SZKOŁA DOKTORSKA
BioMedChem
Uniwersytetu Łódzkiego
i Instytutów Polskiej
Akademii Nauk w Łodzi

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Praca doktorska:

**Aktywność biologiczna i toksyczność nanocząstek srebra pozyskanych na drodze mikrobiologicznej przy udziale grzyba strzępkowego *Gloeophyllum striatum***

Doctoral thesis:

**Biological activity and toxicity of silver nanoparticles synthesized by microbiological method using the filamentous fungus *Gloeophyllum striatum***

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**Łódź, 2025**

## Spis treści:

## I. Źródła finansowania

- Minigranty Szkoły Doktorskiej BioMedChem Uniwersytetu Łódzkiego i Instytutów PAN w Łodzi; lata 2021/2022, 2022/2023 i 2023/2024;
- Projekt NAWA PROM 2023 nr PPI/PRO/2019/1/00035/DEC/01 (rok realizacji 2023, kod projektu: P1910000000028NW00) – krótkoterminowe wyjazdy zagraniczne dla doktorantów.

## II. Spis publikacji wchodzących w skład rozprawy doktorskiej

Na rozprawę doktorską składają się cztery opublikowane prace oryginalne o łącznym IF = 17,5 oraz sumie punktów MEiN = 560:

- **Tończyk Aleksandra**, Niedziałkowska Katarzyna, Lisowska Katarzyna. 2023. *Optimizing the microbial synthesis of silver nanoparticles using Gloeophyllum striatum and their antimicrobial potential evaluation*, Scientific Reports, 13: 21124; DOI: 10.1038/s41598-023-48414-9

  $IF_{2023}$ = 3,8; $IF_{5\text{-letni}}$ = 4,3; MEiN = 140

- **Tończyk Aleksandra**, Niedziałkowska Katarzyna, Nowak-Lange Marta, Bernat Przemysław, Lisowska Katarzyna. 2025. *Mycogenic silver nanoparticles: promising antimicrobials with fungistatic properties.* International Journal of Molecular Sciences, 26: 6639; DOI: 10.3390/ijms26146639

  $IF_{2025}$ = 4,9; $IF_{5\text{-letni}}$ = 5,7; MEiN = 140

- **Tończyk Aleksandra**, Niedziałkowska Katarzyna, Lisowska Katarzyna. 2025. *Ecotoxic effect of mycogenicsilver nanoparticles in water and soil environment*. Scientific Reports, 15: 10815; DOI: 10.1038/s41598-025-95485-x

  $IF_{2025}$ = 3,9; $IF_{5\text{-letni}}$ = 4,3; MEiN = 140

- **Tończyk Aleksandra**, Niedziałkowska Katarzyna, Bernat Przemysław, Lisowska Katarzyna. 2025. *Synergistic activity of Gloeophyllum striatum-derived AgNPs with ciprofloxacin and gentamicin against human pathogenic bacteria*. International Journal of Molecular Sciences, 26: 3529; DOI: 10.3390/ijms26083529

  $IF_{2025}$ = 4,9; $IF_{5\text{-letni}}$ = 5,7; MEiN = 140

## Omówienie celu naukowego i uzyskanych wyników

## III. Omówienie celu naukowego i uzyskanych wyników

### III.1. Wprowadzenie

W roku 1959 amerykański fizyk i laureat Nagrody Nobla Richard Feynman po raz pierwszy zasugerował, że możliwość manipulowania materią na poziomie molekularnym stanie się jednym z kierunków rozwoju przyszłej nauki. Niecałe 20 lat później wprowadzono termin „nanotechnologia", a początek jej dynamicznego rozwoju przypadł na lata 80. XX wieku (Baig i in. 2021). Nanotechnologia jest wszechstronną dziedziną nauki, znajdującą zastosowanie w wielu różnorodnych obszarach, wliczając elektronikę, optykę, mechanikę, katalizę, chemię, kosmetykę, farmację, medycynę i nauki biomedyczne, technologię żywności czy nauki o środowisku (Sati i in. 2025). Funkcjonalną podstawę nanotechnologii stanowią nanomateriały, m.in. nanocząstki. Są to materiały o wielkości mieszczącej się w przedziale od 1 do 100 nanometrów, które mogą być zbudowane z węgla, metali, tlenków metali lub związków organicznych. Niewielki rozmiar oraz wynikający z niego wysoki stosunek powierzchni do objętości warunkują właściwości chemiczne, fizyczne i biologiczne nanocząstek, a nowe właściwości nanomateriałów mogą znacząco różnić się od analogicznych cech ich materiałów wyjściowych (Abbas i in. 2024, Duman i in. 2024, Khan i in. 2022).

Właściwości przeciwdrobnoustrojowe srebra znane są już od czasów starożytnych. Historia medycyny wyraźnie dokumentuje zastosowanie srebra i jego soli na przestrzeni wieków, m.in. w leczeniu różnorodnych infekcji, ran czy oparzeń. W XIX wieku wprowadzono użycie srebrnych nici chirurgicznych, a podczas I wojny światowej stosowano srebrne opatrunki, które zapobiegały zakażeniom ran i przyspieszały ich gojenie. Na początku XX wieku popularność zyskało srebro koloidalne, wykorzystywane w szpitalach jako środek bakteriobójczy (Eker i in. 2024, Rodrigues i in. 2024). Nanocząstki srebra zbudowane są z wielu atomów srebra elementarnego, tworzących struktury rozmiarem odpowiadające nanoskali. Nanocząstki srebra wykazują silniejszą aktywność przeciwdrobnoustrojową niż srebro, a ponadto odznaczają się aktywnością przeciwwirusową, przeciwzapalną i przeciwnowotworową (Eker i in. 2024, Nie i in. 2023, Ong i Nyam 2022). Właściwości biologiczne predysponują nanocząstki srebra do zastosowań w biomedycynie i przemyśle medycznym, przemyśle kosmetycznym, przemyśle tekstylnym, a także w technologiach związanych z pakowaniem i przechowywaniem żywności oraz w zastosowaniach środowiskowych takich jak dezynfekcja wody. Ponadto, ich właściwości fizykochemiczne, m.in. przewodność, sprzyjają ich zastosowaniom

w szeroko rozumianej technice (Tarannum i in. 2019, Shayo i in. 2024, Sati i in. 2025). Tak szerokie możliwości zastosowania nanocząstek srebra sprawiają, że są one jednymi z najczęściej używanych i najbardziej skomercjalizowanych nanomateriałów (Hadi i in. 2024).

Aktywność przeciwdrobnoustrojowa nanocząstek srebra uznawana jest za najsilniejszą wśród przebadanych pod tym kątem nanocząstek metalicznych (Hadi i in. 2024, Yassin i in. 2022). Nanocząstki srebra są skuteczne wobec szerokiego spektrum mikroorganizmów, wliczając w to szczepy bakterii gram-dodatnich i gram-ujemnych, zarówno w postaci izolatów klinicznych jak i szczepów wzorcowych, bakterie z rodziny *Mycobacteriaceae*, grzyby, wirusy. Ponadto wykazują zdolność zwalczania szczepów drobnoustrojów wielolekoopornych (ang. multidrug-resistant, MDR) (Khaldoun i in. 2025, Bruna i in. 2021).

