used for the purposes of the current analysis) was positive (i.e., manifested itself as the increase in growth rate or/and cellular metabolic activity), or it was negative (i.e., manifested itself as a reduction of both aforementioned features). In turn, for some strains exposed to the RMF, we also observed no biological effect. Thus, it can be stated, perverse as that sounds, that the results obtained in the current study are

consistent with all data previously reported by other authors, even though they are often contradictory (Tables 1 and 2).

As already mentioned, so far, the research within the scope of analysis performed in the current study has been carried out with the use of different species or different groups (due to the selected characteristics) of microorganisms, but not with the use of different strains belonging to one species. Thus, the present study proved that by selecting the appropriate strain of the microorganisms, we can draw completely different conclusions concerning the influence of the MF (or RMF, at least) on them. Interestingly, even when we used one clonal type of *S. aureus* represented by several strains belonging to different biotypes, the results of RMF exposure also varied (although the degree of differentiation was lower than for strains representing different clones). This observation additionally emphasizes the sensitivity of individual microorganisms to the activity of MFs. Of course, we are aware that in the present study, we used only one type of MF, but there are no reasons to believe that the results of such experiments would be different if another MF type was applied.

It should be noted that from the microbiological perspective, bacteria show a significant diversity, not only within the species, but even within the strain, and to some extent even within the clone. The general concept of species held by most bacteriologists could be formulated as follows: a species consists of strains of common origin that are more similar to each other than they are to any other strain [36]. The strain can be defined as an isolate or group of isolates of the same species by phenotypic characteristics or genotypic characteristics or both [37]. It should also be noted that bacterial strains can change over time. They undergo mutations, may lose plasmids, and acquire genetic material from other strains in the environment [36,38]. However, strains retain their identity even if their phenotype is changed. The term "clone" (genetically related isolates) is used to denote bacterial isolates that are indistinguishable from each other by a variety of genetic tests [36]. Thus, even strains and clones of one species are not identical in terms of such phenotypic features as, for example, biochemical, pathogenic, or antibiotic-resistant profile [39]. Therefore, taking into account that MFs affect the activity of enzymes involved in the metabolic processes of bacterial cells [40], as well as considering the differences in the phenotype (including the biochemical profile even between closely related strains of a given species), it seems that one should not expect that exposure to any kind of MF would have the same effect on each strain (despite these differences). The recommendations of EUCAST (European Committee on Antimicrobial Susceptibility Testing) [41] and CSLI (Clinical and Laboratory Standards Institute) [42] should be quoted as a significant example of the microbiological perspective. According to these recommendations, a separate examination of bacterial susceptibility to antibiotics should be carried out for each bacterial isolate/strain of a given species. These recommendations are obviously based on the undeniable observation that sensitivity to antimicrobial substances is unique for each strain isolated [43]. Similarly, the ability of bacteria to produce biofilm is well known to be strain dependent [44]. Therefore, it can be assumed that the observed effects of MFs on bacteria (apart from such physical parameters as type, distribution, magnetic induction, frequency, and duration of exposure), reflecting, for example antibiotic type, duration of exposure to the antibiotic, antibiotic concentration (in antibiotic susceptibility testing), or type and composition of the culture medium (in the case of biofilm analyses), cannot depend only on species affiliation or such variables as the structure and shape of bacterial cell, but also on the specific characteristics of each strain. In this context, one could compare here the claim that a specific type of MF stimulates Gram-positive bacteria, or even a specific representative of that group, such as *S. aureus*, and inhibits Gram-negative bacteria (e.g., *P. aeruginosa*) to a situation in which one would assume that an analogous division (G+/G− or *S. aureus*/*P. aeruginosa*) may be applied in determining bacterial resistance to a specific antibiotic (e.g., clindamycin). Obviously, microbiology knows the concept of natural antibiotic resistance covering whole groups of bacteria or only individual species, but the basis of this mechanism is of a different nature,

which would be difficult to expect to manifest itself in the case of the effect observed after exposure to the MF.

The second important achievement resulting from our research was the confirmation that the effect of RMF exposure is additionally differentiated depending on exposure duration. Thus, by selecting the appropriate exposure time, it is also possible to observe a different nature of MF influence. The previous works of our research group [18,20] and reports by other authors [6,14,26] also indicated that the time of magnetic exposure is of key importance for the effect exerted on biological systems. The results of previous studies have shown that, depending on the MF exposure time, the observed effect may be positive (e.g., increased viability of bacteria) [30–32] or negative (e.g., reduced viability of bacteria) [7,10,26]. However, the results obtained in the current study revealed that the effect of the duration of magnetic exposure was additionally different depending on the strains, and in most analyses, no distinct trends were found between different strains of the same species. Therefore, depending on exposure duration, final conclusions from the obtained results may be different (i.e., the effect observed can be positive or negative, or there can be no effect). This observation is even more important if the results are analyzed through the prism of different strains within a given species.

The parameter that determined the effect of the RMF to a lesser extent (compared with the variability between strains and taking into account exposure time) was related to the RMF characteristics. In the current study, analyses included exposure to the RMF generated at two frequencies of the alternating current. In the case of the RMF setup used in the present study, the current frequency determined the intensity of the MF, but above all, it was responsible for the physical characteristics of the MF wave shape. As was shown by the simulative calculations, at 5 Hz, the amplitude of the RMF was characterized by a longer period of magnetic induction (B) maximal strength state that was 50 ms with $B_{\max}$ 16.89 mT. In contrast, the RMF generated at 50 Hz (the highest current frequency that can be used in the setup) was characterized by a shorter period, with 5 ms time of magnetic induction maximal strength state with $B_{\max}$ 17.62 mT (Figure 5; Supplementary Table S20). Simultaneously, the applied AC frequencies (5 Hz https://www.youtube.com/watch?v=aSkb6nAUgz8 and 50 Hz https://www.youtube.com/watch?v=ryiLdqfRnwM) generated magnetic flux rotation around the stator with different synchronous speeds of 150 and 1500 rpm, respectively (calculations performed on the basis of the manufacturer characteristics of the stators). Nevertheless, also in this case, we proved that by selecting the appropriate strain of a microorganism, it is possible to achieve results that will prove that the characteristics of the MF significantly influence the obtained results.

Our observations are essentially very basic, and in a sense, they can be seen as obvious. However, similar studies have not been performed before, and they should be treated as a foundation for a more cohesive approach, which should be undeniably developed if findings on the biological impact of MFs were to be introduced to biotechnology and medical microbiology on a large scale. The majority of previous research conducted by members of our scientific team and by other authors was carried out with the use of a single bacterial strain of a given species (Table 1). In the case of our research team, it took us at least several years to formulate the goals of the current study or, to put it simply, to notice the problem. Before that, like most other authors, we repeated the generally accepted research scheme in which various species of microorganisms, bacteria of different shapes, or of different cell wall structures were scrutinized, but the use of different strains within the same species was neglected. However, with time, not only we gained experience in the subject of the effects of MFs on microorganisms, but also we developed the research team, which currently consists of scientists from various scientific disciplines—clinical microbiologists, biotechnologists, engineers, electricians, and chemists. Currently, we have fully automated laboratories equipped with MF generators along with comprehensive control and measurement equipment. This allows us to conduct analyses with a very high degree of precision, characterized by high repeatability. Such a multidisciplinary approach allowed us to look at the analyzed challenge from a different, broader perspective

and to get an insight into the importance of intraspecies variability. The observations described in the present article are related to the analyses that we are currently carrying out as a part of ongoing scientific projects. These studies concern the analysis of changes in the antibiotic sensitivity of microorganisms to various antibiotics under the influence of the RMF. In these studies, we used a large group of microorganisms of the same species (*S. aureus* and *P. aeruginosa*, mainly), including clinical and reference strains. Conducting the aforementioned analysis, we noticed that the effect of the RMF regarding the changes in antibiotic susceptibility cannot be defined as the same for different strains.

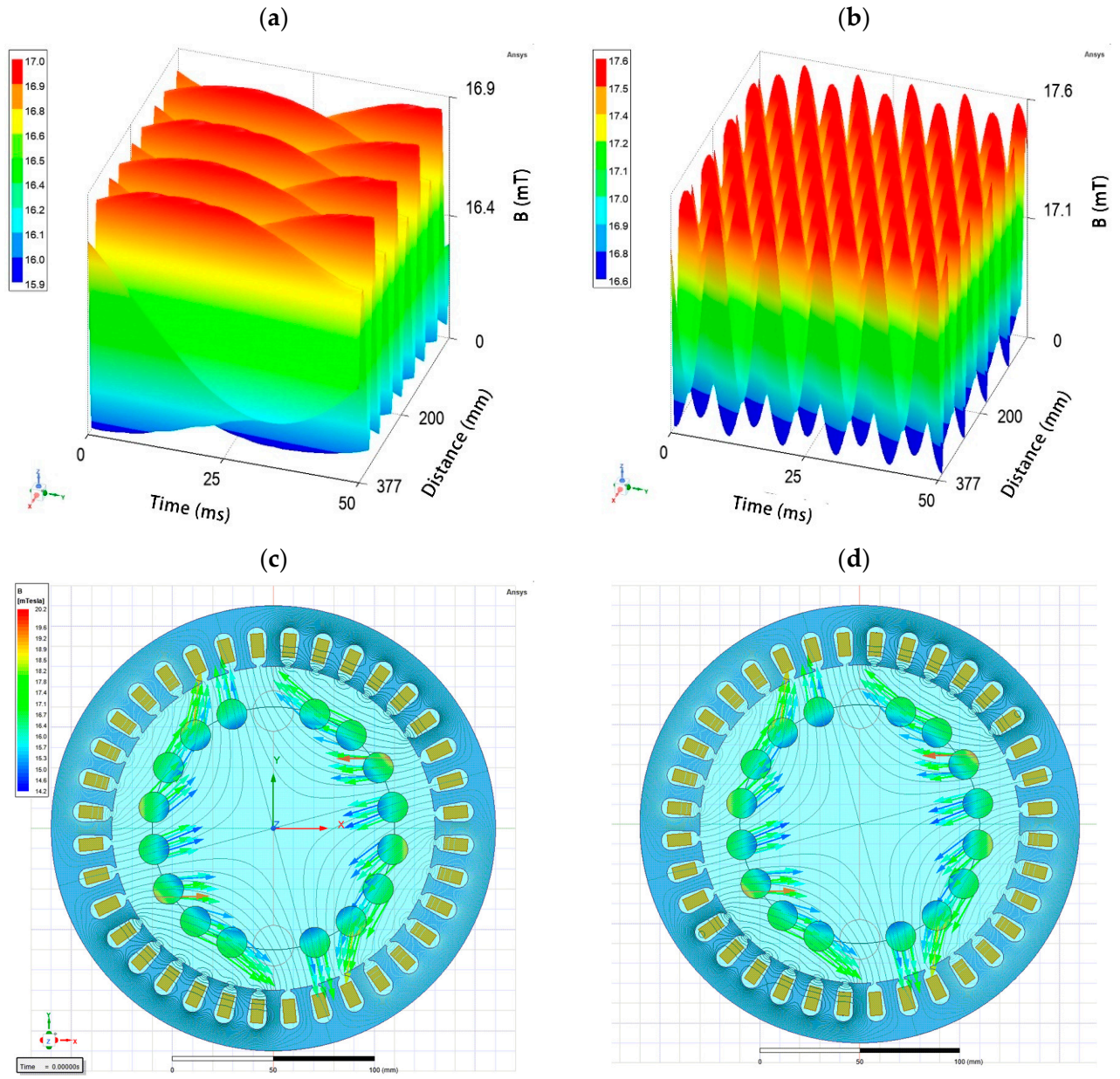

Figure 5. The changes of magnetic flux characteristic depending on the applied AC frequency: (**a**) 5 Hz; (**b**) 50 Hz and visualization of periodical changes in magnetic flux density and the direction of magnetic flux density vectors inside the RMF reactor chamber at (**c**) 5 Hz https://www.youtube.com/watch?v=aSkb6nAUgz8and (**d**) 50 Hz https://www.youtube.com/watch?v=ryiLdqfRnwM. The circles in (**c**,**d**) show the arrangement of tubes with bacterial cultures.

Finally, we would like to point to the fact that the discoveries of recent years proved that, although bacteria are less complex organisms than eukaryotic yeasts or higher animals,

they show a high level of intertwined interactions found in bacterial intra- and extraspecies communication [45–47], expression of virulence factors [48], or coordinated changes in the metabolic activity of community-forming cells [49]. In nature, strains of one species are subjected to a plethora of diverse stimuli, which modify the expression of their genes, resulting in multiple changes in bacterial phenotype and behavior [50]. Thus, bacterial strains of one species cannot be perceived as a number of identical biological automatons (which answer in exactly the same (binary) way to the same stimulus), but rather—similarly as in the case of animal or human communities—the type of answer to a stimulus is more of a Gaussian nature. It means that, if an adequately high population is used in the analysis, the obtained results will take the form of not only one main type of answer (manifested by the majority of strains) but also other types of answers (displayed by the minority of strains). As the issue of growing antimicrobial resistance to antibiotics shows, one should not underestimate these secondary types of reactions, because they may become dominant ones under specific circumstances. Therefore, as explicitly shown in the present study, the fact that intraspecies variability determines the effects exerted by the RMF (and the MF in general) should raise (among others) the question of the number of strains that should be analyzed (exposed to the specific type of MF) to draw proper conclusions and to obtain the desirable effect being the result of exposure. We would like to point out the fact that such a question is more and more frequently asked also in other types of microbiological studies, especially those concerning bacterial biofilm [51] and the use of antiseptics for chronic wound treatment [52,53]. With increasing knowledge concerning complex bacterial genetics, metabolomics, and proteomics, we have become aware that a study performed solely on the reference microorganism provides an answer concerning only this reference microorganism, and extrapolation of such results to the whole species (to which such reference microorganism belongs) should be performed carefully, if at all. Thus, the question of the exact number of strains required to obtain conclusive data on the type and level of biological effect exerted by the MF remains open. However, the answer should be provided in the first place, taking into consideration the possible advantages of the application of the MF in medical microbiology and biotechnology.

4. Materials and Methods

4.1. Experimental Setup

A schematic diagram of the experimental setup with the RMF generator is graphically presented in Figure 6. The base of each RMF reactor was a 3-phase, 4-pole stator with an internal core diameter of 16 cm and a height of 20 cm equipped with 12 groups of 3 coil sets [54]. The alternating current (AC) frequency supplied to the RMF generator was controlled using a Unidrive M200 inverter (Control Techniques, Nidec Industrial Automation, Poznan, Poland). The temperature in the RMF reactor chamber was maintained using a water-fed cooling/heating system monitored by a set of temperature probes with sampling deviation in the accuracy range ± 1.0 °C. The correct temperature distribution in the RMF reactor chamber was ensured by airflow supplied continuously throughout the exposure (2 L/min, 37 °C, RH 60%). The characteristics of the RMF, including the distribution of magnetic induction (B) in the reactor chamber, were performed at 100 V and AC frequencies of 5 and 50 Hz using Ansys Maxwell simulation software ver.19.1 (Ansys, Inc., Canonsburg, PA, USA) and confirmed empirically using a teslameter (SMS-102, Asonik, Tuczno, Poland).

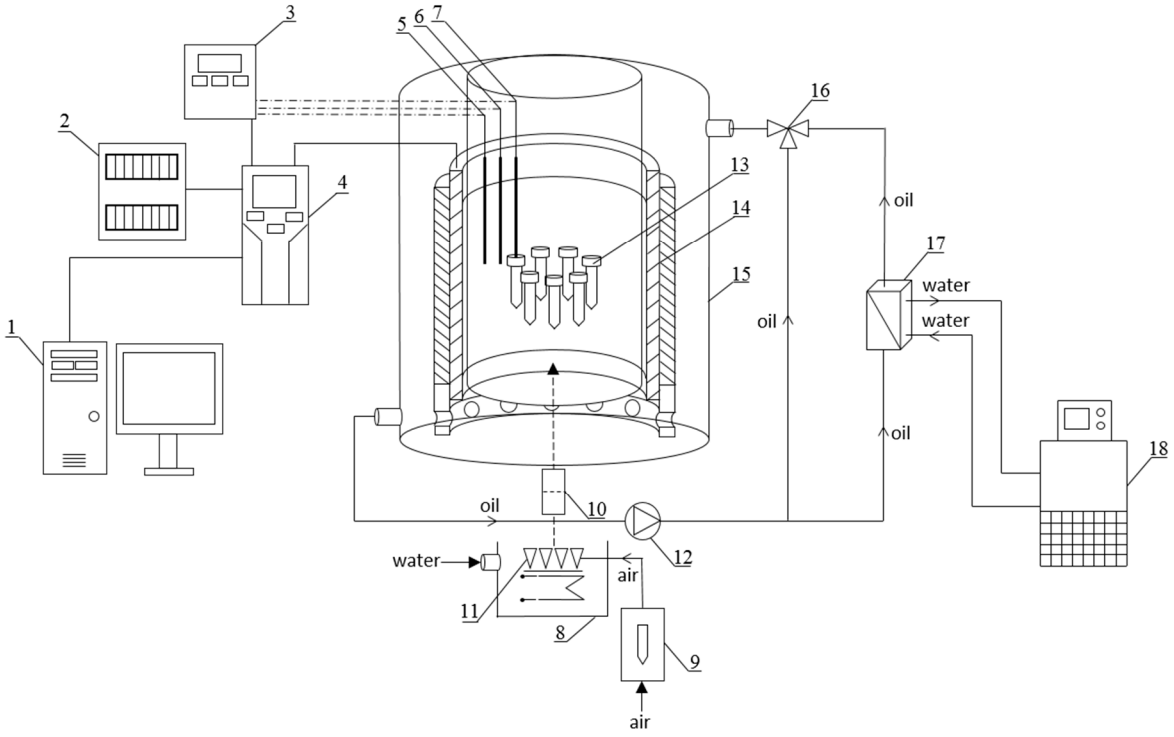

Figure 6. Schematic diagram of the experimental setup with the RMF generator. Experimental setup: 1—computer, 2—electrical switchgear, 3—measuring and control equipment, 4—inverter, 5—temperature probe, 6—RH% probe, 7—sample temperature probe, 8—water bath, 9—rotameter, 10—filter, 11—sparger, 12—circulation pump, 13—sample, 14—RMF generator, 15—cooling jacket, 16—three-way valve, 17—heat exchanger, 18—thermostat.

4.2. Microorganisms and Culture Conditions

Eight species of bacteria, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Moraxella catarrhalis*, and *Bacillus cereus*, were chosen for experimental purposes. Each species, except for *S. aureus*, was represented by 4 different strains. In the case of *S. aureus* (species chosen for an extended analyses), 8 strains were scrutinized. Moreover, an additional group of *S. aureus* belonging to a single clonal type but representing different biotypes was also included in the experiment.

Each species group consisted of 1 reference strain and 3 (7 in the case of *S. aureus*) wild isolates. The group of *S. aureus* representing 1 clonal type belonged to the Strain Collection of the Department of Microbiology and Biotechnology of West Pomeranian University of Technology in Szczecin. The results of the genetic and biochemical analyses for this group of microorganisms were already published [39,55]. The remaining wild isolates belonged to the Strain Collection of the Department of Pharmaceutical Microbiology and Parasitology of Wroclaw Medical University. Species identification was performed, in the first step by macroscopic observation of specific colonies on Columbia agar (Graso Biotech, Jablowo, Poland). Then, colonies were transferred into Mueller-Hinton agar (M-H, Graso Biotech, Jablowo, Poland) and identified using the Becton-Dickinson Phoenix 100 automated system for microorganism detection (Becton-Dickinson, Franklin Lakes, NJ, USA). The following reference strains were used: *Staphylococcus aureus* American Type Culture Collection (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 15442), *Proteus mirabilis* (ATCC 7002), *Klebsiella pneumoniae* (ATCC 70603), *Enterococcus faecalis* (ATCC 29212), *Enterobacter cloacae* Polish Collection of Microorganisms (PCM) 2569, *Moraxella catarrhalis* (PCM 2340), and *Bacillus cereus* (PCM 449).

The species of bacteria chosen for this research were characterized by various shapes, cell wall structures (Gram-negative and Gram-positive), and metabolisms and the ability to move and produce spores (Table 4).

Table 4. Characteristics of bacterial species selected for the study.

	Gram Staining	Shape	Spore	Capsule	Motility	Catalase/ Oxidase	Oxidative/ Fermentative
S. aureus	+ve	cocci	−ve	−ve	−ve	+ve/−ve	F
P. aeruginosa	−ve	rod	−ve	−ve	+ve	+ve/+ve	O
P. mirabilis	−ve	rod	−ve	−ve	+ve	+ve/−ve	F
K. pneumoniae	−ve	rod	−ve	+ve	−ve	+ve/−ve	F
E. faecalis	+ve	cocci	−ve	−ve	−ve	−ve/−ve	F
E. cloacae	−ve	rod	−ve	−ve	+ve	+ve/−ve	F
M. catarrhalis	+ve	cocci	−ve	+ve	−ve	+ve/+ve	U
B. cereus	+ve	rod with square ends	+ve	−ve	+ve	+/−ve	O

U—unreactive (nonsaccharolytic).

The group of *S. aureus* isolates was additionally analyzed according to the biochemical features using VITEK® 2 Compact (bioMérieux, Durham, NC, USA) and genetic diversity using the pulsed-field gel electrophoresis (PFGE) method (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

4.3. Exposure of Bacterial Cultures to the RMF

Initially, bacterial strains were plated on Brain Heart Infusion Agar (Graso Biotech, Jablowo, Poland) and cultivated for 24 h at 37 °C. After incubation, one colony-forming unit (CFU) of each isolate was transferred into 10 mL of Tryptic Soy Broth (TSB, Oxoid, Basingstoke, UK) and incubated for 24 h at 37 °C with shaking. In the next step, cultures (1.0 in McFarland turbidity standard) were diluted 1:10 in TSB. The obtained bacterial suspensions were vortexed and dispensed at a volume of 10 mL into 15 mL plastic tubes with caps featuring seven holes and a specific capillary pore filter membrane with a pore size of 0.2 µm providing gas exchange (CELLSTAR® CELLreactor™, Greiner Bio-One GmbH, Frickenhausen, Germany) in which the bacteria were exposed to the RMF.

The bacteria were exposed to the RMF generated at different AC frequencies (5 and 50 Hz) for 9 h. The test tubes with bacterial cultures were arranged in the RMF generator in a way that allowed the same exposure to the RMF of the whole volume of bacterial culture (Figure 7a,b).

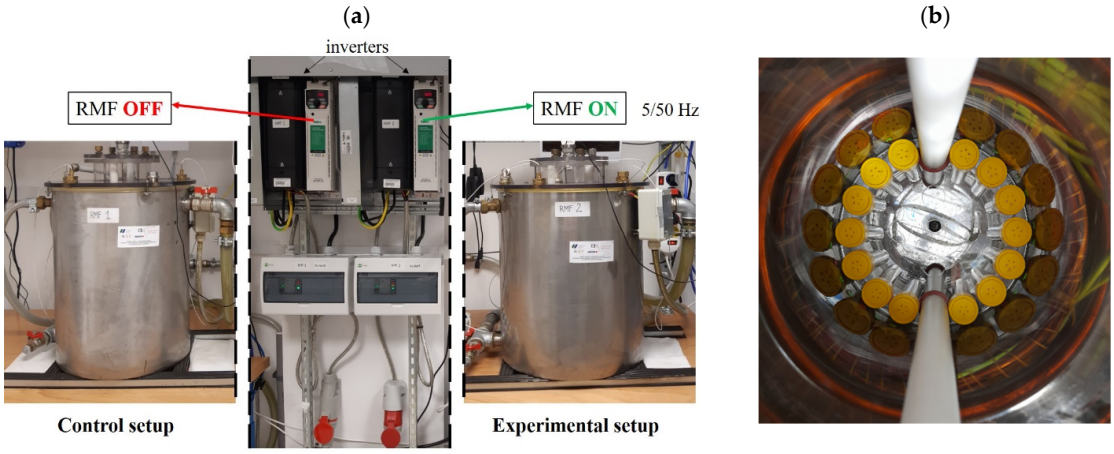

Figure 7. (**a**) The RMF generator and control settings with monitoring and control equipment. (**b**) Arrangement and location of the tubes with bacterial cultures inside the RMF generator.

The same bacteria incubated at the same time and under the same conditions as during the experiment but without exposure to the RMF were used as positive controls (RMF-off controls). The RMF-off controls were incubated in the twin reactor, however, with the RMF generator switched off (Figure 7a). As it was recorded, the fluctuations of the

temperature during the incubation of controls were the same as recorded during exposure of bacteria to the RMF and were less than 1.0 °C. The control RMF-off reactor was placed 2 m from the reactor with the RMF generator on. As measured using a Hall probe (Smart Magnetic Sensor-102, Asonik, Tuczno, Poland), the source of the RMF did not affect the RMF-off controls during the experiment (the magnetic induction in the RMF controls (*B*) was $\leq$0.05 mT).

4.4. Growth Dynamics and Metabolic Activity of Bacterial Cells

The optical density (OD) of bacterial cultures, which indirectly reflects cellular growth, was measured using an Infinite 200 PRO NanoQuant Microplate Reader (Tecan Trading AG, Männedorf, Switzerland) at a wavelength of 600 nm in 96-well plates (NEST Biotechnology Co., Ltd., Wuxi, Jiangsu, China) with 100 µL of each sample of bacterial culture taken after 3, 6, and 9 h of exposure to the RMF (5 and 50 Hz).

The metabolic activity of bacterial cells was determined using Alamar-Blue Cell Viability assay (Thermo Fisher, Eugene, OR, USA). Alamar-Blue Cell Viability Reagent is a ready-to-use resazurin-based solution that functions as a cell viability and metabolic activity indicator. Resazurin, the active ingredient of Alamar-Blue Reagent, is a nontoxic, cell-permeable compound that is blue in color and virtually nonfluorescent. Upon entering living cells, resazurin is reduced to resorufin, a compound that is red in color and highly fluorescent. After 3, 6, and 9 h of exposure to the RMF (5 and 50 Hz), 100 µL of each bacterial culture was transferred into wells of 96-well fluorescence microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany). Next, 10 µL of Alamar-Blue was added, and the plates were incubated for 30 min at 37 °C in the dark. Fluorescence was measured using a microplate fluorescence reader (Synergy HTX, BioTek, Winooski, VT, USA) at wavelengths of 540 and 590 nm for excitation and emission, respectively. As a blank, sterile TSB was used.

The results for both aforementioned assays were shown as the percentage of control values calculated using Equation (1):

$$\% \text{ of control} = (A_{\text{sample}} - A_{\text{background}})/(A_{\text{positive control}} - A_{\text{background}}) \times 100 \quad (1)$$

where A is absorbance.

4.5. Analysis of Molecular Diversity between Strains of S. aureus

The genetic relationship between the examined isolates was analyzed by digestion of chromosomal DNA with *Sma*I enzyme and its separation by pulsed-field gel electrophoresis (PFGE) method, according to the manufacturer of the GenePath Group 6 Reagent Kit Instruction Manual (Bio-Rad Laboratories, Inc., Marnes-la-Coquette, France) and the Centers for Disease Control and Prevention (CDC) protocol (oxacillinresistant *Staphylococcus aureus* on PulseNet (OPN)), laboratory protocol for molecular typing of *S. aureus* to pulsed-field gel electrophoresis (PFGE) [56].

Single colonies of *S. aureus* isolates (Graso Biotech, Jablowo, Poland) were transferred from blood agar plates to 3 mL of Tryptone Soya Broth (TSB, Oxoid, Basingstoke, UK) and incubated for 24 h at 37 °C. Next, 1 mL cultures were transferred to 1.5 mL tubes and centrifuged at 5000 rpm for 5 min, and the resulting cell pellets were mixed with Cell Suspension Buffer (100 µL), 6 µL of lysozyme (0.025 g/mL, Millipore Sigma, Mannheim, Germany), 4 µL of lysostaphin (400 U/mL, DNA Gdańsk, Gdansk, Poland), and 100 µL of 2%, warmed to 55 °C, liquid agarose solution (Bio-Rad Laboratories, Inc., Marnes-la-Coquette, France) and transferred into molds to form blocks, which were further treated with 20 µL of proteinase K (DNA Gdańsk, Gdansk, Poland) in Proteinase K Buffer, and digested with 3 µL of the restriction enzyme *Sma*I in Tango Buffer (Thermo Fisher Scientific, Hennigsdorf, Germany). The above-mentioned steps were carried out separately in two replications for each of the test strains of *S. aureus*.

The plugs were loaded into 1.2% agarose gels and electrophoresed in TBE buffer (Inno-Train Diagnostik GmbH, Kronberg, Germany) using a CHEF-DR III apparatus (Bio-Rad

Laboratories, Inc., Marnes-la-Coquette, France). The run time was 22 h with an initial switch time of 2.2 s and a final switch time of 54.2 s. The ramping factor was linear. The temperature was set at 14 °C, voltage at 6 V/cm, and the included angle at 120°. The gels were stained with ethidium bromide (0.5 µg/mL, Mannheim, Germany) and photographed in an image system, GelDoc-It2 Imager (Upland, CA, USA). Restriction profiles were analyzed using the FPQuest software (Bio-Rad Laboratories, Inc., Marnes-la-Coquette, France). The classification of individual restriction patterns for particular genetic profiles was made using the unweighted pair group method with the arithmetic mean (UPGMA) method (similarity coefficient (SAB) value = 65.7%) and the Dice coefficient (2.0%). The results are presented in the form of a dendrogram.

4.6. Statistical Analysis

Data are presented as the means ± standard errors of the means (SEM) calculated from three repetitions of the experiment (plus three technical repetitions for each measurement). The statistical significance of the differences between RMF-exposed and control cultures, cultures exposed or incubated for different time, cultures of different strains, and cultures exposed to the RMF at 5 and 50 Hz was analyzed by two-way analysis of variance (ANOVA) and Tukey's post hoc test. Differences were considered significant at a level of $p < 0.05$. The statistical analyses were conducted using Statistica 12.5 (StatSoft, Inc., Tulsa, OK, USA).

5. Conclusions

In conclusion, our research has proven that, apart from the previously described factors related primarily to the characteristics of the magnetic field, one of the key parameters affecting the final result of its influence is the specificity of a given microorganism. By the specificity of a microorganism we mean not only the species, cell shape, or cell wall structure, but also the individual set of features specific for the given strain, referred to as the intraspecies variability. We showed that, depending on the exposed strain, the effect exerted by the RMF may be positive (i.e., manifests as the increase in growth rate or/and cellular metabolic activity) or negative (e.g., manifests as the reduction of both aforementioned features) or none. Therefore, the data from the performed analyses explicitly show that biological effects exerted by the magnetic field on a single strain (reference or wild type) cannot be extrapolated to the entire bacterial species this specific analyzed strain belongs to.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pathogens10111427/s1: Table S1: Phenotypic patterns of *S. aureus* strains; Table S2: Statistical differences in growth dynamics between *S. aureus* strains representing different clonal types exposed for 3 h to a rotating magnetic field of 5 Hz; Table S3: Statistical differences in growth dynamics between *S. aureus* strains representing different clonal types exposed for 6 h to a rotating magnetic field of 5 Hz; Table S4: Statistical differences in growth dynamics between *S. aureus* strains representing different clonal types exposed for 9 h to a rotating magnetic field of 5 Hz; Table S5: Statistical differences in growth dynamics of *S. aureus* strains representing different clonal types depending on the duration (3, 6, and 9 h) of rotating magnetic field (5 Hz) exposure; Table S6: Statistical differences in growth dynamics between *S. aureus* strains representing different clonal types exposed for 3 h to a rotating magnetic field of 50 Hz; Table S7: Statistical differences in growth dynamics between *S. aureus* strains representing different clonal types exposed for 6 h to a rotating magnetic field of 50 Hz; Table S8: Statistical differences in growth dynamics between *S. aureus* strains representing different clonal types exposed for 9 h to a rotating magnetic field of 50 Hz; Table S9: Statistical differences in growth dynamics of *S. aureus* strains representing different clonal types depending on the duration (3, 6, and 9 h) of rotating magnetic field (50 Hz) exposure; Table S10: Statistical differences in growth dynamics of *S. aureus* strains representing different clonal types exposed to a rotating magnetic field (5 vs. 50 Hz); Table S11: Statistical differences in cellular metabolic activity between *S. aureus* strains representing different clonal types exposed for 3 h to a rotating magnetic field of 5 Hz; Table S12: Statistical differences in cellular metabolic activity between *S. aureus* strains representing different clonal types exposed for 6 h to a rotating magnetic field of 5 Hz; Table S13:

Statistical differences in cellular metabolic activity between *S. aureus* strains representing different clonal types exposed for 9 h to a rotating magnetic field of 5 Hz; Table S14: Statistical differences in cellular metabolic activity of *S. aureus* strains representing different clonal types depending on the duration (3, 6, and 9 h) of rotating magnetic field (5 Hz) exposure; Table S15: Statistical differences in cellular metabolic activity between *S. aureus* strains representing different clonal types exposed for 3 h to a rotating magnetic field of 50 Hz; Table S16: Statistical differences in cellular metabolic activity between *S. aureus* strains representing different clonal types exposed for 6 h to a rotating magnetic field of 50 Hz; Table S17: Statistical differences in cellular metabolic activity between *S. aureus* strains representing different clonal types exposed for 9 h to a rotating magnetic field of 50 Hz; Table S18: Statistical differences in cellular metabolic activity of *S. aureus* strains representing different clonal types depending on the duration (3, 6, and 9 h) of rotating magnetic field (50 Hz) exposure; Table S19: Statistical differences in cellular metabolic activity of *S. aureus* strains representing different clonal types exposed to a rotating magnetic field (5 vs. 50 Hz); Table S20: The values of magnetic induction inside the RMF generator, at the location of the Petri dishes, depending on the applied AC frequency; Figure S1: Statistical differences in growth dynamics and cellular metabolic activity between *S. aureus* strains representing one clonal type exposed to a rotating magnetic field (5 and 50 Hz) for 3, 6, and 9 h; Figure S2: Statistical differences in growth dynamics and cellular metabolic activity between *P. aeruginosa* strains exposed to a rotating magnetic field (5 and 50 Hz) for 3, 6, and 9 h; Figure S3: Statistical differences in growth dynamics and cellular metabolic activity between *E. faecalis* strains exposed to a rotating magnetic field (5 and 50 Hz) for 3, 6, and 9 h; Figure S4: Statistical differences in growth dynamics and cellular metabolic activity between *E. cloacae* strains exposed to a rotating magnetic field (5 and 50 Hz) for 3, 6, and 9 h; Figure S5: Statistical differences in growth dynamics and cellular metabolic activity between *K. pneumoniae* strains exposed to a rotating magnetic field (5 and 50 Hz) for 3, 6, and 9 h; Figure S6: Statistical differences in growth dynamics and cellular metabolic activity between *B. cereus* strains exposed to a rotating magnetic field (5 and 50 Hz) for 3, 6, and 9 h; Figure S7: Statistical differences in growth dynamics and cellular metabolic activity between *P. mirabilis* strains exposed to a rotating magnetic field (5 and 50 Hz) for 3, 6, and 9 h; Figure S8: Statistical differences in growth dynamics and cellular metabolic activity between *M. catarrhalis* strains exposed to a rotating magnetic field (5 and 50 Hz) for 3, 6, and 9 h.

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The Effect of Rotating Magnetic Field on Susceptibility Profile of Methicillin-Resistant *Staphylococcus aureus* Strains Exposed to Activity of Different Groups of Antibiotics

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Abstract: Methicillin-resistant strains of *Staphylococcus aureus* (MRSA) have become a global issue for healthcare systems due to their resistance to most β-lactam antibiotics, frequently accompanied by resistance to other classes of antibiotics. In this work, we analyzed the impact of combined use of rotating magnetic field (RMF) with various classes of antibiotics (β-lactams, glycopeptides, macrolides, lincosamides, aminoglycosides, tetracyclines, and fluoroquinolones) against nine *S. aureus* strains (eight methicillin-resistant and one methicillin-sensitive). The results indicated that the application of RMF combined with antibiotics interfering with cell walls (particularly with the β-lactam antibiotics) translate into favorable changes in staphylococcal growth inhibition zones or in minimal inhibitory concentration values compared to the control settings, which were unexposed to RMF. As an example, the MIC value of cefoxitin was reduced in all MRSA strains by up to 42 times. Apart from the β-lactams, the reduced MIC values were also found for erythromycin, clindamycin, and tetracycline (three strains), ciprofloxacin (one strain), gentamicin (six strains), and teicoplanin (seven strains). The results obtained with the use of in vitro biofilm model confirm that the disturbances caused by RMF in the bacterial cell walls increase the effectiveness of the antibiotics towards MRSA. Because the clinical demand for new therapeutic options effective against MRSA is undisputable, the outcomes and conclusions drawn from the present study may be considered an important road into the application of magnetic fields to fight infections caused by methicillin-resistant staphylococci.

Keywords: antibiotics; biofilm; β-lactam; methicillin-resistant *Staphylococcus aureus*; rotating magnetic field

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1. Introduction

In the last decades, antimicrobial resistance has become a significant health issue. The increasing tolerance to antibiotics is observed in a variety of bacterial species, regardless of their origin (community or clinical) [1,2]. Methicillin-resistant *Staphylococcus aureus* strains, referred to as MRSA, have acquired resistance to such β-lactam antibiotics as penicillins, cephalosporins (with the exception of ceftaroline and ceftobiprole), and to

carbapenems, commonly considered antibiotics of the last resort in the treatment of hard-to-heal infections. These resistant staphylococcal strains are presently thought to comprise 25–50% of all *S. aureus* strains [3]. MRSA strains are etiological factors of more than 70,000 severe infections annually [4]. The infections caused by MRSA include a variety of disease entities, from skin, soft tissue, and wound inflammation, through alimentary and respiratory tract infections, to bone and biomaterial-related diseases [5,6]. Over time, MRSA strains have also developed resistance to other antibiotic groups, including aminoglycosides, fluoroquinolones or macrolides, thus becoming the first "super-bugs", i.e., multidrug-resistant (MDR) pathogens [7].

Taking into account the fact of high staphylococcal adaptation to diverse physiologic niches [8], combined with the aforementioned resistance mechanism, the development of new treatment algorithms is required to prevent the increasing rate of severe infections of which MRSA is the causative agent [3]. To overcome the challenges related to staphylococcal antibiotic resistance, numerous approaches have been developed and scrutinized, though understood with moderate success, as the introduction of new molecules/treatment routes into clinical practice [9].

One of the solutions that still has not been fully investigated but is promising with regard to the matter in question is the application of various types of magnetic fields (MF) intended as an agent boosting the efficacy of antimicrobial molecules [10–13] or in the character of a self-reliant antimicrobial agent [14,15]. Our research team has long-standing experience in studying the applications of the specific type of magnetic field, referred to as the rotating magnetic field (RMF). In our previous works, we have showed that, in the case of RMF, opposite poles rotate around a certain point; therefore, the charged molecules (e.g., antibiotics) present in a medium move in an unpredictable, Brownian-type motion. Therefore, one of the results of RMF application is high mixing of the medium due to increased particle movement [16]. Moreover, in our earlier publication [10], we indicated that the combined effect of RMF and antimicrobials increases the eradication rate of *S. aureus* biofilm to 50% as compared to biofilms exposed to antimicrobials only. At that time, because our study concerned multi-cellular spatial structures, we assumed that a possible mechanism behind the observed result was related to the mixing effect caused by RMF, translating into higher penetrability of antimicrobials into the deeper layers of biofilm.

The omnidirectional and differentiated effect of RMF on micro-organisms (indicated in our earlier works [17,18]), as well as data presented, among others, by Mega-Tiber et al. (2008) [19], who showed that the application of MF may affect macromolecular synthesis and may cause protein injury in bacteria, moved us into another (the present) investigation line. The goal of this work was to analyze the influence of RMF on the changes in MRSA strains' susceptibility to different classes of antibiotics. Our hypothesis was that RMF could have an impact on the overall antibiotic activity, resulting in a higher rate of eradication of MRSA in vitro. Although such research has never been carried out before, we assumed that at least two main factors may be responsible for such a phenomenon, namely i) the already-mentioned RMF mixing effect resulting in better transportation of antimicrobial agents; such an assumption was backed up by the study of Khoury et al. (1992), Costerton et al. (1994), and Stewart et al. (1999) [20–22], though performed on other types of MF and pathogenic biofilms; ii) RMF-induced alteration of staphylococcal cell functionality; this assumption was, in turn, backed up by the data shown by Golberg et al. (2014) and Alya et al. (2010) [23,24]. However, these authors also applied MFs other than RMF types in their studies.

Moreover, we assumed that other variables may have an impact on these two main possible RMF-related factors. These distinguished variables were MF characteristics, mechanism of activity of the antibiotic towards bacterial cells or the charge of the antibiotic molecule, and, last but not least, the intraspecies variability. Therefore, by using a cohesive set of analytical techniques allowing the observation and interpretation of the phenomena that were a result of the interplay of all aforementioned factors, we aimed to perform

in vitro research, which could be the basis of subsequent analyses aimed at the application of RMF in the clinical practice.

2. Results

2.1. Analysis of Changes in Antibiotic Susceptibility of S. aureus Strains under the Influence of RMF

As mentioned in the methodological section, the zones of growth inhibition, as well as the MIC values for all *S. aureus* strains were measured after the end of exposure to RMF (12 h), and once more after completion of the entire incubation time (18 h—total amount of time consisting of exposure and non-exposure period). The seeded bacterial cultures on agar plates with antibiotics were subjected to the RMF for 12 h because this period allowed a well-developed bacterial lawn to be obtained, which did not change visually throughout further incubation until 18 h. Except for the bacterial growth, the inhibition zones around the antibiotic discs or E-tests were also sharp and clearly visible, so, taking both observations together, there was no reason to extend the exposure duration over the 12 h. These assumptions were reflected in further analyses in which the cultures were exposed to RMF; it was found that the results obtained immediately after RMF exposure and after further incubation without RMF did not differ regardless of the staphylococcal strain analyzed. Therefore, it can be assumed that the exposure to the RMF was long enough to obtain not only a well-developed bacterial lawn, which did not change during further incubation, but also a stable antimicrobial effect. However, it should also be noted that, based on our findings, it cannot be excluded that the time of magnetic exposure could be shorter to obtain the same or at least comparable effects, especially taking into account the different mechanisms of action of the various classes of antibiotics.

2.2. Disc Diffusion Test

The studies showed that, according to the diameters of growth inhibition zones, all MRSA strains were characterized by increased sensitivity to cefoxitin under the influence of RMF (Table 1; Figure 1). The greatest differences and their greatest number compared to the unexposed control were found for exposure to the RMF of 5 Hz. Similar results were obtained in the analyses with amoxicillin but, in this case, RMF frequency was irrelevant for the observed changes, although the largest difference in the zone of growth inhibition compared to the control (4 mm) was obtained as a result of exposure to the RMF of 50 Hz (strains MRSA 1, 3, and 5). It is also worth noting that, for MSSA strain, no differences in changes in susceptibility to both β-lactam antibiotics were noted. Where amoxicillin was combined with clavulanic acid, the differences in the zones of growth inhibition were observed for all MRSA strains, while, in the case of the MSSA strain, the differences were again not detected. Moreover, in this case, a greater effect was observed in the cultures exposed to the RMF of 50 Hz. It was also found that the differences in the zones of growth inhibition were, in each case, substantially greater than the differences observed when amoxicillin without clavulanic acid was used. In the case of erythromycin, differences in the diameters of the zones of growth inhibition were found only for the MSSA and MRSA 7 strain (the zones were enlarged by 2 mm as compared to the control). For clindamycin, no differences were found in the zones of growth inhibition of MRSA strains, regardless of the frequency of RMF used during exposure. Only in the cultures of MSSA strain were the zones enlarged; however, only by 2 mm as compared to the controls. Similar results were obtained for ciprofloxacin, although, in this case, the lack of differences also concerned the MSSA strain. For tetracycline, differences between inhibition zones were found in the cultures of two MRSA strains (MRSA 1 and MRSA 4). The diameters of the growth inhibition zones were increased by 4 mm (MRSA 1) and 2 mm (MRSA 4) as compared to the control conditions. It can also be noted that an increase in the zones of growth inhibition was observed only in strains sensitive to this antibiotic. Most of the differences in the zones of growth inhibition under the influence of RMF, apart from antibiotics from the β-lactam group, were found for gentamicin (a representative of aminoglycosides) and

teicoplanin (a representative of glycopeptides). The differences between inhibition zones around gentamicin discs were found for four MRSA strains, whereas around teicoplanin discs for five strains, including the MSSA. The diameters of inhibition zones were larger by 2–3 mm as compared to the control. Moreover, in the study with gentamicin, the differences were only observed in the cultures exposed to RMF 5 Hz.