Dokładny sposób działania przeciwdrobnoustrojowego nanocząstek srebra nie jest do końca znany. Za ogólną zasadę ich aktywności przyjmuje się przenikanie przez ścianę komórkową, zmianę struktury błony komórkowej w kierunku jej zwiększonej przepuszczalności, produkcję reaktywnych form tlenu (ang. reactive oxygen species, ROS) oraz zakłócanie replikacji DNA (Raza i in. 2023, Bruna i in. 2021). Mechanizm aktywności przeciwdrobnoustrojowej nanocząstek srebra bywa porównywany do działania „konia trojańskiego" – nanocząstki oddziałują z powierzchnią komórki doprowadzając do zmian w przepuszczalności błony komórkowej i zaburzenia procesów oddechowych komórki, po czym wnikają do jej wnętrza, gdzie zachodzi uwalnianie jonów srebra z powierzchni nanocząstek. Jony srebra mogą oddziaływać z mostkami disiarczkowymi lub grupami tiolowymi enzymów komórkowych, głównie związanymi z procesami oddechowymi, a ich inaktywacja może następnie prowadzić do generowania reaktywnych form tlenu i indukcji stresu oksydacyjnego w komórce. Jony srebra mogą także oddziaływać z zasadami azotowymi DNA, powodując zahamowanie jego replikacji oraz zakłócanie procesów transkrypcji i translacji, przez co zaburzona zostaje synteza białek komórkowych (Khaldoun i in. 2025, Yadav i in. 2024, Nicolae-Maranciuc i in. 2022, Mikhailova 2020). Nanocząstki srebra wykazują także aktywność przeciwbiofilmową, zarówno w formie zapobiegania rozwojowi biofilmu jak i skutecznego zwalczania już istniejących form (Shariati i in. 2022). Nanocząstki srebra mogą ograniczać rozwój biofilmu poprzez uniemożliwianie adhezji drobnoustrojów do powierzchni, zaburzanie sił międzycząsteczkowych, hamowanie produkcji zewnątrzkomórkowych polisacharydów lub hamowanie zjawiska *quorum sensing* między bakteriami (Khaldoun i in. 2025, Hamid i in. 2025, Swidan i in. 2022).

Aktywność nanocząstek srebra może różnić się w zależności od organizmu, na który oddziałują, a jedna z najczęściej przytaczanych w literaturze różnic w ich działaniu dotyczy grup bakterii gram-dodatnich i gram-ujemnych i związana jest z odmienną budową ich powłok komórkowych. Przyjmuje się, że bakterie gram-dodatnie są mniej podatne na działanie nanocząstek srebra. Zjawisko to przypisywane jest większej grubości ściany komórkowej, która spowalnia przenikanie jonów srebra do cytoplazmy. Z kolei na większą wrażliwość bakterii gram-ujemnych, poza mniejszą grubością ściany komórkowej w porównaniu do bakterii gram-dodatnich, może mieć wpływ także obecność lipopolisacharydów w osłonach komórkowych, których ujemny ładunek sprzyja adhezji nanocząstek srebra, potęgując ich efekt toksyczny (Mikhailova 2020).

Siła działania przeciwdrobnoustrojowego nanocząstek srebra może być determinowana przez ich właściwości, takie jak rozmiar oraz stabilność, na którą bezpośrednio wpływa ładunek powierzchniowy czy skład powłoki nanocząstek. Przyjmuje się, że nanocząstki o mniejszych rozmiarach wykazują większą aktywność, ze względu na szybsze uwalnianie jonów srebra z ich powierzchni. Ponadto, wraz z malejącym rozmiarem nanocząstek zwiększany jest ich stosunek powierzchni do objętości, co ułatwia interakcję z powłokami komórek drobnoustrojów. Mniejsze nanocząstki łatwiej przenikają przez błonę komórkową, w konsekwencji zaburzając procesy metabolizmu i prowadząc do śmierci drobnoustrojów (Ali i in. 2024). Wysoka stabilność nanocząstek przekłada się na spowalnianie procesu ich aglomeracji, który w istotny sposób osłabia ich aktywność biologiczną. Fakt, że nanocząstki srebra wykazują zmienną aktywność przeciwdrobnoustrojową w zależności od swoich właściwości, pozwala na podejmowanie prób opracowania metod syntezy produktu o zoptymalizowanych właściwościach (Bruna i in. 2021).

Metody syntezy nanocząstek srebra, tak samo jak większości nanomateriałów, można podzielić na fizyczne i chemiczne, określane mianem konwencjonalnych, oraz biologiczne (Dhaka i in. 2023, Naganthran i in. 2022). Metody fizyczne często wymagają zastosowania specjalnych warunków syntezy – promieniowania, ciśnienia lub temperatury, do czego wymagana jest specjalistyczna aparatura oraz wysokie nakłady energii zwiększające koszt procesu. Z kolei metody chemiczne zakładają użycie toksycznych odczynników, przez co generują niebezpieczne odpady poprodukcyjne, a końcowy produkt wymaga często złożonych procesów oczyszczania przed użyciem (Sawalha i in. 2025, Nguyen i in. 2023, Dhaka i in. 2023, Naganthran i in. 2022). Metody biologiczne produkcji nanocząstek srebra zdają się być odpowiedzią na wymienione wady metod

konwencjonalnych. Synteza biologiczna uznawana jest za prostą, przyjazną środowisku i opłacalną metodę produkcji nanocząstek. Polega ona na wykorzystaniu organizmów żywych – przede wszystkim mikroorganizmów, roślin i glonów, lub produktów ich metabolizmu, takich jak alkohole, flawonoidy, alkaloidy, terpenoidy i związki fenolowe, a także egzopolisacharydy, celuloza i enzymy, jako czynniki redukujące, stabilizujące i opłaszczające powstałe nanocząstki, bez konieczności stosowania dodatkowych, niebezpiecznych odczynników chemicznych. Nanocząstki syntetyzowane biologicznie charakteryzują się wysoką stabilnością koloidalną oraz ograniczoną tendencją do aglomeracji w roztworach wodnych. Ponadto, ze względu na występowanie czynników bioaktywnych pochodzenia naturalnego na ich powierzchni, mogą wykazywać zwiększoną aktywność biologiczną (Duman i in. 2024, Dhaka i in. 2023, Nguyen i in. 2023, Naganthran i in. 2022).

Spośród organizmów wykorzystywanych w biosyntezie nanocząstek srebra szczególna uwaga zwrócona jest w kierunku grzybów strzępkowych. Synteza nanocząstek z udziałem grzybów strzępkowych może przebiegać wewnątrzkomórkowo lub zewnątrzkomórkowo. Proces wewnątrzkomórkowy polega na wiązaniu jonów metalu, w tym wypadku srebra, do ściany komórkowej grzyba przy udziale białek lub enzymów. W wyniku powstałego oddziaływania elektrostatycznego i przeniesienia elektronów, jony srebra zostają zredukowane do formy metalicznej. Synteza zewnątrzkomórkowa przebiega bez udziału grzybni, a jony metalu oddziałują z wydzielonymi do środowiska zewnętrznego metabolitami grzybowymi, np. enzymami, cukrami, kwasami organicznymi, flawonoidami czy alkaloidami. Metabolity te pełnią funkcję czynników redukujących, prowadzących do redukcji jonów srebra do formy zero-wartościowej i formowania nanocząstek w roztworze, a także stabilizujących i opłaszczających (Jangid i Kumar 2025, Sawalha i in. 2025). Grzyby strzępkowe wykazują szereg właściwości predysponujących je do zastosowania w biosyntezie nanocząstek srebra na skalę przemysłową. Charakteryzują je proste wymagania żywieniowe, efektywna produkcja biomasy, a grzybnia wykazuje wysoką odporność na stres mechaniczny związany z procesem syntezy. Wyróżnia je także zdolność do produkcji znacznych ilości różnorodnych metabolitów wtórnych, wydzielanych do środowiska wzrostu. Stanowi to dodatkową zaletę w związku z ułatwionym otrzymaniem ekstraktu zawierającego składniki bioaktywne oraz późniejszego uproszczonego procesu obróbki poprodukcyjnej powstałych nanocząstek (El-Deeb i in. 2025, Šebesta i in. 2022, Arif i Uddin 2020).

Gatunki grzybów z rodzajów *Trichoderma*, *Fusarium*, *Aspergillus*, *Penicillium*, *Rhizopus*, *Isaria*, *Rhizoctonia* wykazują zdolność do syntezy nanocząstek srebra o konkretnym kształcie i rozmiarze w zoptymalizowanych warunkach procesu (Guilger-Casagrande i de Lima 2019). Pośród przebadanych pod kątem przydatności do syntezy nanocząstek srebra gatunków grzybów strzępkowych, stosunkowo słabo poznaną grupę stanowią grzyby powodujące rozkład drewna (Zawadzka i in. 2021). Grzyby te wykazują wspomniane wcześniej, korzystne dla procesu biosyntezy właściwości – produkują znaczne ilości różnych, biologicznie czynnych metabolitów, są łatwe w hodowli oraz wykazują się szybkim wzrostem, dzięki czemu możliwe jest wydajne otrzymywanie dużych objętości biomasy (Waszczuk i in. 2022, Kobashigawa i in. 2019). Wykazano, że niektóre gatunki grzybów białej zgnilizny drewna, m.in. *Trametes* sp. i *Phanerochaete* sp., mogą pomyślnie przeprowadzać redukcję jonów srebra do postaci nanocząstek. (Osorio-Echevarria i in. 2021, Gudikandula i in. 2017). Przydatność grzybów z grupy brunatnej zgnilizny drewna do biosyntezy nanocząstek srebra jest dotychczas zweryfikowana w bardzo niewielkim stopniu. Jednakże ze względu na ich inne predyspozycje do zastosowań biotechnologicznych, np. zdolność do dekoloryzacji barwników lub degradacji ksenobiotyków (Purnomo i in. 2020, Purnomo i in. 2019), grzyby te mogą stanowić obiecujące źródło nanocząstek srebra pochodzenia biologicznego. O istotności poszukiwań nowych szczepów grzybowych charakteryzujących się zdolnością syntezy nanocząstek świadczy zależność procesu biosyntezy od danego gatunku i produkowanych przez niego metabolitów, z których każdy może wpływać na właściwości produktu, a tym samym – determinować potencjalne zastosowanie zsyntetyzowanych nanocząstek srebra (Olivera i in. 2025, Murillo- Rábago i in. 2022).

W związku z wysokim potencjałem aplikacyjnym nanocząstki srebra są powszechne w różnych dostępnych produktach konsumenckich, od tekstyliów, przez środki czystości i kosmetyki, po sprzęt medyczny (Kister i in. 2023). Przewiduje się, że w bieżącym roku globalna roczna produkcja nanocząstek srebra przekroczy 800 ton (Zhang i in. 2024). Tak duże zapotrzebowanie na produkty zawierające nanocząstki srebra oraz ich intensywne zużycie spowodowało, że nanocząstki srebra w zwiększonej ilości wykrywane są w środowisku naturalnym (Ihtisham i in. 2021, Radić i in. 2019).

Nanocząstki mogą być uwalniane do środowiska naturalnego z zawierających je produktów na różnych etapach tzw. „cyklu życia" produktu – podczas procesów produkcji, użytkowania, degradacji lub nieodpowiedniego utylizowania (Du i in. 2018, McGillicuddy i in. 2017). Ponadto, mogą przedostawać się bezpośrednio do środowiska jako

składnik preparatów wykorzystywanych w rolnictwie, takich jak nawozy, fungicydy czy pestycydy (Ihtisham i in. 2021). Częstym źródłem występowania nanocząstek srebra w środowisku naturalnym są oczyszczalnie ścieków, zarówno komunalnych, jak i przemysłowych (Lazim i in. 2023). Oczyszczalnie zatrzymują do ok. 90% nanocząstek pochodzących z produktów konsumenckich, a do wód odbierających oczyszczone ścieki mogą nadal przedostawać się znaczne ilości srebra, sięgające nawet kilku nanogramów na litr wody (Mishra i Jang 2025, Gagnon i in. 2021). Skuteczność oczyszczania ścieków z zawartego w nich srebra związana jest z jego akumulacją w osadach ściekowych. Osady, o zawartości srebra wynoszącej nawet do setek miligramów na kilogram osadu, pochodzące z oczyszczalni ścieków, są często wykorzystywane jako nawozy na użytkach rolnych (Mishra i Jang 2025, Courtois i in. 2021). Drogi dystrybucji nanocząstek srebra do środowiska naturalnego wyraźnie wskazują, że najbardziej zagrożone poprzez ich obecność są ekosystemy wodne i glebowe.

Szacowane stężenia nanoczastek srebra w glebie mieszczą się w zakresie od 0,24 do 729,23 ng/kg. Przewiduje się, że do roku 2050 wartości te wzrosną do 10 µg/kg (de Oca-Vásquez i in. 2020). Stężenie srebra w postaci jonowej w naturalnych ekosystemach wodnych waha się od 0,3 do 500 mg/L. Nanocząstki srebra wykrywane są w wodach powierzchniowych w różnych stężeniach, które w krajach europejskich wynoszą od 0,03 do 320 ng/L i wyższych (Lazim i in. 2023). W środowisku nanocząstki srebra mogą ulegać utlenieniu i przekształcać się w formę jonową, która jest bardziej biodostępna, co zwiększa ryzyko toksyczności wobec organizmów w środowisku (Mishra i Jang 2025, Ihtisham i in. 2021). W ekosystemach wodnych nanocząstki srebra oddziałują z organizmami na różnych poziomach troficznych, od alg i zooplanktonu po ryby, powodując stres oksydacyjny, uszkodzenia błon komórkowych oraz zaburzenia reprodukcyjne. W ekosystemach glebowych nanocząstki srebra negatywnie oddziałują na mikroorganizmy glebowe poprzez uszkadzanie błon komórkowych, uszkadzanie DNA, indukowanie stresu oksydacyjnego oraz zaburzanie pracy enzymów komórkowych. W ten sposób zmniejszają ilość i aktywność mikrobioty glebowej, w tym mikroorganizmów wspierających wzrost roślin i wpływających na obieg składników odżywczych, pośrednio oddziałując na wzrost roślin (Mishra i Jang 2025, Jangid i Kumar 2023, Liu i in. 2021, Ottoni i in. 2020, Zhang i in. 2020, Colman i in. 2014). Poza bezpośrednim oddziaływaniem na organizmy ekosystemów wodnych i lądowych, duże zagrożenie zarówno dla środowiska jak i zdrowia ludzi stwarza zdolność nanocząstek srebra do bioakumulacji i biomagnifikacji (Zhu i in. 2024, Jangid i Kumar 2023, Lazim i in. 2023). Jednakże przewidywanie zagrożeń

środowiskowych związanych z występowaniem nanocząstek srebra jest trudne, ponieważ wykazują one różną toksyczność w zależności od organizmu, na który oddziałują. Ponadto, ich oddziaływanie z systemami ekologicznymi jest bardzo złożone i zależy od wielu czynników – zarówno właściwości samych nanocząstek, jak i warunków środowiska (Lazim i in. 2023, Mishra i in. 2021).

Aktywność przeciwdrobnoustrojowa nanocząstek srebra wobec szerokiego spektrum drobnoustrojów jest wyjątkowo obiecująca, szczególnie w kontekście walki z infekcjami wywoływanymi przez mieszane grupy drobnoustrojów. Użyteczność nanocząstek podkreśla także ich aktywność antybiofilmotwórcza – komórki tworzące biofilm mogą wykazywać nawet do 1000 razy wyższą oporność wobec działania standardowych środków przeciwdrobnoustrojowych w porównaniu do form planktonowych (Rodrigues i in. 2024, Ribeiro i in. 2023, Mah 2012). Mimo niewątpliwych zalet wykorzystania nanocząstek srebra jako środka przeciwdrobnoustrojowego, ich wysoka reaktywność, niestabilność, podatność na utlenianie i potencjalna cytotoksyczność wobec komórek, szczególnie związana z wydłużoną ekspozycją na ich aktywne stężenia, może ograniczać możliwości ich bezpiecznego zastosowania (Dinç 2025, Ribeiro i in. 2023, Tripathi i Goshisht 2022). Efekt cytotoksyczny nanocząstek srebra oraz uwalnianych z ich powierzchni jonów srebra może polegać na niszczeniu mitochondriów i lizosomów, degradowaniu błony komórkowej, indukcji stresu oksydacyjnego przez generowanie reaktywnych form tlenu oraz genotoksyczności, a wszystkie te procesy mogą w konsekwencji prowadzić do śmierci komórki. Nanocząstki srebra mogą być wchłanianie do organizmu drogą oddechową, pokarmową lub skórną i swobodnie przedostawać się poprzez układ krwionośny lub limfatyczny do różnych tkanek i narządów. Mogą także przenikać barierę krew-mózg (Ribeiro i in. 2023, Lopez-Carrizales i in. 2018, Paknejadi i in. 2018, Akter i in, 2017). Wykazano, że nanocząstki srebra pochodzenia biologicznego mogą wywierać efekt cytotoksyczny wobec komórek ssaczych (Zawadzka i in. 2021, Khan i in. 2020).