Table 1. Growth inhibition zones (mm) of RMF-exposed staphylococcal strains around antibiotic discs.

Culture Conditions	Staphylococcal Strain								
	ATTC 33591	**ATTC 6538**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Cefoxitin—β-Lactams—Methicillin Resistance Indicator									
Control	12	27	6	7	6	7	9	13	6
RMF (5 Hz)	18 [a]	27	16 [a]	21 [a]	18 [a]	16 [a]	15 [a]	16 [a]	16 [a]
RMF (50 Hz)	15 [b]	27	14 [b]	15 [b]	9 [b]	11 [b]	10 [b]	13 [b]	13 [b]
Amoxicillin—β-lactams									
Control	6	15	6	6 [d]	6	6	6	6	6
RMF (5 Hz)	8 [b]	15	8 [b]	8 [a]	9 [b]	9 [a]	9 [b]	8 [a]	8 [a]
RMF (50 Hz)	9 [a]	15	10 [a]	8 [a]	10 [a]	9 [a]	10 [a]	8 [a]	8 [a]
Amoxicillin + Clavulanic Acid—β-lactams + inhibitor of β-lactamases									
Control	10	32	11	6 [d]	11	13	13	15	9
RMF (5 Hz)	15 [b]	32	17 [b]	13 [b]	19 [a]	19 [b]	20 [a]	18 [b]	17 [a]
RMF (50 Hz)	16 [a]	32	19 [a]	17 [a]	19 [a]	20 [a]	20 [a]	19 [a]	14 [b]
Erythromycin—macrolides									
Control	25	27	6 [c]	6 [c]	6 [c]	6 [c]	6 [c]	6 [c]	10 [c]
RMF (5 Hz)	25	29 [a]	6 [c]	6 [c]	6 [c]	6 [c]	6	6 [c]	12 [ac]
RMF (50 Hz)	25	27	6 [c]	6 [c]	6	6 [c]	6 [c]	6 [c]	10 [c]
Clindamycin—lincosamides									
Control	27	25	6 [c]	6 [c]	6 [c]	6 [c]	6 [c]	27	6 [c]
RMF (5 Hz)	27	27 [a]	6 [c]	6 [c]	6 [c]	6 [c]	6 [c]	27	6 [c]
RMF (50 Hz)	27	27 [a]	6 [c]	6 [c]	6 [c]	6 [c]	6 [c]	27	6 [c]
Ciprofloxacin—fluoroquinolones									
Control	26 [c]	28	6 [c]	6 [c]	6 [c]	6 [c]	6 [c]	27 [c]	6 [c]
RMF (5 Hz)	26 [c]	28	6 [c]	6 [c]	6 [c]	6 [c]	6 [c]	27 [c]	6 [c]
RMF (50 Hz)	26 [c]	28	6 [c]	6 [c]	6 [c]	6 [c]	6 [c]	27 [c]	6 [c]
Tetracycline—tetracyclines									
Control	30	29	32	30	30	30	31	12 [c]	15 [c]
RMF (5 Hz)	30	29	36 [a]	30	30	32 [a]	31	12 [c]	15 [c]
RMF (50 Hz)	30	29	32	30	30	30	31	12 [c]	15 [c]
Gentamicin—aminoglycosides									
Control	25	23	27	23	26	25	26	23	6 [c]
RMF (5 Hz)	25	23	27	25 [a]	28 [a]	27 [a]	26	25 [a]	6 [c]
RMF (50 Hz)	25	23	27	23	26	25	26	23	6 [c]
Teicoplanin—glycopeptide									
Control	18	20	18	19	19	19	18	16	18
RMF (5 Hz)	20 [a]	22 [a]	21 [a]	21 [a]	19	19	18	16	18
RMF (50 Hz)	20 [a]	22 [a]	21 [a]	21 [a]	21 [a]	19	18	16	18

The differences in the diameter of the growth inhibition zones between three repetitions of the experiment did not exceed ±1 mm. The zones that are larger in comparison to the control are marked with letters: "[a]" means a larger zone, "[b]" a smaller one—as compared to each other. "[c]" indicates resistance according to the EUCAST clinical breakpoints (2021) [25]; "[d]" indicates a lack of sensitivity to clavulanic acid.

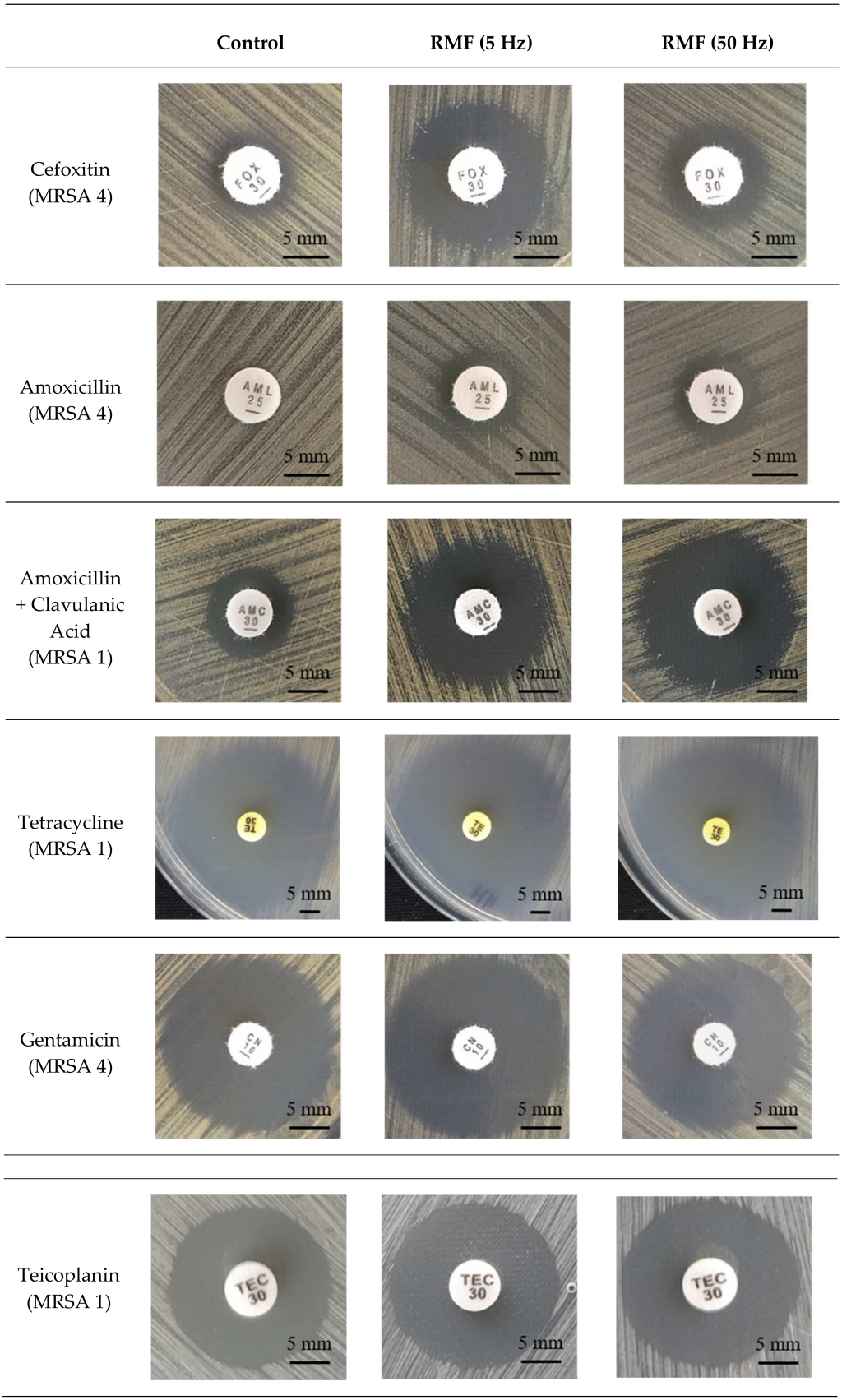


Figure 1. Representative pictures of growth inhibition zones (mm) in control and RMF-exposed MRSA cultures around discs with different antibiotics.

2.3. Gradient MIC Strips (E-Test)

It was found that in each of the RMF-exposed cultures of MRSA strains, a substantial decrease in the MIC value of β-lactam antibiotics occurred as compared to the controls (Table 2, Figure 2). In turn, no differences were found in the cultures of MSSA strains, regardless of whether cefoxitin or amoxycillin was tested. In the analyses with amoxicillin combined with clavulanic acid, differences in MIC values were also observed for all MRSA strains, while, in the case of the MSSA strain, the differences were, again, not detected. Similarly, as found in the disc diffusion tests, it was found that the differences in the MIC values were, in each case, greater than the differences observed when amoxicillin without clavulanic acid was used. The differences in MIC values of erythromycin were found in the cultures of MSSA ATCC 6538 and MRSA 7 strains exposed to the RMF of 5 Hz (these two strains were also reactive in the disc diffusion test). However, in contrast to the disc diffusion test, the use of E-test also allowed the detection of changes in the sensitivity to this antibiotic in the culture of the MRSA ATCC 33591 strain. In the case of clindamycin, lower MIC values as compared to the unexposed controls were found in the cultures of MSSA and MRSA 6 (strain not responding in the disc diffusion test) strains exposed to the RMF. In the cultures of MRSA 6 strain, the difference in MIC of clindamycin was found only when bacteria were exposed to the RMF of 50 Hz. The MRSA 6 strain was also the only one towards which the MIC value of ciprofloxacin changed; however, only when it was exposed to the RMF of 5 Hz. In the analyses with tetracycline, differences in MIC values were recorded in the cultures of MRSA 1 and MRSA 4 strains exposed to the RMF of 5 Hz (these two strains were also reactive in the disc diffusion test) and MRSA 5 strain when the RMF frequency was 50 Hz. Similarly, as found in the disc diffusion tests, most of the differences in MIC values under the influence of RMF, apart from antibiotics from the β-lactam group, were found for gentamicin (a representative of aminoglycosides) and teicoplanin (a representative of glycopeptides). The differences between MIC values of gentamicin were found for six MRSA strains, whereas, of teicoplanin, for seven strains, including the MSSA. It can also be noted that, in contrast to the disc diffusion method, the frequency of RMF was irrelevant for the observed changes in the MIC values of gentamicin.

2.4. Effect of RMF on Release Rate and Diffusion of Antibiotics

It was found that gentamycin, ciprofloxacin, and tetracycline were the antibiotics released from the paper disc into the agar during 120 minutes of exposure to the RMF in higher concentrations as compared to unexposed conditions (taking into account the sum of the concentrations measured in all three zones from which the agar samples were taken) (Figure 3). The obtained results concerning the concentration of those antibiotics released into the agar samples corresponded with the results showing a decrease in their concentrations in the paper discs (Supplementary Figure S1). However, the increase in the release of ciprofloxacin and tetracycline occurred only at the RMF of 50 Hz frequency, and was not observed when the samples were exposed to the RMF of 5 Hz. It is also worth noting that the difference in the concentrations of ciprofloxacin as compared to the unexposed control was determined primarily by their higher concentration in zone 3, i.e., directly under the agar disc (Supplementary Figure S1). In the case of tetracycline, an increased concentration of this antibiotic (except for zone 3) was also observed in agar samples collected from zones 1 and 2. In contrast to the aforementioned antibiotics, the significantly higher concentration of gentamycin released into the agar zones was observed regardless of the RMF frequency, but this finding was determined only by the results from zones 1 and 2 (the results obtained in zone 3 were significantly lower as compared to the control). In the case of the rest of the antibiotics included in the experiment, there was no greater release or increase in the concentration found as a result of RMF exposure as compared to the control conditions, regardless of the zone from which the agar samples were obtained or of the applied RMF frequency. On the contrary, even lower concentrations of antibiotics were detected in most of the samples cut out from the RMF-exposed agar as compared to the unexposed control.

Table 2. MIC values (μg/mL) of β-lactam antibiotics for MRSA strains in control and RMF-exposed cultures.

Culture Conditions	Staphylococcal Strain								
	ATTC 33591	**ATTC 6538**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Cefoxitin—β-Lactams—Methicillin Resistance Indicator									
Control	24	3	256	256	256	96	96	32	256
RMF (5 Hz)	16 [a]	3	24 [a]	6 [a]	12 [a]	16 [a]	16 [a]	16 [a]	64 [b]
RMF (50 Hz)	16 [a]	3	24 [a]	16 [b]	12 [a]	24 [b]	24 [b]	24 [b]	48 [a]
Amoxicillin—β-lactam									
Control	8	0.32	24	24[d]	24	16	16	6	32
RMF (5 Hz)	6 [a]	0.32	6 [a]	12 [a]	4 [b]	8 [b]	6 [b]	2 [a]	24 [a]
RMF (50 Hz)	4 [a]	0.32	6 [a]	12 [a]	2 [a]	6 [a]	4 [a]	3 [b]	24 [a]
Amoxicillin + Clavulanic Acid—β-lactams; aminopenicillins + inhibitor of β-lactamases									
Control	2	0.23	16	24 [d]	16	12	12	3	16
RMF (5 Hz)	1.5 [b]	0.23	4 [a]	8 [b]	3 [a]	4 [a]	4 [b]	1 [a]	12 [a]
RMF (50 Hz)	0.75 [a]	0.23	4 [a]	4 [a]	2 [b]	4 [a]	3 [a]	2 [b]	12 [a]
Erythromycin—macrolides									
Control	0.5	0.25	256 [c]	256 [c]	256 [c]	256 [c]	256 [c]	256 [c]	64
RMF (5 Hz)	0.38 [a]	0.19 [a]	256 [c]	256 [c]	256 [c]	256 [c]	256 [c]	256 [c]	48 [a]
RMF (50 Hz)	0.5	0.25	256 [c]	256 [c]	256 [c]	256 [c]	256 [c]	256 [c]	64
Clindamycin—lincosamides									
Control	0.125	0.94	256 [c]	256 [c]	256 [c]	256 [c]	256 [c]	0.125	0.125
RMF (5 Hz)	0.94	0.64 [a]	256 [c]	256 [c]	256 [c]	256 [c]	256 [c]	0.125	0.125
RMF (50 Hz)	0.94	0.64 [a]	256 [c]	256 [c]	256 [c]	256 [c]	256 [c]	0.094 [a]	0.125
Ciprofloxacin—fluoroquinolones									
Control	0.19	0.19	32 [c]	32 [c]	32 [c]	4 [c]	32 [c]	0.125	32 [c]
RMF (5 Hz)	0.19	0.19	32 [c]	32 [c]	32 [c]	4 [c]	32 [c]	0.094 [a]	32 [c]
RMF (50 Hz)	0.19	0.19	32 [c]	32 [c]	32 [c]	4 [c]	32 [c]	0.125	32 [c]
Tetracycline—tetracyclines									
Control	0.125	0.125	0.125	0.125	0.125	0.125	0.047	48	16
RMF (5 Hz)	0.125	0.125	0.094 [a]	0.125	0.125	0.094 [a]	0.047	48	16
RMF (50 Hz)	0.125	0.125	0.125	0.125	0.125	0.125	0.023 [a]	48	16
Gentamicin—aminoglycosides									
Control	0.25	0.19	0.125	0.38	0.19	0.19	0.19	0.125	256 [c]
RMF (5 Hz)	0.19 [a]	0.19	0.094 [a]	0.25 [a]	0.125 [a]	0.125 [a]	0.19	0.094 [a]	256 [c]
RMF (50 Hz)	0.19 [a]	0.19	0.094 [a]	0.25 [a]	0.19	0.19	0.19	0.125	256 [c]
Teicoplanin—glycopeptide									
Control	0.75	0.38	0.75	0.5	0.75	0.75	0.5	1.0	0.75
RMF (5 Hz)	0.38 [a]	0.25 [a]	0.5 [a]	0.38 [a]	0.5 [a]	0.5 [a]	0.5	1.0	0.75
RMF (50 Hz)	0.38 [a]	0.25 [a]	0.5 [a]	0.38 [a]	0.5 [a]	0.75	0.38 [a]	1.0	0.75

There were no differences in MIC values between three separate experiments. The zones that are larger in comparison to the control are marked with letters: "[a]" means a larger zone, "[b]" a smaller one—as compared to each other; "[c]" indicates resistance according to the EUCAST clinical breakpoints (2021) [25]; "[d]" indicates a lack of sensitivity to clavulanic acid.

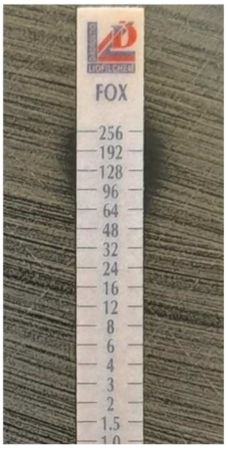
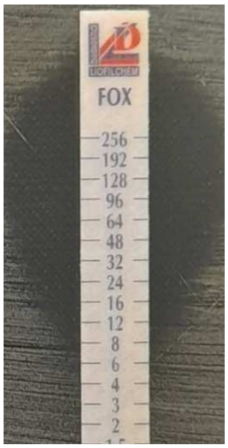
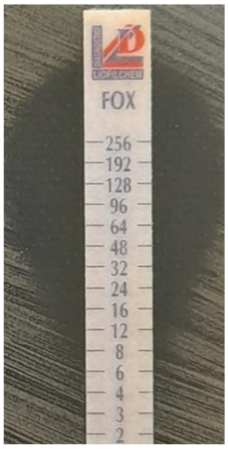
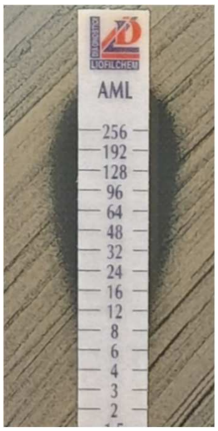
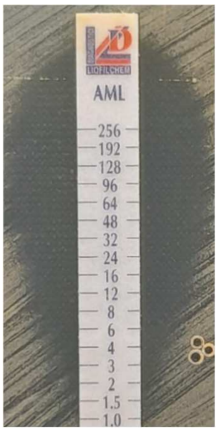
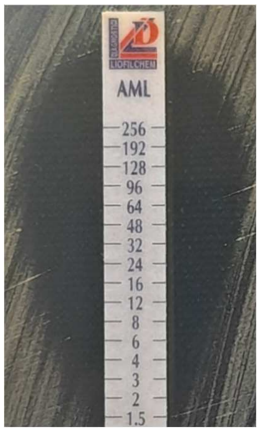
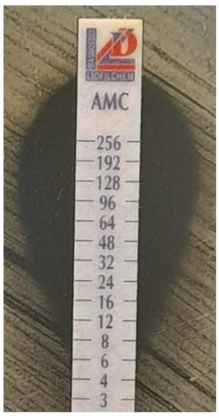
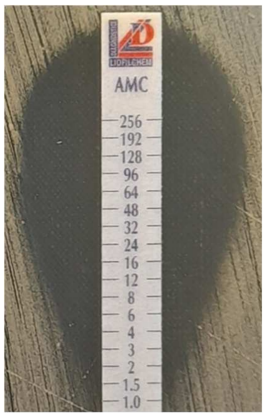
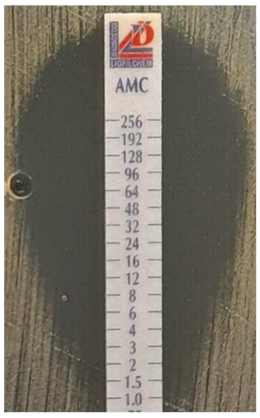
	Control	RMF (5 Hz)	RMF (50 Hz)
Cefoxitin (MRSA 3)			
Amoxicillin (MRSA 3)			
Amoxicillin + Clavulanic Acid (MRSA 5)			

Figure 2. *Cont.*

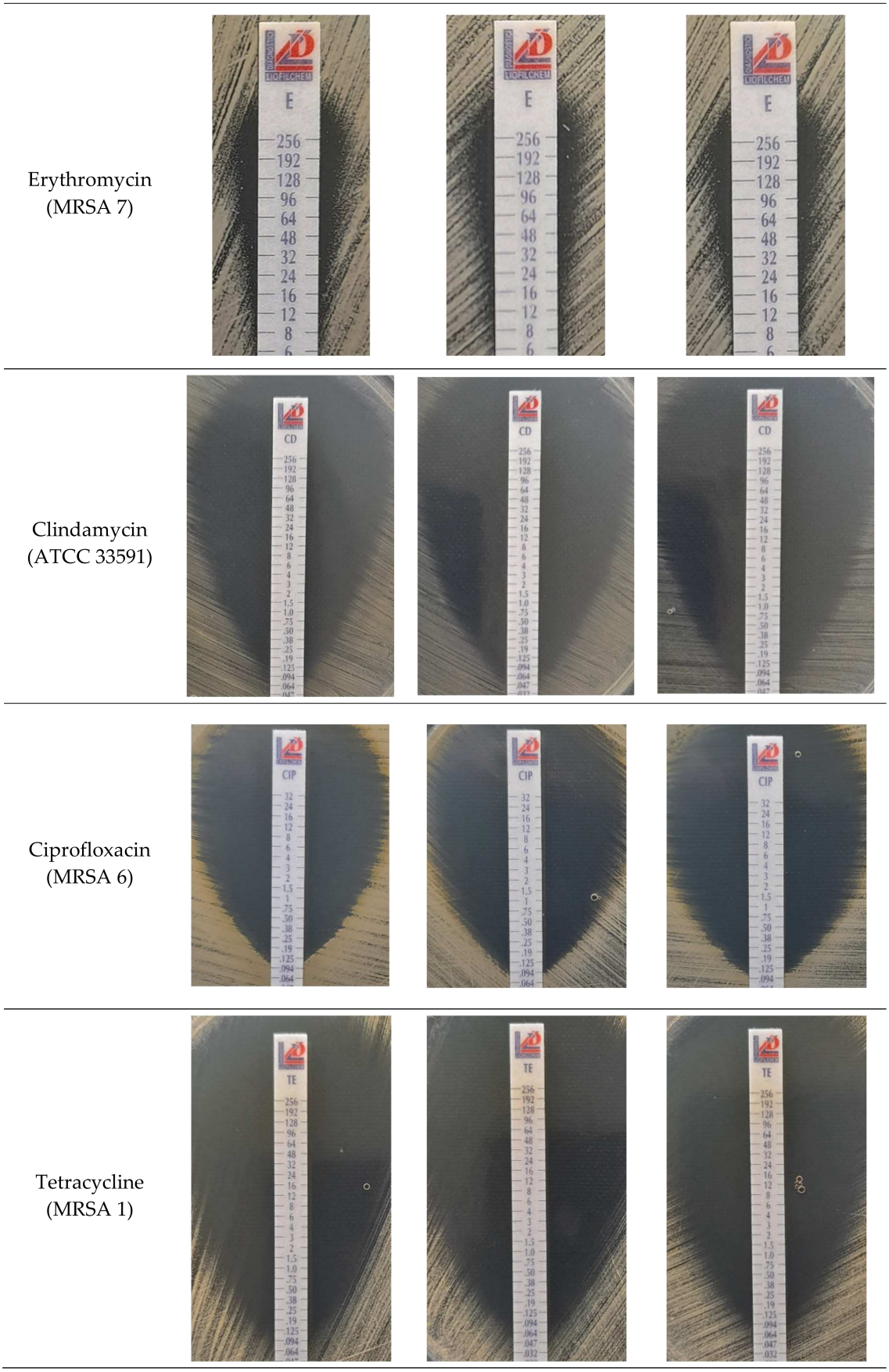


Figure 2. *Cont.*

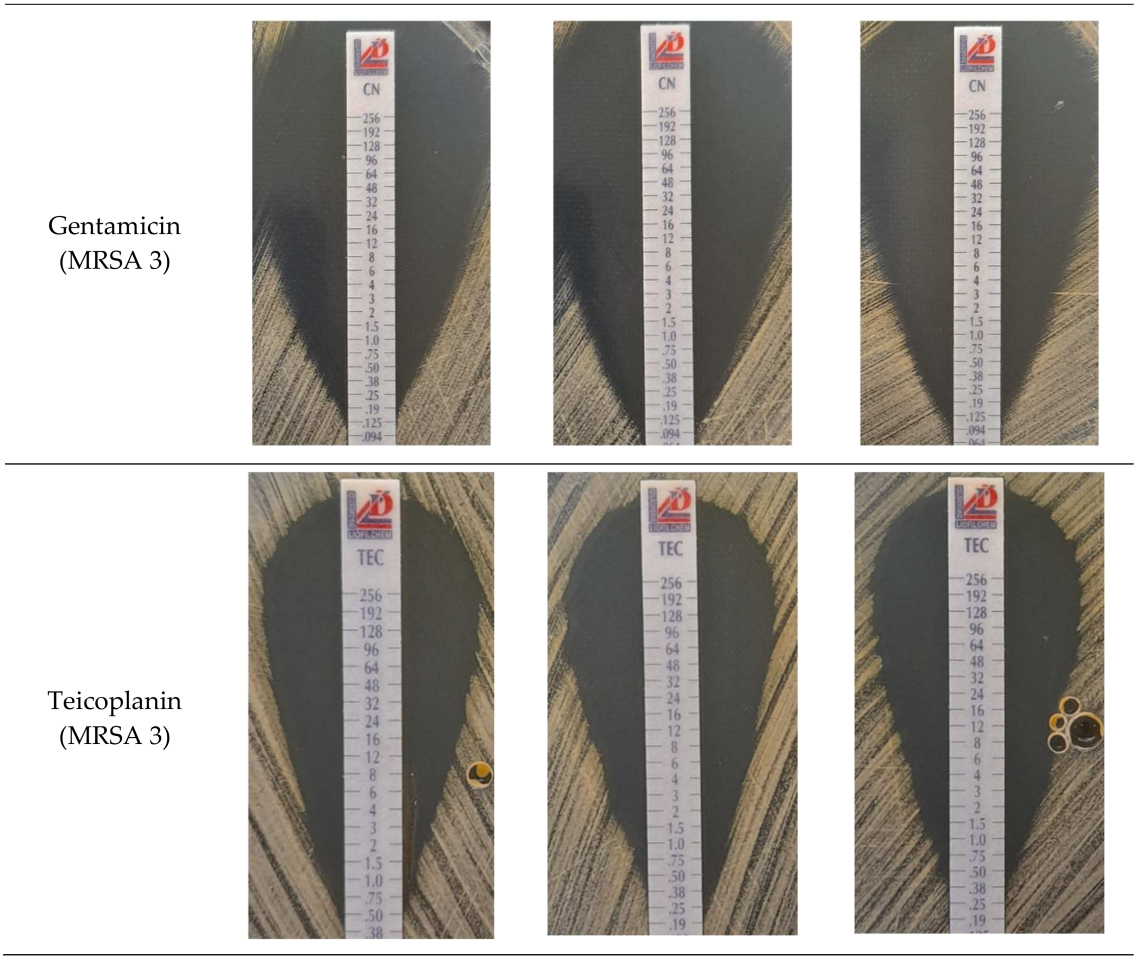


Figure 2. Representative pictures of gradient MIC strips (E-tests) with different antibiotics in control and RMF-exposed cultures of the MRSA strains.

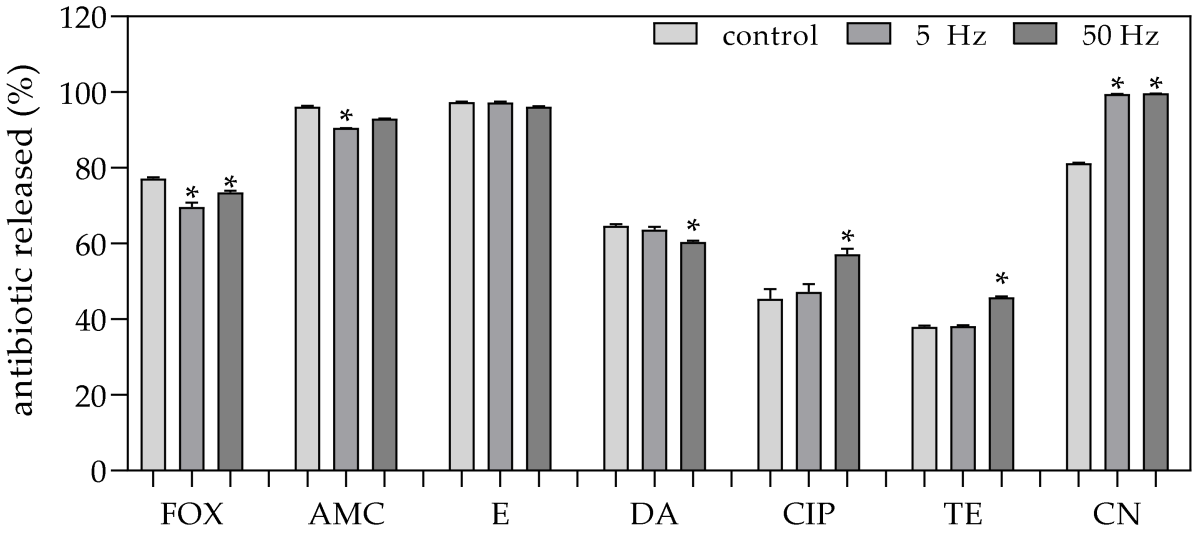


Figure 3. Percentage of antibiotics released from the paper discs during 120 min in control and RMF-exposed (5/50 Hz) settings. The results are presented as a mean ± SEM, calculated using six values (three from each biological replicate). * indicates statistical differences ($p < 0.05$) between control and RMF-exposed settings; FOX—cefoxitin; AMC—amoxicillin; E—erythromycin; DA—clindamycin; CIP—ciprofloxacin; TE—tetracycline; CN—gentamicin.

2.5. Effect of RMF Coupled with β-Lactam Antibiotic on Integrity of Staphylococcal Cell Walls in the Biofilm Model

In the final investigation line, the proof-of-concept experiment, aiming to gain an insight into the potential mechanism of the observed phenomena related with increased susceptibility of staphylococci to antibiotics in the presence of RMF, was performed. Cefoxitin, a β-lactam antibiotic, was chosen for experimental purposes and introduced to biofilm (MRSA ATCC 33591 strain), which is a three-dimensional structure containing cells located in layers. The experimental setting was exposed for 12 h to the RMF of 5 Hz. After exposure, the biofilm was dyed with a combination of SYTO-9 and propidium iodide to discriminate staphylococcal cells with intact walls from cells whose walls were altered (damaged). The subsequent application of confocal microscopy allowed visualization of the extent of cell wall damage in the layers of biofilm (top, middle, and bottom ones) and compare it between the setting where cefoxitin and RMF were applied vs. the setting to which cefoxitin only was introduced (Figure 4a–c). The results showed that the number of cells with altered walls was higher in the setting where both RMF and cefoxitin were applied, as compared to the setting where only cefoxitin was used. Importantly, the comparison of biofilm unexposed to the RMF and the antibiotic to biofilm exposed to the RMF of 5 Hz frequency (Figure 4d) showed not only a higher share of wall-altered cells in the latter of the mentioned experimental settings, but also that these cells were found across the whole vertical cross-section of the biofilm. Thus, the comparison of results presented in Figure 4d (biofilm exposed to the RMF) with the results of the right side of Figure 4a (biofilm treated with cefoxitin and exposed to the RMF) may indicate that the effect displayed by RMF on staphylococci manifests itself in their cell wall alteration, similar to the effect displayed by the cefoxitin, and that the combined effects of these two agents (RMF and cefoxitin) translate into a higher rate of wall-altered staphylococcal cells.

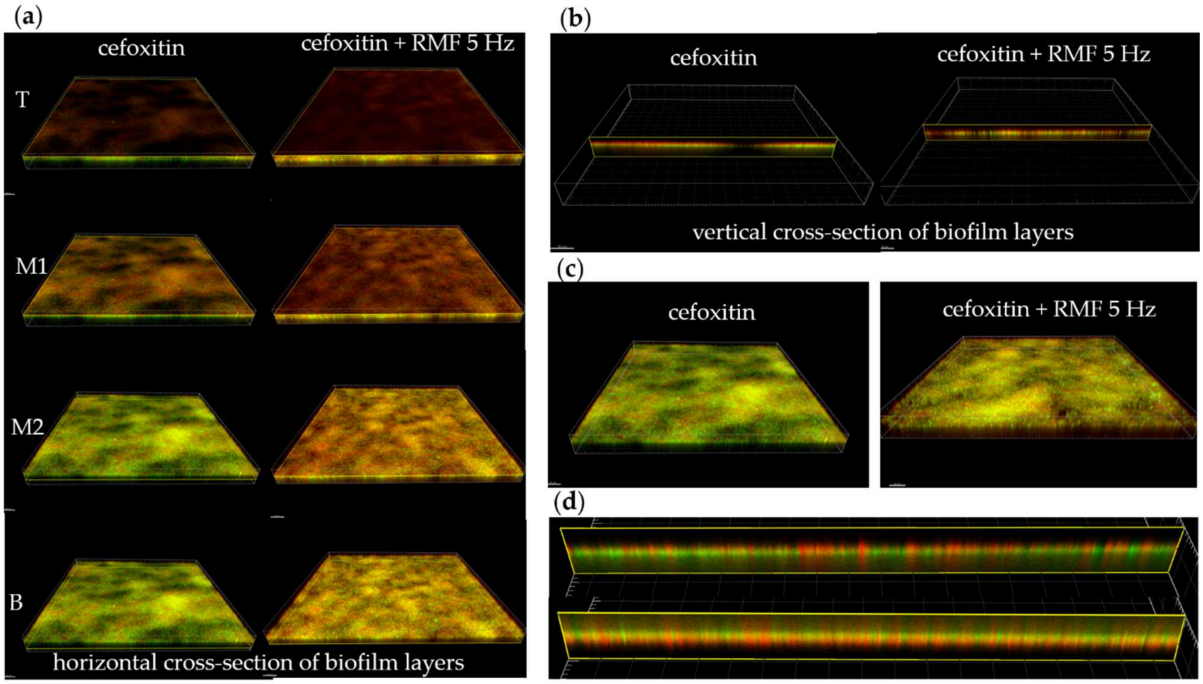


Figure 4. The distribution of staphylococcal cells with intact walls (green color) and with altered walls (red/orange color) in the biofilm of MRSA ATCC 33591 strain; (**a**) horizontal cross-sections of top, middle and bottom (T, M1, M2, B, respectively) layers of biofilm exposed to cefoxitin (left part) or cefoxitin coupled with the RMF of 5 Hz (right part); (**b**) vertical cross-section through biofilm layers treated with cefoxitin only (left part) or cefoxitin and RMF of 5 Hz (right part); (**c**) a stack of recorded cell layers in biofilm in settings where cefoxitin only or cefoxitin with RMF of 5 Hz were used; (**d**) distribution of cells with intact or altered walls in the biofilm unexposed to RMF and the antibiotic (upper part) and exposed to RMF of 5 Hz (lower part).

3. Discussion

The study aimed to assess the possibility of application of RMF to boost the antimicrobial effect exerted by different classes of antibiotics, including β-lactams represented by cefoxitin and amoxicillin (and, additionally, amoxicillin combined with clavulanic acid); aminoglycosides represented by gentamicin; macrolides represented by erythromycin; lincosamides represented by clindamycin; quinolones represented by ciprofloxacin; tetracyclines represented by tetracycline; and glycopeptides represented by teicoplanin, against methicillin-resistant strains of *S. aureus*. The main reason for using two β-lactam antibiotics was that cefoxitin susceptibility testing is a recommended screening method for the detection of methicillin resistance in isolates of *S. aureus* [26]. In turn, application of β-lactams is frequently used together with β-lactamase inhibitors [27,28] and, for this purpose, the most standard combination of such agents (i.e., amoxicillin with clavulanic acid) was used in the present work. Therefore, the amoxicillin applied as a standalone agent served as control for the setting where both β-lactam and β-lactam inhibitor were applied.

The antibiotics selected for the study were characterized by different mechanisms of action, i.e., cefoxitin and amoxicillin—inhibition of cell wall biosynthesis in the bacteria by binding covalently to PBPs in the cytoplasmic membrane [29], gentamicin and tetracycline—inhibition or impairment of protein synthesis by blocking the 30S subunit of the bacterial ribosomes [30,31], erythromycin and clindamycin—inhibition of protein synthesis by binding to the 50S subunit of the bacterial ribosome [32], ciprofloxacin—inhibition of bacterial DNA topoisomerase and RNA gyrase [33], and teicoplanin—inhibition of synthesis of peptidoglycan by binding to amino acids (d-alanyl-d-alanine) in the cell wall [34]. Consequently, due to the aforementioned differences, the resistance mechanisms developed by micro-organisms are also of a different nature between individual antibiotics (Table 3).

Table 3. Mode of action of antibiotic classes selected for the study and related resistance mechanisms [35].

Antibiotic	Mode of Action	Resistance Mechanism
B-lactams, Glycopeptides	Interference with cell wall synthesis	Reduced permeability Reduced affinity for antibiotic target Antibiotic hydrolysis
Macrolides Lincosamides	Inhibition of protein synthesis (binding to 50S ribosomal subunit)	Reduced affinity for antibiotic target Antibiotic hydrolysis Reduced uptake into cells
Aminoglycosides, Tetracyclines	Inhibition of protein synthesis (binding to 30S ribosomal subunit)	Inactivation of antibiotic by enzymatic modification Altered cell permeability Active efflux from cells
Fluoroquinolones	Inhibition of DNA synthesis	Alternation in antibiotic target Decreased cell permeability

It should also be noted that selected antibiotics, apart from various mechanisms of action, were also characterized by different electrical charges (Table 4). Therefore, it was possible to try to determine the impact of another variable on the observed changes (being a result of RMF activity), namely anionic, cationic, or zwitterionic character of the antimicrobials. Numerous literature data [36,37], as well as the previous experience of our research group [16], indicated that RMF influence was related to its interactions with electrically charged molecules. Therefore, it was expected that the charge of individual antibiotics may be of significance, especially taking into account the process of their diffusion in the microbiological medium. Consequently, it was assumed that the character (anionic, cationic, or zwitterionic) of the antibiotic could be one of the possible factors influencing the processes related to the rate of their release from the carrier (e.g., paper disc) and diffusion in the surrounding environment (e.g., agar medium), and thus leading to changes in the antibiotic resistance profile of the tested micro-organisms.

Table 4. Character of antibiotics selected for the study depending on their charges.

Antibiotic	Character
Cefoxitin Teicoplanin	anionic
Erythromycin Clindamycin Gentamicin	cationic
Amoxicillin Tetracycline Ciprofloxacin	zwitterionic

Furthermore, to determine whether the observed changes in antibiotic susceptibility due to the RMF exposure were strain-specific, not only the reference strains, but also seven clinical MRSA isolates were included in the study. It is well established that methicillin resistance level is not identical between staphylococcal strains [38]. For example, methicillin resistance is mediated by the expression of an altered PBP2 protein (called PBP2a) characterized by a low affinity for β-lactam antibiotics, resulting in resistance to most β-lactams [34]. However, PBP2a encoded by the *mecA* gene, which is carried on a mobile genetic element known as a staphylococcal cassette chromosome *mec* (SCC*mec*), can be regulated by two independent regulatory systems (*mecI–mecR–mecR2* and *blaI–blaR*) and multiple chromosomal genes [38]. Additionally, because MRSA strains, in addition to resistance to β-lactam antibiotics, can also be resistant to a number of antibiotics belonging to other classes [7], for comparison purposes, one methicillin-sensitive (lacking the *mecA* gene and showing susceptibility to methicillin in a phenotypic test with a cefoxitin-saturated disc) reference strain was used in the study. Therefore, it was possible to determine whether the changes in antibiotic sensitivity are strictly related to the methicillin resistance mechanism or may also occur in other staphylococci, regardless of their antibiotic resistance profile.

The study showed that among all antibiotic classes, the most promising results were obtained in analyses with the use of cefoxitin as a representative of β-lactams. In all MRSA cultures exposed to the RMF, noticeably larger inhibition zones around the discs with cefoxitin as compared to unexposed conditions were present. These results were also further confirmed in the study with the use of amoxicillin—the trend comparable to the one observed as a result of cefoxitin activity. It can also be seen that, although both antibiotics belong to the same class and are characterized by the same mechanism of action, they have a different charge/character [39,40] (zwitterionic vs. anionic) and, additionally, they act differently as a result of exposure to the RMF. Contrary to the effects observed, with regard to MRSA cultures, when a methicillin-sensitive strain was analyzed, no differences in the size of inhibition zones were found, regardless of whether cefoxitin or amoxicillin was used. Therefore, particularly taking into account the results obtained in the presence of cefoxitin, which is the indicator of methicillin resistance, at this stage of the experiment, it was assumed that the observed changes could be related to this specific resistance mechanism. This first assumption was additionally related to the observations from the analyses with the use of the remaining classes of antibiotics. Apart from glycopeptides represented by teicoplanin, only a few differences in the sizes of inhibition zones around the discs with erythromycin (2), clindamycin (1), tetracycline (2), ciprofloxacin (0), and gentamicin (4) between RMF-exposed and control cultures were observed (Table 1). Moreover, the differences were noticeably smaller as compared to β-lactams and, in any case, did not exceed 4 mm. Furthermore, no correlation between the charge of the antibiotic and the observed effect was found. Importantly, the mechanism of action of none of the above-mentioned antibiotics involves the disturbance of cell wall structure or synthesis (Table 3). In contrast, in the case of teicoplanin, whose mechanism of action relies on the inhibition of cell wall synthesis [41] (and, thus, with regard to the site of activity and the effect, resembles the activity of β-lactam antibiotics), a change in inhibition

zone diameters was observed in cultures of five strains exposed to the RMF (Table 1). The differences were not as large as in the case of β-lactam antibiotics (they did not exceed 2 mm in comparison to the control). However, it should be noted that vancomycin-resistant strains were not used in the studies and, thus, even in the control cultures, the zones of growth inhibition were relatively large. In this context, in the case of β-lactams applied against MSSA, the differences in the inhibition zones were not visible at all.

The next part of the study aimed to determine whether the observed changes (or their lack) in sensitivity to antibiotics caused as a result of the RMF exposure can be related to the specific concentration of antimicrobial used in the disc diffusion test. It was assumed that a too low concentration of an antibiotic could be a possible reason for the lack of differences in the zones of growth inhibition found in the cultures of MSSA strains or the remaining MRSA when antibiotics other than β-lactams were applied. The results of this part of the study confirmed that the lack of the influence of the RMF observed in some of the cultures tested with erythromycin, clindamycin, tetracycline, ciprofloxacin, gentamicin, as well as teicoplanin using the disc diffusion assay could be related to a relatively low concentration of these antimicrobials in the carrier. Although this observation did not apply to all strains, at least in some of the cultures, reduced MIC values were observed in comparison to unexposed controls (although no changes in the diameters of inhibition zones were found in the disc diffusion method). Nevertheless, the exposure to RMF did not change the susceptibility level of the MSSA ATCC 6538 strain to both β-lactams included in the experiment, even when the E-tests were used.