W związku z zagrożeniami płynącymi z wykorzystywania nanocząstek srebra jako środka o aktywności biologicznej, poszukiwane są metody ograniczenia ich ilości i obniżenia aktywnych stężeń, bez utraty skuteczności wobec drobnoustrojów. Jednym z możliwych rozwiązań jest równoczesne stosowanie nanocząstek srebra z innymi środkami przeciwdrobnoustrojowymi, które będą wykazywały wobec siebie aktywność synergistyczną, pozwalającą na obniżenie skutecznych dawek, a w konsekwencji ograniczenie efektu toksycznego wywieranego przez składniki stosowanej mieszaniny środków bioaktywnych (Ribeiro i in. 2023, Haji i in. 2022). Nanocząstki srebra wykazują

zdolność wzmacniania aktywności konwencjonalnych środków przeciwdrobnoustrojowych, zarówno w sposób synergistyczny jak i addytywny. Połączenie antybiotyków takich jak ampicylina, kanamycyna, chloramfenikol, enoksacyna, neomycyna czy tetracyklina z nanocząstkami srebra, tak samo podawanych w formie koniugatów lub podawanych jednocześnie, może istotnie wzmacniać aktywność przeciwdrobnoustrojową obu środków, nawet jeśli antybiotyk czy nanocząstki srebra podawane osobno wykazywały ograniczoną skuteczność. Mechanizm działania nanocząstek srebra osłabia metabolizm komórek bakteryjnych ich mechanizmy obrony przed środkami przeciwdrobnoustrojowymi, zwiększając podatność na ich działanie, nawet w przypadku szczepów opornych (Casals i in. 2025, Hadi i in. 2024, Ribeiro i in. 2023, Rodrigues i in. 2022, Ipe i in. 2020). Możliwe staje się więc obniżenie wymaganej skutecznej dawki zarówno antybiotyków, jak i nanocząstek srebra stosowanych razem, zredukowanie ich potencjalnego efektu toksycznego oraz poszerzenie spektrum działania (Ribeiro i in. 2023). Właściwość ta jest nie tylko obiecująca w kontekście ograniczania toksyczności nanocząstek srebra, ale także zwalczania problemu antybiotykooporności.

W niniejszej pracy doktorskiej podjęto próbę biosyntezy nanocząstek srebra wykorzystując nieopisany dotąd pod tym kątem szczep grzyba strzępkowego brunatnej zgnilizny drewna *Gloeophyllum striatum* DSM 9592. Podczas optymalizacji syntezy zastosowano cztery warianty warunków przeprowadzania procesu różniące się zastosowaną temperaturą i napowietrzaniem w celu weryfikacji, czy różnice te wpływać będą na właściwości biologiczne zsyntetyzowanych nanocząstek. Biorąc pod uwagę fakt, że nanocząstki srebra mogą wykazywać zróżnicowaną aktywność biologiczną, zarówno w kontekście pożądanego działania przeciwdrobnoustrojowego, jak i potencjału toksycznego, w zależności od swoich właściwości oraz organizmów, na które oddziałują, szeroka analiza aktywności biologicznej nowo zsyntetyzowanych nanocząstek srebra wydała się zasadna. Dalsze badania w ramach pracy doktorskiej objęły więc swoim zakresem analizę potencjału przeciwdrobnoustrojowego, cytotoksycznego oraz ekotoksycznego nanocząstek srebra pochodzących od *G. striatum* DSM 9592. Ponadto, w celu dopełnienia kompleksowej analizy potencjału aplikacyjnego zsyntetyzowanych nanocząstek srebra, podjęto próbę oceny ich aktywności synergistycznej z konwencjonalnymi środkami przeciwdrobnoustrojowymi.

## III. 2. Cel pracy

Celem niniejszej pracy doktorskiej było:

1) Opracowanie wydajnej metody syntezy mikrobiologicznej nanocząstek srebra z wykorzystaniem grzyba strzępkowego brunatnej zgnilizny drewna *Gloeophyllum striatum* oraz charakterystyka fizykochemiczna uzyskanych nanomateriałów;
2) Ocena aktywności przeciwdrobnoustrojowej uzyskanych nanocząstek srebra wobec mikroorganizmów: bakterii gram-ujemnych i gram-dodatnich, w tym bakterii beztlenowych, oraz drożdży i grzybów strzępkowych;
3) Określenie potencjału cytotoksycznego zsyntetyzowanych mikrobiologicznie nanocząstek srebra wobec komórek ludzkich;
4) Kompleksowa ocena toksyczności środowiskowej obejmująca organizmy z różnych poziomów troficznych ekosystemów szczególnie narażonych na zwiększoną ekspozycję na nanocząstki srebra;
5) Określenie potencjalnej aktywności synergistycznej nanocząstek srebra zsyntetyzowanych przy użyciu *G. striatum* w połączeniu z konwencjonalnie stosowanymi antybiotykami.

### III.3. Hipotezy badawcze

Badania realizowane w ramach niniejszej pracy doktorskiej miały na celu weryfikację następujących hipotez badawczych:

1) Grzyb strzępkowy brunatnej zgnilizny drewna *Gloeophyllum striatum* zdolny jest do wydajnej produkcji nanocząstek srebra.
2) Warunki syntezy wpływają na aktywność biologiczną i toksyczność nanocząstek srebra pozyskanych na drodze mikrobiologicznej przy udziale *G. striatum*.
3) Zastosowanie różnych warunków syntezy nanocząstek srebra pozwala uzyskać nanocząstki o wysokiej skuteczności przeciwdrobnoustrojowej przy jednoczesnej niskiej toksyczności i nieznacznej toksyczności środowiskowej.

### III.4. Metodologia badań

**Tabela 1.** Podsumowanie technik badawczych wykorzystanych podczas realizacji pracy doktorskiej.

| Technika | Zakres badań |
|---|---|
| Skaningowa mikroskopia elektronowa (SEM) | • Analiza właściwości fizykochemicznych zsyntetyzowanych nanocząstek srebra. |
| Spektroskopia w podczerwieni z transformacją Fouriera (FTIR) | • Analiza właściwości fizykochemicznych zsyntetyzowanych nanocząstek srebra. |
| Wizualizacja i analiza wielkości nanocząstek zawieszonych w cieczy techniką NTA (Nanoparticle Tracking Analysis) | • Analiza właściwości fizykochemicznych zsyntetyzowanych nanocząstek srebra. |
| Spektrofotometria | • Analiza właściwości fizykochemicznych zsyntetyzowanych nanocząstek srebra;<br>• Ocena wpływu nanocząstek srebra na wzrost wybranych szczepów chorobotwórczych bakterii gram-dodatnich, gram-ujemnych, tlenowych i beztlenowych oraz bakterii glebowych;<br>• Ocena wpływu nanocząstek srebra na wzrost wybranych szczepów chorobotwórczych drożdży i grzybów strzępkowych oraz strzępkowych grzybów glebowych;<br>• Ocena wpływu nanocząstek srebra, antybiotyków oraz ich mieszaniny na produkcję biofilmu przez wybrane szczepy chorobotwórczych bakterii;<br>• Ocena potencjału hemolitycznego nanocząstek srebra;<br>• Ocena aktywności cytotoksycznej nanocząstek srebra wobec linii komórkowej fibroblastów ludzkich. |
| Mikroskopia konfokalna | • Ocena zmian w przepuszczalności błony komórkowej wybranego szczepu drożdży pod wpływem nanocząstek srebra. |
| Chromatografia cieczowa sprzężona z tandemową spektrometrią mas, LC-MS/MS | • Analiza zmian profilu fosfolipidowego wybranych szczepów grzybów w obecności nanocząstek srebra;<br>• Analiza zmian profilu fosfolipidowego wybranych szczepów bakterii w obecności |

| | |
|---|---|
| | nanocząstek srebra, antybiotyków lub ich mieszaniny. |
| Chromatografia gazowa sprzężona ze spektrometrią mas, GC-MS | • Analiza zmian profilu kwasów tłuszczowych wybranych szczepów bakterii w obecności nanocząstek srebra, antybiotyków lub ich mieszaniny. |
| Spektrofluorymetria | • Ocena toksyczności środowiskowej nanocząstek srebra wobec wybranych szczepów bakterii środowisk wodnych;<br>• Ocena zmian w płynności błony komórkowej wybranego szczepu drożdży pod wpływem nanocząstek srebra. |
| Testy toksykologiczne typu Toxkit:<br>Daphtoxkit F<br>Arthroxkit<br>Phytotoxkit<br>Duckweed | • Ocena toksyczności środowiskowej nanocząstek srebra wobec wybranych gatunków skorupiaków wodnych oraz roślin wodnych i lądowych. |
| Analiza statystyczna | • Analiza statystyczna otrzymanych wyników z wykorzystaniem oprogramowania Microsoft Excel. |