Apart from the aforementioned analyses of different groups of antibiotics, the current research also included the use of a β-lactam antibiotic (amoxicillin) combined with an inhibitor of β-lactamases, referred to as clavulanic acid. This part of the study was carried out to potentially elucidate the fact of the relatively small (as compared to cefoxitin) differences in inhibition zones for amoxicillin observed in the cultures of RMF-exposed and control staphylococcal strains. As was demonstrated by Harrison et al. (2019) [42], such β-lactamase inhibitors as clavulanic acid may find potential application in the eradication of infections caused by MRSA strains thanks to the property of clavulanic acid manifesting itself in an increase in the affinity of PBP2a for β-lactams. For these reasons, we assumed that the use of β-lactam in combination with β-lactamase inhibitor may provide a good model to determine whether the changes in the sensitivity of MRSA strains, observed under the influence of RMF, are related to the mechanism of action of β-lactam antibiotics. First of all, the results obtained under control conditions allowed one strain (MRSA 2) insensitive to the presence of clavulanic acid (the use of amoxicillin in combination with clavulanic acid did not affect its growth inhibition zones as compared to the test with amoxicillin only) to be selected (Table 1). For the remaining strains, the zones of growth inhibition were enlarged due to the presence of clavulanic acid and, hence, all these strains were defined as sensitive to the presence of β-lactamase inhibitors. It was also found that, due to the RMF exposure, the differences in the zones of growth inhibition were, in each case, greater than the differences observed when amoxicillin without clavulanic acid was used. However, in the case of the MSSA strain, the differences were, again, not detected. This finding indicates that the observed changes under the influence of RMF may be related to the mechanism of resistance to β-lactam antibiotics. Moreover, it can be noted that the differences were also found in the case of the culture of the MRSA 2 strain for which the effect of clavulanic acid was not detected in the control cultures. This offers the potential for further use of clavulanic acid, not only in the case of MRSA susceptible to its presence (to further boost the effect caused by the β-lactamase inhibitor), but also in the case of resistant strains.

The research previously conducted by our research group [17,18,43], as well as the studies of several other authors [36,44,45], revealed that the strength of MF impact (regardless of its type or the phenomenon analyzed in its presence) depends also on the intensity and/or the frequency of MF, because these two factors determine the physical characteristics of the magnetic signal [46,47]. Therefore, the potential impact of these variables was

also analyzed with regard to the observed changes in the antibiotic susceptibility profiles between the analyzed strains. In the case of the RMF set-up used in the present study, the frequency of AC determines the MF intensity and, importantly, it is responsible for the physical characteristics of the magnetic wave shape. For this reason, the analyses including the AC frequencies of 5 and 50 Hz at which the RMF was generated allowed an MF of different parameters to be obtained. As shown by the simulation calculations, at 5 Hz (https://www.youtube.com/watch?v=2dxP7nzEThA accessed on 18 October 2021), the amplitude of the RMF was characterized by a longer period between magnetic induction maximal strength state (100 ms with B_{max} 8.1 mT) as compared to the RMF generated at 50 Hz (the highest current frequency in the applied set-up). The RMF generated at 50 Hz (https://www.youtube.com/watch?v=4xggMktw3ho accessed on 18 October 2021) was characterized by a shorter period, with 10 ms between magnetic induction maximal strength state with B_{max} 8.5 mT (Figure 5; Supplementary Figure S3).

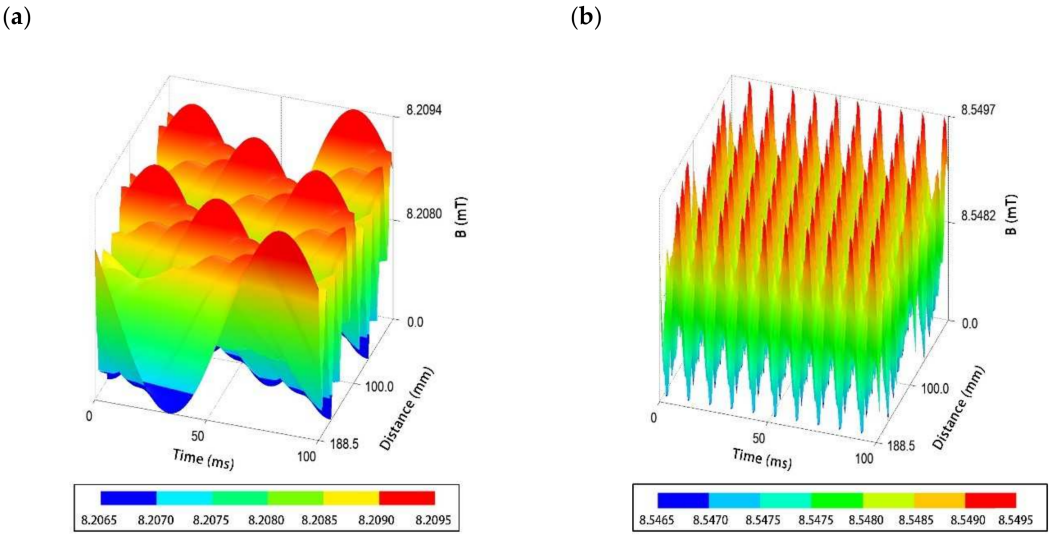


Figure 5. Changes in magnetic flux characteristic depending on the applied AC frequency: (**a**) 5 Hz; (**b**) 50 Hz.

Simultaneously, the applied AC frequencies generated magnetic flux rotation around the stator with different synchronous speeds of 150 rpm and 1500 rpm, respectively (calculations performed on the basis on the manufacturer characteristics of the stators). In line with our assumptions, it was shown that the results obtained during the analysis using the disc diffusion method and E-tests were determined by the characteristics of the generated RMF. For example, it can be seen that, for cefoxitin and gentamicin, the diameters of the zones of growth inhibition were larger as a result of the exposure to RMF of 5 Hz, compared to RMF of 50 Hz. Similar trends were found in the analyses with E-tests—although, in the cultures of the three strains, the MIC did not differ depending on the RMF frequency, the remaining strains were characterized by lower MIC values as a result of exposure to the RMF of 5 Hz. On the other hand, in the case of amoxicillin alone or coupled with clavulanic acid, the tendency was the opposite, which means that RMF of 50 Hz was more effective. In turn, in the analyses with teicoplanin, the changes occurred regardless of the frequency used. For the remaining antibiotics, no recurring trend could be found due to the relatively low number of RMF-reactive cultures. Nevertheless, due to the lack of connection of the obtained results with the charge/character and the mechanism of action of individual antibiotics, at this stage, apart from noting their presence, it is impossible to explain their nature. However, the obtained results allow the conclusion that the frequency of the generated RMF should be adjusted primarily to a specific antibiotic (not only with regard to its class), and also, although to a lesser extent, individually to each bacterial strain/isolate.

Summarizing the previous stages of the analyses, the most promising results related to the possibility of using RMF to change the antibiotic resistance of MRSA strains concern two groups of antibiotics, β-lactams and glycopeptides, the common feature of which is the site of their antimicrobial activity, i.e., the bacterial cell wall. We are aware that, although the general mechanism of action of β-lactam antibiotics is similar, there are some differences in their specific activity, e.g., related to different binding sites with the PBP2a protein or to the binding energy value [38,42]. Similarly, in a group of glycopeptide antibiotics, the relationship between vancomycin and teicoplanin minimum inhibitory concentration (MIC) is not clearly defined [48,49]. Despite some correlation between vancomycin and teicoplanin MIC for *S. aureus*, the reports have highlighted the importance of both species and strain-specific MIC differences and that the microbiological activity of the two compounds cannot be considered to be unequivocally equal [50]. For this reason, at this stage, we are not able to conclude that our results would apply to the whole group of β-lactams or glycopeptides. Nevertheless, the positive results obtained in the present study encourage us to perform the next experiments, in which the findings of the present study will be investigated further by, among others, analyzing a higher number of representatives of different groups of antibiotics, different modes of bacterial exposures to the RMF, as well as optimization of exposure duration.

The next line of investigation was performed to analyze another crucial variable in the applied experimental system, namely the differences in antibiotics' release and diffusion, which may potentially occur between bacterial cultures exposed and unexposed to the RMF. Considering our previously published data regarding, e.g., mixing efficiency under the influence of the RMF [16,51], it can be noticed that MF influence was related to its interactions with electrically charged molecules. Therefore, we aimed to investigate whether magnetic exposure alters the release of the antibiotics from the paper discs and their diffusion in the agar medium. Moreover, the study aimed to determine whether the effect of applying RMF is dependent on the charge of the antibiotic molecules (Table 4) and correlated with the results of the changes in the antibiotic sensitivity observed in the biological study (Tables 1 and 2).

Although gentamycin, ciprofloxacin and tetracycline were the antibiotics released from the paper disc into the agar during 120 minutes of exposure to the RMF in higher concentrations as compared to unexposed conditions, in the case of ciprofloxacin, it did not translate into changes in the antibiotic susceptibility profile with regard to any staphylococcal strain. In the case of tetracycline, the changes were found only in two staphylococcal strains. On the other hand, the difference in the concentration of ciprofloxacin (in the RMF-exposed setting as compared to unexposed control) was determined primarily by its higher concentration in zone 3, i.e., directly under the agar disc. It could not have determined the assumed effects in the disc diffusion method and E-tests. It is also worth noting that a significantly higher concentration of gentamycin released into the agar zones was observed regardless of the applied RMF frequency, while, in the case of diffusion tests, more differences (in inhibition zones and MIC values) were found under the influence of RMF of 5 Hz. It should also be noted that LC-MS/MS analyses showed lower concentrations of β-lactams (antibiotics for which the most significant changes under the influence of RMF were found) in the analyzed zones of agar samples as compared to the unexposed control.

The above observations provide a strong premise that the observed effect of changes in the susceptibility of staphylococcal strains is not related to the direct impact of RMF on the particles of these antibiotics' molecules (at least not in the applied diffusion tests). If this is the case, the RMF must react directly with the staphylococcal cells (of MRSA strains, particularly). Indeed, the team of Oncul et al. (2016) [52] indicated that time-varying MFs may interact with the physicochemical potential of such microbial cells' external structures as cell membranes and walls, leading to their physiological alterations and changes in the level of formed free radicals. Following this lead, we performed a proof-of-concept experiment in which we exposed the biofilm of MRSA to β-lactam antibiotic (cefoxitin),

coupled or not with RMF of 5 Hz frequency. The untreated biofilm of the same strain and the biofilm exposed to RMF only served as control settings in this experiment. The images of cell-wall-compromised and intact biofilm-forming cells were captured in *x*, *y*, and *z* axis and visualized using image processing software. The rationale behind using a biofilm in vitro model for this experiment was the fact that this spatial microbial community is a three-dimensional structure, consisting of layers formed by immobilized cells [53]. Thus, we made use of biofilm as the cells' carrier. Such an approach allowed us to omit the challenges related to the application of other immobilizers (various types of hydrogels) whose presence could be another variable, potentially affecting cell walls' integrity. We hypothesized that, because RMF is the factor of wave-like and not of corpuscular properties [54], its activity against staphylococci should not be hindered by such physical obstacles as biofilm height or the cellular density within particular layers. The above-mentioned features are considered the major factors (together with the presence of extracellular matrix) impeding the activity of antibiotics targeted against biofilm [55]. Our primary observation (Figure 4) was that the structure of biofilm unexposed to RMF and/or antibiotics contains a certain number of cells of altered wall integrity, mostly in the top and bottom parts of its structure. The highest cellular density (and the highest number of cells with intact cell walls) was observed in the middle part of the biofilm. The horizontal and vertical cross-sections (of ~2µm thickness) of biofilm treated with cefoxitin revealed that this antibiotic was able to disintegrate the walls of cells located within the top and upper-middle layer of the biofilm. At the same time, the lower-middle and bottom layers remained mostly intact. Such observation is consistent with the generally accepted statement of highly elevated tolerance of biofilms against antibiotics [55–57]. In turn, when cefoxitin was coupled with RMF of 5 Hz frequency, a high number of wall-compromised cells was observed within the top and upper-middle, but also in the lower-middle and in the bottom layers of the biofilm. The question that should, thus, once again be addressed is whether this observed, increased number of wall-compromised cells (regardless of their spatial position in biofilm) is a result of increased penetrability of cefoxitin (caused by the RMF presence) through biofilm layers or the observed effect is induced by the RMF itself (directly). The data, already presented in this work, show no increased diffusion of β-lactams in agar medium in the presence of RMF, although we are aware of potential biases related with comparisons of results obtained from different methodological approaches. In turn, a direct comparison of unexposed biofilm to biofilm exposed to RMF (Figure 4d, upper and lower part, respectively) shows a higher number of wall-compromised cells in the later setting, regardless of their position (height) in the biofilm structure. Such observation, if further confirmed on a larger number of strains, matches the wave properties of magnetic field [58], which is not constrained by the already-mentioned physical factors constituting significant impediments for antimicrobial molecules applied against biofilm. At this moment, we cannot conclusively exclude the possibility of increased permeability of antibiotics through the biofilm layers in the presence of RMF because the different models of biofilms cultured in vitro may display various characteristics translating into different results in this regard [59]. Nevertheless, analyzing data obtained from the biofilm model applied in this particular study, we may conclude that the increased susceptibility of MRSA cells to β-lactam antibiotic is caused by the activity of RMF, affecting the structure of staphylococcal cell walls (which are also the target size of cefoxitin). Undoubtedly, further research is required to determine whether the observed effect is of additive or synergistic character. Nevertheless, this observation brings us closer to the nature of this effect, as it allows focus on the particular component of the staphylococcal cell, namely the cell wall.

The goal of the present research was to analyze the effect of rotating magnetic field on the susceptibility profile of methicillin-resistant *S. aureus* strains to different groups of antibiotics. This seemingly easy-to-perform analysis was thus planned as consisting of barely three main variables, namely RMF, staphylococcal strains, and antibiotics. However, within the course of the study, these variables developed into a subsequent high number of factors, all crucial with regard to the matters analyzed. First of all, it occurred that

RMF specifics should be calibrated thoroughly to obtain repeatable conditions of exposure. Based on our previous experience [18], two frequencies (5 and 50 Hz) were chosen. The second major variable, the staphylococcal strains, involved many more aspects to take into account. The significance of intraspecies variability is presently more and more stressed, especially with regard to studies of antimicrobial efficacy [60]. Therefore, we decided to analyze the impact of antibiotics and RMF on not only the reference staphylococcal strain, but we also included clinical isolates to the experiment. Indeed, we observed a spectrum of answers to the same antimicrobial (coupled with the RMF), depending on which staphylococcal strain it was applied against. This phenomenon, of pivotal significance in the studies on MF impact on micro-organisms, is often neglected, i.e., only one reference strain as an example of a given species is analyzed [61–63]. This specific methodological approach requires a separate line of investigation if proper conclusions on the observed effects are to be drawn. Moreover, although the experimental group consisted of staphylococcal strains of methicillin resistance pattern (and single MSSA strain provided as a control strain), it should be once more reminded that this specific resistance mechanism is conditioned by different binding sites and energy value of β-lactam antibiotics to altered PBP proteins [38,42]. Thus, the effects, after exposure to the RMF and the antibiotic, were expected to differ as well; the estimation of the impact of the above-mentioned factors related to binding of β-lactam to the cell wall is presently beyond the scope of this research. The other variable, related to both the micro-organisms and the antimicrobials, was the experimental setting. From the macro-perspective, the applied agar plate should be considered a semi-solid surface. From the micro-perspective, the hydrocolloid agar, in which diffusion of antibiotics from antibiotic-containing disc or E-test occurs, consists in ~98% of water. While the agarose, which represents approximately two-thirds of the natural agar composition is of neutral charge, the remaining one-third of agar consists of agaropectin, which is negatively charged due to the presence of pyruvate and sulfate groups [64]. The agar polymer forms mesh-like structures of pore size inversely proportional to the concentration of the agar (approximately 70 nm radius in the case of agar applied in this study) [65]. Presently, there are no data conclusively indicating a correlation between the pace of diffusion of the specific antibiotic through such negatively charged, water-filled nano-pores of agar and the antibiotic molecular mass, hydrophobicity, electric charge, and concentration. Only general assumptions on these aspects can be made, while the details, of potentially high significance, are still obscure. The above-mentioned remarks were presented to highlight the fact that the number of unknowns and variables revealed during the performed studies was increasing, along with the number of techniques performed and the amount of data collected.

Nevertheless, the outcomes of this study indicate explicitly that the major component standing behind the increased susceptibility of methicillin-resistant staphylococci to antibiotics is these microbes' cell wall. This statement can be elucidated not only from the fact that the application of antibiotics for which the cell wall is the target site correlated with the largest inhibition zones of staphylococcal growth (although their sizes were modified by strain-dependent variability) in the presence of RMF, but also from the fact that these changes were observed in MRSA but not in MSSA strains. Having indirectly proven that RMF's mechanism of action is related to staphylococcal cell wall, we confirmed it directly by microscopic observation of a high number of cells with altered walls as a result of exposure to the RMF.

We believe that our findings significantly narrow the number of possible drawbacks related to the application of MFs against bacterial infections, such as, in particular, the yet unresolved mechanism of interaction. The data presented in this research are another premise that future studies on the mechanism of RMF impact on staphylococci should be focused on the interaction of MF with the septation machinery correlated to peptidoglycan and/or the peptidoglycan-modifying enzymes [13].

From the clinical perspective, it should be stated explicitly that our observations may be applied only to the RMF-responsive strains of methicillin-resistant *S. aureus* (due to

the intraspecies variability, some strains may not react to exposure to RMF) and/or to the specific antibiotic used. Nevertheless, it is reasonable to speculate that the application of RMF coupled with β-lactams may, at some point, become an attractive treatment option with regard to infections caused by recurring staphylococcal strains, as is the case in chronic bone infections [66]. Nevertheless, before such treatment can be applied, the mechanism standing behind the impact of MFs needs to be fully elucidated. We believe that the high amount of data presented in this work, pointing to the staphylococcal cell wall as the primary target of RMF action, allows to ask the final question, namely how the RMF affects the staphylococcal cell wall structure. Therefore, this work may be considered an important step into the application of MFs in future clinical practice to fight staphylococcal-based infections.

4. Materials and Methods

4.1. Microorganisms

Two reference staphylococcal strains, including one MRSA—American Type Culture Collection (ATCC 33591) and one MSSA (ATCC 6538), and seven clinical MRSA isolates (MRSA 1—MRSA 7) were used for experimental purposes. All analyzed clinical isolates were provided from the Strain Collection of the Department of Pharmaceutical Microbiology and Parasitology of Wroclaw Medical University. All these strains were previously isolated from chronic wounds of patients treated in the Teaching Hospital of Wroclaw Medical University (Wroclaw, Poland) during another project, approved by the Bioethical Committee of Wroclaw Medical University, protocol # 8/2016. The strains' species affiliation was confirmed using the automated Becton-Dickinson Phoenix 100 system (Franklin Lakes, NJ, USA), whereas methicillin resistance was measured according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021) [67] using the disc diffusion method with cefoxitin antibiotic (30 μg), as well as by detection of the *mecA* gene using primers and PCR conditions previously described by Oliveira and de Lencastre (2002) [68].

4.2. Rotating Magnetic Field Generator

The core of the RMF bioreactor (Figure 6) was a 3-phase, 4-pole stator with an internal diameter of 16 cm and height of 20 cm, consisting of 12 groups of three coil sets [69]. The alternating current (AC) frequency supplied to the RMF generator was regulated using the Unidrive M200 inverter (Control Techniques, Nidec Industrial Automation, Poznan, Poland). The temperature in the RMF reactor chamber was controlled using a water-fed cooling/heating system equipped with several temperature probes with sampling deviation in the accuracy range ±1.0 °C. The correct temperature distribution in the RMF bioreactor was maintained by air flow provided during exposure (2 L/min, 35 °C, RH 60%). The distribution of magnetic induction (*B*) in the reactor chamber was performed at an initial voltage of AC of 100 V and AC frequencies of 5 Hz and 50 Hz using the Ansys Maxwell simulation software ver.19.1 (ANSYS, Inc., Canonsburg, PA, USA) and using a tesla meter (SMS-102, Asonik, Tuczno, Poland).

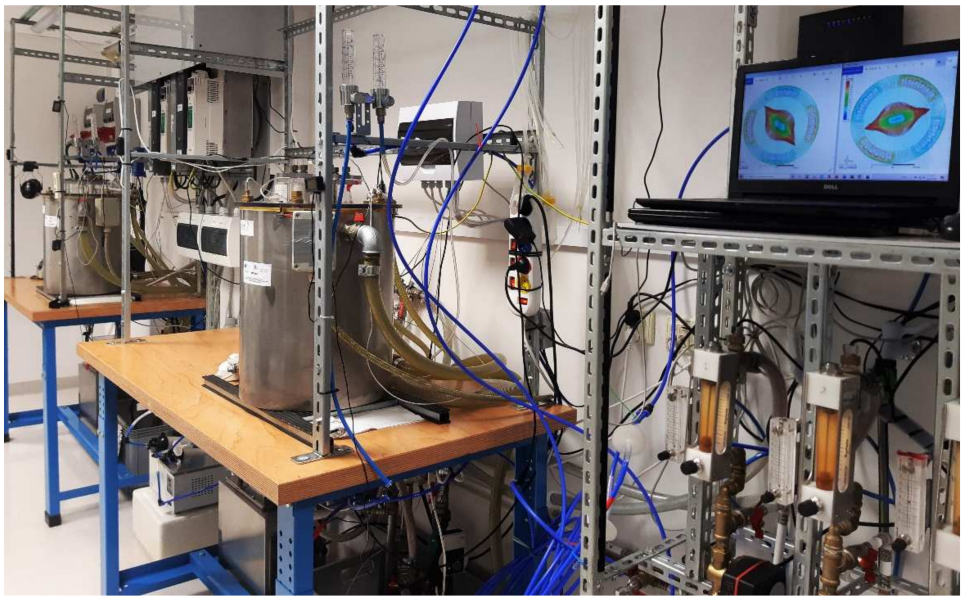


Figure 6. RMF generator with monitoring and control equipment.

4.3. Analysis of the Impact of RMF on Changes in Antibiotic Susceptibility

4.3.1. Disc Diffusion Method

In the first stage of the study, the impact of exposure to the RMF generated at AC frequencies of 5 and 50 Hz on the changes in susceptibility to different classes of antibiotics, including β-lactams, aminoglycosides, macrolides, lincosamides, quinolones, and glycopeptides, was analyzed.

Additionally, analyses of the influence of RMF on changes in bacterial strain sensitivity to antibiotics in combination with an inhibitor of β-lactamase enzymes were performed. A commonly used variant of active substances (amoxicillin with clavulanic acid) was used.

The antibiotic susceptibility assessment was performed according to EUCAST guidelines (2021) [67]. The bacterial cultures were adjusted to 0.5 McFarland standard, which corresponds to 1–2 $\times$ 10^8 CFU/mL, and spread evenly over the surface of M–H agar (Graso Biotech, Jablowo, Poland) plates using sterile cotton swabs. After the application of antimicrobial discs on the M–H plates, the bacteria were exposed to the RMF for 12 h. After completion of the exposure time, the cultures with the antibiotic discs were taken out from the RMF generator and incubated at 35 $\pm$ 1 °C without RMF until 18 h of incubation were completed (total amount of time consisting of exposure and non-exposure period). The zones of growth inhibition were measured after the end of exposure to RMF, and once more after completion of entire incubation time.

The same bacterial cultures, incubated under the same conditions but without exposure to the RMF, were used as a control setting. Both in the RMF generator and the incubator, the same temperature (35 $\pm$ 1 °C) and relative humidity RH (60%) were maintained throughout the entire experiment.

The following antibiotic discs were used: cefoxitin (30 µg/disc), amoxicillin (25 µg/disc), amoxicillin/clavulanic acid (20/10 µg/disc), erythromycin (15 µg/disc), clindamycin (2 µg/disc), ciprofloxacin (5 µg/disc), gentamycin (2 µg/disc), tetracycline (30 µg/disc), and teicoplanin (30 µg/disc) (Oxoid, Basingstoke, UK).

4.3.2. Gradient MIC Strips (E-Test)

Because the disc diffusion method only allows the analysis of the impact of a single concentration of an antibiotic, in the second stage of the experiment, the E-test strips with exponentially decreasing antibiotic concentrations were applied for all of the antibiotics that were previously used in the disc diffusion test.

The cultures with the E-tests were exposed and incubated in the same way as described for the disc diffusion method. The E-tests containing cefoxitin, amoxicillin, amoxicillin/clavulanic acid, erythromycin, clindamycin, tetracycline, ciprofloxacin, gentamicin, and teicoplanin were obtained from Liofilchem (Roseto degli Abruzzi, Italy). The analyses were performed on M–H agar in accordance with the E-test manufacturer's recommendations.

The location of the Petri dishes in the RMF reactor chamber and the location of antibiotic discs and E-tests in Petri dishes are presented in Figure 7.

4.4. Analysis of the Impact of RMF on the Diffusion of Antibiotics in the Agar Medium

In order to analyze the impact of the RMF on antibiotic diffusion, the same discs as the ones used for the analysis of antibiotic resistance were placed on Petri dishes with 1.7% agar (Graso Biotech, Jablowo, Poland) (agar concentration was equal to the concentration of M–H agar applied in previous experiments) and exposed to the RMF generated at 5 and 50 Hz. After 120 min, the plates were removed from the RMF generator, the paper discs with antibiotics were removed, and, using a cork borer, cylindrical agar samples 6 mm in diameter were cut out (4 samples from the proximal zone (zone 1), 8 samples from the distal zone (zone 2), representing 50% of the total agar volume in each zone) and, additionally, 1 sample was obtained from the agar where the antibiotic disc was placed (zone 3) (Figure 8). To extract the antibiotic, the paper discs and agar samples were placed in 0.5 mL of methanol (Stanlab, Lublin, Poland) in deionized water (1:1) and incubated with shaking (250 rpm; Biosan, Riga, Latvia) for 3 h. Next, the methanol–water mixtures with the extracted antibiotics were filtered through a syringe filter (0.22 µm pore diameter) and analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) technique (1260 Infinity II Series Liquid Chromatograph, Agilent, Santa Clara, CA, USA). An InfinityLab Poroshell 120 EC-C18 column (Agilent, Santa Clara, CA, USA) with a particle diameter of 2.7 µm equipped with a guard column was used for chromatographic separation. A mass spectrometer (Ultivo G6465B, Agilent, Santa Clara, CA, USA) coupled to the chromatograph was used to detect and identify the assessed antibiotics. The quantitative analysis was based on calibration curves prepared with the use of antibiotic standards (Millipore Sigma, St. Louis, MO, USA). The results were converted and presented as the concentration of antibiotic remaining in the disc and released to each zone.

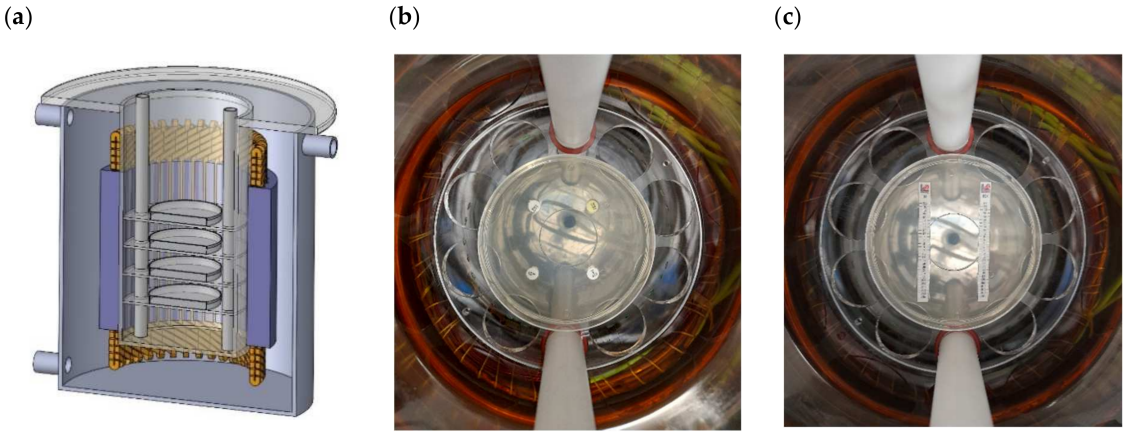


Figure 7. Location of Petri dishes (**a**) with antibiotic discs (**b**) and E-tests (**c**) in RMF bioreactor.

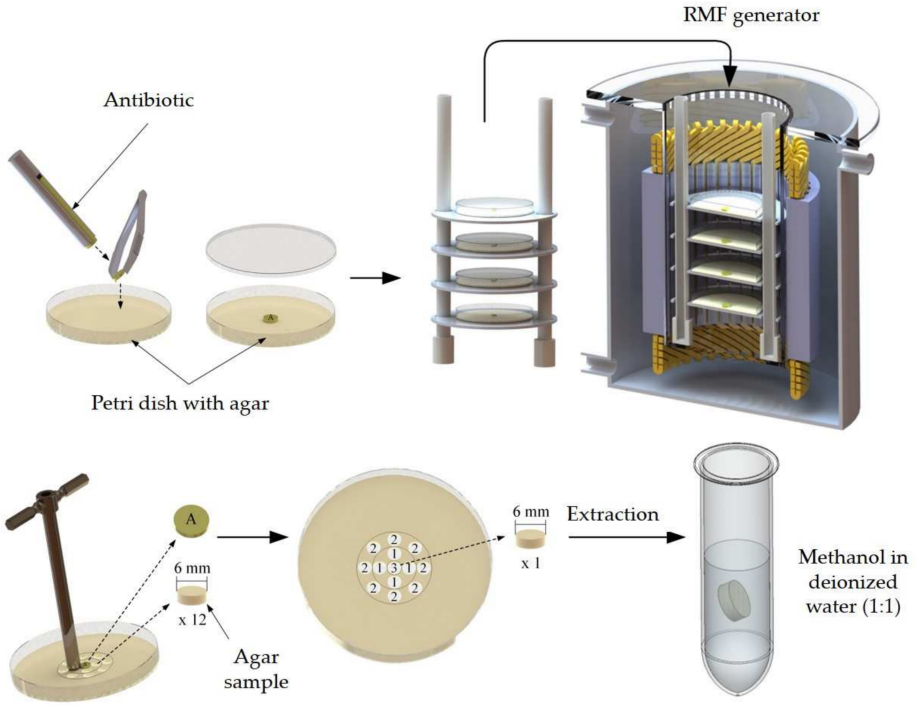


Figure 8. Schematic presentation of the analysis of the impact of RMF on the diffusion of antibiotics in the agar medium. 1—agar sample from the proximal zone (zone 1); 2—agar sample from the distal zone (zone 2); 3—agar sample under the antibiotic paper disc (zone 3); A—antibiotic paper disc.

4.5. Visualization of the Impact of RMF and β-Lactam Antibiotic on the Integrity of Staphylococcal Cell Walls

Initially, MRSA reference strain (ATCC 33591) was plated onto Columbia agar with 5% sheep blood (Graso Biotech, Jablowo, Poland) and cultivated for 24 h at 37 °C. After incubation, one colony-forming unit (CFU) was transferred into 10 mL of tryptic soy broth (TSB, Graso Biotech, Jablowo, Poland) and incubated another 24 h at 37 °C with shaking (200 rpm). Next, cultures were diluted in TSB supplemented with 1% glucose to obtain bacterial suspension equal to 1×10^5 CFU/mL. In the next step, 1 mL of the bacterial suspension was added to a 24-well plate (1 mL into each well), (VWR, Radnor, PA, USA). To obtain biofilm, the plates with bacterial suspension were incubated for 48 h at 37 °C.

Prior to the addition of the β-lactam antibiotic (cefoxitin, Pol-Aura, Olsztyn, Poland), the TSB medium was removed and the wells with biofilm washed with PBS buffer. Then, 200 µL of PBS containing cefoxitin (8 mg/mL) was added to each well of a 24-well plate. As it was determined in the initial step of the study, such a concentration of cefoxitin caused approximately a 50% reduction in growth of biofilm-forming bacteria. After the application of antimicrobial, the biofilms were exposed to the RMF of 5 Hz for 12 h at 36 ± 1 °C.

The MRSA ATCC 33591 biofilms exposed to the RMF only or unexposed to any of the investigated factors (RMF or cefoxitin) served as control settings.

After the above-mentioned procedures, the medium from biofilm-containing wells of 24-well plates was removed and replaced with 200 µL of Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit (Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) solution and incubated at room temperature for 15 min. After incubation, the solution was removed and the wells were gently rinsed 3 times with sterile water. Next, the water was removed. The biofilms were analyzed using a confocal microscope Leica SP8 (Wetzlar, Germany) with a 25× water dipping objective using 488 nm laser line and 500–530 nm emission to visualize SYTO-9 and 552 nm laser line and 575–627 nm emission to visualize propidium iodide (PI), in a sequential mode. Images are maximum intensity projections obtained from confocal Z stacks with ~2 µm spacing in Z dimension. PI is represented in red/orange and SYTO-9 in

green color. The obtained biofilm images were further analyzed using Imaris 9 (Abingdon, UK) software.

4.6. Statistical Analysis

The data obtained in this study concerning the changes in antibiotic concentrations in paper discs and agar samples in the control and RMF-exposed settings were presented as means ± standard errors of the means (SEM) obtained from three different measurements (plus technical repetitions). Statistical differences between RMF-exposed and control, unexposed settings were determined by one-way analysis of variance (ANOVA) and Tukey's post hoc test. Differences were considered significant at a level of $p<0.05$. The statistical analyses were conducted using GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA).

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Rotating Magnetic Field Increases β-Lactam Antibiotic Susceptibility of Methicillin-Resistant *Staphylococcus aureus* Strains

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Abstract: Methicillin-resistant strains of *Staphylococcus aureus* (MRSA) have developed resistance to most β-lactam antibiotics and have become a global health issue. In this work, we analyzed the impact of a rotating magnetic field (RMF) of well-defined and strictly controlled characteristics coupled with β-lactam antibiotics against a total of 28 methicillin-resistant and sensitive *S. aureus* strains. The results indicate that the application of RMF combined with β-lactam antibiotics correlated with favorable changes in growth inhibition zones or in minimal inhibitory concentrations of the antibiotics compared to controls unexposed to RMF. Fluorescence microscopy indicated a drop in the relative number of cells with intact cell walls after exposure to RMF. These findings were additionally supported by the use of SEM and TEM microscopy, which revealed morphological alterations of RMF-exposed cells manifested by change of shape, drop in cell wall density and cytoplasm condensation. The obtained results indicate that the originally limited impact of β-lactam antibiotics in MRSA is boosted by the disturbances caused by RMF in the bacterial cell walls. Taking into account the high clinical need for new therapeutic options, effective against MRSA, the data presented in this study have high developmental potential and could serve as a basis for new treatment options for MRSA infections.

Keywords: antibiotics; antibiotic resistance; MRSA; *Staphylococcus aureus*; rotating magnetic field

1. Introduction

In recent years, antimicrobial resistance has become a major public health issue. Methicillin-resistant strains of *Staphylococcus aureus* (referred to as MRSA) have developed

resistance to most β-lactam antibiotics including penicillins, cephalosporins (with the exception of ceftaroline and ceftobiprole) and carbapenems [1]. The frequency of occurrence of these resistant strains (compared to methicillin-sensitive *S. aureus* strains, referred to as MSSA) has been reported for 25% and, in certain geographical regions, even over 50% of all SA strains. The Centers for Disease Control and Prevention estimates that MRSA is responsible for more than 70,000 severe infections and 9000 deaths [2]. MRSA has been identified as the most common etiological factor of skin and soft tissue infections in United States intensive care hospital units and it is also associated with numerous other disease entities, including biomaterial-related diseases [3,4]. It is believed that even ongoing development of new (other than β-lactam) antibiotics and advances in infection prevention will not prevent MRSA from remaining one of the most prominent pathogens with persistently high mortality [5]. The main reason behind the above-mentioned evolutionary success of this pathogen in the nosocomial and, in recent years, community environments, is the fact that MRSA harbors the *mecA* gene which codes alternative penicillin-binding protein, PBP2a, responsible for the resistance mechanism in question. Although other gene variants, namely *mecB* and *mecC*, occur less frequently in the staphylococcal genome, their presence is also related with resistance to β-lactams [6,7]. The native PBP protein is the essential enzyme in the process of bacterial cell wall synthesis. β-lactam antibiotics, against which MRSA strains are resistant, exert their antibacterial activity by binding and inactivating PBPs. Importantly, β-lactam antibiotics display a low affinity for alternative PBP2a, so the process of cell wall synthesis mediated by this protein remains undisturbed. This results in the survival of MRSA strains in the presence of β-lactam antibiotics [8]. Currently, numerous alternative strategies have been developed to overcome the issues related to microbial resistance [9]. Among them, the application of various types of magnetic fields (MFs) has been proposed to boost the activity of standard antimicrobial agents [10–13].

The first studies on the influence of magnetic fields (MFs) on organisms started at the end of the 19th century. This line of investigation was intensified in subsequent decades, mostly fueled by a growing interest in the impact of fields generated by electric and telecommunication networks on the health and behavior of humans and such animals as bees and birds [14]. At present, it is well-established that MFs may affect structure and functional processes in microorganisms. Regarding the direct effects of MFs on microorganisms, the literature indicates possible modifications in the physiology and shape of the cells, changes in the chemical-physical characteristics of the cellular membrane [15], and in membrane permeability [16,17]. The most commonly observed exposure effects concern changes in growth dynamics [18–21], the ability of bacteria to adhere and form biofilms [22,23], gene transcription [24,25] and sensitivity to antimicrobial substances [26], as well as irreversible damage to the microorganisms, mostly due to the loss of integrity of the cellular wall/membrane [27–31].

Despite the existence of seemingly extensive scientific literature on the topic, the results of studies of the influence of various types of MFs on microorganisms are not unequivocal. Some authors reported an antibacterial effect of MFs [32–35], while others suggested the lack of any significant impact of MFs on microbial growth [36,37], biochemical activity [38] or bacterial adhesion [39]. Other research teams demonstrated a stimulating effect of MFs on microbial cell growth and cell viability [40–42]. Such contradictory results led to the recognition that MFs may exert a whole spectrum of biological effects (from none through inhibitory/negative to stimulatory/positive), depending on the bacterial species analyzed, the nature of the emitted magnetic signals and the time of magnetic exposure [32,35,43]. Therefore, one of the explanations for the aforementioned discrepancies in the results obtained by different authors relates to the variety of the applied MF-generating systems, resulting in the diversity of the generated MFs, as well as to the multitude of species of microorganisms (or strains within a particular species) exposed to MFs [44]. Unfortunately, the above lack of methodological consistency in the research on the impact of MFs on microorganisms prevents this innovative approach from crossing the border separating in vitro analyses from preclinical studies. Therefore, in the present work, we attempted to

take a step towards systematization of the knowledge in this area, focusing on only one type of MF, i.e., rotating magnetic field (RMF, a field where opposite poles rotate around a central point or axis), as well as on one species of bacteria (*S. aureus*) and one group of antibiotics (β-lactams).

Our research team has long-standing experience in studying RMF applications. We previously demonstrated the impact of RMF on the growth, metabolic activity and biofilm formation of several different species of microorganisms [20,22]. Moreover, in a distinct line of investigation, we analyzed the combined effect of RMF and different (non-β-lactam) antibiotics and antiseptics against *S. aureus* and *Pseudomonas aeruginosa* biofilms [10]. The obtained results indicated that the reduction of biofilms exposed to RMF and antimicrobials was 50% higher compared to biofilms exposed only to antimicrobials. We assumed that the observed effect could be related to the mixing effect caused by the RMF, which allowed antimicrobials to effectively reach deeper layers of biofilm.

The purpose of this study was to investigate the influence of the RMF on the changes in MRSA strains' susceptibility to β-lactam antibiotics. We hypothesized that RMF could have an impact on the antibiotics' activity and penetrability through the bacterial cell membrane, resulting in a higher rate of eradication displayed by the antibiotics. This assumption was backed up by the results of the experiments performed by Golberg et al. (2014) [45] and Tagourti et al. (2010) [46], where other types of electromagnetic fields (EMFs) were used to permanently damage cell membranes (presumably by membrane irreversible electroporation). Assuming that β-lactams can disturb cell wall synthesis in MRSA (even if these alterations are of minor character and do not lead to cell destruction), it can be expected that, together with RMF-induced alterations, a kind of booster effect may be observed. To investigate the above hypotheses we aimed to answer the following questions: is it possible to increase the susceptibility of MRSA strains towards β-lactam antibiotics in the presence of the RMF?; is the observed effect connected to the influence of the RMF on the antibiotic molecules, bacterial cells or both?; does the observed effect depend on the bacterial strain and apply to the entire group of β-lactams?; is the observed effect of a permanent character or it occurs only in the presence of the RMF? Furthermore, the associations between RMF parameters, as well as exposure conditions and the changes in antibiotic susceptibility, were analyzed.

2. Results and Discussion

2.1. Analysis of Changes in Antibiotic Susceptibility of S. aureus Strains under the Influence of RMF

The performed analyses confirmed that the bacterial strains used in the experiment belonged to *S. aureus* species. Resistance to cefoxitin (determining resistance to methicillin), and the presence of the *mecA* gene were confirmed for all analyzed MRSA strains. Accordingly, all strains which were included to the study as MSSA were susceptible to cefoxitin and did not harbor the *mecA* gene (Supplementary Figure S1).

The reference MRSA strain (ATCC 33591) and two clinical isolates (namely MRSA 1 and MRSA 2) were selected for the first phase of the analyses, which aimed to assess the changes in the aforementioned strains' susceptibility to cefoxitin in the presence of RMF. Because resistance to cefoxitin indicates resistance to most other β-lactams, three other antibiotics from this group were included in this experimental line. The research previously conducted by our research group, as well as the studies of several other authors, revealed that the strength of MF impact (regardless of its type or the phenomenon analyzed in its presence) depends on exposure duration as well as on the intensity and/or the frequency of MF [19,20,32,43,47], because these two factors determine the physical characteristics of the magnetic signal [48,49].

It should be explained here that in the case of the RMF set-up used in the present study, the frequency of AC determines the MF intensity and, importantly, it is responsible for the physical characteristics of the magnetic wave shape. For this reason, the first analyses were carried out to identify the AC frequency at which the generated RMF induced the greatest changes in antibiotic susceptibility. As the optimal exposure time to the RMF has not been

established at this stage of the experiment, seeded bacterial cultures on agar plates with the antibiotic were subjected to RMF for 18 h, because this is the time recommended by EUCAST (2021) [50] guidelines for antibiogram preparation.

It was shown that the strongest effect in terms of changes in growth inhibition zone diameter was observed at the lowest current frequency enabling the provision of the magnetic characteristic for the RMF (5 Hz) in the applied setup (Table 1). As shown by the simulative calculations, at 5 Hz the amplitude of the RMF was characterized by a longer period between magnetic induction maximal strength state (100 ms with B_{max} 8.1 mT). In contrast, a significantly lower effect in terms of the changes in antibiotic susceptibility was observed when RMF at 50 Hz (the highest current frequency in applied set up) was used. The RMF at this AC frequency was characterized by a shorter period, with 10 ms time between magnetic induction maximal strength state with B_{max} 8.5 mT (Figure 1). For the intermediate AC frequencies applied (10 Hz and 25 Hz), the changes in inhibition zones were also observed for most of the antibiotics included in the experiment, although the diameters of the zones were, in most cases, smaller, and in no case greater than those observed at the RMF generated at 5 Hz.