## III.5. Omówienie wyników badań przedstawionych w cyklu publikacji wchodzących w skład rozprawy doktorskiej

Cykl tematycznie spójnych publikacji naukowych, wchodzących w skład niniejszej pracy doktorskiej, otwiera artykuł zatytułowany **„*Optimizing the microbial synthesis of silver nanoparticles using Gloeophyllum striatum and their antimicrobial potential evaluation*”** (Tończyk A., Niedziałkowska K., Lisowska K., 2023. Scientific Reports, IF = 3,8, MEiN = 140). W publikacji przedstawiono opracowaną metodę biologicznej syntezy nanocząstek srebra wykorzystującą płyn pohodowlany grzyba strzępkowego brunatnej zgnilizny drewna *Gloeophyllum striatum* DSM 9592. Synteza została przeprowadzona w czterech różnych wariantach warunków procesu, różniących się zastosowaną temperaturą i napowietrzaniem (28°C bez napowietrzania, 28°C z napowietrzaniem, 4°C bez napowietrzania i 4°C z napowietrzaniem). Uzyskane nanocząstki srebra, w zależności od zastosowanych warunków syntezy, były odpowiednio oznaczane 28ns AgNPs, 28s AgNPs, 4ns AgNPs i 4s AgNPs.

Pierwszym etapem badań zsyntetyzowanych mikrobiologicznie nanocząstek srebra była ich analiza fizykochemiczna. Na podstawie zgromadzonych danych stwierdzono, że wszystkie z uzyskanych typów nanocząstek srebra wykazały charakterystyczną dla tych nanomateriałów wartość powierzchniowego rezonansu plazmowego (SPR), potwierdzając tym samym skuteczność zawartych w płynie pohodowlanym *G. striatum* metabolitów w redukcji jonów srebra do postaci metalicznej. Analiza fizykochemiczna nanocząstek srebra potwierdziła, że warunki biosyntezy mogą determinować właściwości powstałych nanomateriałów. Wykazano, że potencjał zeta przyjmował wyższe wartości w przypadku nanocząstek syntetyzowanych w temperaturze 28°C, a mniejszym rozmiarem charakteryzowały się nanomateriały syntetyzowane bez napowietrzania. Ponadto stwierdzono, że w zależności od zastosowanych warunków syntezy różniła się wydajność produkcji nanocząstek srebra, która była największa w przypadku 28s AgNPs.

Kolejnym etapem badań była ocena aktywności przeciwbakteryjnej zsyntetyzowanych nanocząstek srebra. Do tego celu wybrano dziesięć szczepów bakterii, w tym bakterie gram-dodatnie, gram-ujemne, zarówno tlenowe, beztlenowe oraz mikroaerofilne: *Escherichia coli* ATCC 25922, *Proteus hauseri* ATCC 15442, *Pseudomonas aeruginosa* ATCC 27853, *Campylobacter jejuni* ATCC 33560, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 6538, *Staphylococcus*

*aureus* ATCC 780699 oraz *Listeria monocytogenes* ATCC 19115. Wybrane szczepy wzorcowe reprezentowały różne grupy bakterii chorobotwórczych dla człowieka, dzięki czemu możliwa była kompleksowa ocena potencjału nowo zsyntetyzowanych nanocząstek srebra w zwalczaniu patogenów ludzkich. Badania zostały przeprowadzone według odpowiednich dla danego szczepu bakterii norm CLSI (ang. *Clinical and Laboratory Standards Institute*) w zakresie stężeń nanocząstek srebra wynoszącym od 0,098 do 25 μg/ml, dobranym w taki sposób, aby obejmował wartości MIC dla wszystkich badanych szczepów drobnoustrojów. Uzyskane wyniki oraz wyznaczone na ich podstawie wartości MIC (ang. *minimal inhibitory concentration*) i MBC (ang. *minimal bactericidal concentration*) dowiodły, że wszystkie zsyntetyzowane rodzaje nanocząstek srebra wykazały skuteczność wobec badanych szczepów bakteryjnych, a ich aktywność różniła się w zależności od zastosowanych warunków syntezy oraz modelu badawczego. Nie wykazano jednak wyraźnie wyższej skuteczności jednego wariantu nanocząstek srebra wobec wszystkich badanych szczepów. Wyniki badania potwierdziły opisywane w literaturze tendencje dotyczące działania przeciwbakteryjnego nanocząstek srebra – zsyntetyzowane przy udziale *G. striatum* nanocząstki były bardziej efektywne wobec bakterii gram-ujemnych w porównaniu do bakterii gram-dodatnich oraz bakterii tlenowych w porównaniu do beztlenowych. Najmniejsza wartość MIC wykazana została dla szczepu bakterii gram-ujemnych *P. aeruginosa* i wynosiła 1,56 μg/ml dla wszystkich badanych wariantów nanocząstek srebra. W przypadku bakterii gram-dodatnich, najniższa wartość MIC, wynosząca 6,25 μg/ml została wykazana dla szczepu *S. aureus* ATCC 780699 dla wszystkich badanych typów nanocząstek, dla szczepu *S. aureus* ATCC 43300 dla nanocząstek syntetyzowanych w 28°C, a dla szczepów *S. aureus* ATCC 29213 i *S. aureus* ATCC 6538 dla 28ns AgNPs. Gram-dodatnie bakterie beztlenowe *L. monocytogenes* wykazały najniższą wrażliwość na działanie wszystkich rodzajów nanocząstek srebra, które hamowały wzrost komórek bakterii w najwyższym ze stosowanych stężeń wynoszącym 25 μg/ml. Tak zróżnicowany między modelami badawczymi efekt działania nanocząstek srebra może wynikać z różnic w budowie osłon komórkowych bakterii gram-dodatnich i gram-ujemnych oraz szybszego uwalniania jonów srebra z powierzchni nanocząstek w warunkach tlenowych. Spośród badanych szczepów najwyższą podatnością na działanie wszystkich rodzajów nanocząstek srebra charakteryzował się wspomniany już *P. aeruginosa*. W związku z tym, poszerzono badanie tego szczepu o analizę wpływu zsyntetyzowanych nanocząstek na zdolność do produkcji biofilmu. *P. aeruginosa* znany jest

z silnej aktywności biofilmotwórczej oraz zdolności do kolonizacji powierzchni sprzętów medycznych, przez co może stanowić zagrożenie dla zdrowia. Wykazano, że zastosowanie wszystkich typów nanocząstek w stężeniu odpowiadającym wyznaczonej wcześniej wartości MIC, zahamowało wytwarzanie biofilmu bakteryjnego. Natomiast zastosowanie stężenia równego połowie wartości MIC, wynoszącego 0,78 μg/ml, które ograniczało, ale nie hamowało całkowicie wzrostu bakterii, spowodowało wyraźną stymulację aktywności biofilmotwórczej *P. aeruginosa*. Formy biofilmu bakteryjnego uznawane są za bardziej oporne wobec działania środków przeciwdrobnoustrojowych, stąd wykryte zjawisko może być związane z odpowiedzią badanego szczepu bakterii na nieprzyjazne warunki środowiska wzrostu spowodowane obecnością nanocząstek srebra.

W związku z masowym wykorzystaniem nanocząstek srebra ukierunkowanym na zastosowania związane z ich właściwościami biologicznymi, wzrastają obawy dotyczące bezpieczeństwa ich eksploatacji. Wątpliwości potęguje coraz szersza znajomość mechanizmu ich aktywności, którego podstawą jest ogólne, nieukierunkowane na konkretny rodzaj komórek działanie cytotoksyczne. Biorąc pod uwagę potencjalne zagrożenie, po potwierdzeniu skuteczności zsyntetyzowanych z udziałem *G. striatum* nanocząstek srebra wobec szczepów bakterii chorobotwórczych, podjęto próbę oceny ich potencjału toksycznego wobec komórek ludzkich. W tym celu wykonano analizę aktywności hemolitycznej nanocząstek oraz toksyczności wobec linii komórkowej fibroblastów ludzkich. Na podstawie wyników stwierdzono, że wszystkie badane typy nanocząstek srebra w stężeniu 12,5 μg/ml powodowały co najmniej 60-procentowy efekt hemolityczny wobec ludzkich erytrocytów. Natomiast stężenia wszystkich rodzajów nanocząstek odpowiadające wartościom MIC uzyskanym dla bakterii gram-ujemnych *P. aeruginosa*, *E. coli* oraz stężenia nanocząstek 28ns AgNPs, 28s AgNPs i 4s AgNPs odpowiadające wartości MIC dla gram-dodatniego szczepu *S. epidermidis*, mieszczące się w zakresie od 1,56 μg/ml do 3,125 μg/ml, nie powodowały efektu hemolitycznego. Efekt toksyczny badanych nanocząstek srebra wobec linii komórkowej fibroblastów był silniejszy – trzy z czterech typów nanocząstek srebra powodowały około 60-procentowe zahamowanie przeżywalności komórek w stężeniu 1,56 μg/ml. Jednakże, w przypadku 4ns AgNPs efekt toksyczny w podanym stężeniu był słabszy. Uzyskane wyniki badań potwierdziły zatem, że warunki biosyntezy mogą determinować nie tylko pożądaną aktywność przeciwdrobnoustrojową nanocząstek srebra pochodzenia mikrobiologicznego, ale także ich potencjalne działanie niepożądane wobec komórek ludzkich. Nanocząstki syntetyzowane w 4°C bez

napowietrzania w stężeniu 1,56 μg/ml wykazały skuteczność wobec szczepu bakterii *P. aeruginosa*, zarówno w kontekście aktywności hamującej wzrost bakterii, jak i produkcję biofilmu bakteryjnego, nie powodując w tym samym stężeniu efektu hemolitycznego wobec erytrocytów ludzkich i wywierając najsłabszy efekt toksyczny na komórki fibroblastów ludzkich w porównaniu do innych rodzajów nanocząstek.

Dopełnieniem analizy potencjału przeciwdrobnoustrojowego zsyntetyzowanych nanocząstek srebra było badanie ich aktywności przeciwgrzybowej, którego wyniki zostały opublikowane w drugiej publikacji naukowej, wchodzącej w skład niniejszej pracy doktorskiej, zatytułowanej **„*Mycogenic silver nanoparticles: promising antimicrobials with fungistatic properties*"** (Tończyk A., Niedziałkowska K., Nowak-Lange M., Bernat P., Lisowska K., 2025, International Journal of Molecular Sciences, IF = 4,9, MEiN = 140).

Badanie aktywności przeciwgrzybowej nanocząstek przeprowadzono wykorzystując dwa szczepy drożdży i dwa szczepy grzybów strzępkowych chorobotwórczych dla człowieka – *Candida albicans* ATCC 10231, *Malassezia furfur* DSM 6170, *Aspergillus flavus* ATCC 9643 i *Aspergillus fumigatus* ATCC 204305. Badania przeprowadzone zostały według odpowiednich dla danego szczepu norm CLSI w zakresie stężeń korespondujących do zastosowanych w badaniu aktywności przeciwbakteryjnej zsyntetyzowanych nanocząstek. Na podstawie wyników badań stwierdzono, że wybrane szczepy drożdży były bardziej podatne na działanie wszystkich typów nanocząstek srebra w porównaniu do szczepów grzybów strzępkowych. Zsyntetyzowane nanocząstki srebra były najbardziej aktywne wobec szczepu drożdży *M. furfur*, a wyznaczone wartości MIC nie różniły się między badanymi rodzajami nanocząstek i wynosiły 0,39 μg/ml. Wszystkie typy nanocząstek w podanym stężeniu nie wywoływały efektu hemolitycznego, a oba rodzaje nanocząstek zsyntetyzowanych w temperaturze 4°C wykazały słabsze działanie toksyczne wobec linii komórkowej fibroblastów ludzkich w porównaniu z pozostałymi rodzajami nanocząstek. Ponadto, 4ns AgNPs wykazały także skuteczność wobec drożdży *C. albicans* w stężeniu równym 1,56 μg/ml. Porównując wartości MIC wyznaczone dla wszystkich badanych szczepów drobnoustrojów, zarówno bakterii jak i grzybów, stwierdzono, że nanocząstki srebra zsyntetyzowane przy udziale *G. striatum* wykazały silniejszą aktywność przeciwgrzybową. Jednak biorąc pod uwagę wyznaczone wartości MFC (ang. *minimal fungicidal concentration*) należy podkreślić, że aktywność ta miała charakter fungistatyczny, a całkowity efekt bójczy wobec badanych szczepów grzybowych nie został osiągnięty w zastosowanym zakresie stężeń nanocząstek.

Kolejnym etapem pracy była ocena wpływu uzyskanych nanocząstek srebra na właściwości błony komórkowej wybranych modeli badanych. W tym celu przeprowadzono analizę szczepów *A. flavus* i *C. albicans* pod kątem zmian w ich profilu fosfolipidowym, które mogą stanowić wskaźnik zaburzeń integralności błon komórkowych. Układy badane zostały dobrane na podstawie wcześniejszej analizy potencjału przeciwgrzybowego nanocząstek srebra – w przypadku wybranych szczepów najbardziej widoczne były różnice w aktywnościach 28ns AgNPs i 4s AgNPs. Wybrane stężenia nanocząstek wynosiły odpowiednio 1,56 μg/ml i 0,19 μg/ml dla *A. flavus* oraz *C. albicans* i zostały dobrane w taki sposób, by ograniczały, ale nie hamowały całkowicie wzrostu komórek. Obecność badanych typów nanocząstek srebra wpłynęła na zawartość fosfatydylocholin i fosfatydyloetanoloamin zarówno w przypadku *A. flavus* jak i *C. albicans*, jednak tylko w przypadku *A. flavus* zmiany były istotne statystycznie. W celu określenia zmian właściwości błon komórkowych wybranych modeli badawczych pod wpływem nanocząstek srebra wyznaczono wartości stosunku fosfatydylocholin do fosfatydyloetanoloamin (PC/PE) oraz indeksu DBI (ang. *double-bond index*). Stwierdzono, że w przypadku *A. flavus* zarówno wartość stosunku PC/PE jak i DBI wzrosła w obecności obu badanych typów nanocząstek srebra, w przeciwieństwie do *C. albicans*, gdzie nie zaobserwowano zmian w wartości indeksu DBI pod wpływem badanych nanocząstek. Stwierdzony u *A. flavus* przyrost wartości indeksu DBI może sugerować wzrost płynności błon komórkowych wywołanych obecnością nanocząstek srebra. Poszerzone o spektroskopię fluorescencyjną i mikroskopię konfokalną badania drożdży *C. albicans* potwierdziły, że mimo braku zmian w wartości indeksu DBI, zarówno płynność jak i przepuszczalność błony komórkowej badanego szczepu została zwiększona w obecności nanocząstek srebra syntetyzowanych mikrobiologicznie. Rezultat ten sugeruje, że poza fosfolipidami istotną rolę w zmianach właściwości błony komórkowej mogą odgrywać także inne składniki błon, takie jak sterole, sfingolipidy czy białka błonowe.

Wspomniana już masowa produkcja nanocząstek srebra i powszechne wykorzystanie produktów je zawierających stały się źródłem wątpliwości dotyczących ich bezpieczeństwa środowiskowego. Nanocząstki srebra mogą przedostawać się do środowiska naturalnego na różnych etapach cyklu życia produktu, od produkcji, przez transport, dystrybucję i samo użytkowanie, do utylizacji. Biorąc pod uwagę możliwe drogi dystrybucji nanocząstek srebra do środowiska uznaje się, że najbardziej narażonymi na ich zwiększoną obecność i oddziaływanie są ekosystemy wodne i glebowe. Ze względu na swój złożony i ogólny sposób działania, nanocząstki srebra mogą wywierać negatywny wpływ na organizmy

wymienionych środowisk. Wcześniej uzyskane rezultaty analizy potencjału cytotoksycznego nanocząstek srebra zsyntetyzowanych z użyciem *G. striatum* potwierdziły, że nanomateriały te, obok aktywności przeciwdrobnoustrojowej, wykazują działanie toksyczne wobec komórek ludzkich. W celu dopełnienia oceny bezpieczeństwa zsyntetyzowanych w badaniu nanocząstek srebra, w kolejnym etapie realizacji pracy doktorskiej podjęto próbę analizy ich potencjału ekotoksycznego wobec organizmów najbardziej zagrożonych ekosystemów. Uzyskane wyniki opublikowane zostały w trzecim artykule naukowym wchodzącym w skład rozprawy, zatytułowanym ***„Ecotoxic effect of mycogenicsilver nanoparticles in water and soil environment"*** (Tończyk A., Niedziałkowska K., Lisowska K., 2025, Scientific Reports, IF = 3,9, MEiN = 140).