Table 1. Growth inhibition zones (mm) of MRSA strains around discs with β-lactam antibiotics depending on RMF frequency (Hz).

Strain	RMF Frequency				
	C	**5 Hz**	**10 Hz**	**25 Hz**	**50 Hz**
			Cefoxitin		
ATTC 33591	12	18	15	16	15
MRSA 1	6	16	14	16	14
MRSA 2	7	21	15	17	15
			Cefepime		
ATTC 33591	10	16	15	16	14
MRSA 1	6	19	14	18	13
MRSA 2	6	18	6 (14p.)	6 (16p.)	6
			Cefuroxime		
ATTC 33591	6	22	20	20	18
MRSA 1	6	23	19	21	18
MRSA 2	6	15	6 (14p.)	14	6
			Ceftriaxone		
ATTC 33591	6	18	15	17	16
MRSA 1	6	15	14	15	13
MRSA 2	6	15	6 (13p.)	15	6

Differences in the diameter of the growth inhibition zones between three repetitions of the experiment did not exceed ±1 mm. C—unexposed control culture; "p"—partial growth of bacteria around the antibiotic discs.

When the MRSA 2 strain was exposed to RMF at 10 Hz in the presence of cefepime, cefuroxime and ceftriaxone, a zone of partial growth around the antibiotic discs was formed. This zone consisted of a bacterial layer, thinner and distinctly demarcated from the staphylococcal lawn grown on the rest of the agar plate (Supplementary Figure S2). It was confirmed that the discussed layer did not consist of contaminating species but of cells of the staphylococcal strain. A similar result (presence of partial growth zone around the antibiotic disc) was also observed when the same MRSA 2 strain was challenged against cefepime in the presence of RMF at 25 Hz. In turn, when RMF at 50 Hz and three of the aforementioned antibiotics were applied, the staphylococcal lawn formed by MRSA 2 reached the edge of antibiotic discs (no inhibition or reduced growth zones were noticed). It can be thus assumed that the observed effect could be related to RMF activity, because its frequencies (5, 25, 50 Hz) were the only variables in this experiment. However, no such effect of RMF was observed for MRSA 1 and ATCC 33591 strains. At 25 Hz RMF, the zones

of their growth inhibition were clean and of similar size as the zones observed at 5 Hz. Such results may be related to the specific features of a given strain exposed to the RMF. It is well-known that sensing stress factors, bacteria switch on new pathways aimed at cell preservation [51]. In particular, the transposition, which represents an important source of genetic variability, can be induced in bacteria exposed to environmental stress, e.g. to the MF [49,52,53]. By this means, bacteria try to adapt using intra-strain variability. This allows enhancement of the persistence of bacteria and promotion of the selection of clones adapted to the particular stress conditions [54], e.g., RMF of specific characteristics. The conclusion which can be drawn from the discussed results is that RMF potential to induce changes in the antibiotic susceptibility of MRSA strains is not restricted only to the RMF generated at 5 Hz. Nevertheless, because the most favorable results were obtained at 5 Hz, further studies and analyses were performed in RMF using this frequency.

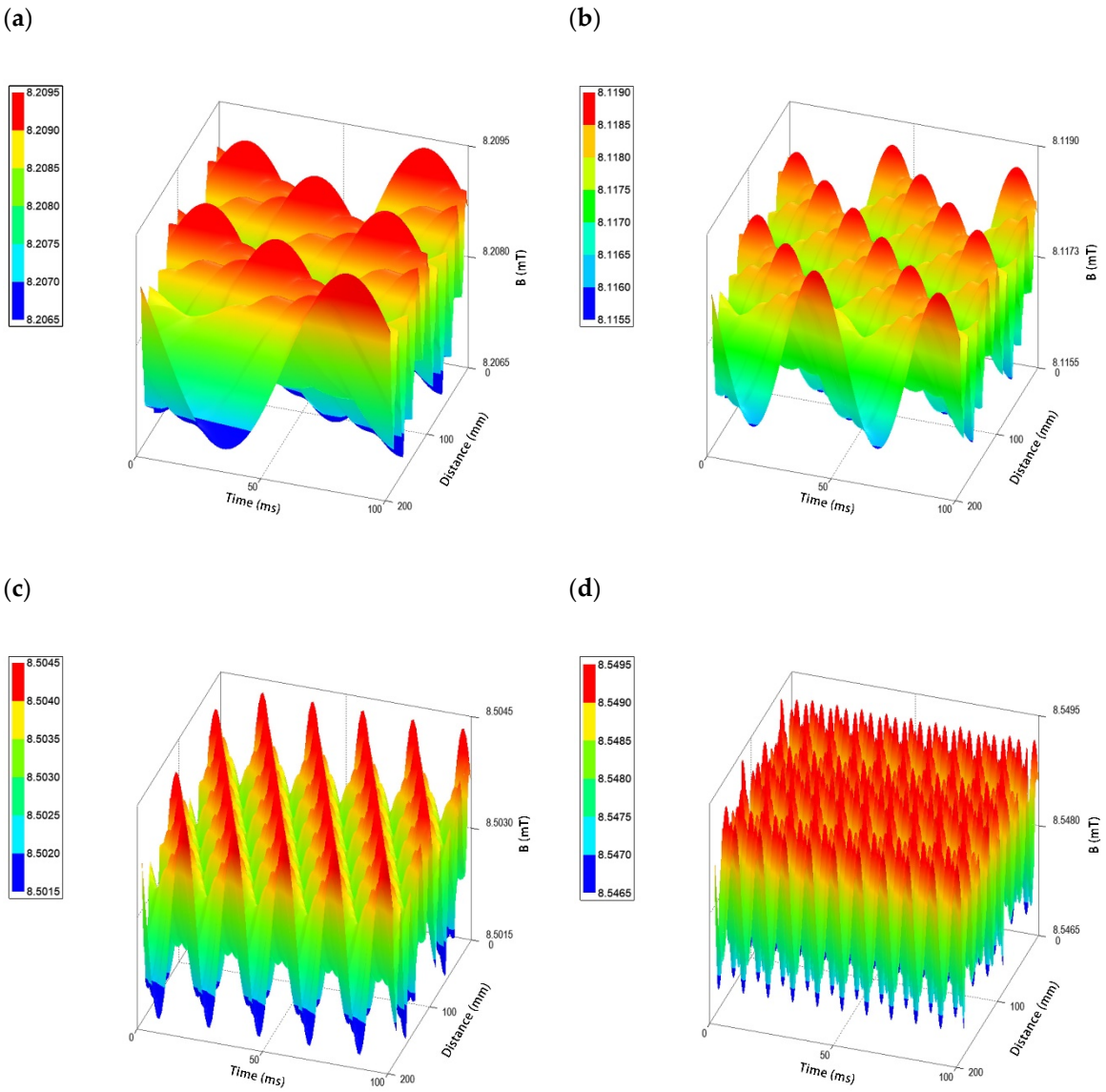


Figure 1. Changes of magnetic flux characteristic depending on the applied AC frequency: (**a**) 5 Hz; (**b**) 10 Hz; (**c**) 25 Hz; (**d**) 50 Hz.

It is worth noting that in our previous work, a completely different pattern of changes in antimicrobial activity was observed with regard to the applied RMF frequency [10]. In the above work, we aimed to assess the activity of various antimicrobials (antibiotics and antiseptics) against staphylococcal and pseudomonal biofilms exposed to the RMF

generated in the range from 10 to 50 Hz, in which the higher the frequency, the greater the antimicrobial effect observed. We assumed that this effect could be caused by the direct correlation between magnetic induction and mixing effect within the biofilm matrix, which was immersed in a liquid microbiological medium. Our assumption was that the more active the MF (please refer to Figure 1), the more particles of the antimicrobial could reach and deactivate bacterial cells within biofilm layers. These results are in opposition to those presented in Table 1. However, the previous study used not only a different biological model (biofilm) but also analyzed other antimicrobials. The above additionally emphasizes the impact of the applied variables on the final outcome of the experiment. The findings of the present study agree, in principle, with the report of Stepanian et al. (2000) [55] who revealed that the percentage of cell survival was proportional to the increase in EMF frequency. The highest percentage (53%) was observed at 50 Hz and the lowest (20%) at 4 Hz. In other words, the lower the frequency of the EMF, the lower the bacterial survival rate. Therefore, although the results of research of the effects of MFs on microorganisms frequently suggest that the effect is proportional to the intensity or frequency of the generated field, it should not be taken as a binding principle. Contradicting results may be explained, e.g., by the differences in MF wave characteristics at lower and higher frequencies, which act on the bacterial cells as a stress factor and consequently cause disturbances in their development; the exposed cells are alternately (however, with different frequency) subjected to weaker and stronger MF influences. As was already indicated, the amplitude of the RMF at a lower frequency is characterized by a longer period between magnetic induction maximal strength state, whereas at higher frequencies this period is shorter. Simultaneously, the applied AC frequencies (5 Hz https://youtu.be/YXH5CkArdQ0, accessed on 1 September 2021, 10 Hz https://youtu.be/4TGUOQOVOLo, accessed on 1 September 2021, 25 Hz https://youtu.be/8r1AcS0dIP0, accessed on 1 September 2021, 50 Hz https://youtu.be/gfr7AYqCyh8, accessed on 1 September 2021—please click the links to watch the simulation) generated magnetic flux rotation around the stator with different synchronous speeds of 150 rpm, 300 rpm, 750 rpm and 1500 rpm, respectively (calculations performed on the basis on the manufacturer's characteristics of the stators).

Previous studies have also indicated the ability of different MF types to disturb microbial structures and to permanently damage cell membranes, presumably by their irreversible electroporation [45,46]. Fojt et al. (2004) [32] explained that a drop in bacterial viability after exposure to MF was caused by an increase in the permeability of ion channels in the cytoplasmic membranes or by the formation of free radicals in bacterial cells. The relationship between the changes in the induction of the MF and the formation of active oxygen and free radicals in bacterial cells was previously demonstrated by Kohno et al. (2000), Fojt et al. (2004) and Jin et al. (2009) [32,56,57]. Therefore, it can be expected that in the case of a fairly homogeneous MF, e.g., the RMF generated at 50 Hz, which is characterized by relatively low MF strength fluctuations, bacteria could adapt more easily to the stressor. Such a mechanism was previously reported by Mittenzwey et al. (1996) [58] who showed high resistance of different bacteria to MFs due to the intracellular repair systems and self-regulatory mechanisms. The data reported by the above-mentioned researchers, together with the results presented in Table 1 and Figure 1, indicate the possibility of an additive interaction between β-lactam antibiotics and RMF. At this stage of the experiment, we assumed that the potential mechanisms behind the observed interaction may include a direct influence of the RMF on antibiotic molecules, bacterial membrane/cell wall or metabolic intercellular processes.

The next stage of the experiment aimed to determine the optimal RMF exposure time to obtain the highest increase in antibiotic susceptibility. Previous papers of our research group [19,20] as well as reports of other authors [44,59], indicated that the time of magnetic exposure (apart from the intensity, frequency and characteristics of the field wave) is also of key importance with regard to the effect exerted on biological systems. Depending on the exposure time, the MF may have a different effect on bacterial viability, i.e., it may increase it [40–42] or reduce it [32,34,35]. In the current study, the cultures with antibiotic

discs were exposed to the RMF for a specified time, ranging from 1 to 12 h, and then the plates were transferred to the incubator until the 18 h period of incubation was completed. It was revealed that the zones of bacterial growth inhibition increased with the length of exposure time up to 12 h in most cases (Table 2). However, in the case of MRSA 1 exposed to ceftriaxone and MRSA 2 exposed to cefuroxime, the increase of growth inhibition stopped after 5 h of magnetic exposure. In turn, the first increase in the diameter of the growth inhibition zones around the antibiotic discs was observed after 2 h of the strain's exposure to RMF compared to an unexposed strain. This effect was observed in *S. aureus* strains ATCC 33591 and MRSA 2 subjected to the activity of cefoxitin, cefepime and ceftriaxone. Nevertheless, in most cases the differences in inhibition zones were visible after 4 h of exposure to the RMF. It should also be explained here that in each of the above cases, the period of exposure to the RMF was shorter than the time needed to observe bacterial growth on the medium. However, in the case of cultures exposed longer, at least by the time after which bacterial growth on the media was already observed (>6 h), it was possible to measure the zones of growth inhibition even before transferring the plates to the incubator. It was found that in cultures exposed for more than 6 h, after further incubation w/o RMF, the inhibition zones were only slightly reduced (<2 mm) compared to the zones measured immediately after exposure to the RMF (Supplementary Table S1). It was also noticed that in a few cases, after magnetic exposure lasting for less than 6 h, a small number of individual colonies was also observed within the growth inhibition zones (thus they were referred to as zones of partial growth inhibition) (Supplementary Figure S3). No differences between the zones of growth inhibition measured immediately after RMF exposure compared to the zones remeasured after further incubation w/o RMF was observed only when the exposure lasted at least 10–11 h (depending on the staphylococcal strain), when the cultures were already well-developed (Table 2). For this reason, the optimal RMF exposure time ensuring the possibility of observing a stable effect was defined for 12 h.

Table 2. Growth inhibition zones (mm) of MRSA strains around discs with β-lactam antibiotics depending on the RMF exposure (5 Hz) duration.

	Strain																							
	ATCC 33591								**MRSA 1**								**MRSA 2**							
	FOX		**FEP**		**CXM**		**CRO**		**FOX**		**FEP**		**CXM**		**CRO**		**FOX**		**FEP**		**CXM**		**CRO**	
t	**C**	**RMF**	**C**	**RMF**	**C**	**RMF**	**C**	**RMF**	**C**	**RMF**	**C**	**RMF**	**C**	**RMF**	**C**	**RMF**	**C**	**RMF**	**C**	**RMF**	**C**	**RMF**	**C**	**RMF**
1	13	13	10	10	6	6	6	6	6	6	6	6	6	6	6	6	7	7	6	6	6	6	6	6
2	12	13	9	11	6	6	6	10	6	6	6	6	6	6	6	6	6	10	6	6	6	6	6	6
3	12	13	9	12	6	16	6	12	6	11	6	6	6	6	6	6	7	12	6	6	6	6	6	6
4	12	14	10	12	6	15	6	12	6	14	6	14	6	19	6	14	7	13	6	13	6	14	6	6
5	12	15	9	13	6	16	6	13	6	15	6	13	6	20	6	15	7	14	6	15	6	15	6	13
6	12	15	9	13	6	17	6	13	6	15	6	15	6	19	6	15	7	14	6	17	6	15	6	13
7	13	16	10	14	6	17	6	13	6	16	6	15	6	21	6	15	7	13	6	17	6	15	6	13
8	12	16	10	15	6	18	6	13	6	16	6	15	6	20	6	15	7	14	6	16	6	15	6	12
9	12	16	10	14	6	18	6	15	6	16	6	16	6	20	6	15	7	16	6	18	6	15	6	13
10	12	17	10	15	6	19	6	16	6	16	6	17	6	21	6	15	7	18	6	18	6	15	6	13
11	12	18	10	16	6	20	6	17	6	16	6	18	6	22	6	15	7	20	6	18	6	15	6	14
12	12	18	10	16	6	22	6	18	6	16	6	19	6	23	6	15	7	21	6	18	6	15	6	15
18 *	12	18	10	16	6	22	6	18	6	16	6	19	6	23	6	15	7	21	6	18	6	15	6	15

Cultures with antibiotic discs were exposed to the RMF for a specified time (*t*), ranging from 1 to 12 h, and then the plates were transferred to the incubator until the 18 h period of incubation was completed. 18 *—constant exposure to the RMF for 18 h. C—control culture unexposed to RMF. The differences in the diameter of the growth inhibition zones between three repetitions of the experiment did not exceed ±1 mm. FOX—cefoxitin, FEP—cefepime, CXM—cefuroxime, CRO—ceftriaxone.

The next part of the experiment focused on changes in the susceptibility of the RMF-exposed bacteria to the various β-lactam antibiotics, including different classes of cephalosporins, carbapenems and penicillins. Although the general mechanism of action of β-lactam antibiotics is similar, there are some differences in their specific activity, e.g., related to different binding sites with the PBP2a protein or the binding energy value [60]. Harrison et al. (2019) [61] demonstrated that aminopenicillins (e.g. amoxicillin) bind better to PBP2a than cephalosporins. The size of the particles of individual antibiotics, as well as their charge, may also be important, especially taking into account the possible

effects of the MF on charged particles, and thus on the process of their diffusion in the microbiological medium [62,63]. Our findings showed that an increase in antibiotic susceptibility in all RMF-exposed cultures was obtained for seven out of the eleven β-lactam antibiotics included in the experiment (Table 3, Figure 2, Supplementary Figure S4). A lack of increase in the diameters of growth inhibition zones in RMF was found only for ceftazidime and amoxicillin (for all three strains analyzed); cefradin (in the case of ATCC 33591 and MRSA 2 strains) and cephalexin (MRSA 2 strain). Unfortunately, we were unable to establish any pattern explaining the relationship between the properties, mechanism of action of the specific types of antibiotics and the observed changes in the zones of growth inhibition under the influence of RMF.

Table 3. Growth inhibition zones (mm) of MRSA strains around discs with different β-lactam antibiotics in RMF-exposed (5 Hz) and control, unexposed cultures.

Strain	C	RMF	C	RMF	C	RMF	C	RMF
	Cefazolin		**Cefotetan**		**Cefradine**		**Cefalexin**	
ATCC 33591	11	18	9	12	16	16	10	15
MRSA 1	6	18	6	11	6	12	6	9
MRSA 2	6	12	6	14	6	6	6	6
	Ceftaroline		Meropenem		Imipenem		Ertapenem	
ATCC 33591	21	23	9	15	31	35	6	16
MRSA 1	19	22	6	18	6	22	6	15
MRSA 2	20	22	6	15	10	20	6	17
	Ceftazidime		Doripenem		Amoxicilin			
ATCC 33591	6	6	6	13	6	6		
MRSA 1	6	6	6	12	6	6		
MRSA 2	6	6	6	12	6	6		

Differences in the diameter of the growth inhibition zones between three repetitions of the experiment did not exceed ±1 mm. C—control culture unexposed to RMF.

The next part of the study aimed to determine whether the observed effect of increased sensitivity of β-lactam antibiotics caused by RMF exposure depends on the specific concentration of antimicrobial used. Since the disc diffusion method is limited to only one concentration of the antibiotic/disc, for the purposes of this experiment, gradient MIC strips (E-tests) were used. The use of E-tests enabled assessment of the changes in antibiotics susceptibility of bacterial cultures exposed to the RMF (5 Hz) depending on the concentration of the antimicrobials. Eight antibiotics were selected for the study, for which in the disc diffusion test the greatest and the lowest differences in the zones of growth inhibition compared to the unexposed control cultures were obtained. It was found that in each (except one) of the RMF-exposed cultures, a substantial decrease in the MIC value (by at least one order of the antibiotic concentration value marked on the E-test strips) occurred compared to the controls (Table 4, Figure 3, Supplementary Figure S5). The only exception was the RMF-exposed *S. aureus* ATCC 33591 cultures with ceftazidime, for which the MIC value was the same as for the control. In turn, the decrease in MIC values of cefoxitin, ceftriaxone, cefuroxime, ceftazidime and cefepime was particularly significant in the case of MRSA 1 and MRSA 2 strains exposed to RMF. The MIC values measured in the control settings were 256 μg/mL, while in the RMF-exposed cultures, MIC values ranged from 6 to 96 μg/mL, depending on the strain and antibiotic. Importantly, the use of more precise E-test methodology allowed to detect the effect displayed by RMF in the case of these antibiotics for which no or minimal changes in growth inhibition zones were observed when the disc diffusion method was applied. These antibiotics were, e.g., amoxicillin (in all RMF exposed cultures) and ceftazidime (in MRSA 1 and MRSA 2 cultures but not in the ATCC 33591 strain). To summarize this part of the results, it should be noted that the lack of the influence of the RMF, observed in the case of several antibiotics in the disk diffusion assay, was related to a low concentration of these antimicrobials in the test discs. Neverthe-

less, the exposure to RMF did not change the susceptibility level of the ATCC 33591 strain to ceftazidime. This reveals not only the importance of intra-species variability in their answer to the same stimuli but also the necessity of testing a high number of microbial strains to draw proper conclusions from the observed phenomena.

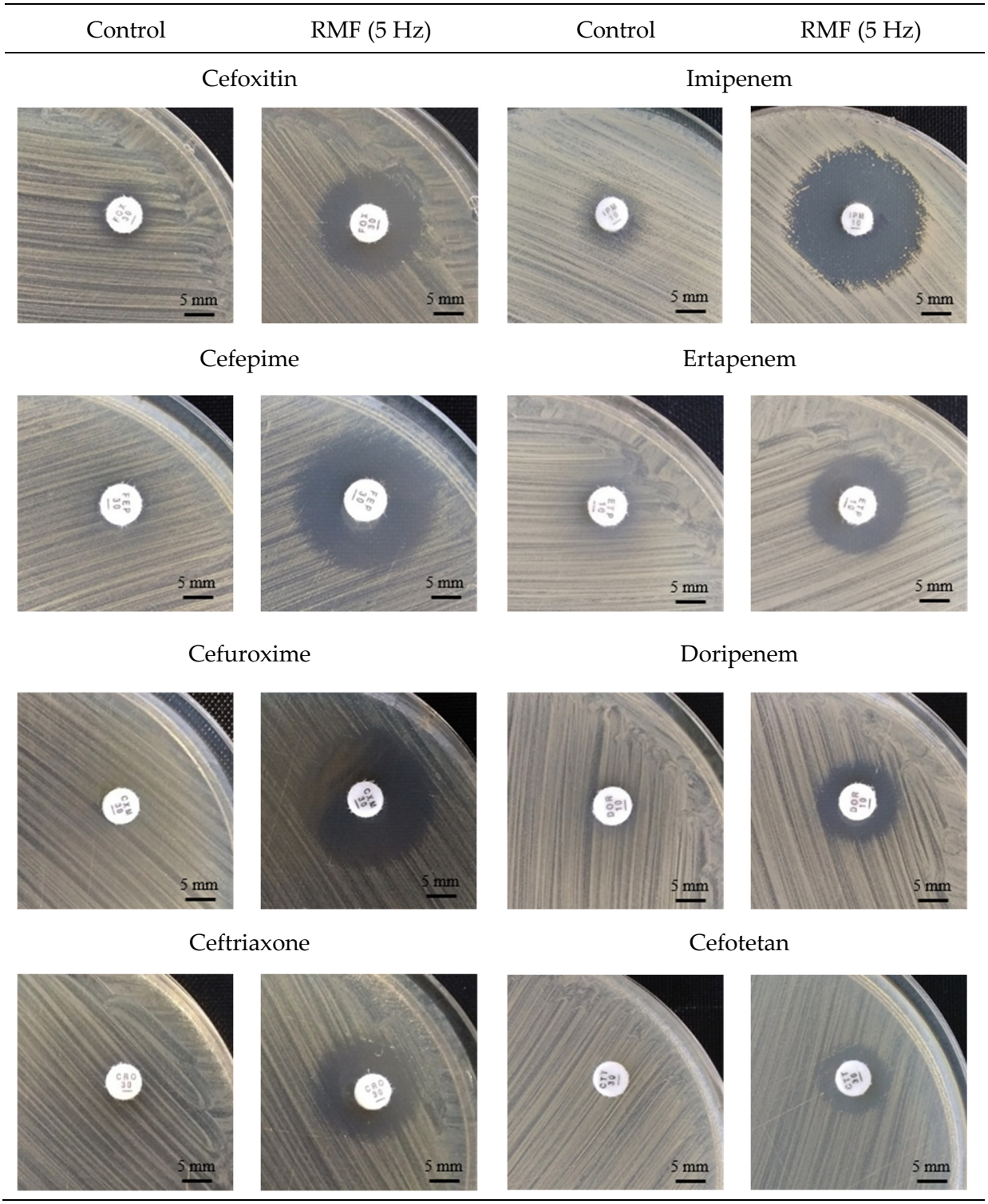


Figure 2. Representative pictures of growth inhibition zones (mm) in control and RMF-exposed (5 Hz) MRSA 1 cultures around discs with β-lactam antibiotics.

Table 4. MIC values (μg/mL) of β-lactam antibiotics for MRSA strains in control and RMF-exposed (5 Hz) cultures.

	Strain					
	ATCC 33591		MRSA 1		MRSA 2	
	C	RMF	C	RMF	C	RMF
Cefoxitin	24	16	256	24	256	6
Amoxicilin	8	6	24	6	24	12
Imipenem	1.5	1	32	0.19	32	1.5
Meropenem	1	0.75	32	1.5	32	4
Ceftriaxone	32	24	256	16	256	24
Cefuroxime	12	8	256	8	256	12
Ceftazidime	32	32	256	32	256	96
Cefepime	32	16	256	16	256	64

C—control culture unexposed to RMF; there were no differences in MIC values between three separate experiments.

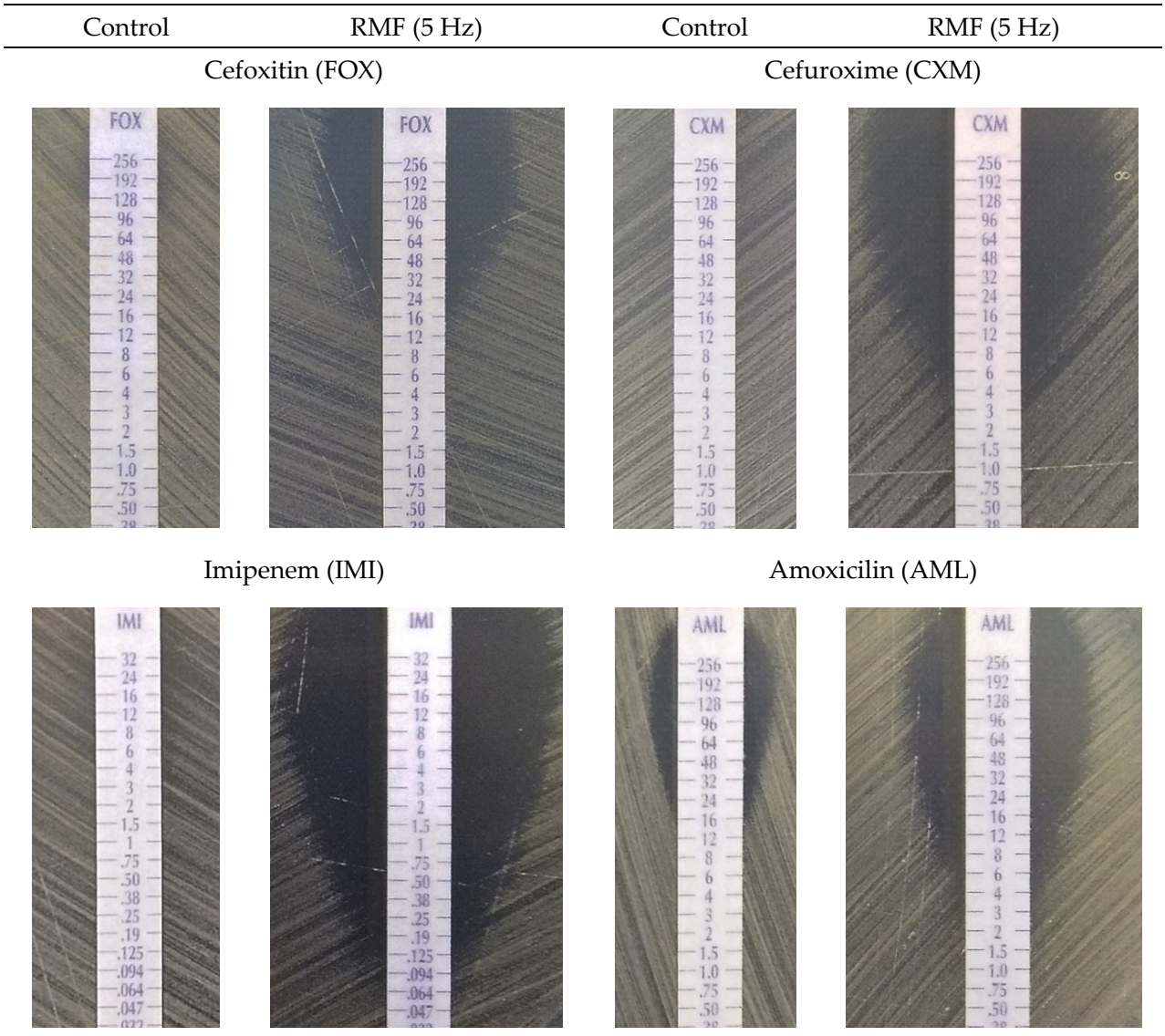


Figure 3. Representative pictures of gradient MIC strips (E-tests) with β-lactam antibiotics in control and RMF-exposed (5 Hz) cultures of the MRSA 1 strain.

The additional finding from the antibiotic diffusion-based assays was the observation that the zones of growth inhibition obtained in the RMF-exposed cultures retained their characteristic shape of a circle in the disc diffusion method and an ellipse when the MIC strips were used (Figures 2 and 3, respectively). Importantly, in the case of E-tests, the zones of growth inhibition measured in a straight line from the edge of the strip across to the edge of the bacterial lawn in the RMF-exposed cultures were larger than in the control settings, approximately in the same manner as was previously observed when the disc diffusion test was applied (Figure 4). This observation is particularly important in the context of the basic methodological assumptions for the applied diffusion-based tests. A demonstration of the disturbances in the diffusion of antibiotics under the influence of RMF at this level of analysis would significantly hinder the correct interpretation of the obtained results. On the other hand, if such a phenomenon occurred, it would provide precious data on a possible interaction of RMF with antibiotics.

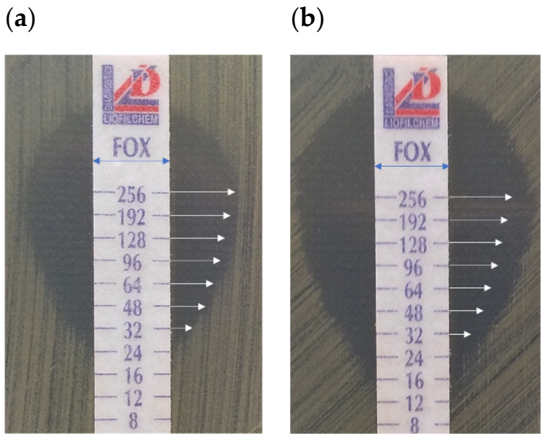


Figure 4. Representative pictures of growth inhibition zones around E-test strips with cefoxitin in (**a**) control and (**b**) RMF-exposed (5 Hz) *S. aureus* ATCC 33591 cultures. The arrows at a certain antibiotic concentration represent sections of the same length.

To determine whether the observed effect of elevated antibiotic susceptibility in RMF is strain-specific, the research was extended to include another 21 MRSA strains. It is well-established that the MRSA mechanism is mediated by the expression of an alternative of PBP2 protein (called PBP2a) characterized by a low affinity for β-lactam antibiotics, resulting in resistance to most β-lactams. However, PBP2a encoded by the *mecA* gene which is carried on a mobile genetic element known as a staphylococcal cassette chromosome *mec* (SCC*mec*) can be regulated by two independent regulatory systems (*mecI-mecR-mecR2* and *blaI-blaR*) and multiple chromosomal genes. As shown by other authors, also PBP1-4 and PBP2a structures are not identical between staphylococcal strains [60]. Moreover, β-lactam resistance in most MRSA is heterogeneous, meaning that while most cells in a population have low MICs, some fraction can survive at much higher MICs [64]. Despite the naturally occurring differences between strains and their different susceptibility to cefoxitin (confirmed on the basis of the results obtained in control cultures), our studies showed that all analyzed MRSA strains displayed elevated susceptibility to cefoxitin (Table 5). In the case of eight of the 20 strains, the diameter of the inhibition zones increased at least by 10 mm, while in the others by at least 4 mm. Of note was the observation that the smaller the zone of growth inhibition under the control conditions, the greater the change in its diameter as a result of RMF exposure.

Table 5. Zones of growth inhibition (mm) of MRSA strains around cefoxitin discs in control and RMF-exposed (5 Hz) cultures.

Strain	C	RMF	Strain	C	RMF
ATCC 33591	12	18	MRSA 12	12	16
MRSA 1	6	16	MRSA 13	6	20
MRSA 2	7	21	MRSA 14	9	15
MRSA 3	6	17	MRSA 15	12	18
MRSA 4	6	18	MRSA 16	6	14
MRSA 5	6	16	MRSA 17	7	18
MRSA 6	6	16	MRSA 18	11	16
MRSA 7	6	21	MRSA 19	6	14
MRSA 8	12	16	MRSA 20	6	16
MRSA 9	6	13	MRSA 21	15	19
MRSA 10	6	19	MRSA 22	6	14
MRSA 11	12	19	MRSA 23	6	12

Differences in the diameter of the growth inhibition zones between three repetitions of the experiment did not exceed ±1 mm. C—control culture unexposed to RMF.

The next stage of the study aimed to demonstrate whether the changes in susceptibility to β-lactam antibiotics are related to the MRSA mechanism based on the presence of PBP2a protein (whether the effect of changes in susceptibility to β-lactams is observed only in MRSA strains, while in the case of MSSA strains, the changes are not observed) or whether the observed effect is strictly related to the presence of the antibiotic in the microbial culture (changes in antibiotic susceptibility occur in the presence of the antibiotic in the RMF-exposed culture, regardless of the lack of methicillin resistance mechanism). For the purposes of this research, five *S. aureus* strains lacking the *mecA* gene and showing susceptibility to methicillin in a phenotypic test with the cefoxitin-saturated disc were used (Supplementary Figure S1). The findings did not show any changes in the diameters of growth inhibition of RMF-exposed (5 Hz) cultures when the disk diffusion test was applied (Table S2). However, when E-tests were used, for all analyzed MSSA strains differences in MIC values were found between the control and RMF-exposed cultures with cefuroxime and cefepime and, in the case of four strains cultivated with ceftriaxone (in all cases, by one order of concentration value) (Table 6). In turn, no differences were found for cefoxitin. Therefore, it can be assumed that the observed changes in susceptibility to β-lactam antibiotics observed in the case of MRSA strains under the influence of RMF may, at least to some extent, be related to the presence of the alternative PBP2a protein. On the other hand, it can also be noted that the MIC values in the control cultures were relatively low; therefore, it cannot be excluded that the effect of RMF concerning the changes in antibiotic susceptibility has its limitations in the case of strains showing high susceptibility to the analyzed antibiotics.

Table 6. MIC values (μg/mL) for β-lactam antibiotics in control and RMF-exposed (5 Hz) MSSA cultures.

Strain	Cefoxitin		Cefepime		Cefuroxime		Ceftriaxone	
	C	RMF	C	RMF	C	RMF	C	RMF
ATCC 6538	3	3	3	2	1.5	1	3	3
MSSA 1	3	3	4	3	1	0.75	2	2
MSSA 2	3	3	4	3	2	1	6	3
MSSA 3	3	3	4	3	1.5	1	6	4
MSSA 4	3	3	3	2	1	0.75	3	2
MSSA 5	3	3	8	4	2	1.5	8	6

C—control culture unexposed to RMF. There were no differences in MIC values between three separate experiments.

2.2. Effect of RMF on Changes in Number of Culturable Bacteria

The conducted studies also included an assessment of the influence of the RMF on the changes in the number of bacterial cells having ability to form colonies on the M-H agar. The study was performed to detect the possible bactericidal impact of RMF exposure. Although there are numerous literature data presenting the influence of MFs on the growth and viability of bacterial cells [21,32,56,65], the obtained results did not show any significant differences in the CFU number in RMF-exposed vs. control settings (Figure 5). There were also no differences found in the morphology or size of bacterial colonies formed (Supplementary Figure S6). However, in the context of the current work, it is worth noting that our previous research [19,20,22] and the research of most other authors (e.g. Kohno et al., 2000 [56]; Kermanshahi and Sailani, 2005 [65]) were carried out with the use of bacterial cultures in liquid media. Therefore, considering our previously published data regarding, e.g., mixing efficiency under the influence of the RMF [63], it can be noticed that the physical phenomena occurring under the influence of the MFs significantly differs depending on the type of the medium in/on which the bacteria are cultivated during exposure. It should therefore be concluded that for the purposes of the present study, the most important finding was the absence of a bactericidal effect of the RMF on bacterial cells.

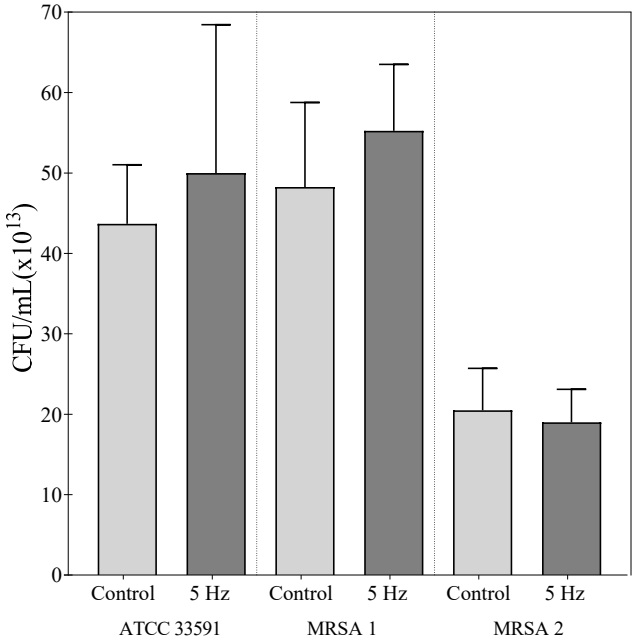


Figure 5. The number of culturable bacteria, expressed as a CFU/mL, after 12 h of RMF exposure (5 Hz) and in control, unexposed setting. The results are presented as a mean ± SEM calculated using six values (three from each biological replicate). The results show no statistically significant differences at $p < 0.05$.

2.3. Effect of RMF on Changes in Relative Number of Live and Dead Bacterial Cells

The data presented in Figure 5 show that the number of culturable bacteria did not differ between RMF-exposed vs unexposed cultures. Nevertheless, the results of quantitative culturing did not provide data on the physiological state of the cells after exposure. Therefore, in the next assay, RMF exposed and unexposed bacterial cultures were stained with a combination of propidium iodide and SYTO9 dyes and visualized by means of fluorescence microscopy. This approach allows not only to distinguish live (dyed green) from dead (dyed red) cells, but also to show changes in the structure of the bacterial cell wall because of the properties of the dye components, which are able to penetrate these cells of bacteria which are living but display a compromised integrity of the cell wall [66]. The further post hoc processing of fluorescent pictures allowed us to change the intensity

of red/green fluorescence into a value referred to as Mean Grey Value (MGV) and, using this parameter, to analyze the changes in the relative number of live (noncompromised) and dead (compromised) cells exposed or unexposed to the RMF.

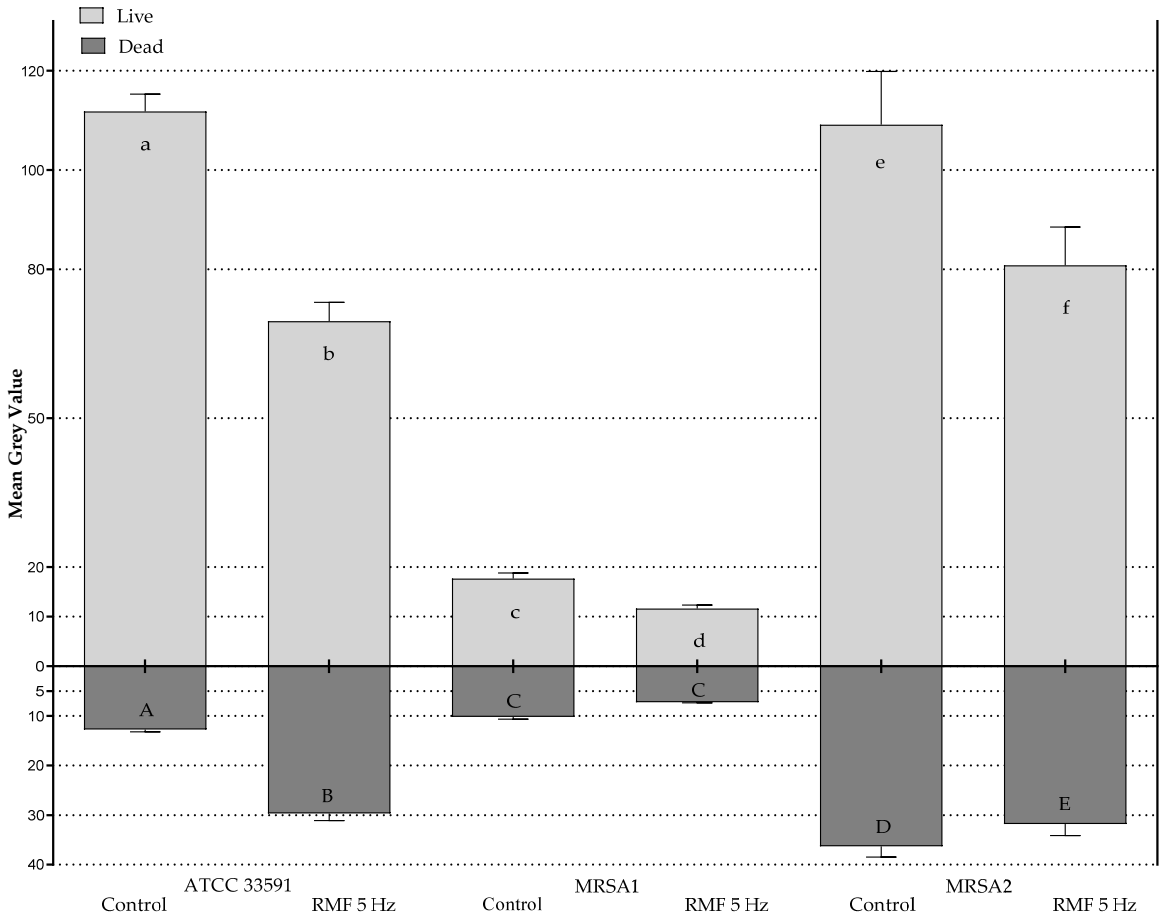
The results presented in Figure 6a show significantly higher intensity of green fluorescence (expressed as MGV), corresponding to the number of live (noncompromised) cells in the cultures unexposed (Figure 6b) compared to the cells exposed to RMF at 5 Hz (Figure 6c). This phenomenon was observed for all investigated MRSA strains. Noteworthy is that the MGV measured for live/non-compromised cells in the control setting differed between strains (with an average MGV value of 111.83 vs. 17.83 vs. 109.14 for strain ATCC 33591, MRSA 1 and MRSA 2, respectively), showing species-specific differences in the ability to multiply on an agar surface. Noteworthy is that the average reduction in the relative number of live cells (calculated from the mean value from the 3 strains analyzed) being the result of exposure to the RMF was 32.64 $\pm$ 6.07% (the MGV value recorded for unexposed strains was considered 100%). The relatively low standard deviation of the mean reduction value shows that, regardless of the strain applied and its ability to multiply on agar, a comparable relative number of cells was affected by the activity of the RMF to the level which can be measured by the applied technique. During the 12 h of exposure to the RMF, the seeded cells form multilayered aggregates on the agar surface and display differentiated properties regarding their metabolic activity and division rate. As was already explained, in this manner, bacteria can try to adapt through intra-strain variability, so that better adapted variants can persist [54]. Such an explanation is in line with the observation of single colonies or partial growth inhibition zones when the antibiotics disc diffusion test was applied. It can also be hypothesized that the reduction in the intensity of green fluorescence (expressed as a drop of MGV) caused by exposure to the RMF, should correlate with a synchronic boost in the number of dead (dyed with propidium iodide) cells. Nevertheless, such a phenomenon occurred only in the case of the ATCC 33591 strain, which displayed the highest value of MGV in the control setting among the analyzed microorganisms (Figure 6a). In the case of MRSA 1 and MRSA 2, the opposite trend was observed (although devoid of statistical significance), namely a drop in red signal intensity coming from the dead (compromised) cells in RMF-exposed cultures. Such a fact may be caused by specific methodological stages applied for L/D dyeing. Propidium iodide (red dye) is incorporated into cells with compromised membranes (dead cells or damaged cells). However, the subsequent stages of rinsing and centrifugation performed during the dyeing procedure cause further damage to the already altered cells and, in effect, lead to their removal (together with the incorporated propidium iodide dye) from the reaction environment. By contrast, the green fluorescence of the SYTO9 dye is microscopically recorded only in live cells (of intact cell walls). Because such cells are less prone to damage, due to rinsing/centrifugation procedures, this results in their higher share (comparing to the cells dyed with propidium iodide) in the pellet later applied for microscopic analyses [67].

2.4. Effect of RMF on Diffusion of Antibiotics

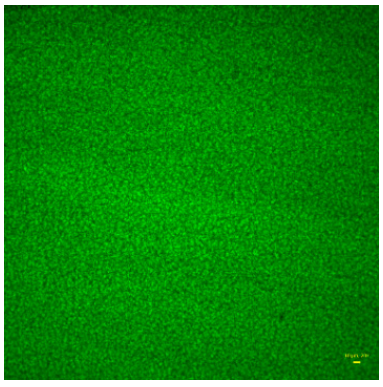
This line of investigation was performed to analyze another variable in the applied experimental system, namely the differences in antibiotic diffusion which may potentially occur between bacterial cultures exposed and unexposed to the RMF. Numerous literature data [32,62], as well as the previous experience of our research group [63] indicated that RMF influence was related to its interactions with electrically charged molecules. Therefore, we aimed to investigate whether magnetic exposure alters the diffusion of the antibiotic in the agar medium. The findings obtained in this part of the study were especially important for the proper interpretation of the results obtained by means of the disk-diffusion method and E-tests. Noteworthy is that the results obtained from the application of the above-mentioned tests allowed drawing a preliminary conclusion that, during exposure to the RMF, the diffusion of antibiotics in the agar medium was not significantly altered. Specifically, the zones of growth inhibition (in the disk-diffusion method) maintained their

oval shape, while the zones in the E-test method remained symmetrical on both sides of the strip soaked with the antibiotic gradient (Figures 2–4).

(**a**)



(**b**)



(**c**)

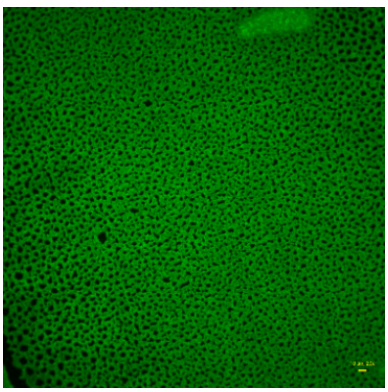


Figure 6. (**a**) The average intensity of fluorescence of dead and live MRSA cells after 12 h of RMF exposure (5 Hz) and in the control, unexposed setting, presented as differences in Mean Grey Value (MGV). (**b**) Picture of dyed with SYTO9 staphylococcal ATCC 33591 cells unexposed and (**c**) exposed to RMF (5 Hz). The results presented in (**a**) are shown as a mean MGV ± SEM calculated using 24 Regions of Interest (ROIs) obtained from four biological replicates. Different letters indicate statistical differences ($p < 0.05$) between RMF-exposed and unexposed cultures of the same staphylococcal strain.

For a more detailed analysis of the potential influence of the RMF on antibiotic diffusion in the agar medium, the concentrations of these antimicrobials in specific parts of the agar plate were measured using the LC-MS/MS method (Supplementary Figure S7). The obtained results did not show any increase in the concentration of antibiotics in the agar due to the RMF exposure (Figure 7). On the contrary, even lower concentrations of antibiotics were detected in the samples cut out from the RMF-exposed agar as compared to the unexposed control. As mentioned before, in our previous work we demonstrated the influence of the RMF on mixing efficiency [63,68]. However, such tests were always carried out in liquids (including liquid microbiological media), while in the case of the analyses performed in the current study, the diffusion took place in 1.7% agar. Thus, it can be assumed that the application of a relatively weak MF (B_{max} 8.3 mT at 5 Hz and B_{max} 8.5 mT at 50 Hz) did not act on the antibiotic particles strongly enough to relocate them significantly through the pores of the agar gel.

2.5. Effect of RMF on Cell Morphology

In the present study we confirmed that the observed RMF effect is related to changes in antibiotic susceptibility but not to the direct bactericidal feature of the RMF (measured by means of quantitative culturing). Moreover, we showed that the presence of RMF does not correlate with changes in the diffusion of antibiotics in the agar medium. The above results encouraged us to perform analyses capable of detection of such subtle phenomena caused by RMF as minor cell damage that could weaken the cell structure without completely destroying it. RMF-exposed cells displayed significant alterations compared to their unexposed counterparts. These changes included mostly the shape and size of cells; also collapse of cell walls and cellular leakage was observed (Figure 8a,b, Supplementary Figure S8). Noteworthy is that with the exception of the last type of alteration, of a rather irreversible character, one may assume that changes in such altered cells as those pictured in Figure 8a (and marked with numbers 2 and 3) could be reversed [69] if the cells were seeded in a fresh microbiological medium and cultivated without RMF presence. Such a phenomenon largely explains the lack of changes in the number between cultivable bacteria exposed and unexposed to RMF (Figure 5) and a drop in live cells (dyed with SYTO9) in the RMF-exposed vs unexposed setting (Figure 6).

The results related to durability of the effect of exposure to RMF (post-exposure effect) showed that bacterial cultures previously exposed to RMF behaved in the same way as the control unexposed cultures, i.e., the antibiotic susceptibility level was the same for both types of cultures (Supplementary Table S3). Thus, despite the inevitable biases related to the applied methodology, the results of this part of the investigation suggest that RMF acts by partial disintegration of staphylococcal cell walls. This statement is particularly important in the context of issues presented in this article (Tables 1–6) because it satisfactorily explains the increased efficacy of β-lactam antibiotics acting against already weakened (as a result of RMF exposure) staphylococcal cell walls. Therefore, another SEM analysis was performed to visualize this hypothetical, boosting effect of the RMF on cefoxitin activity. Figure 8c shows staphylococcal population (resistant to cefoxitin) grown on agar with the aforementioned antibiotic applied. Figure 8d shows the same resistant staphylococcal strain grown on agar with cefoxitin introduced, but additionally exposed to the RMF (5 Hz). It can also be seen that the application of the RMF together with cefoxitin translated into explicit morphological changes of staphylococcal cells, including alteration of cell shape, collapse of cell wall, decreased turgor and reduced size (probably effected by cytoplasmic leakage). Noteworthy is that these changes were more ubiquitous in the discussed setting (RMF + antibiotic) than in a setting when only RMF was applied (please compare Figure 8a with Figure 8d).

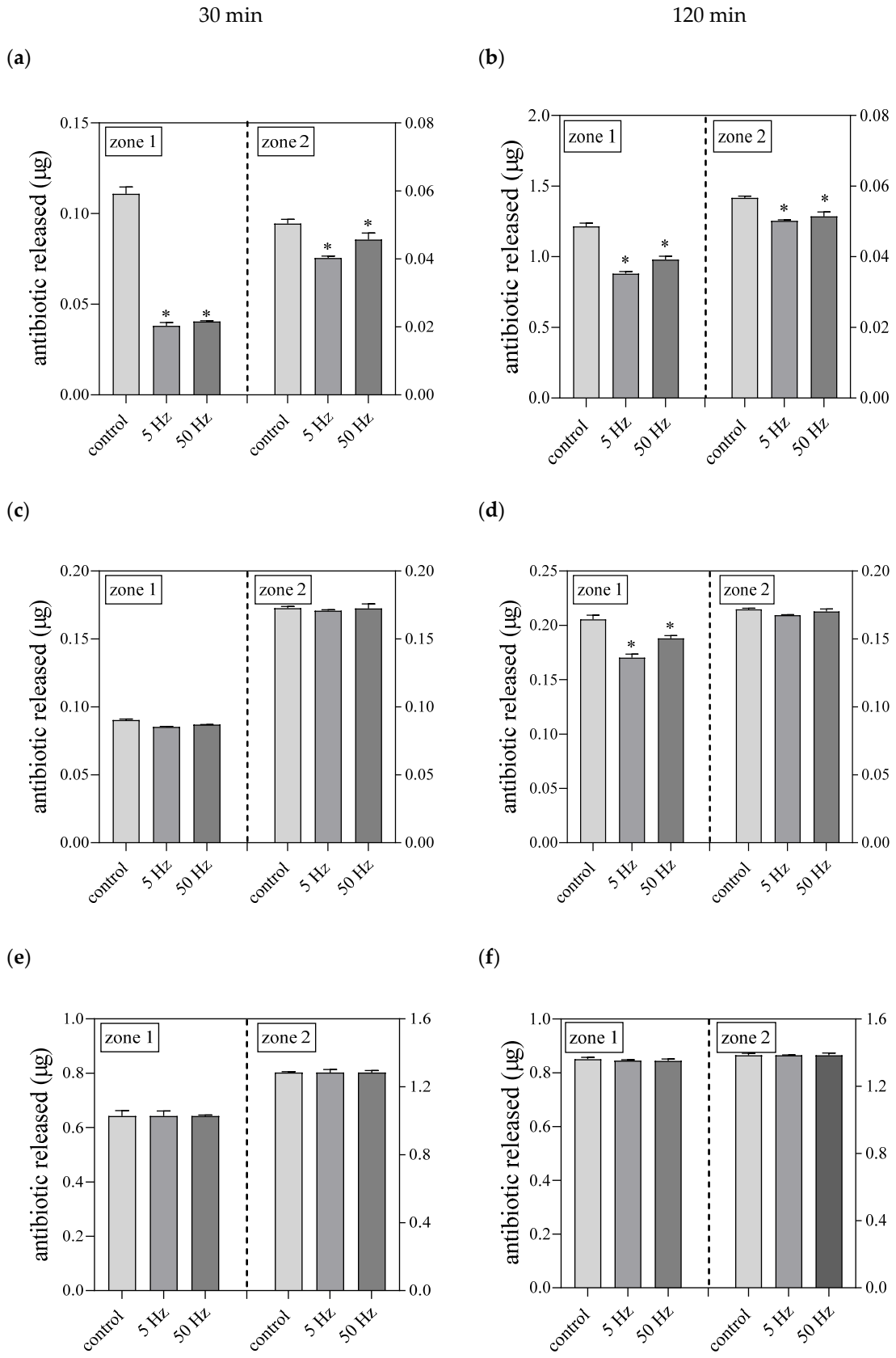


Figure 7. Concentrations of antibiotics in agar in control and RMF-exposed (5/50 Hz) settings; (**a**,**b**) cefoxitin; (**c**,**d**) cefepime; (**e**,**f**) imipenem. The results are presented as mean ± SEM calculated using six values (three from each biological replicate). *—indicates statistical differences ($p < 0.05$) between control and RMF-exposed settings.

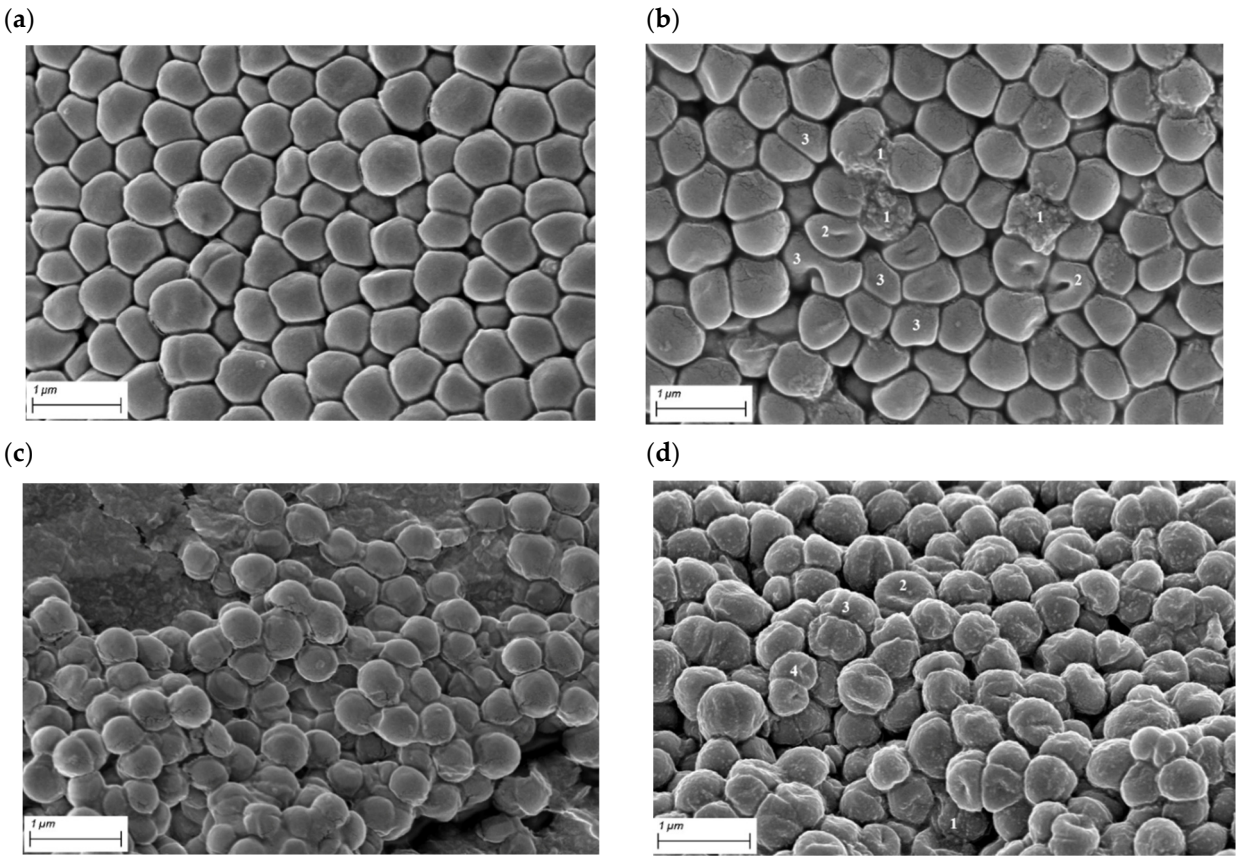


Figure 8. Scanning electron microscopy images: (**a**) MRSA 1 cells unexposed to the RMF; (**b**) MRSA 1 cells exposed to the RMF (5 Hz); (**c**) MRSA 2 cells seeded on an agar plate in the presence of a disc containing cefoxitin; (**d**) MRSA 2 cells seeded on an agar plate in the presence of a disc containing cefoxitin and exposed to the RMF (5 Hz). 1—cellular content leakage; 2—collapse of cell wall; 3—strong deformation of cellular shape, 4—cell size reduction. Scale bars represent 1 μm.

To get additional data concerning RMF impact on staphylococcal cell morphology, cross-sections of bacterial cells (exposed and unexposed to the RMF) were performed using transmission electron microscopy. In Figure 9 and Supplementary Figure S9, representative pictures of the clinical strain MRSA 1 are presented. Pictures a,b of Figure 9 show bacterial cells unexposed to RMF, with an oval shape typical for staphylococci, whereas the RMF-exposed staphylococcal cells present deformed morphology, particularly well-visible in pictures c,d of Figure 9. The deformation pattern included cell elongation (loss of oval shape, picture c of Figure 9) or formation of bulges (indicated with red arrows in Figure 9d,f). As seen in Figure 9a,b, the walls of unexposed cells are evenly contrasted and strongly distinguished from the cytoplasm, while in RMF-exposed cells a lower contrast was observed between the cytoplasm and the cell wall (marked with green arrows in Figure 9), indicating a loss of wall density. In the case of RMF-exposed bacteria, contraction of the cytoplasm is also visible, manifested by an uneven border between the cytoplasm and the cell wall (blue arrows in Figure 9) suggesting lower cytoplasm density. Such a cytoplasmic condensation induced by membrane damage was recently observed in Gram-negative *E. coli* by other authors [70]. The above-mentioned observations suggest that RMF activity translates into a reduction in cell wall density, which in turn manifests itself by cell shape deformations and (at least) partial leakage of cytoplasm.

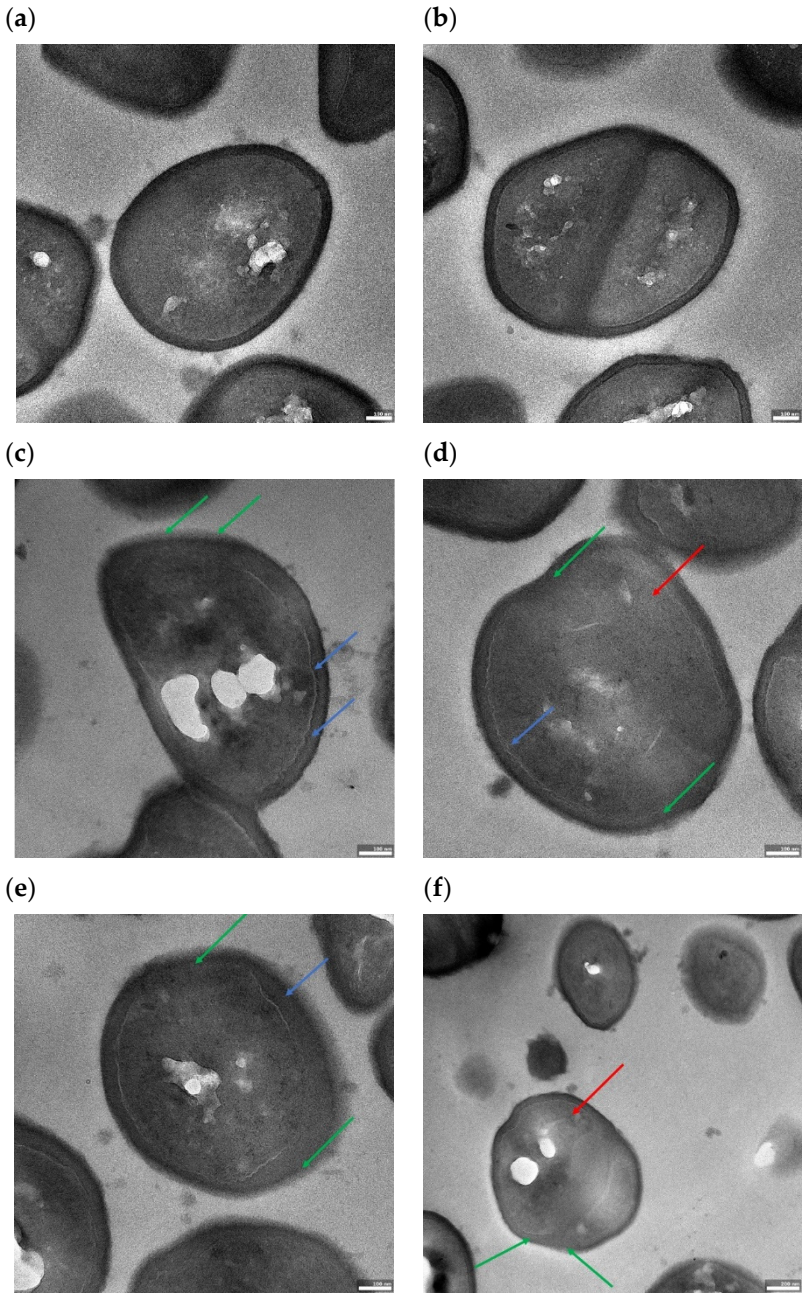


Figure 9. Transmission electron microscopy cross-section of (**a**,**b**) MRSA 1 cells unexposed to the RMF; (**c**–**f**) MRSA 1 cells exposed to the RMF (5 Hz). Red arrows indicate deformation of shape of staphylococcal cells after exposure to RMF. Green arrows indicate loss of cell wall density after exposure to RMF. Blue arrows indicate contraction of cytoplasm after exposure to RMF. Scale bars represent 100 nm (picture **a**–**e**) and 200 nm (picture **f**). Magnified regions of interest are additionally presented in Supplementary Figure S9. The cellular shapes of ca. 400 nm diameter seen in the image 9f are staphylococcal cells cut in their apex parts.

The following hypothesis is thus worth considering. As already mentioned, MRSA strains carrying the *mecA* gene encoding an alternative form of PBP, called PBP2a, have a reduced (to a varying extent) affinity for β-lactam antibiotics [61,71]. Therefore, PBP2 and PBP2a are not the same in different strains (the polymorphism of the *mecA* complex may affect the function of these genes and methicillin resistance mechanism) and thus these proteins can show different affinity for the same β-lactam antibiotic. The EUCAST defines the presence of methicillin resistance when the inhibition zone is ≥22 mm, not

only when there is no inhibition zone. This means that binding of β-lactam antibiotics to PBP2a protein occurs and translates to a certain level of inhibition of peptidoglycan synthesis in MRSA strains. The process of binding, and the aforementioned inhibition, are significantly less effective compared to MSSA strains expressing unmodified PBP protein, as well as compared to the competitive reaction with peptidoglycan chains. Thus, given the competitive mechanism, a large proportion of the PBP2a is not blocked and is involved in the cross-linking of the peptidoglycan chains, and so the cell survives (at least in relatively low concentrations of the β-lactam antibiotic). Considering the observations using SEM, TEM and fluorescence microscopy suggesting that the RMF induced disturbances in the structure of the bacterial cell wall, it can be assumed that the small amount of β-lactam antibiotic, which blocks the activity of some of the entire PBP2/PBP2a protein present in the MRSA cell, is sufficient to induce further structural changes in the cell wall, which, in effect, makes it impossible to maintain the intracellular osmotic pressure and leads to cell disintegration. Additionally, considering one of the RMF interaction mechanisms associated with increasing the mixing efficiency [63], which apply to both the external and internal environment of the cell culture and cells, it can also be assumed that in the RMF-exposed bacteria there is an additional increase in the pressure of the external and intracellular liquid on the cell wall that may lead to its further damage.

3. Conclusions

The presence of MF is one of the most basic properties of our physical reality. It escapes our sensual experience and can be described only by the use of mathematical formulas. Noteworthy is that the specific RMF created for the purposes of this study, has no reflection in the natural environment. It is a type of stimulus unknown (contrary to the geomagnetic field) to the bacterial pathogens we challenged by its means. The results of our study indicate that the analyzed reference and clinical *mecA*-positive MRSA isolates were prone to this stimulus, which manifested itself in the substantially elevated susceptibility to most of β-lactam antibiotics. By means of differentiated techniques and approaches we were able to indicate that this susceptibility is most likely related to RMF-induced changes in staphylococcal cell walls. These alterations were subtle enough to be missed when basic microbiological techniques (e.g., quantitative culturing) were applied. However, they were revealed by means of, among others, SEM or TEM microscopy. Nevertheless, the data obtained by means of basic microbiological techniques allowed refinement of the spectrum of possibilities standing behind the observed phenomenon and brought us to the conclusions presented above.

The constantly growing number of methicillin-resistant strains (of nosocomial and community origin) warrants the search for new treatment options. In the light of the results presented in this study, the application of the RMF together with β-lactam antibiotics could be a promising direction to follow. Taking into account the high clinical need for the provision of new therapeutic options effective against methicillin-resistant strains, the data presented in this study have high developmental potential and could provide the basis for new treatment options for MRSA infections.

4. Materials and Methods

4.1. Microorganisms

Two reference staphylococcal strains (one MRSA-American Type Culture Collection (ATCC 33591) and one MSSA (ATCC 6538)) and two clinical MRSA isolates (MRSA 1 and MRSA 2) were used for experimental purposes. In the course of the investigation, an additional 24 clinical isolates including 19 MRSA (MRSA 3–MRSA 21) and 5 MSSA (MSSA 1–MSSA 5) were used in specific experiments. All analyzed clinical isolates belonged to the Strain Collection of the Department of Pharmaceutical Microbiology and Parasitology of Wroclaw Medical University. These strains originated from chronic wounds of patients treated in the Teaching Hospital of Wroclaw Medical University (Wroclaw, Poland). The strains' species affiliation was confirmed in the first step by macroscopic

observation of the specific colonies (yellow/golden oval shapes with distinct β-hemolysis zones) on Columbia agar (Graso Biotech, Jablowo, Poland). Then, the colonies were transferred into Müller-Hinton agar (M-H, Graso Biotech, Jablowo, Poland) and analyzed using the automated Becton-Dickinson Phoenix 100 system (Franklin Lakes, NJ, USA) for microorganisms' biochemical identification. Next, all *S. aureus* isolates were tested for methicillin resistance according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021) [50], using the disk diffusion method with cefoxitin antibiotic (30 µg). The resulting zones of growth inhibition were interpreted according to the breakpoint tables (EUCAST, 2021) [72] in which zone diameter ≥22 mm indicates a strain's susceptibility to methicillin, while zone diameter <22 mm indicates the strain's resistance to methicillin.

4.2. Rotating Magnetic Field Generator

The base of each RMF reactor (Figure 10) was a 3-phase, four-pole stator with an internal core diameter of 16 cm and height of 20 cm equipped with twelve groups of three coils sets [73]. The alternating current (AC) frequency supplied to the RMF generator was controlled using a Unidrive M200 inverter (Control Techniques, Nidec Industrial Automation, Poznan, Poland). The temperature in the RMF reactor chamber was maintained using a semiautomatic water fed cooling/heating system monitored by a set of temperature probes with sampling deviation in the accuracy range ±1.0 °C. The correct temperature distribution in the RMF reactor chamber was ensured by air flow supplied continuously throughout the exposure (2 L/min, 35 °C, RH 60%). The characteristics of the RMF, including the distribution of magnetic induction (*B*) in the reactor chamber were performed at an initial voltage of 100 VAC and AC frequencies of 5, 10, 25 and 50 Hz using Ansys Maxwell simulation software ver.19.1 (ANSYS, Inc., Canonsburg, PA, USA) and confirmed empirically using a teslameter (SMS-102, Asonik, Tuczno, Poland) equipped with a transverse probe.

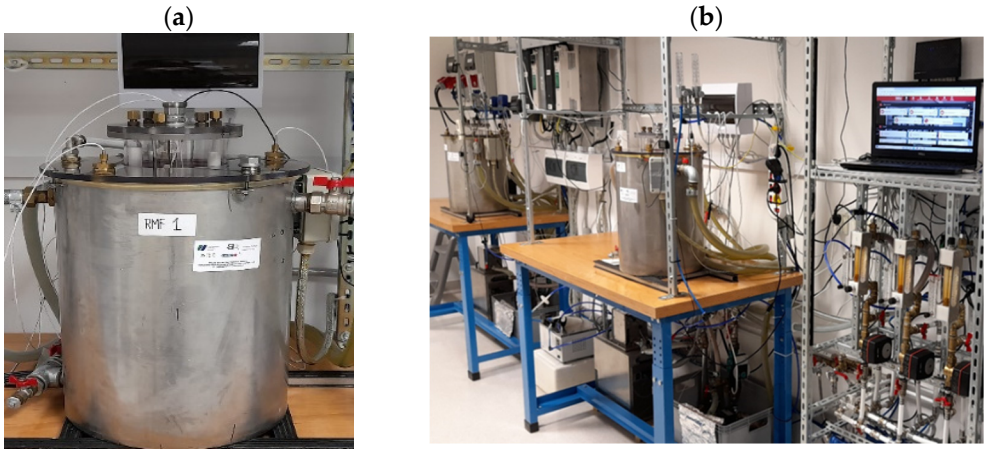


Figure 10. (**a**) RMF generator with (**b**) monitoring and control equipment.

4.3. Detection of mecA Gene

Total DNA was extracted from bacterial cultures using a DNA Bacterial & Yeast Genomic Purification Kit (EURx, Gdansk, Poland), according to the manufacturer's instructions. The presence of the *mecA* gene was detected by the PCR method using primers previously described by Oliveira and de Lencastre (2002) [74]. The PCR reaction mixture (25 µL) consisted of 6.25 µL 2x PCR Mix Plus (A&A Biotechnology, Gdansk, Poland), 0.4 µM of each primer, and 20–50 ng of DNA. The PCR conditions were as follows: initial denaturation of DNA at 95 °C for 2 min, 30 cycles (95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min) and final extension at 72 °C for 5 min [75]. PCR products were analyzed using 2% agarose gel (Prona Agarose, Burgos, Spain) electrophoresis in 1x Tris-borate-EDTA buffer (Thermo Scientific, Vilnius, Lithuania). The DNA was stained with ethidium bromide

(Merck, Darmstadt, Germany), visualized under UV light and analyzed using GeneTools software (Syngene, Cambridge, UK). *S. aureus* control strains included ATCC 33591 (*mecA* positive) [76] and ATCC 6538 (*mecA* negative) [77]. PCR for non-template controls was also performed in each analysis to exclude potential DNA contamination.

4.4. Analysis of the Impact of RMF on Changes in Antibiotic Susceptibility

4.4.1. Disc Diffusion Method

In the first stage of the study, the impact of continuous exposure to the RMF generated at specific AC frequencies (5, 10, 25 and 50 Hz) for 18 h on the change in susceptibility to β-lactam antibiotics, including cefoxitin, was analyzed. The location of the Petri dishes in the RMF reactor chamber and the location of antibiotic discs in Petri dishes is presented in Figure 11.

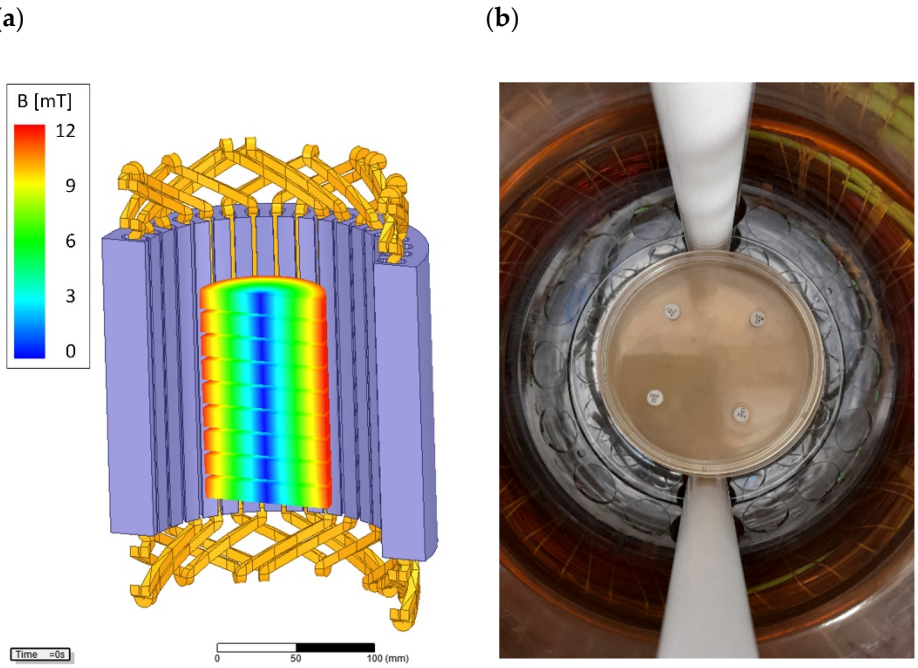


Figure 11. (**a**) Arrangement and location of the Petri dishes and (**b**) antibiotic discs in RMF reactor chamber.

The rationale behind the use of cefoxitin is the recommendation of this antibiotic by EUCAST (2021) [72] as the indicator of methicillin resistance, which, in turn, is interpreted as resistance to a prevailing majority of β-lactam antibiotics. To demonstrate whether the observed changes and analyzed dependencies in susceptibility may be applied to cephalosporins other than cefoxitin, cefepime, cefuroxime and ceftriaxone were also selected and analyzed in the same way as it was performed for cefoxitin.

In the second stage of the study, the efficient exposure time to the RMF (generated at optimal AC frequency, which was defined on the basis of the results of the first stage), ranging from 1 to 12 h, was determined. After completion of the exposure time, the plates containing staphylococcal cultures with antibiotic discs were taken out from the RMF generator and incubated at 35 ± 1 °C without RMF until 18 h of incubation were completed (total amount of time consisting of exposure and nonexposure period). The zones of growth inhibition were measured after incubation. If inhibition zones were visible after the end of exposure to RMF, they were measured at this time point and once more after completion of entire incubation time.

In the third stage of the study, the impact of RMF on the changes in susceptibility of different groups of β-lactams, including penicillins, cephalosporins (1st–5th generation) and carbapenems was analyzed.

In the fourth stage of the research, the impact of the RMF on the changes in the susceptibility to cefoxitin was examined with the use of 21 MRSA strains. The rationale behind this approach was to exclude the possibility that the observed effect occurred only in three MRSA strains applied in the previous experimental stages.

In the fifth stage, five MSSA clinical isolates and one MSSA reference strain (ATCC 6538) were exposed to the RMF in the presence of a cefoxitin-saturated disc. The aim of this experimental line was to discriminate whether the earlier observed changes in β-lactam susceptibility in the presence of RMF were related to the interaction of the RMF and antibiotics or were related to the mechanism of resistance to β-lactam antibiotics (based on the presence of the alternative PBP2a protein), which MSSA strains do not possess.

In stages from three to five, the optimal RMF frequency (the frequency causing the greatest and stable changes in the antibiotic susceptibility of the analyzed MRSA strains) and the optimal exposure time (exposure time after which the maximal and lasting zones of growth inhibition were observed) were applied using data obtained in the previous stages of the research.

Antibiotic susceptibility testing was carried out according to the guidelines of EUCAST (2021) [50]. The bacterial cultures were adjusted to a 0.5 McFarland standard, which approximately corresponds to 1–2 $\times$ 10^8 CFU/mL. A sterile cotton swab was used to spread inoculums evenly over the surface of the M-H agar (Graso Biotech, Jablowo, Poland) plates. After the application of antimicrobial discs on the agar surface, the bacteria were exposed to RMF in accordance with the aforementioned variants. The same bacterial cultures, incubated under the same conditions but without exposure to the RMF, were used as a control setting. Both in the RMF generator and the incubator, the same temperature (35 $\pm$ 1 °C) and relative humidity RH (60%) were maintained throughout the entire experiment.

The following antibiotic discs were used: cefoxitin (30 μg/disc), amoxicillin (10 μg/disc), ceftazidime (10 μg/disc), cephradine (30 μg/disc), cephalexin (30 μg/disc), ceftaroline (5 μg/disc), cefepime (30 μg/disc), cefuroxime (30 μg/disc), ceftraxone (30 μg/disc), cefotetan (30 μg/disc), meropenem (10 μg/disc), imipenem (10 μg/disc), ertapenem (10 μg/disc), doripenem (10 μg/disc), cefazolin (30 μg/disc) (Oxoid, Basingstoke, UK).

4.4.2. Gradient MIC Strips (E-Test)

Because the disk diffusion method allows to analysis of the impact of a single concentration of an antibiotic/disk, in this experiment E-test strips with exponentially decreasing antibiotic gradient were applied. In this investigation line, the antibiotics were selected whose application, coupled with exposure to RMF, caused the largest and the smallest differences in the zones of growth inhibition in the disc diffusion method (compared to unexposed controls). The E-tests containing cefoxitin, amoxicillin, imipenem, ceftriaxone, cefuroxime, ceftazidime and cefepime were obtained from Liofilchem (Roseto degli Abruzzi, Italy), whereas the E-test containing meropenem was purchased from BioMérieux (Craponne, France).

Similarly, as in the case of the disc diffusion method, the optimal RMF frequency and exposure time, as well as unexposed controls were used. The analyses were performed on M-H agar in accordance with the E-test manufacturer's recommendations.

4.5. Analysis of the Impact of RMF on Changes in the Number of Culturable Bacteria

The conducted studies also included assessment of the influence of the RMF on the number of bacterial cells expressing an ability to form colonies on the M-H agar. For this purpose, bacterial cultures prepared as for antibiotic susceptibility testing were exposed to the RMF generated at the optimal frequency and exposure time. Next, 2 mL of sterile PBS was poured onto each culture plate, mixed thoroughly with a spreader, and transferred into a sterile test tube. The process was repeated three times to remove bacteria from the agar plate. In the next step, decimal dilutions of the obtained suspension were made, inoculated on BHI (Graso Biotech, Jablowo, Poland) agar and incubated for 24 h at 37 °C. After incubation, the colonies were counted and expressed as number of CFU/mL.

4.6. Analysis of the Impact of RMF on Changes in the Relative Number of Live and Dead Bacterial Cells Using Fluorescence Microscopy and Picture Processing

The microbial cultures were prepared and exposed to the RMF at the optimal frequency and exposure time as performed in the analyses of the number of culturable bacteria. Staphylococcal liquid cultures were centrifuged, and the obtained cell-free supernatant was removed and replaced with 1 mL of LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Invitrogen, Thermo Fisher Scientific, Bend, OR, USA) solution. The samples were then vortex-mixed, spin-centrifuged and incubated at room temperature for 15 min. After incubation, the samples were again centrifuged, and cell-free LIVE/DEAD (Live, *L*-SYTO9 dye; Dead, *D*-propidium iodine dye) solution was removed. The dyed staphylococcal pellet was resuspended in 100 µL of sterile water and introduced to the wells of a 24-well plate (VWR, Randor, PA, USA). The samples were left to dry at room temperature in darkness while shaking at 100 rpm (Lab Companion IST 30-075, Oxfordshire, UK). The rationale behind this stage was even propagation of bacterial cells throughout the surface of the plate's well. Subsequently, the pictures of dyed staphylococcal cells were captured using Lumascope 620 (Etaluma, Carlsband, CA, USA) with 20× magnification objective Olympus IPC phase (Shinjuku, Japan). The field of vision recorded was 0.49 mm and frame size was 1200 × 1200 pixels. The excitation/emission wavelengths for SYTO9 and propidium iodide were 480/500 nm and 490/635 nm, respectively. Next, the pictures were processed using ImageJ (National Institutes of Health, Bethesda, MD, USA) software. The whole captured picture was treated as the Region of Interest (ROI). The mean grey value (MGV) of each ROI was recorded for green and red fluorescence channels and served as an estimator of changes in the number of live and dead cells, respectively. The MGV was considered the sum of gray values of all the pixels in the selection divided by the number of pixels. For RGB images recorded for the purpose of this analysis, the MGV was calculated by converting each pixel to grayscale using the Equation (1) [78]:

$$\text{gray} = 0.299\text{red} + 0.587\text{green} + 0.114\text{blue} \quad (1)$$

In total, 24 ROIs from each channel were recorded for each sample.

4.7. Analysis of the Impact of RMF on the Diffusion of Antibiotics in the Agar Medium

To analyze the potential influence of the RMF on the changes in antibiotic diffusion, the same discs as those used for the analysis of antibiotic resistance were placed on Petri dishes with 1.7% agar (agar concentration was equal to the concentration of M-H agar applied in previous experiments), and exposed to the RMF generated at 5 and 50 Hz for 30 and 120 min. After each time-point, one plate was removed from the RMF generator and, using a cork borer, cylindrical agar samples 6 mm in diameter were cut out (four samples from the proximal zone (zone 1), and eight samples from the distal zone (zone 2), representing 50% of the total agar volume in each zone). The agar samples were cut out radially starting from the edge of the antibiotic disc towards the edge of the Petri dish (Figure S7). To extract the antibiotic, the agar samples were placed in 0.5 mL of methanol (Stanlab, Lublin, Poland) in deionized water (1:1) and incubated in a shaker (250 rpm; Biosan, Riga, Latvia) at room temperature for 2 h. Then, the samples were removed, the methanol-water mixtures with the extracted antibiotics were filtered through a syringe filter (0.22 µm pore diameter) and analyzed by the liquid chromatography, tandem mass spectrometry (LC-MS/MS) technique (1260 Infinity II Series Liquid Chromatograph, Agilent, Santa Clara, CA, USA). An InfinityLab Poroshell 120 EC-C18 column (Agilent, Santa Clara, CA, USA) with a particle diameter of 2.7 µm equipped with a guard column was used for the chromatographic separation. The mass spectrometer (Ultivo G6465B, Agilent, Santa Clara, CA, USA) coupled to the chromatograph was used to detect and identify the tested analytes. Quantitative analysis was performed based on calibration curves prepared with the use of high purity antibiotic standards (Millipore Sigma, St. Louis, MO, USA). The results were converted and presented as the total concentration of antibiotic released to proximal zone 1 and distal zone 2.

4.8. Analysis of the Impact of RMF on Staphylococcal Cell Morphology

To assess the influence of the RMF on bacterial cell morphology, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analyses were performed. First, the cultures were prepared and exposed to the RMF as described for analyses of the number of culturable bacteria (no antibiotics were used). After exposure, cylindrical samples with a diameter of 6 mm were cut out from the M-H agar medium with the bacterial culture using a cork borer. Four samples, at least 2 cm apart, were cut out from each culture. In the second part of the experiment, the samples were prepared during the disc diffusion-based tests related to the changes in antibiotic susceptibility to cefoxitin. For this purpose, in the control setting (RMF-unexposed cultures with no inhibition zones), the agar samples were cut out next to the antibiotic discs and, in the case of the RMF-exposed samples, from the edge of the inhibition zones.

4.8.1. Scanning Electron Microscopy

All collected samples were washed gently with PBS and fixed by immersion in 3% glutaraldehyde (POCH, Gliwice, Poland) for 15 min at room temperature. The samples were rinsed twice with PBS to remove the fixative. Dehydration in increasing concentrations of ethanol (25, 50, 60, 70, 80, 90, and 100% POCH) was performed for 10 min per solution. The ethanol was then rinsed off, and the samples were dried at room temperature. Next, the samples were covered with gold and palladium (60:40; sputter current, 40 mA; sputter time, 50 s) using a Quorum machine (Quorum International, Fort Worth, TX, USA) and examined under a Zeiss EVO MA25 scanning electron microscope (SEM) (Carl Zeiss, Jena, Germany).

4.8.2. Transmission Electron Microscopy

The samples were fixed in 2% glutaraldehyde (POCH) and centrifuged (5 min, 50 μf). Contrasting was performed with 2% uranyl acetate (MicroShop, Piaseczno, Poland) (8 h) and 2% osmium tetroxide (Agar Scientific, Stansted, UK) (2 h) in the dark. The material was then passed through an ascending alcohol series (POCH from 30% to 99.8%) and embedded in epoxy resin (Agar Scientific, Stansted, UK). Sections of 60 nm thickness were prepared from the resin blocks using an UltraMicrotome Leica EMUC7 (Leica, Wetzlar, Germany) and placed on copper grids (400 Mesh) with formvar film and carbon coating (Agar Scientific, Stansted, UK). Imaging was performed using a JEOL 1200, (JEOL, Tokyo, Japan) microscope.