W ramach oceny potencjału ekotoksycznego przeanalizowano wpływ wszystkich typów zsyntetyzowanych nanocząstek srebra na organizmy różnych poziomów troficznych ekosystemów wodnych i glebowych, w tym szczepy mikroorganizmów glebowych *Pseudomonas moorei* DSM 12647, *Pseudomonas putida* DSM 291, *Trichoderma reesi* QM 9414 i *Trichoderma virens* DSM 1963, szczep bakterii środowisk wodnych *Aliivibrio fischeri* DSM 7151, gatunki skorupiaków słodkowodnych *Daphnia magna* i słonowodnych *Artemia franciscana* oraz gatunki roślin wodnych i uprawnych *Spirodela polyrhiza*, *Sorgho saccharatum*, *Lepidium sativum* i *Sinapis alba*. Badania przeprowadzone zostały zgodnie z dostępnymi protokołami standaryzowanych testów toksyczności lub odpowiednich norm ISO. Na podstawie uzyskanych wyników stwierdzono, że warunki biosyntezy nanocząstek srebra mogą determinować ich właściwości biologiczne, a ich aktywność może różnić się w zależności od organizmu, na który oddziałują.

Spośród badanych mikroorganizmów glebowych, to szczepy bakterii wykazały większą niż grzyby strzępkowe wrażliwość na obecność wszystkich badanych typów nanocząstek srebra. Wyznaczona wartość MIC nanocząstek dla szczepów bakterii *P. moorei* i *P. putida* wynosiła 1,56 μg/ml i korespondowała z aktywnymi stężeniami nanocząstek wobec bakterii oraz grzybów chorobotwórczych, z wyjątkiem szczepu drożdży *M. furfur.* Badane szczepy grzybów strzępkowych z rodzaju *Trichoderma* wykazały niewielką wrażliwość na obecność nanocząstek srebra, które spowodowały maksymalnie około 50% inhibicji wzrostu grzybów w najwyższym badanym stężeniu nanocząstek równym 25 μg/ml.

W przypadku szczepu bakterii środowiska wodnego *A. fischeri* zaobserwowano różnice w aktywności nanocząstek srebra w zależności od zastosowanych warunków przeprowadzania procesu biosyntezy. Na podstawie wyznaczonych wartości $EC_{50}$, określających stężenie nanocząstek srebra powodujące 50% zahamowanie bioluminescencji

komórek bakterii, stwierdzono, że nanocząstki syntetyzowane w warunkach napowietrzania wykazały większą aktywność w porównaniu do pozostałych typów nanocząstek srebra. Najsilniejszym działaniem po 30 minutach ekspozycji charakteryzowały się 4s AgNPs, a najsłabszym – 28ns AgNPs, a wyznaczone wartości $EC_{50}$ wynosiły odpowiednio 7,096 μg/ml oraz 8,191 μg/ml.

Podobne różnice w aktywności wszystkich typów nanocząstek srebra zaobserwowano w przypadku skorupiaków słonowodnych – ponownie wykazano wyższą toksyczność nanocząstek syntetyzowanych w warunkach napowietrzania, tym razem wobec *A. franciscana*. Na podstawie uzyskanych wyników stwierdzono, że po 48 godzinach ekspozycji to 28s AgNPs wykazały najsilniejsze działanie wobec badanego gatunku skorupiaków, a wyznaczona wartość $EC_{50}$ wynosiła 10,965 μg/ml. Aktywność ta była o ponad 1,5 razy silniejsza od aktywności najsłabiej działającego w tym przypadku rodzaju nanocząstek – 28ns AgNPs, gdzie wartość $EC_{50}$ po 48 godzinach inkubacji wynosiła 26,162 μg/ml. Skorupiaki słodkowodne *D. magna* wykazały co najmniej 400-krotnie większą wrażliwość na obecność nanocząstek srebra w środowisku w porównaniu do badanego gatunku słonowodnego. Po 48 godzinach inkubacji wyznaczone wartości $EC_{50}$ były niższe niż 0,03 μg/ml dla wszystkich badanych typów nanocząstek i nie różniły się znacząco między badanymi wariantami. Tak duża różnica w aktywności nanocząstek srebra wobec badanych gatunków skorupiaków może być spowodowana wpływem ich środowiska bytowania na właściwości nanocząstek – środowisko słonowodne może ograniczać uwalnianie jonów srebra z powierzchni nanocząstek, przez co zmniejszana jest ich aktywność.

Badania roślin uprawnych nie wykazały żadnego wpływu zsyntetyzowanych nanocząstek srebra na ich wzrost. Jednakże negatywne działanie oraz różnice w aktywności nanocząstek zależne od warunków syntezy zostały wykryte w przypadku słodkowodnej rośliny *S. polyrhiza*. Na podstawie wyznaczonych wartości $EC_{50}$ wskazano, że tym razem to nanocząstki srebra syntetyzowane bez napowietrzania były bardziej toksyczne wobec badanego organizmu, a najsilniejszą aktywność wykazały 4ns AgNPs. W tym przypadku wyznaczona wartość $EC_{50}$ po 72 godzinach inkubacji równa była stężeniu 0,671 μg/ml nanocząstek, co stanowi wartość o ponad połowę niższą od aktywnego stężenia wybranego typu nanocząstek srebra wobec bakterii *P. aeruginosa* i drożdży *C. albicans*. Podane stężenie było natomiast wyższe od wartości MIC wskazanego rodzaju nanocząstek wobec drożdży

*M. furfur*, co dowiodło, że 4ns AgNPs nie wywierały efektu ekotoksycznego na *S. polyrhiza* w stężeniu aktywnym wobec wymienionego szczepu.

Uzyskane wyniki dowiodły, że zarówno pożądana aktywność biologiczna, jak i potencjał toksyczny i ekotoksyczny nanocząstek srebra pochodzenia mikrobiologicznego, mogą różnić się w zależności od warunków zastosowanych podczas procesu biosyntezy lub rodzaju organizmu, na który oddziałują. Na podstawie przedstawionych wyników można stwierdzić, że jeden typ zsyntetyzowanych nanocząstek srebra, 4ns AgNPs, zasługuje na szczególną uwagę, ze względu na wysoką aktywność wobec szczepu bakterii gram-ujemnych *P. aeruginosa* oraz badanych szczepów drożdży, przy zachowaniu najniższej toksyczności wobec komórek ludzkich spośród wszystkich uzyskanych typów nanocząstek. Analiza potencjału ekotoksycznego wykazała, że wskazany rodzaj nanocząstek w stężeniu korespondującym z wartością MIC wobec drożdży *M. furfur* może oddziaływać toksycznie jedynie na skorupiaki słodkowodne *D. magna*, a w stężeniu korespondującym z wartością MIC wobec drożdży *C. albicans* i bakterii gram-ujemnych *P. aeruginosa* dodatkowo wobec rośliny słodkowodnej *S. polyrhiza* oraz bakterii glebowych. Jednocześnie wybrany typ nanocząstek we wskazanych stężeniach aktywnych wobec szczepów drobnoustrojów chorobotwórczych dla człowieka nie wykazywał efektu toksycznego wobec badanych grzybów glebowych, bakterii środowisk wodnych, skorupiaków słonowodnych i roślin uprawnych.