4.9. Analysis of the RMF Post-Exposure Effect

This experiment aimed to assess whether the changes in antibiotic susceptibility occur only during the RMF exposure, or whether they remain fixed as a result of the exposure performed prior to the antibiotic testing. Liquid bacterial cultures of cell density equal to 0.5 of McFarland turbidity standard prepared in a M-H medium were exposed to RMF at the optimal frequency and exposure time. Next, the cultures were centrifuged, the pellet resuspended in PBS to obtain the initial cell density and used as an inoculum for the antibiotic (cefoxitin, cefuroxime, cefepime and ceftriaxone) susceptibility test. Growth inhibition zones were compared with the zones obtained in the control cultures performed using the inoculum never exposed to the RMF.

4.10. Statistical Analysis

The data obtained in this study (number of culturable bacteria, mean grey value and concentrations of antibiotics in agar in the control and RMF-exposed settings) were presented as means ± standard errors of the means (SEM) obtained from at least three different measurements (plus technical repetitions). Statistical differences between RMF-exposed and control, unexposed settings were determined by one-way analysis of variance (ANOVA) and Tukey's post hoc test. Differences were considered significant at a level of $p < 0.05$. The statistical analyses were conducted using Statistica 12.5 (StatSoft, Inc. Tulsa, OK, USA).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms222212397/s1.

Author Contributions: Conceptualization, K.F and M.W. (Marta Woroszyło); methodology, K.F.; M.W. (Marta Woroszyło) and A.J; formal analysis, K.F.; M.W. (Marta Woroszyło) and A.J.; investigation, M.W. (Marta Woroszyło); D.C.-J.; A.J.; R.D.; M.W. (Marcin Wardach); P.M.; P.S.-Z. and D.S.; resources, A.J.; data curation K.F.; writing—original draft preparation, K.F. and M.W. (Marta Woroszyło); writing—review and editing, K.F. and A.J; visualization, M.W. (Marta Woroszyło), D.C.-J.; M.W. (Marcin Wardach); supervision, K.F.; project administration, K.F.; funding acquisition, K.F. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Załącznik 2

Kopie suplementów publikacji naukowych wchodzących w skład cyklu stanowiącego rozprawę doktorską

Marta Woroszyło

Rozprawa doktorska

ANALIZA WPŁYWU WIRUJĄCEGO POLA MAGNETYCZNEGO NA EFEKTYWNOŚĆ DZIAŁANIA SUBSTANCJI PRZECIWDROBNOUSTROJOWYCH WZGLĘDEM BAKTERII PATOGENNYCH

Supplementary Material

The Impact of Bacterial Intra-species Variability on Biological Effects Exerted by Rotating Magnetic Field

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Table S1. Phenotypic patterns of *S. aureus* strains.

Phenotypic pattern	Strain number							
	1	2	3	4	5	6	7	ATCC 6538
AMY	-	-	-	-	-	-	-	-
APPA	-	-	-	-	-	-	-	-
LeuA	-	-	-	-	-	-	-	-
AlaA	-	-	-	-	-	-	-	-
dRIB	-	-	-	-	-	-	+	-
NOVO	-	+	-	-	-	-	+	-
dRAF	-	-	-	-	-	-	-	-
OPTO	+	+	+	+	+	+	+	+
PIPLC	-	-	-	-	-	-	-	-
CDEX	-	-	-	-	-	-	-	-
ProA	-	-	-	-	-	-	-	-
TyrA	-	-	-	-	-	-	-	-
ILATk	+	-	+	-	-	+	+	+
NC6.5	+	+	+	+	+	+	+	+
O129R	+	+	+	+	+	+	+	-
dXYL	-	-	-	-	-	-	-	-
AspA	-	-	-	-	-	-	-	-
BGURr	-	-	-	-	-	-	-	-
dSOR	-	-	-	-	-	-	-	-
LAC	-	-	+	-	-	-	-	-
dMAN	+	+	+	+	+	+	+	+
SAL	-	-	-	-	-	-	-	-
ADH1	+	+	+	+	+	+	+	+
BGAR	-	-	-	-	-	-	-	-
AGAL	-	-	-	-	-	-	-	-
URE	-	-	-	-	-	-	-	-
NAG	+	+	+	-	+	+	+	+

dMNE	+	+	+	+	+	+	+	+
SAC	+	+	+	+	+	+	+	+
BGAL	+	-	+	-	+	-	-	-
AMAN	-	+	-	-	-	-	-	-
PyrA	+	+	+	+	+	+	+	+
POLYB	+	+	+	+	+	+	+	+
dMAL	+	+	+	+	+	+	+	+
MBdG	+	-	-	+	+	+	+	-
dTRE	+	+	+	+	+	+	+	+
AGLU	+	+	-	+	+	+	+	-
PHOS	+	+	+	+	+	+	+	+
BGUR	-	-	-	-	-	-	-	-
dGAL	+	+	+	-	+	-	-	+
BACI	+	+	+	+	+	+	+	+
PUL	-	-	-	-	-	-	-	-
ADH2s	+	-	-	-	-	-	-	+

AMY - D-Amygdalin; APPA - Ala Phe Pro arylamidase; LeuA - Leucine arylamidase; AlaA - Alanine arylamidase; dRIB - D-Ribose; NOVO - Novobiocin resistance ; dRAF - D-Raffinose; OPTO - Optochin resistance; PIPLC - Phosphatidylinositol phospholipase C; CDEX - Cyclodextrin; ProA - L-Proline arylamidase; TyrA - Tyrosine arylamidase; ILATk - L-Lactate alkalinization; NC6.5 - Growth in 6.5% NaCl; O129R - O/129 Resistance (comp. vibrio.); dXYL - D-Xylose; AspA - L-Aspartate arylamidase; BGURr - β-Glucaronidase; dSOR - D-Sorbitol; LAC - Lactose; dMAN - D-Mannitol; SAL - Salicin; ADH1 - Arginine dihydrolase 1; BGAR - β-Galactopyranosidase; AGAL - α-Galactosidase; URE - Urease; NAG - *N*-Acetyl-D-glucosamine; dMNE - D-Mannose; SAC - Saccharose/sucrose; BGAL - β-Galactosidase; AMAN - α-Mannosidase; PyrA - L-Pyrrolidonyl-arylamidase; POLYB - Polymixin B resistance; dMAL - D-Maltose; MBdG - Methyl-β-D-glucopyranoside; dTRE - D-Trehalose; AGLU - α-Glucosidase; PHOS - Phosphatase; BGUR - β-Glucaronidase; dGAL - D-Galactose; BACI - Bacitracin resistance; PUL - Pullulan; ADH2s - Arginine dihydrolase 2;

The study of growth dynamics and cellular metabolic activity – statistical analyses

Table S2. Statistical differences in growth dynamics between *S. aureus* strains representing different clonal types exposed for 3 h to rotating magnetic field of 5 Hz.

	ATCC 6538	1	2	3	4	5	6	7	control
ATCC 6538	x	-	-	-	-	-	-	-	-
1	-	x	-	-	-	-	***	-	-
2	-	-	x	-	-	-	-	-	-
3	-	-	-	x	-	-	*	-	-
4	-	-	-	-	x	-	**	-	-
5	-	-	-	-		x	-	-	-
6	-	***	-	*	**	-	x	**	****
7	-	-	-	-	-	-	**	x	-
control	-	-	-	-	-	-	****	-	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S3. Statistical differences in growth dynamics between *S. aureus* strains representing different clonal types exposed for 6 h to rotating magnetic field of 5 Hz.

	ATCC 6538	1	2	3	4	5	6	7	control
ATCC 6538	x	**	****	-	-	-	*	-	*
1	**	x	-	-	-	-	-	-	-
2	****	-	x		*	*	-	*	-
3	-	-		x	-	-	-	-	-
4	-	-	*	-	x		-	-	-
5	-	-	*	-	-	x	-	-	-
6	*	-		-	-	-	x	-	-
7	-	-	*	-	-	-	-	x	-
control	*	-	-	-	-	-	-	-	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S4. Statistical differences in growth dynamics between *S. aureus* strains representing different clonal types exposed for 9 h to rotating magnetic field of 5 Hz.

	ATCC 6538	1	2	3	4	5	6	7	control
ATCC 6538	x	-	****	****	*	-	****	***	****
1	-	x	****	**	-	-	****	*	***
2	****	****	x	-	****	****	-	**	**
3	****	**	-	x	-	**	-	-	-
4	*	-	****	-	x	-	-	-	-
5	-	-	****	**	-	x	***	-	**
6	****	****	-	-	-	***	x	-	-
7	***	*	**	-	-	-	-	x	-
control	****	***	**	-	-	**	-	-	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S5. Statistical differences in growth dynamics of *S. aureus* strains representing different clonal types depending on the duration (3, 6 and 9 h) of rotating magnetic field (5 Hz) exposure.

	***S.aureus* strain**		
	ATCC 6538		
	3 h	6 h	9 h
3 h	x	****	****
6 h	****	x	-
9 h	****	-	x
	1		
	3 h	6 h	9 h
3 h	x	-	-
6 h	-	x	***
9 h	-	***	x
	2		
	3 h	6 h	9 h
3 h	x	-	-
6 h	-	x	-
9 h	-	-	x

	3		
	3 h	6 h	9 h
3 h	x	-	-
6 h	-	x	-
9 h	-	-	x
	4		
	3 h	6 h	9 h
3 h	x	-	-
6 h	-	x	-
9 h	-	-	x
	5		
	3 h	6 h	9 h
3 h	x	-	-
6 h	-	x	-
9 h	-	-	x
	6		
	3 h	6 h	9 h
3 h	x	**	-
6 h	**	x	-
9 h	-	-	x
	7		
	3 h	6 h	9 h
3 h	x	-	-
6 h	-	x	-
9 h	-	-	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S6. Statistical differences in growth dynamics between *S. aureus* strains representing different clonal types exposed for 3 h to rotating magnetic field of 50 Hz.

	ATCC 6538	1	2	3	4	5	6	7	control
ATCC 6538	x	-	-	-	-	-	-	-	-
1	-	x	-	-	**	-	-	-	-
2	-	-	x	-	***	-	-	-	*
3	-	-	-	x	-	-	***	-	-
4	-	**	***	-	x	***	****	-	-
5	-	-	-	-	***	x	-	-	*
6	-	-	-	***	****	-	x	-	****
7	-	-	-	-	-	-	-	x	-
control	-	-	*	-	-	*	****	-	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S7. Statistical differences in growth dynamics between *S. aureus* strains representing different clonal types exposed for 6 h to rotating magnetic field of 50 Hz.

	ATCC 6538	1	2	3	4	5	6	7	control
ATCC 6538	x	****	***	*	-	-	-	-	-
1	****	x	-	-	-	-	-	***	*
2	***	-	x	-	-	-	-	*	-
3	*	-	-	x	-	-	-	-	-
4	-	-	-	-	x	-	-	-	-
5	-	-	-	-	-	x	-	-	-
6	-	-	-	-	-	-	x	-	-
7	-	***	*	-	-	-	-	x	-
control	-	*	-	-	-	-	-	-	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S8. Statistical differences in growth dynamics between *S. aureus* strains representing different clonal types exposed for 9 h to rotating magnetic field of 50 Hz.

	ATCC 6538	1	2	3	4	5	6	7	control
ATCC 6538	x	-	****	***	-	-	****	**	***
1	-	x	****	***	-	-	****	**	***
2	****	****	x	-	****	**	-	-	*
3	***	***	-	x	-	-	-	-	-
4	-	-	****	-	x	-	*	-	-
5	-	-	**	-	-	x	-	-	-
6	****	****	-	-	*	-	x	-	-
7	**	**	-	-	-	-	-	x	-
control	***	***	*	-	-	-	-	-	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S9. Statistical differences in growth dynamics of *S. aureus* strains representing different clonal types depending on the duration (3, 6 and 9 h) of rotating magnetic field (50 Hz) exposure.

	***S.aureus* strain**		
	ATCC 6538		
	3 h	6 h	9 h
3 h	x	**	****
6 h	**	x	-
9 h	****	-	x
	1		
	3 h	6 h	9 h
3 h	x	-	****
6 h	-	x	****
9 h	****	****	x
	2		
	3 h	6 h	9 h
3 h	x	-	-
6 h	-	x	-
9 h	-	-	x

		3	
	3 h	6 h	9 h
3 h	x	-	-
6 h	-	x	-
9 h	-	-	x
		4	
	3 h	6 h	9 h
3 h	x	-	-
6 h	-	x	-
9 h	-	-	x
		5	
	3 h	6 h	9 h
3 h	x	-	**
6 h	-	x	-
9 h	**	-	x
		6	
	3 h	6 h	9 h
3 h	x	***	-
6 h	***	x	-
9 h	-	-	x
		7	
	3 h	6 h	9 h
3 h	x	-	-
6 h	-	x	-
9 h	-	-	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S10. Statistical differences in growth dynamics of *S. aureus* strains representing different clonal types exposed to rotating magnetic field (5 Hz v. 50 Hz).

S.aureus strain/Time of exposition	5 Hz v. 50 Hz	*S.aureus* strain/Time of exposition	5 Hz v. 50 Hz
ATCC 6538/3h	-	4/3h	-
ATCC 6538/6h	-	4/6h	-
ATCC 6538/9 h	-	4/9h	-
1/3h	-	5/3h	-
1/6h	-	5/6h	-
1/9h	-	5/9h	-
2/3h	-	6/3h	-
2/6h	-	6/6h	-
2/9h	-	6/9h	-
3/3h	-	7/3h	-
3/6h	-	7/6h	-
3/9h	-	7/9h	-

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S11. Statistical differences in cellular metabolic activity between *S. aureus* strains representing different clonal types exposed for 3 h to rotating magnetic field of 5 Hz.

	ATCC 6538	1	2	3	4	5	6	7	control
ATCC 6538	x	-	**	****	****	****	-	*	-
1	-	x	-	*	****	****	-	****	-
2	**	-	x		****	****	**	****	-
3	****	*	-	x	****	****	****	****	**
4	****	****	****	****	x	-	****	-	****
5	****	****	****	****		x	****	-	****
6	-	-	**	****	****	****	x	-	-
7	*	****	****	****	-	-	-	x	****
control	-	-	-	**	****	****	-	****	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S12. Statistical differences in cellular metabolic activity between *S. aureus* strains representing different clonal types exposed for 6 h to rotating magnetic field of 5 Hz.

	ATCC 6538	1	2	3	4	5	6	7	control
ATCC 6538	x	-	-	-	**	****	****	****	-
1	-	x	-	-	**	****	***	****	-
2	-	-	x	-	**	****	***	****	-
3	-	-	-	x	***	****	****	****	-
4	**	**	**	***	x	****	-	-	-
5	****	****	****	****	****	x	****	****	****
6	****	***	***	****	-	****	x	-	-
7	****	****	****	****	-	****	-	x	**
control	-	-	-	-	-	****	-	**	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S13. Statistical differences in cellular metabolic activity between *S. aureus* strains representing different clonal types exposed for 9 h to rotating magnetic field of 5 Hz.

	ATCC 6538	1	2	3	4	5	6	7	control
ATCC 6538	x	**	-	-	*	*	****	****	-
1	**	x	-	-	-	****	****	****	***
2	-	-	x	-	-	****	****	****	-
3	-	-	-	x	-	****	****	****	-
4	*	-	-		x	****	****	****	**
5	*	****	****	****	****	x	****	****	***
6	****	****	****	****	****	****	x	-	****
7	****	****	****	****	****	****	-	x	****
control	-	***	-	-	**	***	****	****	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S14. Statistical differences in cellular metabolic activity of *S. aureus* strains representing different clonal types depending on the duration (3, 6 and 9 h) of rotating magnetic field (5 Hz) exposure.

	***S.aureus* strain**		
	ATCC 6538		
	3 h	6 h	9 h
3 h	x	**	-
6 h	**	x	-
9 h	-	-	x
	1		
	3 h	6 h	9 h
3 h	x	-	*
6 h	-	x	-
9 h	*	-	x
	2		
	3 h	6 h	9 h
3 h	x	-	-
6 h	-	x	-
9 h	-	-	x
	3		
	3 h	6 h	9 h
3 h	x	-	-
6 h	-	x	-
9 h	-	-	x
	4		
	3 h	6 h	9 h
3 h	x	****	****
6 h	****	x	****
9 h	****	****	x
	5		
	3 h	6 h	9 h
3 h	x	-	*
6 h	-	x	****
9 h	*	****	x
	6		
	3 h	6 h	9 h

3 h	x	-	****
6 h	-	x	****
9 h	****	****	x
		7	
	3 h	6 h	9 h
3 h	x	-	****
6 h	-	x	****
9 h	****	****	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S15. Statistical differences in cellular metabolic activity between *S. aureus* strains representing different clonal types exposed for 3 h to rotating magnetic field of 50 Hz.

	ATCC 6538	1	2	3	4	5	6	7	control
ATCC 6538	x	-	****	****	**	-	-	**	****
1	-	x	****	****	**	-	-	**	****
2	****	****	x	-	****	****	*	****	-
3	****	****	-	x	****	****	**	****	-
4	**	**	****	****	x	***	****	-	****
5	-	-	****	****	***	x	-	***	***
6	-	-	*	**	****	-	x	****	-
7	**	**	****	****	-	***	****	x	****
control	****	****	-	-	****	***	-	****	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S16. Statistical differences in cellular metabolic activity between *S. aureus* strains representing different clonal types exposed for 6 h to rotating magnetic field of 50 Hz.

	ATCC 6538	1	2	3	4	5	6	7	control
ATCC 6538	x	-	-	-	-	****	*	***	-
1	-	x	-	-	-	****	-	**	-
2	-	-	x	-	-	****	-	*	-
3	-	-	-	x	-	****	-	***	-
4	-	-	-	-	x	****	-	-	-
5	****	****	****	****	****	x	****	**	****
6	*	-	-	-	-	****	x	-	-
7	***	**	*	***	-	**	-	x	-
control	-	-	-	-	-	****	-	-	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S17. Statistical differences in cellular metabolic activity between *S. aureus* strains representing different clonal types exposed for 9 h to rotating magnetic field of 50 Hz.

	ATCC 6538	1	2	3	4	5	6	7	control
ATCC 6538	x	***	-	-	****	***	-	-	-
1	***	x	-	**	-	-	****	**	-
2	-	-	x	-	****	-	****	-	-
3	-	**	-	x	****	*	-	-	-
4	****	-	****	****	x	**	****	****	****
5	****	-	-	*	**	x	****	**	-
6	-	****	****	-	****	****	x	-	****
7	-	**	-	-	****	**	-	x	-
control	-	-	-	-	****	-	****	-	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S18. Statistical differences in cellular metabolic activity of *S. aureus* strains representing different clonal types depending on the duration (3, 6 and 9 h) of rotating magnetic field (50 Hz) exposure.

	***S.aureus* strain**		
	ATCC 6538		
	3 h	6 h	9 h
3 h	x	****	-
6 h	****	x	***
9 h	-	***	x
	1		
	3 h	6 h	9 h
3 h	x	****	****
6 h	****	x	-
9 h	****	-	x
	2		
	3 h	6 h	9 h
3 h	x	-	-
6 h	-	x	-
9 h	-	-	x
	3		
	3 h	6 h	9 h
3 h	x	-	*
6 h	-	x	*
9 h	*	*	x
	4		
	3 h	6 h	9 h
3 h	x	****	****
6 h	****	x	****
9 h	****	****	x
	5		
	3 h	6 h	9 h
3 h	x	-	****
6 h	-	x	****
9 h	****	****	x

	6		
	3 h	6 h	9 h
3 h	x	-	-
6 h	-	x	*
9 h	-	*	x
	7		
	3 h	6 h	9 h
3 h	x	****	****
6 h	****	x	-
9 h	****	-	x

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table S19. Statistical differences in cellular metabolic activity of *S. aureus* strains representing different clonal types exposed to rotating magnetic field (5 Hz v. 50 Hz).

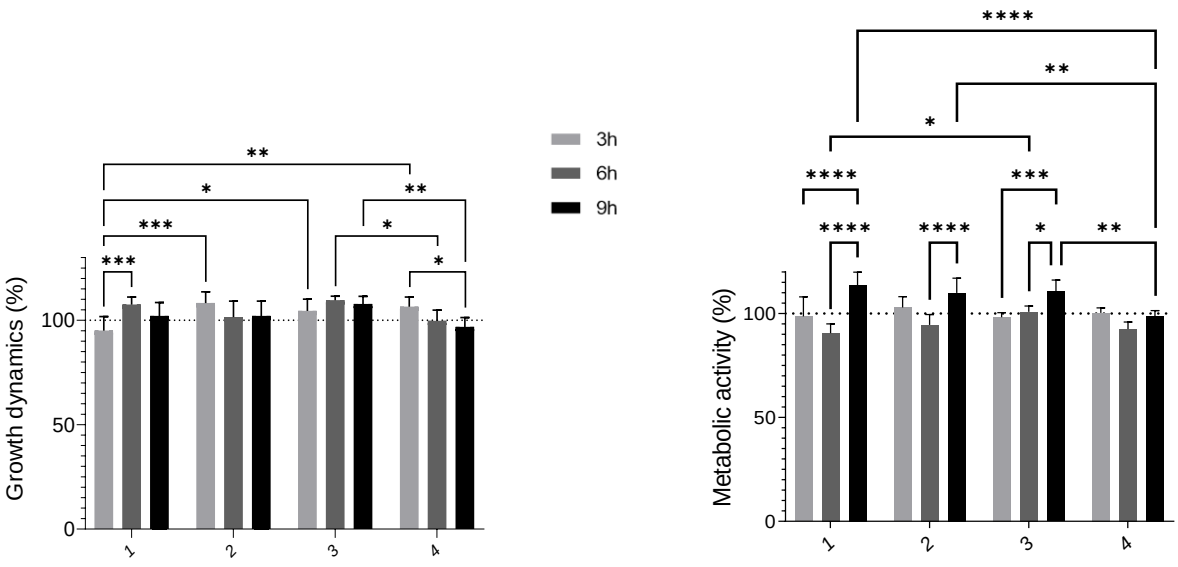
S. aureus strain/Time of exposition	5 Hz v. 50 Hz	*S. aureus* strain/Time of exposition	5 Hz v. 50 Hz
ATCC 6538/3h	-	4/3h	-
ATCC 6538/6h	-	4/6h	-
ATCC 6538/9 h	-	4/9h	-
1/3h	**	5/3h	*
1/6h	-	5/6h	-
1/9h	-	5/9h	****
2/3h	-	6/3h	-
2/6h	-	6/6h	-
2/9h	-	6/9h	*
3/3h	-	7/3h	-
3/6h	-	7/6h	-
3/9h	-	7/9h	****

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Table 20. The values of magnetic induction inside the RMF generator, at the location of the Petri dishes, depending on the applied AC frequency.

Magnetic induction [mT]	5 Hz	50 Hz
MIN	17.23	17.95
MAX	21.88	22.77
Average	19.20	19.99

5 Hz



50 Hz

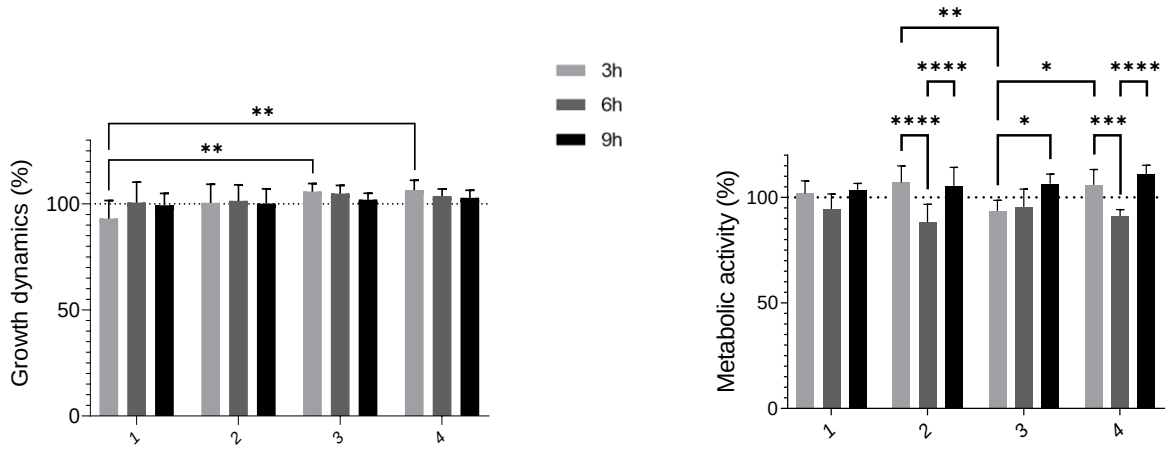
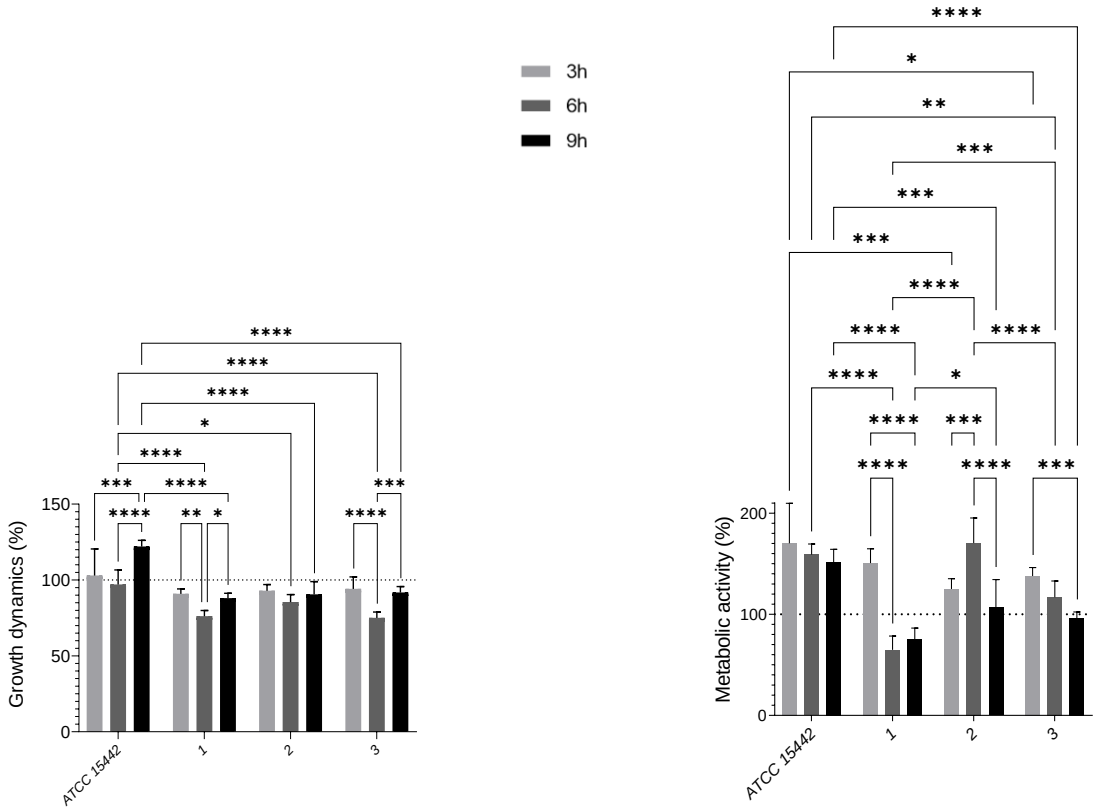


Figure S1. Statistical differences in growth dynamics and cellular metabolic activity between *S.aureus* strains representing one clonal type exposed to rotating magnetic field (5 Hz and 50 Hz) for 3, 6 and 9 h.

Data are expressed as % of control. The results are presented as a mean ± SEM calculated using six values (three from each biological replicate); * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

5 Hz


3h
6h
9h
Growth dynamics (%)
Metabolic activity (%)
ATCC 15442
1
2
3

50 Hz

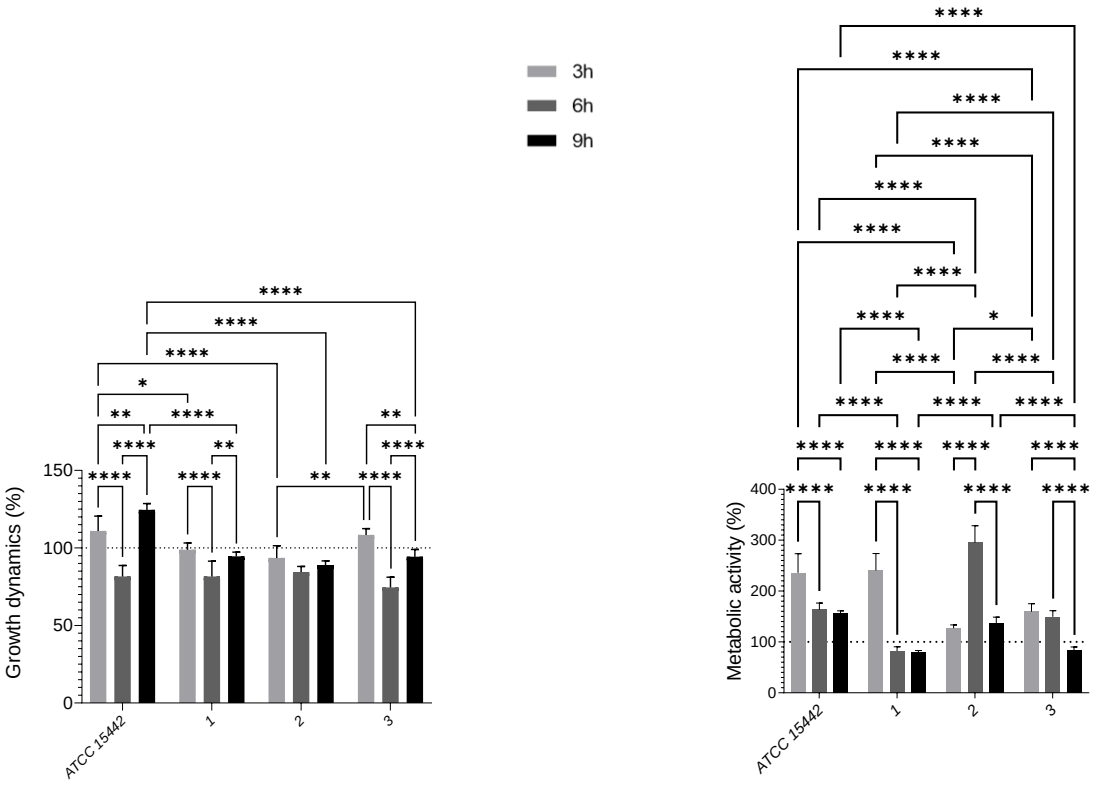

3h
6h
9h
Growth dynamics (%)
Metabolic activity (%)
ATCC 15442
1
2
3

Figure S2. Statistical differences in growth dynamics and cellular metabolic activity between *P.aeruginosa* strains exposed to rotating magnetic field (5 Hz and 50 Hz) for 3, 6 and 9 h.

Data are expressed as % of control. The results are presented as a mean ± SEM calculated using six values (three from each biological replicate); * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

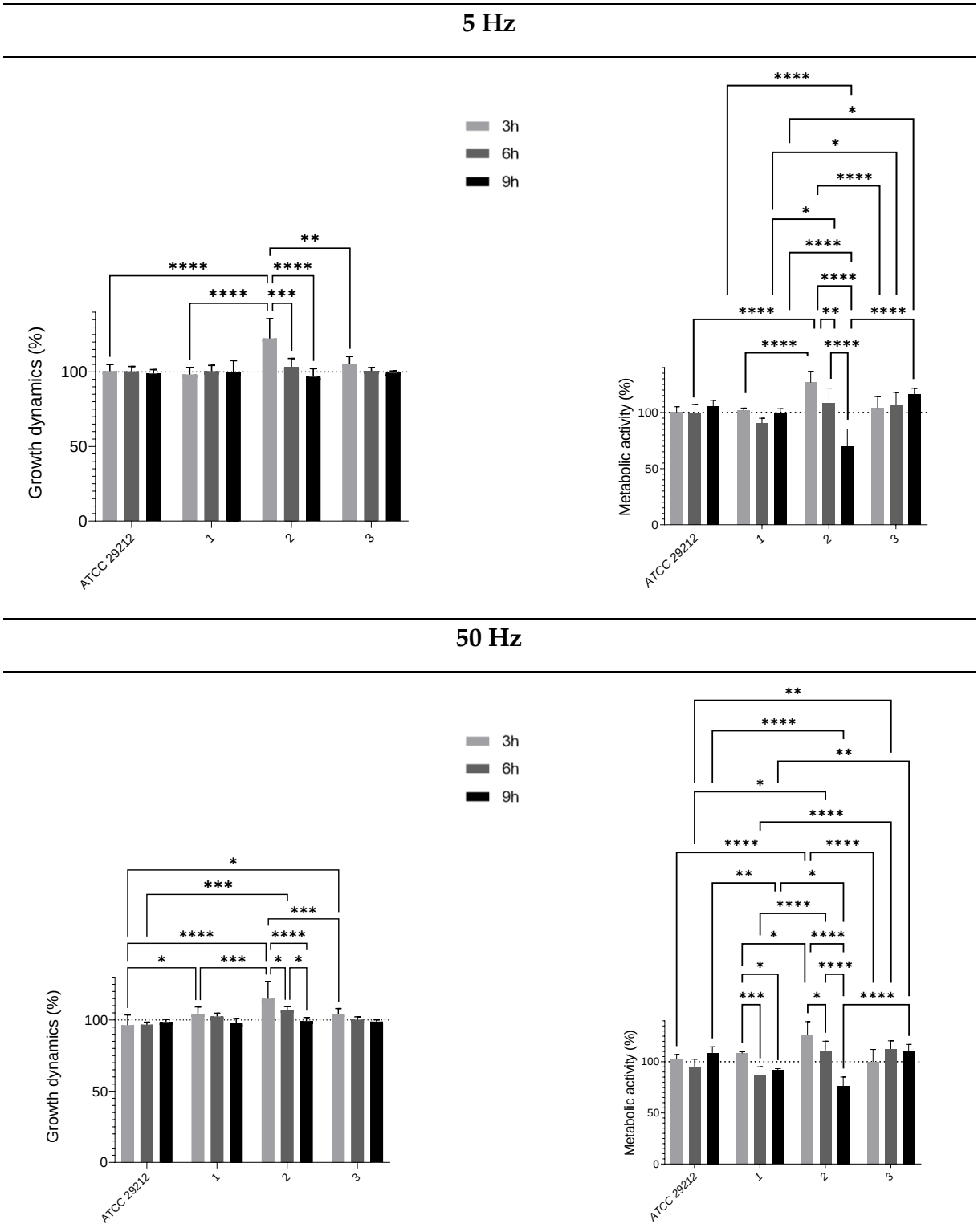


Figure S3. Statistical differences in growth dynamics and cellular metabolic activity between *E. faecalis* strains exposed to rotating magnetic field (5 Hz and 50 Hz) for 3, 6 and 9 h.

Data are expressed as % of control. The results are presented as a mean ± SEM calculated using six values (three from each biological replicate); * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

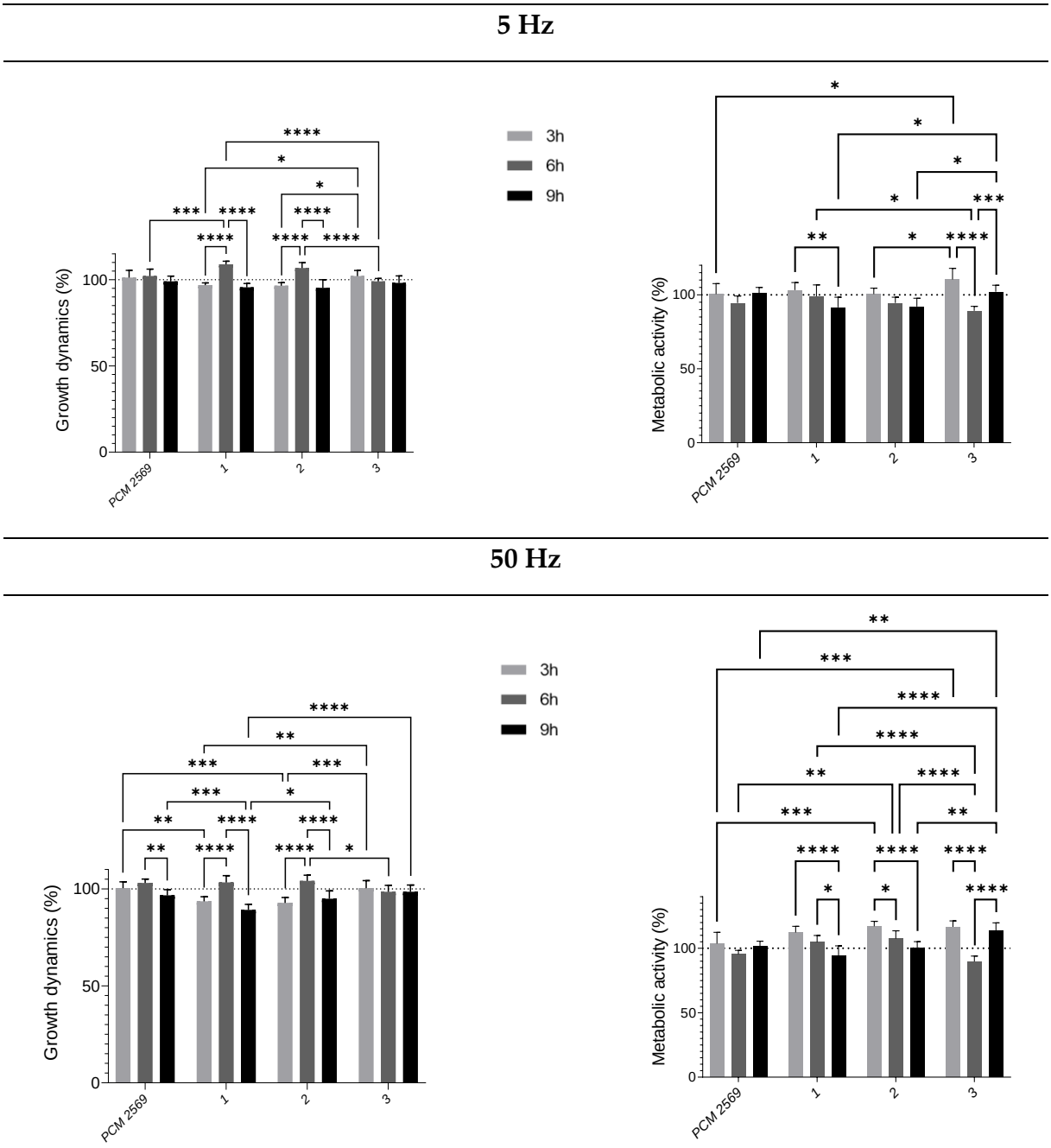


Figure S4. Statistical differences in growth dynamics and cellular metabolic activity between *E. cloacae* strains exposed to rotating magnetic field (5 Hz and 50 Hz) for 3, 6 and 9 h.

Data are expressed as % of control. The results are presented as a mean ± SEM calculated using six values (three from each biological replicate); * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

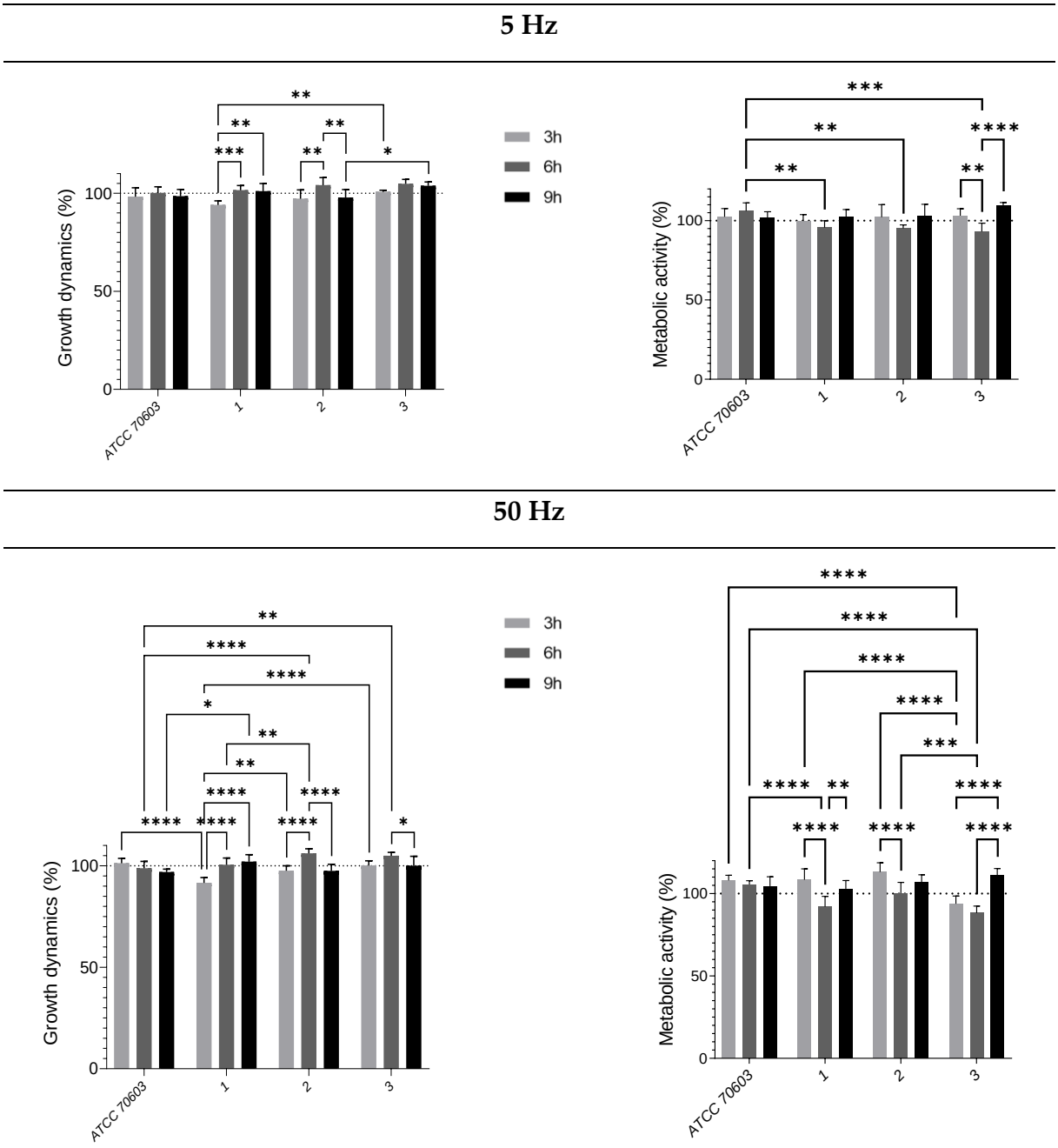


Figure S5. Statistical differences in growth dynamics and cellular metabolic activity between *K. pneumoniae* strains exposed to rotating magnetic field (5 Hz and 50 Hz) for 3, 6 and 9 h.

Data are expressed as % of control. The results are presented as a mean ± SEM calculated using six values (three from each biological replicate); * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

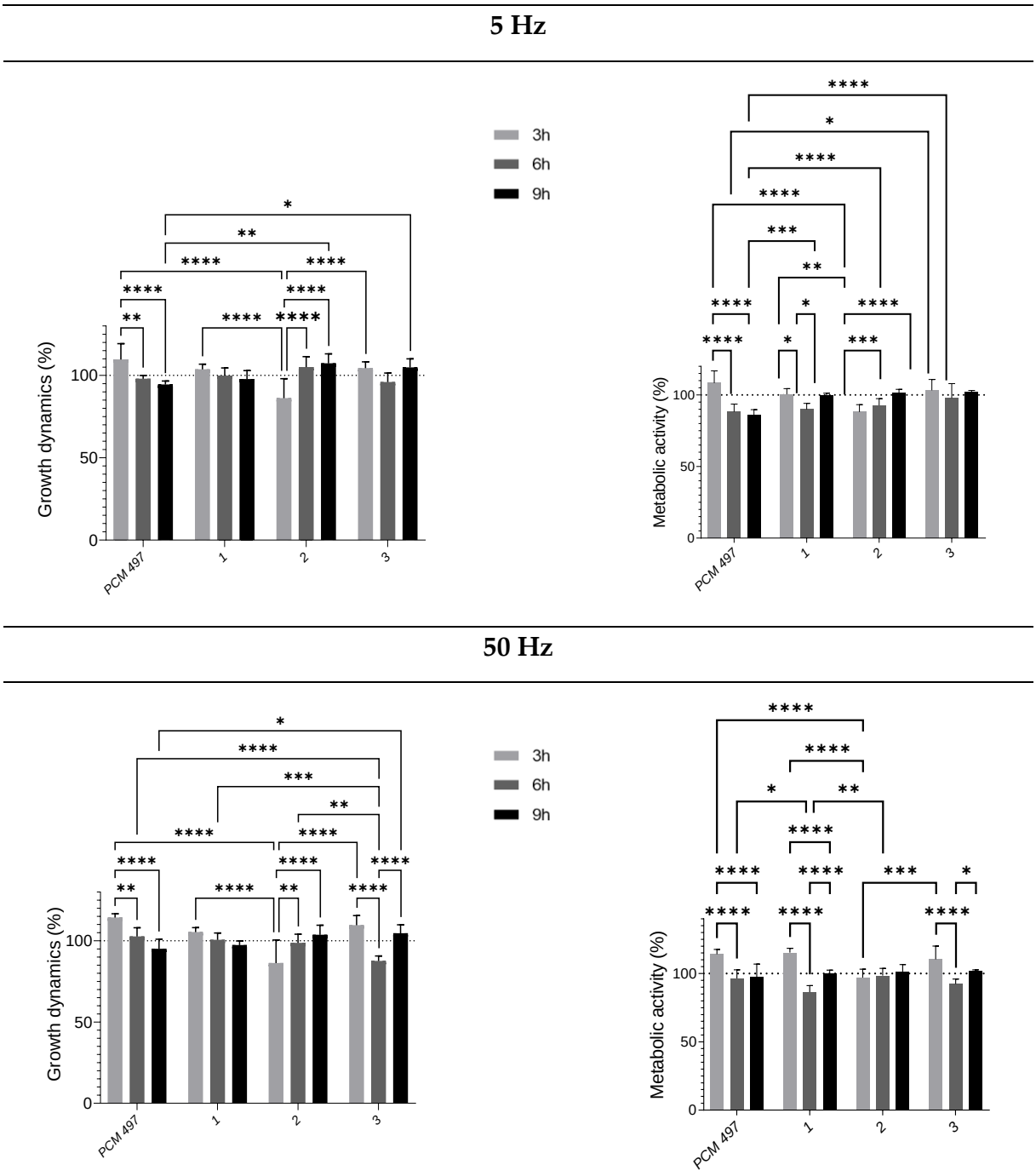


Figure S6. Statistical differences in growth dynamics and cellular metabolic activity between *B. cereus* strains exposed to rotating magnetic field (5 Hz and 50 Hz) for 3, 6 and 9 h.

Data are expressed as % of control. The results are presented as a mean ± SEM calculated using six values (three from each biological replicate); * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

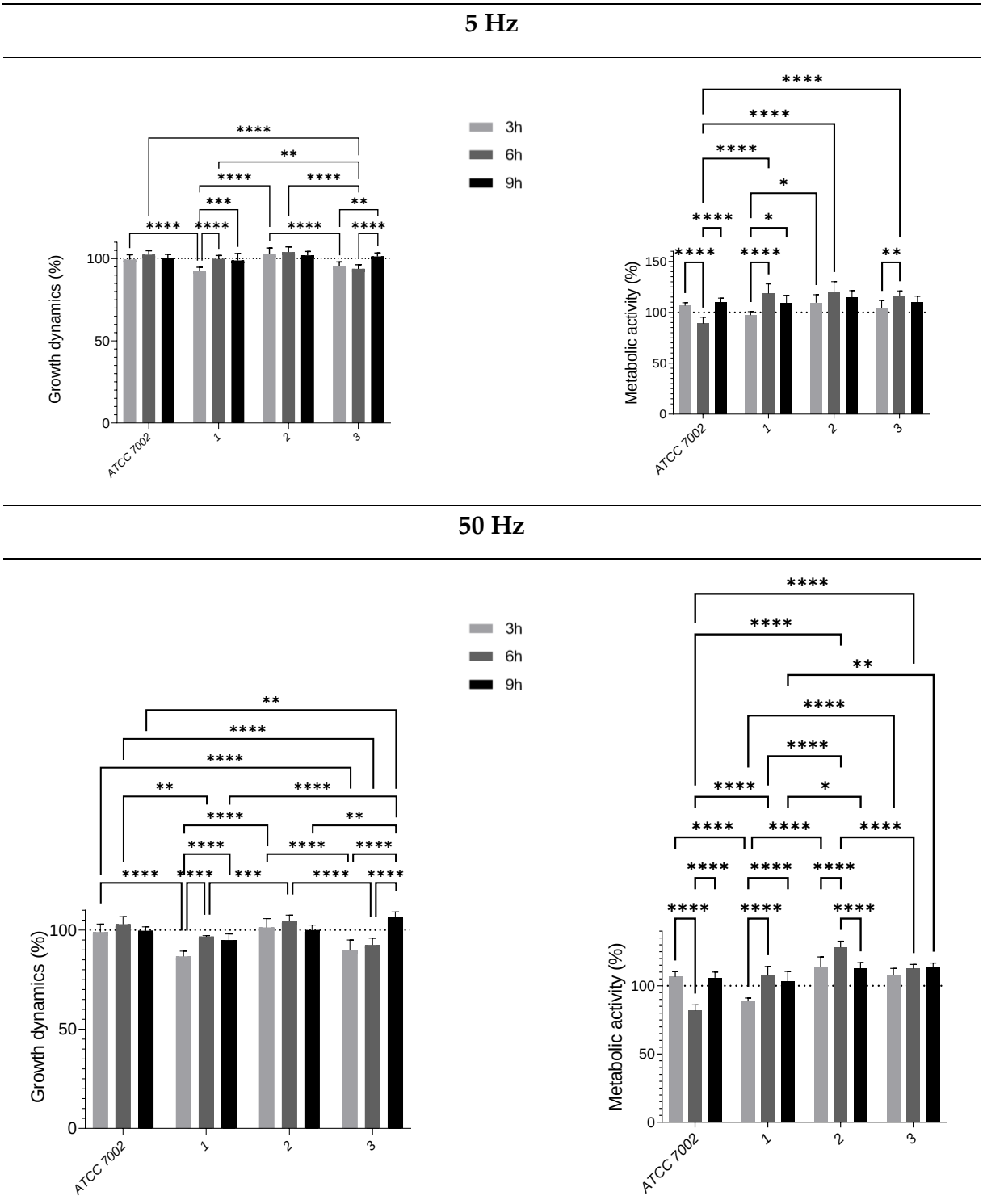


Figure S7. Statistical differences in growth dynamics and cellular metabolic activity between *P. mirabilis* strains exposed to rotating magnetic field (5 Hz and 50 Hz) for 3, 6 and 9 h.

Data are expressed as % of control. The results are presented as a mean ± SEM calculated using six values (three from each biological replicate); * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

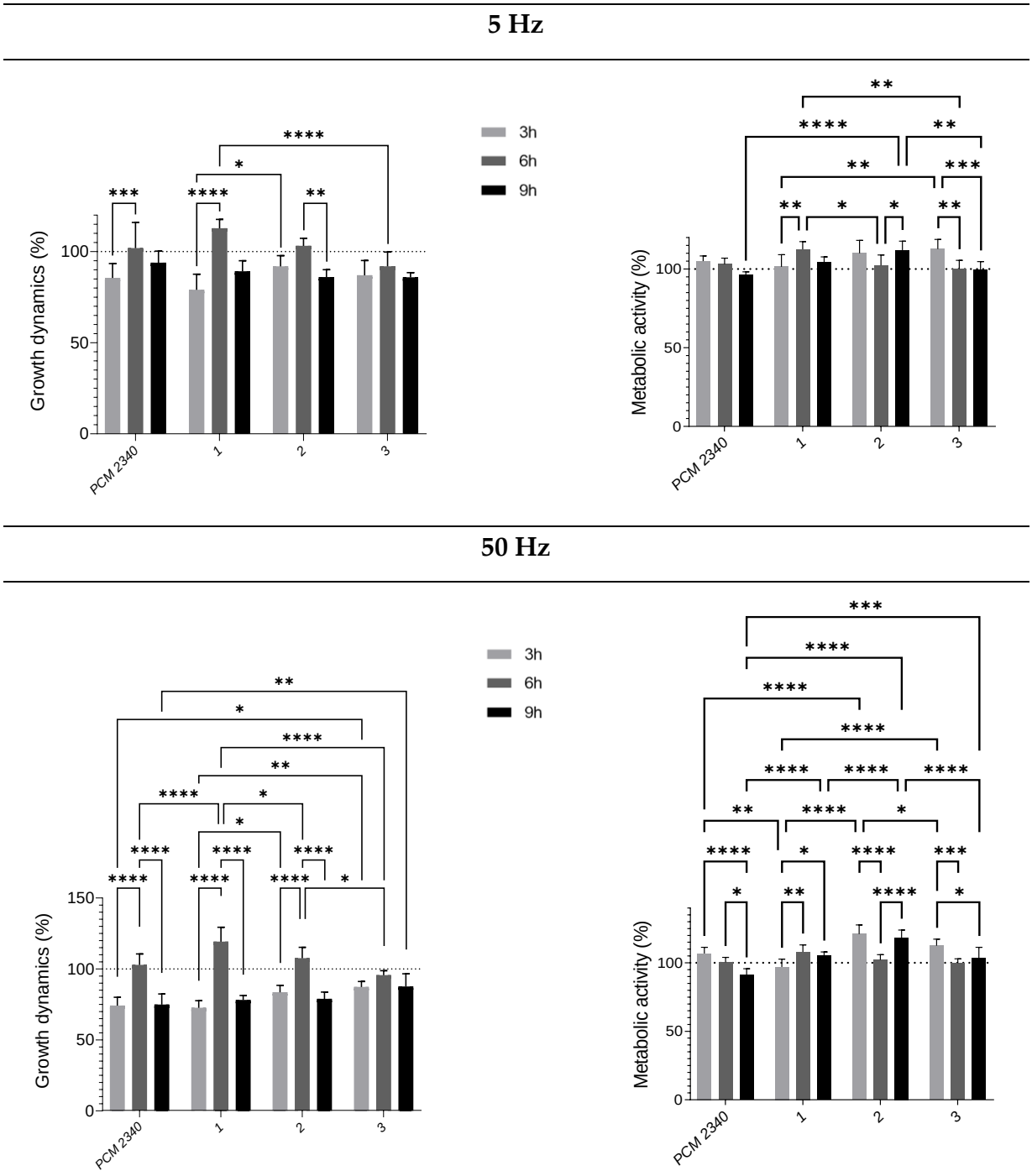


Figure S8. Statistical differences in growth dynamics and cellular metabolic activity between *M. catarrhalis* strains exposed to rotating magnetic field (5 Hz and 50 Hz) for 3, 6 and 9 h.

Data are expressed as % of control. The results are presented as a mean ± SEM calculated using six values (three from each biological replicate); * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Supplementary Material

The Effect of Rotating Magnetic Field on Susceptibility Profile of Methicillin Resistant *Staphylococcus aureus* Strains Exposed to Activity of Different Groups of Antibiotics

Marta Woroszyło[1], Daria Ciecholewska-Juśko[1], Adam Junka[2,3*], Marcin Wardach[4], Grzegorz Chodaczek[5], Bartłomiej Dudek[6], Karol Fijałkowski[1*]

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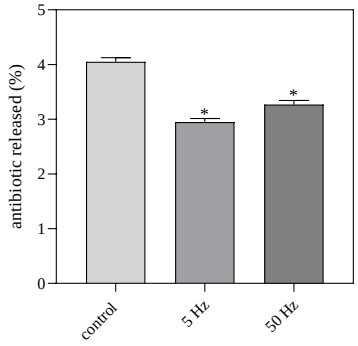
CEFOXITIN

Zone 1



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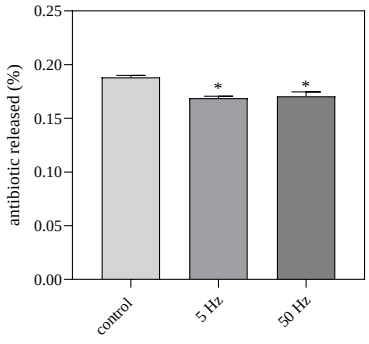

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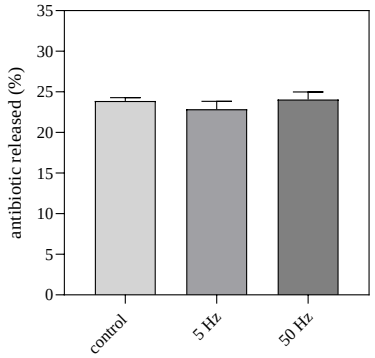

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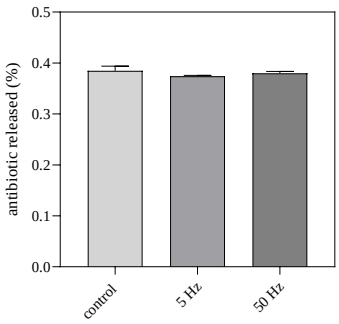

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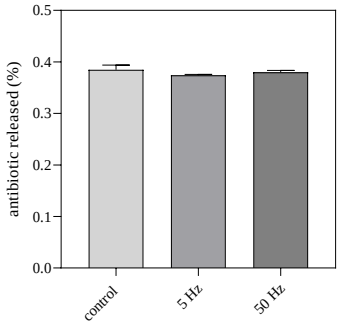

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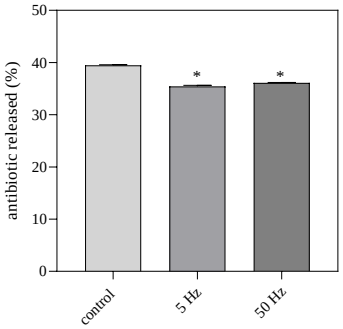

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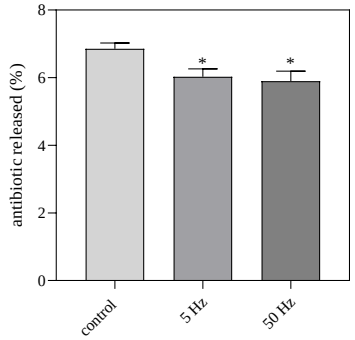

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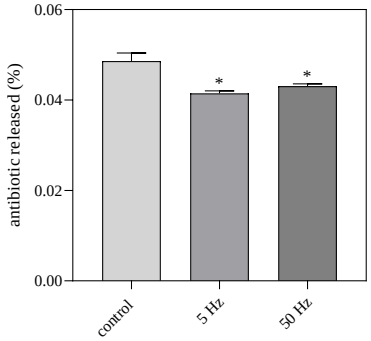

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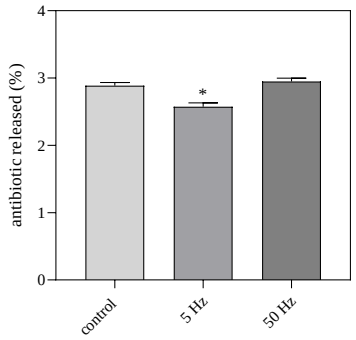

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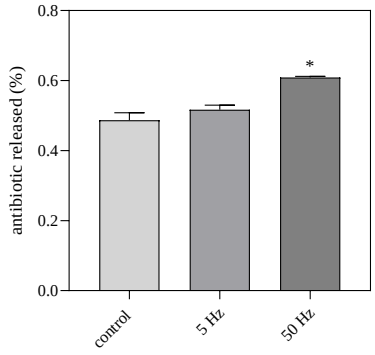

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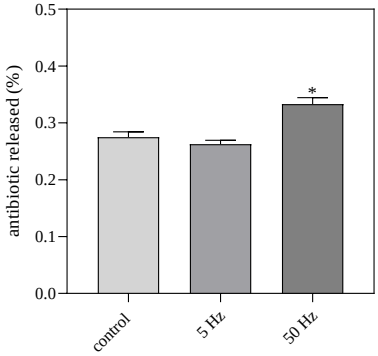

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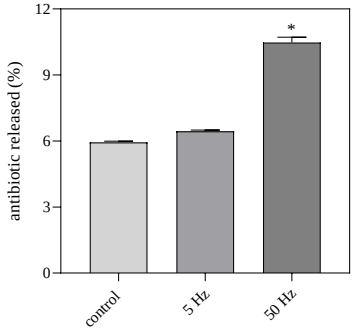

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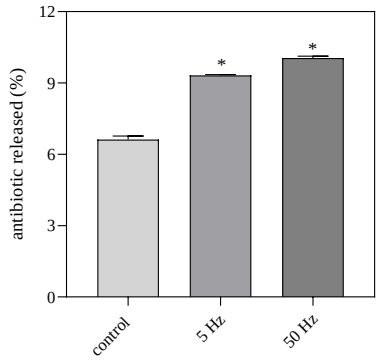

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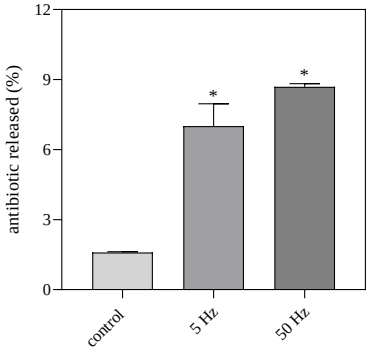

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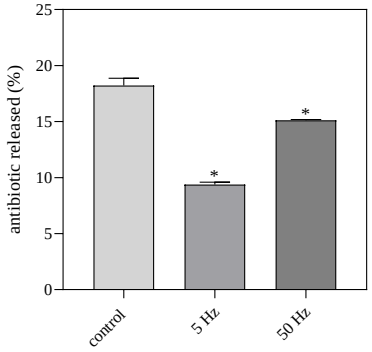

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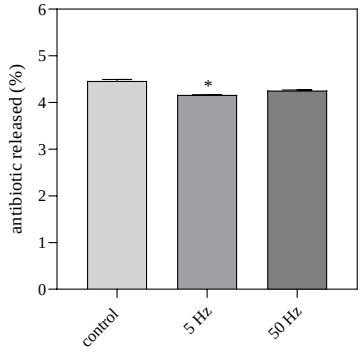
Zone 1



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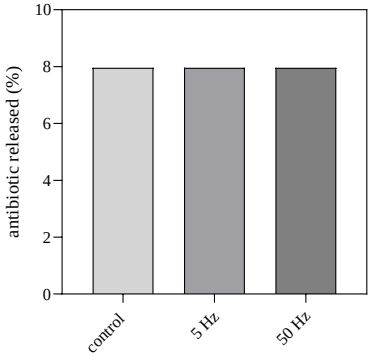

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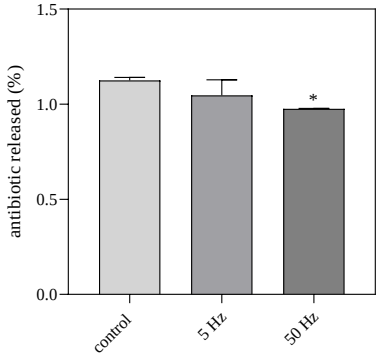

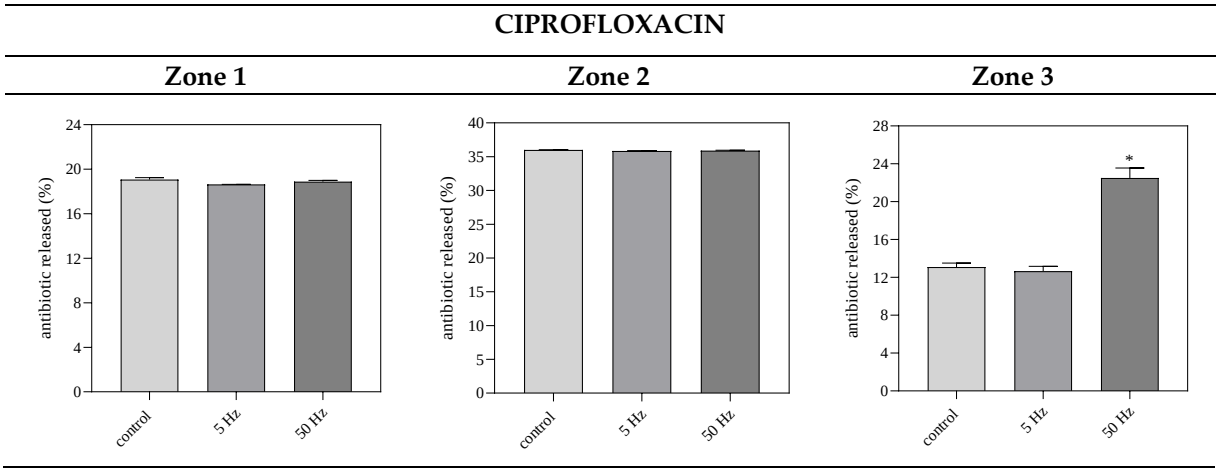

Figure S1. Percentage of antibiotic released from the paper disc during 120 min in control and RMF-exposed (5/50 Hz) settings. The results are presented as a mean ± SEM calculated using six values (three from each biological replicate). * - indicates statistical differences ($p<0.05$) between control and RMF-exposed settings.

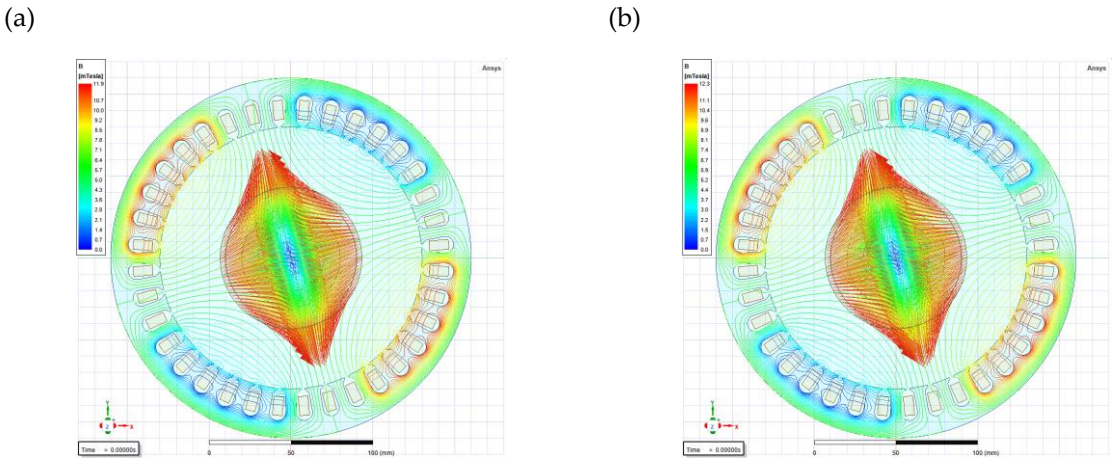

Figure S2. Visualization of generated magnetic flux rotation around the stator inside the RMF reactor chamber at (**a**) 5 Hz and (**b**) 50 Hz (please click the underlined RMF frequencies to watch the simulation).

Supplementary material

Rotating Magnetic Field increases β-lactam antibiotic susceptibility of methicillin-resistant *Staphylococcus aureus* strains

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KF: karol.fijalkowski@zut.edu.pl; + 48 91-449-6714;
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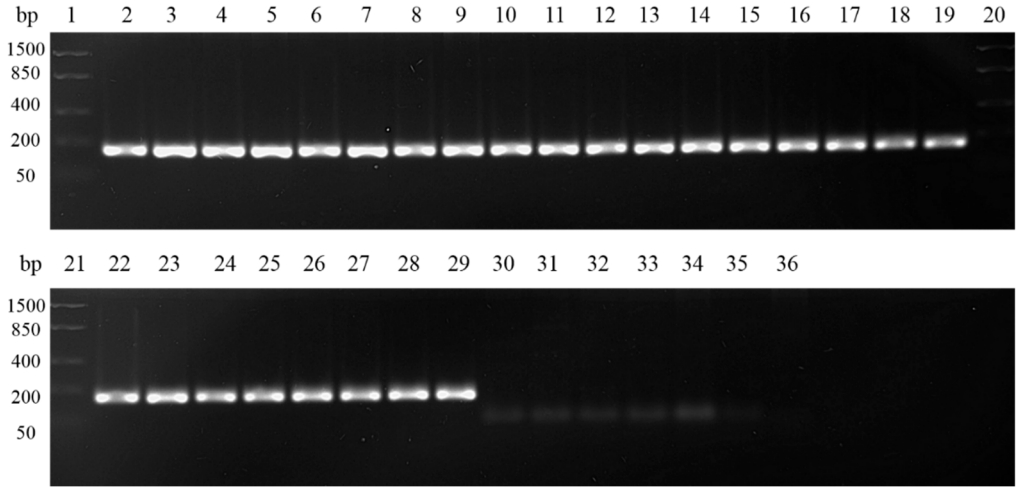


Figure S1. Detection of *mecA* gene.
1, 20, 21 - FastRuler DNA Ladder; 2, 19, 22 - *S. aureus* ATCC 33591 (*mecA* positive control); 3-18 - MRSA; 23-29 - MRSA; 30-34 - MSSA; 35 - no template control; 36 - *S. aureus* ATCC 6538 (*mecA* negative control).

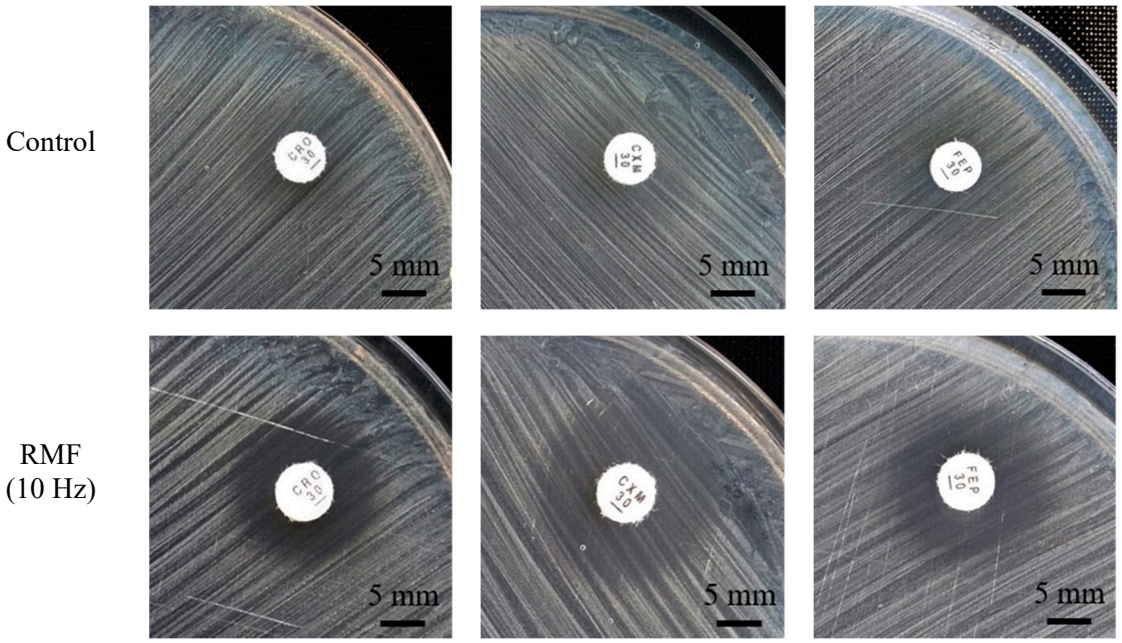


Figure S2. Partial growth around the antibiotic discs of MRSA 2 strain exposed to the RMF (10 Hz).
CRO - ceftriaxone, CXM - cefuroxime, FEP - cefepime.

Table S1. Growth inhibition zones (mm) of MRSA strains measured after exposure to the RMF (5 Hz) and after further (to 18th hour) incubation w/o RMF (5 Hz/Inc).

	Strain																							
	ATCC 33591								MRSA 1								MRSA 2							
t	FOX		FEP		CXM		CRO		FOX		FEP		CXM		CRO		FOX		FEP		CXM		CRO	
	5 Hz	5 Hz/Inc	5 Hz	5 Hz/Inc	5 Hz	5 Hz/Inc	5 Hz	5 Hz/Inc	5 Hz	5 Hz/Inc	5 Hz	5 Hz/Inc	5 Hz	5 Hz/Inc	5 Hz	5 Hz/Inc	5 Hz	5 Hz/Inc	5 Hz	5 Hz/Inc	5 Hz	5 Hz/Inc	5 Hz	5 Hz/Inc
7	17	16	15	14	18	17	14	13	17	16	16	15	21	21	16	15	14	13	17	17	16	15	13	13
8	17	16	15	15	18	18	14	13	17	16	16	15	21	20	16	15	15	14	17	16	15	15	13	12
9	17	16	15	14	18	18	16	15	16	16	16	16	21	20	15	15	16	16	18	18	15	15	13	13
10	18	17	15	15	19	19	17	16	16	16	17	17	21	21	15	15	18	18	18	18	15	15	13	13
11	18	18	16	16	20	20	17	17	16	16	18	18	22	22	15	15	20	20	18	18	15	15	14	14
12	18	18	16	16	22	22	18	18	16	16	19	19	23	23	15	15	21	21	18	18	15	15	15	15
18*	18	-	16	-	22	-	18	-	16	-	19	-	23	-	15	-	21	-	18	-	15	-	15	-

t - time (h) of exposure to the RMF. 18* - constant exposure to the RMF for 18 h. The differences in the diameter of the growth inhibition zones between three repetitions of the experiment did not exceed ± 1 mm. FOX - cefoxitin, FEP - cefepime, CXM - cefuroxime, CRO – ceftriaxone.

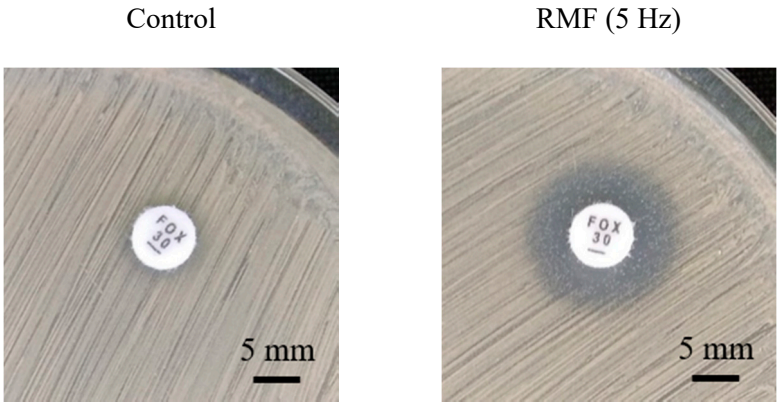
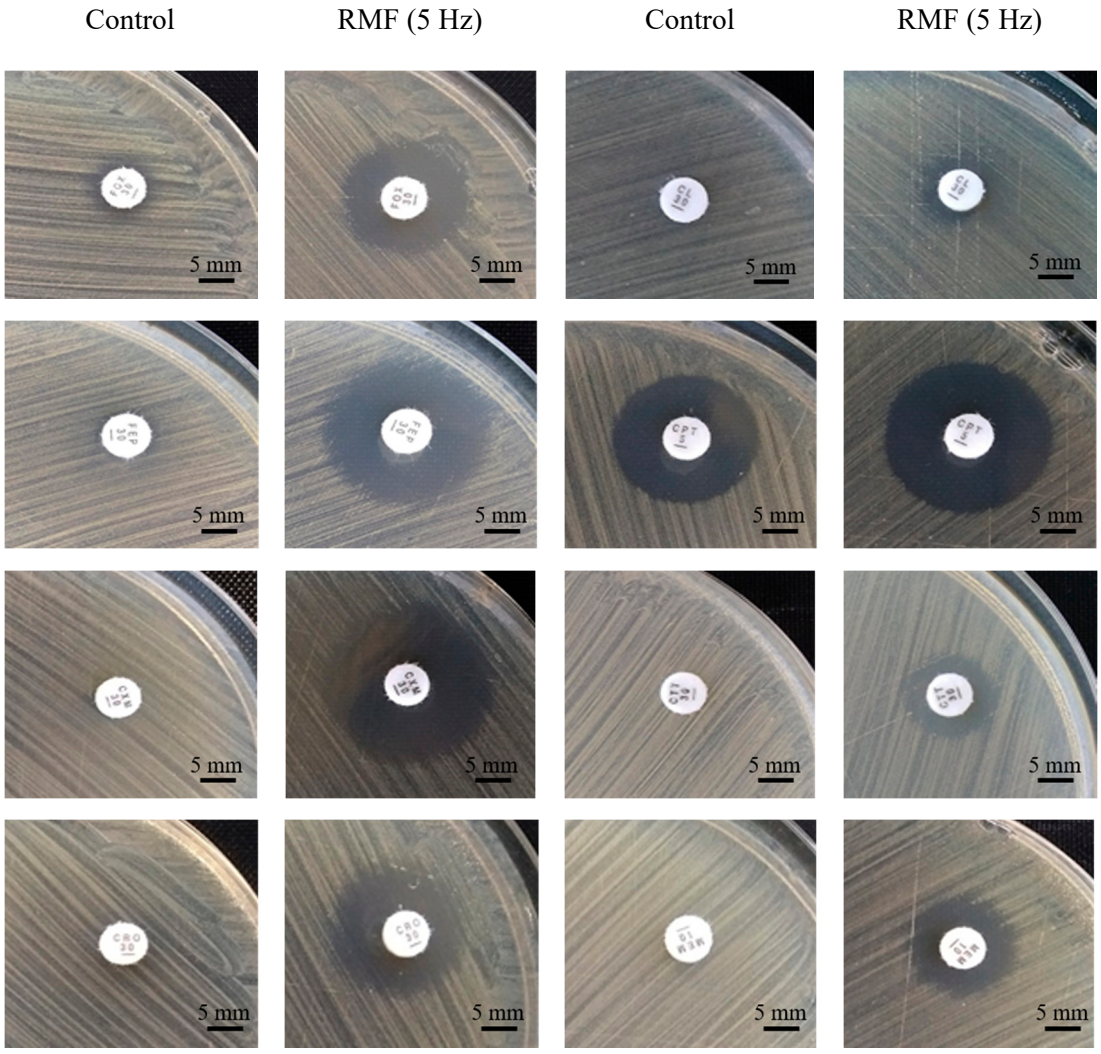


Figure S3. Partial growth around the antibiotic discs with cefoxitin (FOX) of MRSA 1 strain after exposure to the RMF (5 Hz) for 2 h.



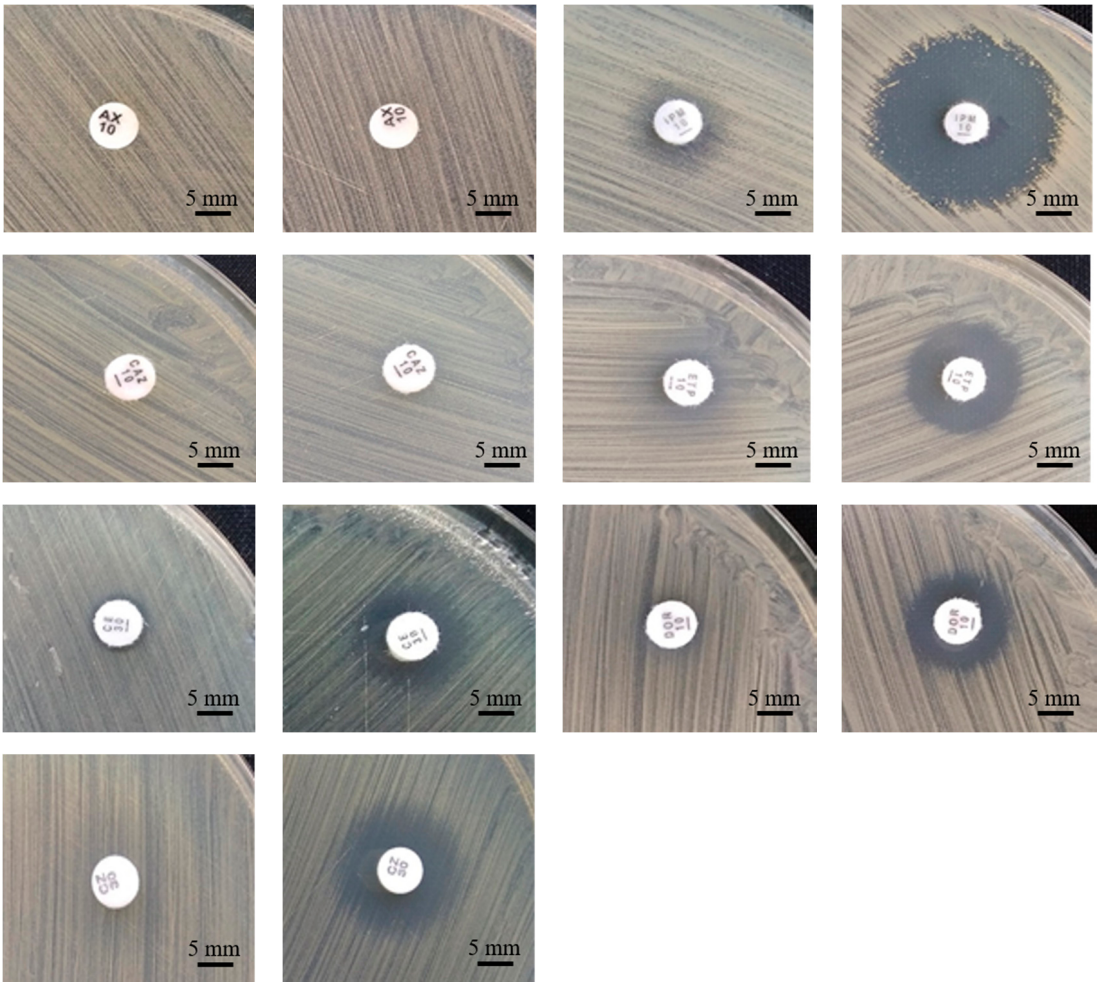
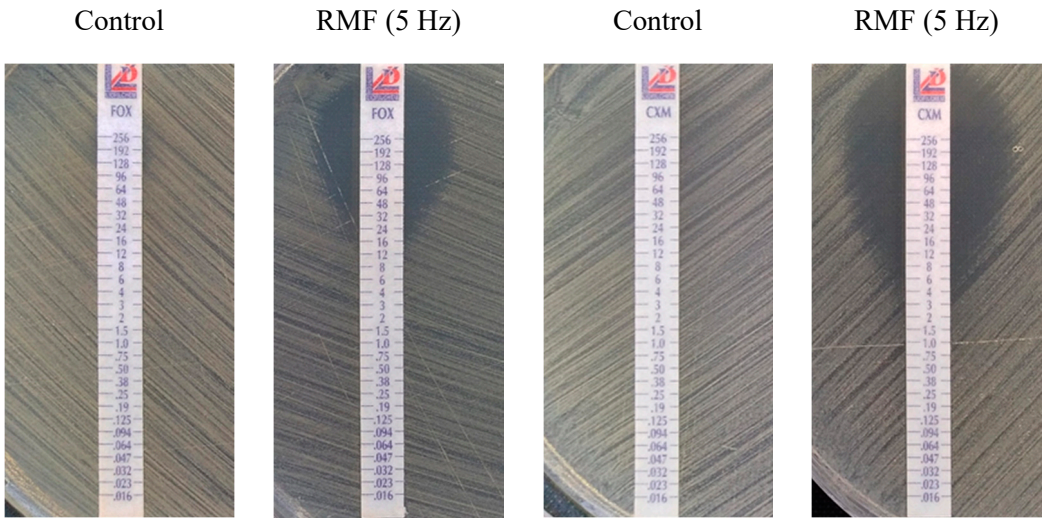


Figure S4. Representative pictures of growth inhibition zones in control and RMF-exposed (5 Hz) MRSA 1 cultures around discs with β-lactam antibiotics.
FOX - cefoxitin, FEP - cefepime, CXM - cefuroxime, CRO - ceftriaxone, AX - amoxicillin, CAZ - ceftazidime, CE - cephradine, CZ - cefazolin, CL - cephalexin, CPT - ceftaroline, CTT - cefotetan, MEM - meropenem, ETP - ertapenem, DOR - doripenem.



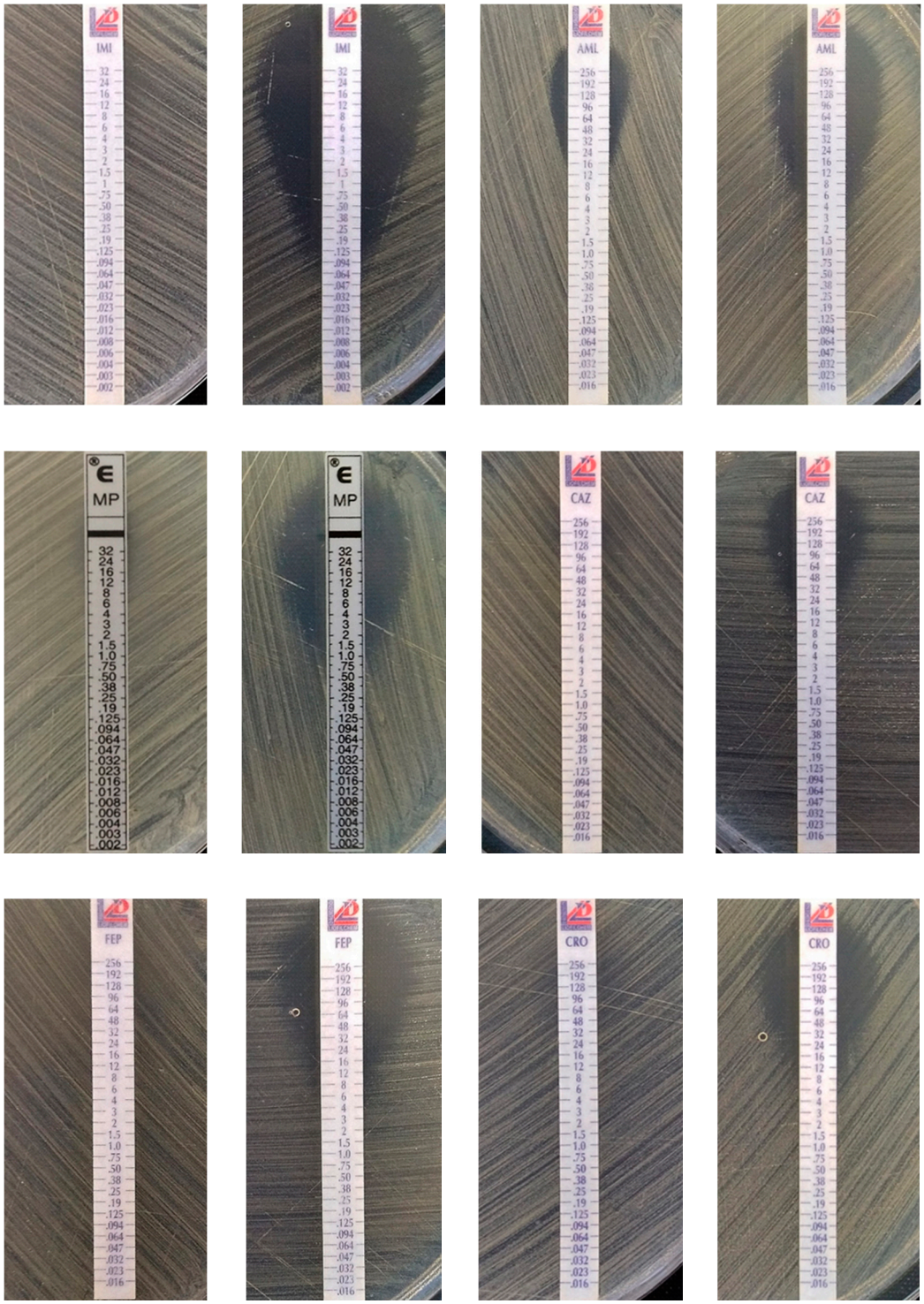
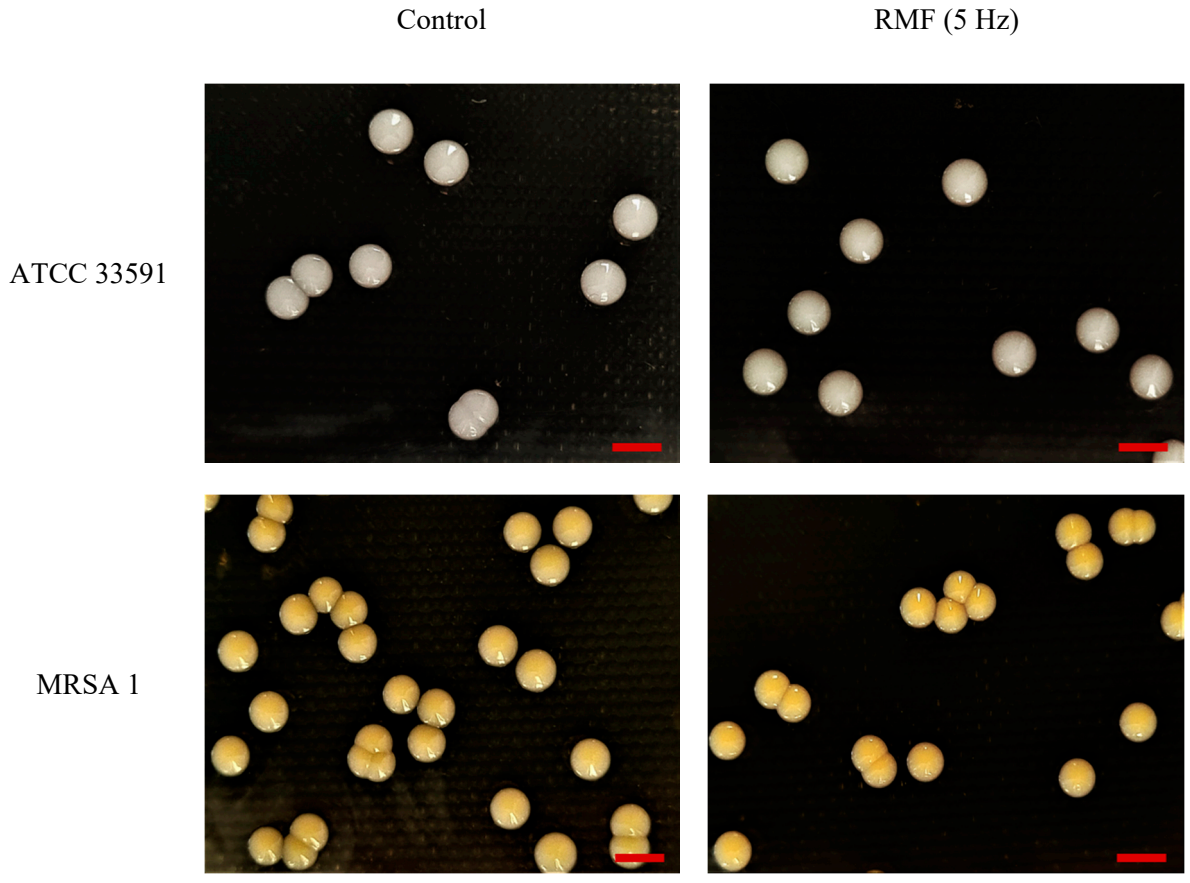


Figure S5. Representative pictures of gradient MIC strips (E-tests) with β-lactam antibiotics in control and RMF-exposed (5 Hz) cultures of the MRSA 1 strain.
FOX - cefoxitin, IMI - imipenem, MP - meropenem, FEP - cefepime, CXM - cefuroxime, AML - amoxicilin, CAZ - ceftazidime, CRO - ceftriaxone.

Table S2. Zones of growth inhibition (mm) for β-lactam antibiotics in control and RMF-exposed (5 Hz) MSSA cultures.

	Cefoxitin		**Cefepime**		**Cefuroxime**		**Ceftriaxone**	
	C	RMF	C	RMF	C	RMF	C	RMF
ATCC 6538	27	27	22	22	26	26	24	24
MSSA 1	29	29	26	26	34	34	29	29
MSSA 2	26	26	22	22	27	27	23	23
MSSA 3	24	24	26	26	29	29	24	24
MSSA 4	28	28	28	28	34	34	29	29
MSSA 5	28	28	26	26	34	34	27	27

The differences in the diameter of the growth inhibition zones between three repetitions of the experiment did not exceed ± 1 mm. C - control culture un-exposed to RMF.



MRSA 2

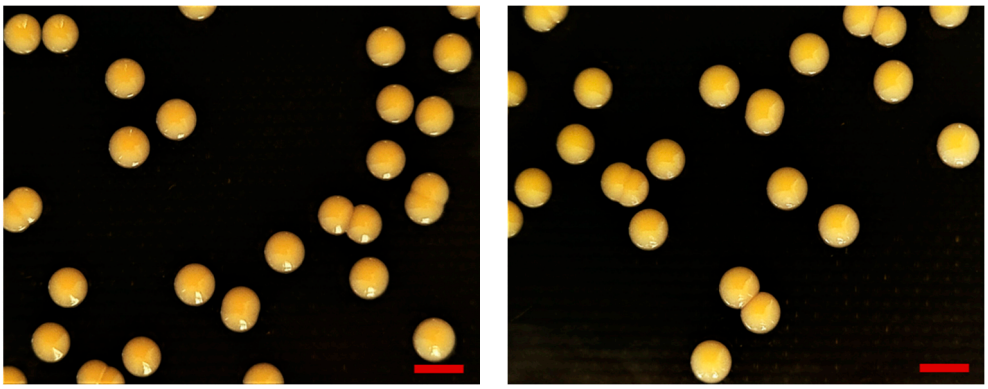


Figure S6. Morphology of the bacterial colonies of MRSA strains formed after 12 h of RMF exposure (5 Hz) in comparison to control cultures un-exposed to RMF.
Scale bars represent 2 mm.

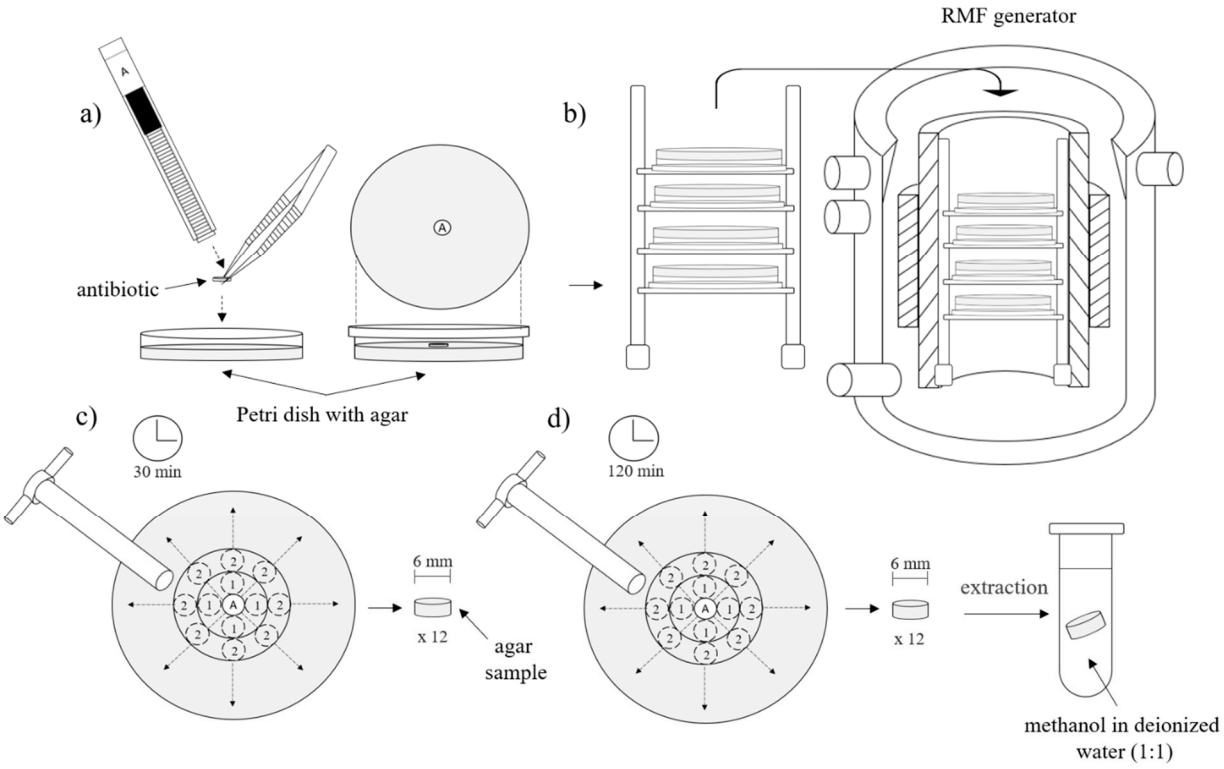


Figure S7. Schematic presentation of the analysis of the impact of RMF on the diffusion of antibiotics in the agar medium.
1 - agar disc from proximal zone (zone 1); 2 - agar disc from the distal zone (zone 2).

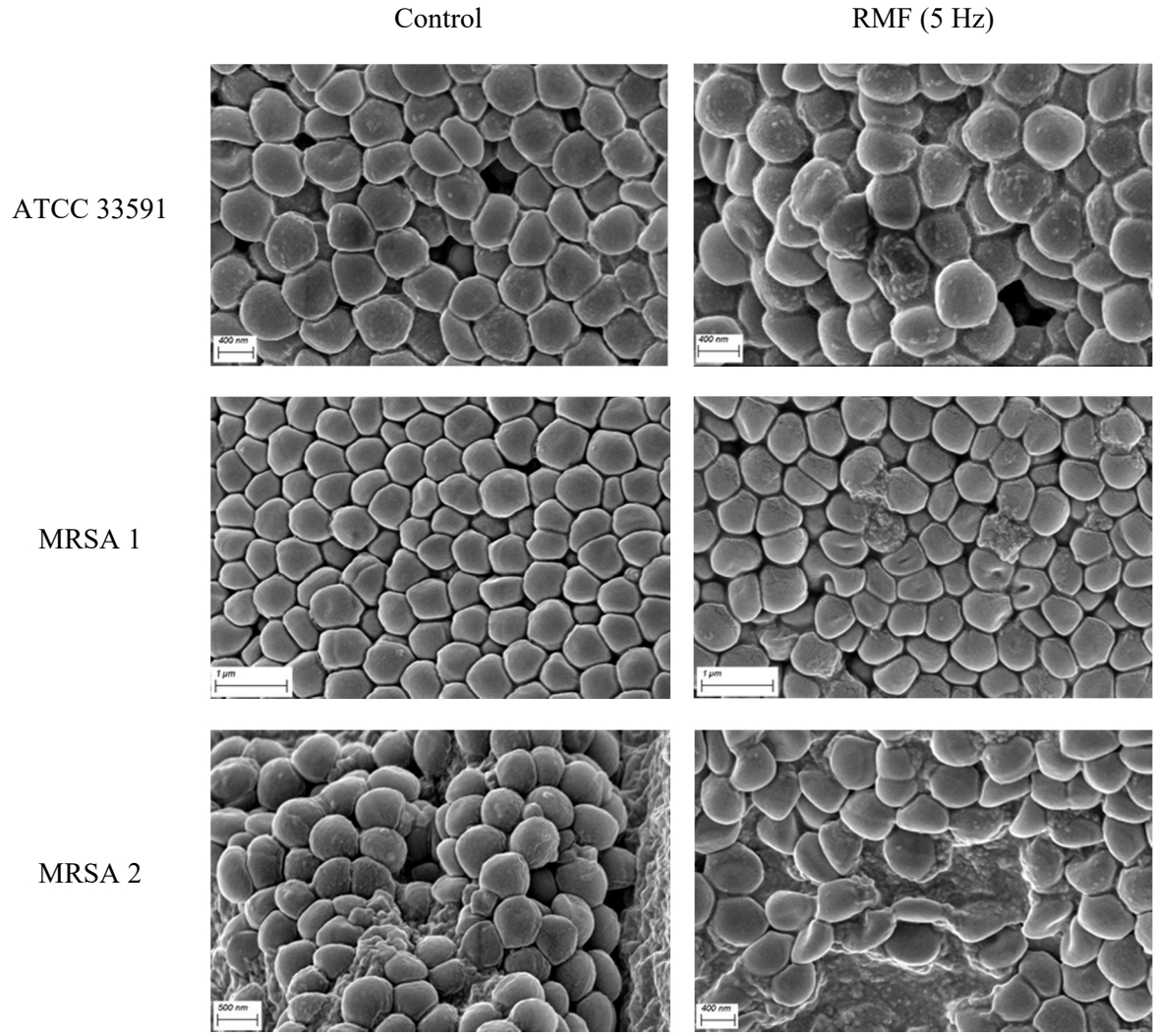
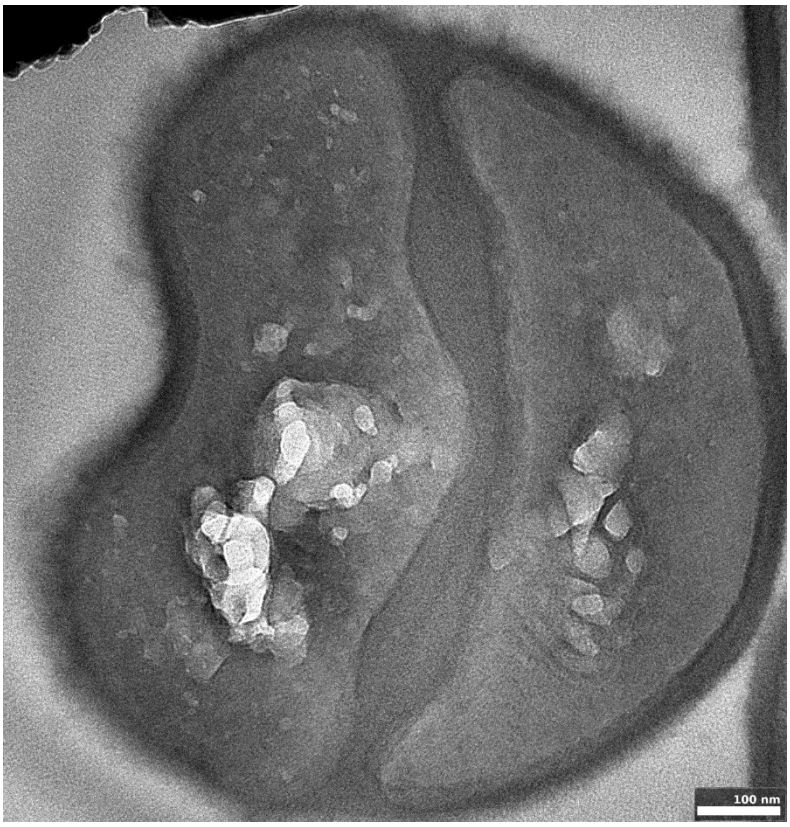


Figure S8. Scanning electron microscopy images of MRSA cells un-exposed and exposed to the RMF (5 Hz).



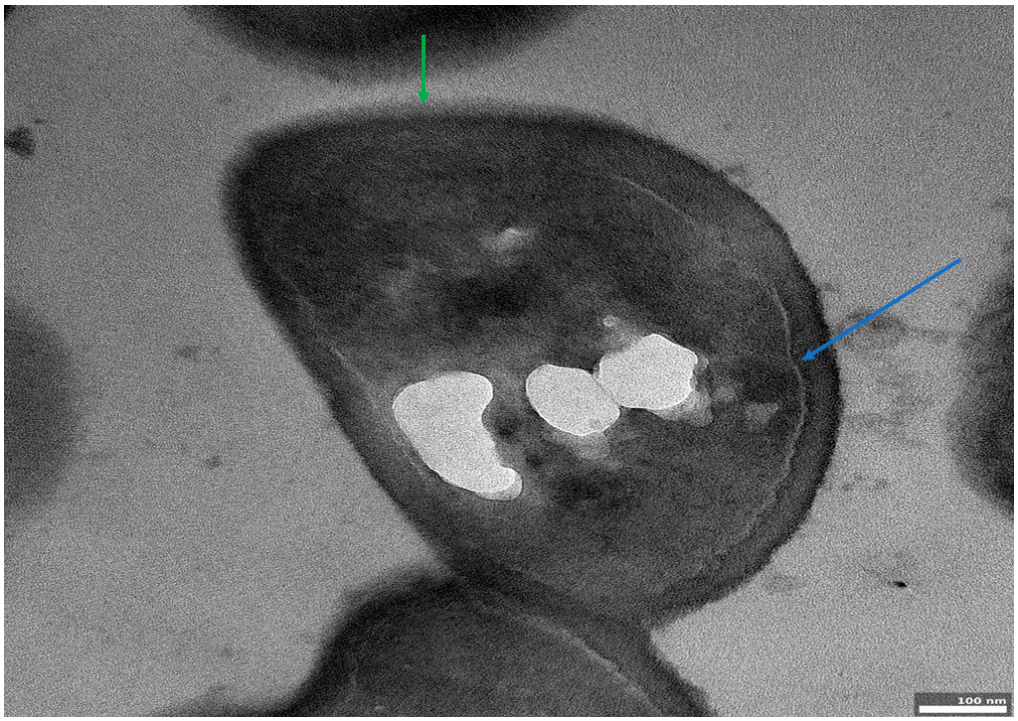


Figure S9. The magnified regions showing impact of RMF on staphylococcal cell.

Upper picture – change of cell's morphology; lower picture, blue arrow – contraction of cytoplasm; green arrow – loss of cell wall density.

Table S3. RMF post-exposure effect.

Strain	Cefoxitin		Cefuroxime	
	C	RMF	C	RMF
ATTC 33591	12	12	6	6
MRSA 1	6	6	6	6
MRSA 2	7	7	6	6
	Cefepime		Ceftriaxone	
	C	RMF	C	RMF
ATTC 33591	10	10	6	6
MRSA 1	6	6	6	6
MRSA 2	6	6	6	6

Liquid bacterial cultures of the cell density equal to 0.5 of McFarland turbidity standard prepared in a M-H medium were exposed to the RMF (5 Hz) for 12 h. Next, the cultures were centrifuged, the pellet resuspended in PBS to obtain the initial cell density and used as an inoculum for antibiotic susceptibility test. The growth inhibition zones (mm) were compared with the zones obtained in the control cultures performed using the inoculum never exposed to the RMF.

Załącznik 3

Oświadczenia współautorów publikacji naukowych wchodzących w skład cyklu stanowiącego rozprawę doktorską wraz z określeniem ich indywidualnego udziału

Marta Woroszyło

Rozprawa doktorska

ANALIZA WPŁYWU WIRUJĄCEGO POLA MAGNETYCZNEGO NA EFEKTYWNOŚĆ DZIAŁANIA SUBSTANCJI PRZECIWDROBNOUSTROJOWYCH WZGLĘDEM BAKTERII PATOGENNYCH

Szczecin, 14.06.2023

dr hab. inż. Karol Fijałkowski, prof. ZUT
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Zachodniopomorski Uniwersytet Technologiczny w Szczecinie
al. Piastów 45
70-311 Szczecin

Oświadczenie

Oświadczam, że jestem współautorem prac wchodzących w skład rozprawy doktorskiej mgr inż. Marty Woroszyło:

1. The impact of intraspecies variability on growth rate and cellular metabolic activity of bacteria exposed to rotating magnetic field. Pathogens. 10 (11), 1427. DOI: 10.3390/pathogens10111427.

   Mój wkład w powstanie tej pracy polegał na udziale w opracowaniu koncepcji i metodologii badań, udziale w analizie uzyskanych wyników, udziale w napisaniu oraz przygotowaniu manuskryptu (jako autor do korespondencji), a także koordynacji prac zespołu jako kierownik projektu (Opus 14), w ramach realizacji którego powstała praca.

2. The effect of rotating magnetic field on susceptibility profile of methicillin-resistant *Staphylococcus aureus* strains exposed to activity of different groups of antibiotics. International Journal of Molecular Sciences. 22 (21), 11551. DOI: 10.3390/ijms222111551.

   Mój wkład w powstanie tej pracy polegał na udziale w opracowaniu koncepcji i metodologii badań, udziale w analizie uzyskanych wyników, udziale w napisaniu oraz przygotowaniu manuskryptu (jako autor do korespondencji), a także koordynacji prac zespołu jako kierownik projektu (Opus 14), w ramach realizacji którego powstała praca.

3. Rotating magnetic field increases β-lactam antibiotic susceptibility of methicillin-resistant *Staphylococcus aureus* strains. International Journal of Molecular Sciences. 22 (22), 12397. DOI: 10.3390/ijms222212397.

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Podpis współautora

.....

dr hab. inż. Karol Fijałkowski, prof. ZUT

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Wydział Farmaceutyczny
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50-534 Wrocław

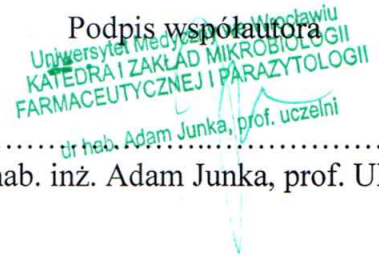
Oświadczenie

Oświadczam, że jestem współautorem prac wchodzących w skład rozprawy doktorskiej mgr inż. Marty Woroszyło:

1. The impact of intraspecies variability on growth rate and cellular metabolic activity of bacteria exposed to rotating magnetic field. Pathogens. 10 (11), 1427. DOI: 10.3390/pathogens10111427.

   Mój wkład w powstanie tej pracy polegał na udziale w opracowaniu metodyk, dostarczeniu części szczepów bakterii *S. aureus*, przeprowadzeniu części analiz statystycznych oraz udziale w opracowaniu końcowej wersji manuskryptu.

Podpis współautora



.....

dr hab. inż. Adam Junka, prof. UMW

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Oświadczenie

Oświadczam, że jestem współautorem prac wchodzących w skład rozprawy doktorskiej mgr inż. Marty Woroszyło:

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   Mój wkład w powstanie tej pracy polegał na udziale w opracowaniu metodyk, dostarczeniu części szczepów bakterii *S. aureus*, udziale w przeprowadzeniu analiz z wykorzystaniem mikroskopu konfokalnego, udziale w interpretacji wyników oraz udziale w opracowaniu końcowej wersji manuskryptu.

Podpis współautora

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Oświadczenie

Oświadczam, że jestem współautorem prac wchodzących w skład rozprawy doktorskiej mgr inż. Marty Woroszyło:

3. Rotating magnetic field increases β-lactam antibiotic susceptibility of methicillin-resistant *Staphylococcus aureus* strains. International Journal of Molecular Sciences. 22 (21). DOI: 10.3390/ijms222212397.

   Mój wkład w powstanie tej pracy polegał na udziale w opracowaniu metodyk, dostarczeniu części szczepów bakterii *S. aureus*, udziale w przeprowadzeniu analiz z wykorzystaniem mikroskopu fluorescencyjnego, udziale w interpretacji wyników oraz udziale w opracowaniu końcowej wersji manuskryptu.

Podpis współautora

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Szczecin, 14.06.2023

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ul. Sikorskiego 37
70-313 Szczecin

Oświadczenie

Oświadczam, że jestem współautorem prac wchodzących w skład rozprawy doktorskiej mgr inż. Marty Woroszyło:

1. The impact of intraspecies variability on growth rate and cellular metabolic activity of bacteria exposed to rotating magnetic field. Pathogens. 10 (11), 1427. DOI: 10.3390/pathogens10111427.

   Mój wkład w powstanie tej pracy polegał na wykonaniu wizualizacji związanych z charakterystyką wirującego pola magnetycznego.

2. The effect of rotating magnetic field on susceptibility profile of methicillin-resistant *Staphylococcus aureus* strains exposed to activity of different groups of antibiotics. International Journal of Molecular Sciences. 22 (21), 11551. DOI: 10.3390/ijms222111551.

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3. Rotating magnetic field increases β-lactam antibiotic susceptibility of methicillin-resistant *Staphylococcus aureus* strains. International Journal of Molecular Sciences. 22 (22), 12397. DOI: 10.3390/ijms222212397.

   Mój wkład w powstanie tej pracy polegał na wykonaniu analiz i wizualizacji związanych z charakterystyką wirującego pola magnetycznego.

Podpis współautora

.....

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Dr inż. Daria Ciecholewska-Juśko

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Zachodniopomorski Uniwersytet Technologiczny w Szczecinie

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Oświadczenie

Oświadczam, że jestem współautorem prac wchodzących w skład rozprawy doktorskiej mgr inż. Marty Woroszyło:

1. The impact of intraspecies variability on growth rate and cellular metabolic activity of bacteria exposed to rotating magnetic field. Pathogens. 10 (11), 1427. DOI: 10.3390/pathogens10111427.

   Mój wkład w powstanie tej pracy polegał na opracowaniu statystycznym wyników oraz ich przedstawieniu w formie wykresów.

2. The effect of rotating magnetic field on susceptibility profile of methicillin-resistant *Staphylococcus aureus* strains exposed to activity of different groups of antibiotics. International Journal of Molecular Sciences. 22 (21), 11551. DOI: 10.3390/ijms222111551.

   Mój wkład w powstanie tej pracy polegał na udziale w przeprowadzeniu analiz związanych z oceną uwalniania antybiotyków z krążków bibułowych oraz ich dyfuzji w agarze pod wpływem wirującego pola magnetycznego i opracowaniu grafik.

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Podpis współautora

Daria Ciecholewska-Juśko

Dr inż. Daria Ciecholewska-Juśko

Szczecin, 18.05.2023

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Oświadczenie

Oświadczam, że jestem współautorem pracy wchodzącej w skład rozprawy doktorskiej mgr inż. Marty Woroszyło:

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   Mój wkład w powstanie tej pracy polegał na udziale w analizach polimorfizmu długości fragmentów restrykcyjnych (RFLP).

Podpis współautora

ADIUNKT
Zakładu Medycyny Laboratoryjnej
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Oświadczenie

Oświadczam, że jestem współautorem pracy wchodzącej w skład rozprawy doktorskiej mgr inż. Marty Woroszyło:

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Podpis współautora

ADIUNKT
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51-148 Wrocław

Oświadczenie

Oświadczam, że jestem współautorem prac wchodzących w skład rozprawy doktorskiej mgr inż. Marty Woroszyło:

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   Mój wkład w powstanie tej pracy polegał na udziale w przeprowadzeniu analiz z wykorzystaniem mikroskopu konfokalnego.

Podpis współautora

Dudek

.....

dr Bartłomiej Dudek

Wrocław, 30.05.2023

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Oświadczenie

Oświadczam, że jestem współautorem pracy wchodzącej w skład rozprawy doktorskiej mgr inż. Marty Woroszyło:

1. The effect of rotating magnetic field on susceptibility profile of methicillin-resistant *Staphylococcus aureus* strains exposed to activity of different groups of antibiotics. International Journal of Molecular Sciences. 22 (21), 11551. DOI: 10.3390/ijms222111551.

   Mój wkład w powstanie tej pracy polegał na udziale w wykonaniu analiz z użyciem mikroskopu konfokalnego.

Podpis współautora

Grzegorz Chodaczek

.....

dr hab. Grzegorz Chodaczek

Wrocław, 30.05.2023

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Wydział Mechaniczny
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Oświadczenie

Oświadczam, że jestem współautorem pracy wchodzącej w skład rozprawy doktorskiej mgr inż. Marty Woroszyło:

1. Rotating magnetic field increases β-lactam antibiotic susceptibility of methicillin-resistant *Staphylococcus aureus* strains. International Journal of Molecular Sciences. 22 (21). DOI: 10.3390/ijms222212397.

   Mój wkład w powstanie tej pracy polegał na udziale w wykonaniu analiz z użyciem skaningowego mikroskopu elektronowego.

Podpis współautora

dr inż. Patrycja Szymczyk-Ziółkowska

Szczecin, 13.06.2023

dr inż. Radosław Drozd
Katedra Mikrobiologii i Biotechnologii
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70-311 Szczecin

Oświadczenie

Oświadczam, że jestem współautorem pracy wchodzącej w skład rozprawy doktorskiej mgr inż. Marty Woroszyło:

1. Rotating magnetic field increases β-lactam antibiotic susceptibility of methicillin-resistant *Staphylococcus aureus* strains. International Journal of Molecular Sciences. 22 (22), 12397. DOI: 10.3390/ijms222212397.

   Mój wkład w powstanie tej pracy polegał na udziale w wykonaniu analiz chromatograficznych (chromatografia cieczowa z tandemową spektrometrią mas).

Podpis współautora

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dr inż. Radosław Drozd

Szczecin, 15.06.2023

dr Daniel Styburski
Laboratorium Chromatografii i Spektroskopii Mas
Wydział Biotechnologii i Hodowli Zwierząt
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Oświadczenie

Oświadczam, że jestem współautorem pracy wchodzącej w skład rozprawy doktorskiej mgr inż. Marta Woroszyło:

1. Rotating magnetic field increases β-lactam antibiotic susceptibility of methicillin-resistant *Staphylococcus aureus* strains. International Journal of Molecular Sciences. 22 (22), 12397. DOI: 10.3390/ijms222212397.

   Mój wkład w powstanie tej pracy polegał na wykonaniu analiz chromatograficznych (chromatografia cieczowa z tandemową spektrometrią mas).

Podpis współautora

.....

dr Daniel Styburski

Wrocław, 30.05.2023

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Oświadczenie

Oświadczam, że jestem współautorem pracy wchodzącej w skład rozprawy doktorskiej mgr inż. Marty Woroszyło:

1. Rotating magnetic field increases β-lactam antibiotic susceptibility of methicillin-resistant *Staphylococcus aureus* strains. International Journal of Molecular Sciences. 22 (21). DOI: 10.3390/ijms222212397.

   Mój wkład w powstanie tej pracy polegał na udziale w wykonaniu analiz z użyciem skaningowego i transmisyjnego mikroskopu elektronowego.

Podpis współautora

.....

dr inż. Paweł Migdał

Załącznik 4

Sumaryczne zestawienie dorobku naukowego

Marta Woroszyło

Rozprawa doktorska

ANALIZA WPŁYWU WIRUJĄCEGO POLA MAGNETYCZNEGO NA EFEKTYWNOŚĆ DZIAŁANIA SUBSTANCJI PRZECIWDROBNOUSTROJOWYCH WZGLĘDEM BAKTERII PATOGENNYCH

Mgr inż. Marta Woroszyło – dorobek naukowy

Prace dyplomowe

1. **<u>Październik 2011 – czerwiec 2014 – studia licencjackie</u>**
   Kierunek: mikrobiologia stosowana
   Miejsce realizacji pracy: Katedra Mikrobiologii Stosowanej i Fizjologii Żywienia Człowieka (dawniej – Katedra Mikrobiologii i Biotechnologii Stosowanej), Wydział Nauk o Żywności i Rybactwa, Zachodniopomorski Uniwersytet Technologiczny w Szczecinie
   Temat pracy dyplomowej: Czynniki bakteryjne w etiopatogenezie reumatoidalnego zapalenia stawów
   Promotor: dr hab. inż. Elżbieta Bogusławska-Wąs, prof. ZUT

2. **<u>Marzec 2015 – czerwiec 2017 – studia magisterskie</u>**
   Kierunek: mikrobiologia stosowana
   Miejsce realizacji pracy: Katedra Mikrobiologii i Biotechnologii (dawniej – Katedra Immunologii, Mikrobiologii i Chemii Fizjologicznej), Wydział Biotechnologii i Hodowli Zwierząt, Zachodniopomorski Uniwersytet Technologiczny w Szczecinie
   Temat pracy dyplomowej: Analiza zdolności wytwarzania biofilmu przez gronkowce koagulazoujmne wyizolowane z przetworów mięsnych
   Promotor: dr hab. inż. Karol Fijałkowski, prof. ZUT

3. **<u>Październik 2014 – luty 2018 – studia inżynierskie</u>**
   Kierunek: Zarządzanie bezpieczeństwem i jakością żywności
   Miejsce realizacji pracy: Katedra Mikrobiologii Stosowanej i Fizjologii Żywienia Człowieka (dawniej – Katedra Mikrobiologii i Biotechnologii Stosowanej), Wydział Nauk o Żywności i Rybactwa, Zachodniopomorski Uniwersytet Technologiczny w Szczecinie
   Temat pracy dyplomowej: Horyzontalny transfer genów i jego wpływ na zmienność bakterii
   Promotor: dr inż. Alicja Dłubała

4. **<u>Październik 2018 – obecnie – studia doktoranckie</u>**
   Miejsce realizacji pracy: Katedra Mikrobiologii i Biotechnologii (dawniej – Katedra Immunologii, Mikrobiologii i Chemii Fizjologicznej), Wydział Biotechnologii i Hodowli Zwierząt, Zachodniopomorski Uniwersytet Technologiczny w Szczecinie
   Temat pracy dyplomowej: Analiza wpływu wirującego pola magnetycznego na efektywność działania substancji przeciwdrobnoustrojowych względem bakterii patogennych
   Promotor: dr hab. inż. Karol Fijałkowski, prof. ZUT

Publikacje naukowe

a) Publikacje naukowe wchodzące w skład cyklu stanowiącego rozprawę doktorską:

1. Woroszyło M., Ciecholewska-Juśko D., Junka A., Pruss A., Kwiatkowski P., Wardach M., Fijałkowski K. (2021). The impact of intraspecies variability on growth rate and cellular metabolic activity of bacteria exposed to rotating magnetic field. Pathogens. 10 (11), 1427. doi: 10.3390/pathogens10111427.

IF – 4,531; 100 pkt. MEiN

2. Woroszyło M., Ciecholewska-Juśko D., Junka A., Wardach M., Chodaczek G., Dudek B., Fijałkowski K. (2021). The effect of rotating magnetic field on susceptibility profile of methicillin-resistant *Staphylococcus aureus* strains exposed to activity of different groups of antibiotics. International Journal of Molecular Sciences. 22 (21), 11551. doi: 10.3390/ijms222111551.

IF – 6,208; 140 pkt. MEiN

3. Woroszyło M., Ciecholewska-Juśko D., Junka A., Drozd R., Wardach M., Migdał P., Szymczyk-Ziółkowska P., Styburski D., Fijałkowski K. (2021). Rotating magnetic field increases β-lactam antibiotic susceptibility of methicillin-resistant *Staphylococcus aureus* strains. International Journal of Molecular Sciences. 22 (22), 12397. doi: 10.3390/ijms222212397.

IF – 6,208; 140 pkt. MEiN

b) Pozostałe:

4. Woroszyło M., Pendrak K., Ciecholewska D., Padzik N., Szewczuk M., Karakulska J. (2018). Investigation of biofilm formation ability of coagulase-negative staphylococci isolated from ready-to-eat meat. Acta Scientiarum Polonorum Zootechnica. 17 (4), 27-34. DOI: 10.21005/asp.2018.17.4.04.

40 pkt. MEiN

5. Krasowski G., Migdał P., Woroszyło M., Fijałkowski K., Chodaczek G., Czajkowska J., Dudek B., Nowicka J., Oleksy-Wawrzyniak M., Kwiek B., Paleczny J., Brożyna M., Junka A. (2022). The assessment of activity of antiseptic agents against biofilm of *Staphylococcus aureus* measured with the use of processed microscopic images. International Journal of Molecular Sciences. 23 (21), 13524. DOI: 10.3390/ijms232113524.

IF – 6,208; 140 pkt. MEiN

6. Karakulska J., Woroszyło M., Szewczuk M., Fijałkowski K. (2022). Identification, superantigen toxin gene profile and antimicrobial resistance of staphylococci isolated from Polish primitive sheep breeds. Animals. 12 (16), 2139. DOI: 10.3390/ani12162139.

IF – 3,231; 100 pkt. MEiN

7. Ciecholewska-Juśko D., Żywicka A., Junka A., Woroszyło M., Wardach M., Chodaczek G., Szymczyk-Ziółkowska, P., Migdał, P., Fijałkowski, K. (2022). The effects of rotating magnetic field and antiseptic on in vitro pathogenic biofilm and its milieu. Scientific Reports. 12 (1), 1-19. DOI: 10.1038/s41598-022-12840-y.

IF – 4,380; 140 pkt. MEiN

Łącznie: 30,766 IF, 800 pkt. MEiN

Udział w projektach i zleceniach badawczych

1. *Analiza mechanizmów zwiększonej efektywności substancji przeciwdrobnoustrojowych względem biofilmów w obecności wirującego pola magnetycznego*; okres realizacji 10.2018-04.2022; OPUS 14, Narodowe Centrum Nauki, OPUS 2017/27/B/NZ6/02103; projekt realizowany w ramach konsorcjum z instytutem naukowo-badawczym Sieć Badawcza Łukasiewicz – PORT (Polski Ośrodek Rozwoju Technologii – wykonawca.

2. *Testowanie w warunkach rzeczywistych innowacyjnych maseczek ochronnych (NanoBioCell) z bionanocelulozy*; okres realizacji 04.2021-09.2021; projekt badawczo-rozwojowy „Odpowiedzialny społecznie Proto_Lab" realizowany w ramach Regionalnego Programu Operacyjnego Województwa Zachodniopomorskiego 2014-2020; Proto_lab/K2/2021/U/7 – wykonawca.

3. Realizacja pracy badawczej dotyczącej oceny wpływu pola magnetycznego o częstotliwości w zakresie 0 do 2000 Hz, na zmiany w efektywności działania antyseptyku na bazie dichlorowodorku oktenidyny wobec bakterii *S. aureus* w formie hodowli planktonicznych; okres realizacji 06.2022-09.2022; nr pracy badawczej – 515-01-083-10217-05/15; badania wykonano na zlecenie firmy Azyro S.A. we współpracy z firmą Scout Scientific Outsourcing – wykonawca.

4. Realizacja pracy badawczej dotyczącej oceny wpływu pola magnetycznego o częstotliwości w zakresie 0 do 2000 Hz, na zmiany w efektywności działania antyseptyku na bazie dichlorowodorku oktenidyny wobec bakterii *S. aureus* w

formie biofilmu i hodowli planktonicznych; okres realizacji 11.2022-01.2023; nr pracy badawczej – 515-01-083-10579-05/15; badania wykonano na zlecenie firmy Azyro S.A. we współpracy z firmą Scout Scientific Outsourcing – wykonawca.

5. Realizacja pracy badawczej dotyczącej oceny wpływu pola magnetycznego o częstotliwości w zakresie 0 do 2000 Hz, na zmiany w efektywności działania antyseptyku na bazie poliheksanidu wobec bakterii *S. aureus* w formie biofilmu i hodowli planktonicznych; okres realizacji 06.2023-09.2023; nr pracy badawczej – w przygotowaniu; badania wykonywane są na zlecenie firmy Azyro S.A. we współpracy z firmą Scout Scientific Outsourcing – wykonawca.

Udział w konferencjach

1. 01-02.04.2017, VII Kopernikańskie Sympozjum Studentów Nauk Przyrodniczych, III Toruńskie Sympozjum Doktorantów Nauk Przyrodniczych, Toruń, „*Zdolność do tworzenia biofilmu oraz antybiotykooporność u gronkowców koagulazoujemnych wyizolowanych z przetworów mięsnych*" (komunikat ustny).

2. 28-31.10.2020, FEMS Online Conference on Microbiology, „*Influence of the rotating magnetic field on the viability, proliferation and metabolic activity of different bacterial species and strains*" (komunikat ustny).

3. 20-22.09.2021, XIV Kopernikańskie Seminarium Doktoranckie, Toruń, „*Zwiększenie wrażliwości metycylinoopornych szczepów Staphylococcus aureus na antybiotyki β-laktamowe pod wpływem wirującego pola magnetycznego*" (komunikat ustny).

4. 21-23.05.2021, 9. Międzyuczelniane Sympozjum Biotechnologiczne „Symbioza", „*Rotating magnetic field exposure increases antibiotic susceptibility of methicillin-resistant Staphylococcus aureus strains*" (komunikat ustny).

5. 20-24.06.2021, World Microbe Forum Online Conference, „*Influence of rotating magnetic field on changes in susceptibility of methicillin-resistant Staphylococcus aureus strains to beta-lactam antibiotics*" (plakat).

6. 22-23.09.2022, 1st Baltic Symposium on Polymer and Biomaterials Science – Baltic Biomat, „*Testing of innovative, biodegradable masks based on a modified bacterial cellulose-based filter in health care units*" (plakat).

Szkolenia i warsztaty

1. 01.2021 – ukończenie szkolenia w zakresie obsługi cytometru przepływowego wraz z oprzyrządowaniem i oprogramowaniem (BC AccuriTM C6 Plus, BD CSamplerTM Plus, BD Accuri C6 Plus Software, BD CSampler Plus Software) – potwierdzone certyfikatem (organizator – Becton Dickinson).

2. 10.2022 – ukończenie warsztatów on-line pt. „Sekrety techniki ELISA” – potwierdzone certyfikatem (organizator – Merck Life Science Sp. z o.o.).

3. 10.2022 – ukończenie szkolenia on-line pt. „Przydatne techniki w prowadzeniu hodowli komórkowych” – potwierdzone certyfikatem (organizator – Merck Life Science Sp. z o.o.).

4. 03.2023 – ukończenie szkolenia on-line pt. „Dodatki do medium hodowlanego” – potwierdzone certyfikatem (organizator – Merck Sp. z o.o).

5. 05.2023 – ukończenie szkolenia on-line pt. „Jakość w testach immunoenzymatycznych” – potwierdzone certyfikatem (organizator – Merck Sp. z o.o).

6. 06.2023 – ukończenie szkolenia on-line pt. „Optymalizacja testów opartych o hodowle” – potwierdzone certyfikatem (organizator – Merck Sp. z o.o).

Praktyki i staże

1. 07.2014 – praktyka zawodowa w Katedrze Mikrobiologii i Biotechnologii Stosowanej ZUT.

2. 11.2014 – 01.2015 – praktyka zawodowa w dziale jakości w Carlsberg Polska Oddział Browar Bosman w Szczecinie.

3. 07.2020 – staż naukowy w Katedrze Mikrobiologii Farmaceutycznej i Parazytologii Uniwersytetu Medycznego we Wrocławiu.

Inne

1. 05.2015 – 02.2019 – członek Studenckiego Koła Naukowego Mikrobiologów w Katedrze Mikrobiologii i Biotechnologii ZUT.

2. 05.2020 – obecnie – członek Polskiego Towarzystwa Mikrobiologów.