Wyniki te umożliwiły zatem wytypowanie jednego rodzaju uzyskanych nanocząstek srebra, 4ns AgNPs, jako najbardziej obiecującego w kontekście wysokiej aktywności przeciw mikroorganizmom chorobotwórczym dla człowieka przy zachowaniu niewielkiej toksyczności i ekotoksyczności. Ostatni etap przeprowadzonych badań zakładał próbę oceny potencjalnej aktywności synergistycznej wybranego rodzaju nanocząstek srebra z konwencjonalnie stosowanymi antybiotykami wobec wybranych bakterii chorobotwórczych. Badania te miały na celu weryfikację, czy możliwe będzie obniżenie stosowanej dawki nanocząstek srebra przy zachowaniu ich aktywności przeciwdrobnoustrojowej, co mogłoby zminimalizować ich efekt toksyczny. Wyniki tego badania opublikowane zostały w ostatnim artykule naukowym wchodzącym w skład niniejszej pracy doktorskiej **zatytułowanym „*Synergistic activity of Gloeophyllum striatum-derived AgNPs with ciprofloxacin and gentamicin against human pathogenic bacteria*"** (Tończyk A., Niedziałkowska K., Bernat P., Lisowska K., 2025, International Journal of Molecular Sciences, IF = 4,9, MEiN = 140).

Ocena aktywności synergistycznej 4ns AgNPs została przeprowadzona z użyciem dwóch antybiotyków – gentamycyny i ciprofloksacyny, wobec szczepów bakterii gram-dodatnich *S. aureus* ATCC 29213 i gram-ujemnych *P. aeruginosa* ATCC 27853. Celem badania była weryfikacja, czy wybrane środki przeciwdrobnoustrojowe zastosowane we wcześniej dobranych eksperymentalnie stężeniach, będą wzmacniać swoją skuteczność wobec badanych szczepów bakteryjnych. Aktywność taka skutkowałaby możliwością obniżenia aktywnych stężeń nanocząstek srebra przy jednoczesnym zachowaniu ich efektywności wobec organizmów patogennych i zminimalizowaniu niepożądanego efektu toksycznego. Uzyskane wyniki wykazały, że jednoczesne podanie nanocząstek srebra i gentamycyny zwiększało aktywność zastosowanych środków przeciwdrobnoustrojowych wobec obu badanych szczepów bakteryjnych. W przypadku *S. aureus*, jednoczesne podanie najmniejszego z testowanych stężeń gentamycyny (0,125 µg/ml) oraz najwyższego z testowanych stężeń nanocząstek srebra (3,125 µg/ml) powodowało całkowite zahamowanie wzrostu bakterii, czterokrotnie zmniejszając aktywną dawkę nanocząstek srebra. Natomiast dla *P. aeruginosa* jednoczesne podanie najwyższego z badanych w tym przypadku stężeń antybiotyku (5 µg/ml) z nanocząstkami srebra w stężeniu 0,39 µg/ml powodowało ok. 90% inhibicję wzrostu komórek bakteryjnych w porównaniu do kontroli biotycznej, znacznie wzmacniając aktywność nanocząstek. Jednoczesne podanie ciprofloksacyny i wybranego typu nanocząstek srebra zwiększyło aktywność badanych środków przeciwdrobnoustrojowych jedynie wobec bakterii *S. aureus* – przy jednoczesnym podaniu 0,125 µg/ml antybiotyku i 3,125 µg/ml nanocząstek srebra uzyskano ponad dwa razy silniejszy efekt hamujący wzrost bakterii w porównaniu do działania samych nanocząstek w podanym stężeniu.

Na podstawie badania aktywności przeciwbiofilmowej, stwierdzono wzmocnienie działania środków przeciwdrobnoustrojowych przy jednoczesnym podaniu gentamycyny lub ciprofloksacyny z nanocząstkami srebra w przypadku bakterii *S. aureus*. Dla *P. aeruginosa* połączenie ciprofloksacyny z nanocząstkami srebra nie wzmacniało działania hamującego wytwarzanie biofilmu wywołanego przez sam antybiotyk. Natomiast połączenie gentamycyny i nanocząstek srebra (w stężeniach poniżej wartości MIC nanocząstek stosowanych osobno) widocznie nasiliło efekt stymulacji aktywności biofilmotwórczej *P. aeruginosa*. Użycie kombinacji najwyższego stężenia antybiotyku z wyższymi stężeniami nanocząstek (0,39 µg/ml i 0,78 µg/ml) ograniczało zaobserwowane zjawisko wzmożenia produkcji biofilmu, ale nie spowodowało zahamowania jego wytwarzania.

Uzyskane wyniki dotyczące aktywności synergistycznej 4ns AgNPs i antybiotyków ciprofloksacyny i gentamycyny dowiodły, że połączenie wybranych środków przeciwdrobnoustrojowych umożliwia obniżenie skutecznej dawki nanocząstek srebra pochodzenia mikrobiologicznego. W przypadku *S. aureus* wartość aktywnego stężenia nanocząstek srebra zmalała czterokrotnie przy równoczesnym zastosowaniu z gentamycyną w porównaniu do wartości MIC wyznaczonej dla nanocząstek podanych osobno. Dla *P. aeruginosa* zaobserwowano podobne wzmocnienie aktywności nanocząstek srebra przy jednoczesnym podaniu z antybiotykiem – około 90% zahamowanie wzrostu bakterii osiągnięte zostało przy zastosowaniu nanocząstek w stężeniu 0,39 μg/ml, stanowiącym wartość cztery razy mniejszą niż ustalona wartość MIC nanocząstek działających samodzielnie. Wybrany rodzaj nanocząstek srebra w podanym stężeniu nie wykazywał efektu hemolitycznego, jego toksyczne działanie wobec komórek ludzkich było najniższe spośród wszystkich typów uzyskanych nanocząstek, a potencjał ekotoksyczny dotyczył jedynie słodkowodnych skorupiaków *D. magna*. Tym samym potwierdzono, że jednoczesne podanie nanocząstek srebra i konwencjonalnie stosowanych antybiotyków może spowodować obniżenie aktywnej dawki nanocząstek i ograniczyć potencjalne niepożądane efekty ich działania zarówno w kontekście bezpośredniego oddziaływania na komórki ludzkie, jak i na organizmy ekosystemów narażonych na wzmożoną ekspozycję na nanocząstki srebra.

Dopełnieniem przeprowadzonych badań była analiza zmian w profilu lipidowym *S. aureus* i *P. aeruginosa* wywołanych obecnością badanych środków przeciwdrobnoustrojowych, mająca na celu ocenę odpowiedzi wybranych szczepów na zmienione warunki środowiska wzrostu. Stwierdzono, że w przypadku bakterii *S. aureus* obecność wszystkich zastosowanych czynników antybakteryjnych, samodzielnie lub w kombinacji, powodowała wzrost zawartości nasyconych kwasów tłuszczowych przy jednoczesnym zmniejszeniu zawartości kwasów rozgałęzionych. Uzyskane wyniki wskazały, że obecność testowanych środków promowała zmiany w składzie błony komórek bakteryjnych, prowadząc do zmniejszonej przepuszczalności i płynności. Taka zmiana właściwości błony w kierunku większego uporządkowania uznawana jest za odpowiedź bakterii *S. aureus* na stres związany z obecnością czynnika przeciwbakteryjnego. W przypadku *P. aeruginosa* zaobserwowano odwrotny efekt wywołany obecnością wszystkich środków przeciwdrobnoustrojowych z wyjątkiem samej ciprofloksacyny, która powodowała nieznaczne ograniczenie wzrostu bakterii – zawartość kwasów nasyconych zmniejszała się przy jednoczesnym wzroście zawartości kwasów tłuszczowych

o rozgałęzionych łańcuchach. Zmiany te spowodowały wzrost płynności błony komórek *P. aeruginosa* w odpowiedzi na obecność stosowanych czynników przeciwdrobnoustrojowych.

## III. 6. Podsumowanie

Podczas badań realizowanych w ramach niniejszej pracy doktorskiej przeprowadzono mikrobiologiczną syntezę nanocząstek srebra (AgNPs) z wykorzystaniem grzyba strzępkowego brunatnej zgnilizny drewna *G. striatum* DSM 9592. Synteza ta miała charakter zewnątrzkomórkowy z wykorzystaniem płynu pohodowlanego *G. striatum*. Dodatkowo przeprowadzono optymalizację procesu produkcji AgNPs, wybierając ostatecznie cztery warianty warunków przeprowadzania biosyntezy, różniące się temperaturą i napowietrzaniem. Biosynteza nanocząstek przebiegła efektywnie we wszystkich badanych warunkach procesu, potwierdzając tym samym po raz pierwszy skuteczność wybranego szczepu grzyba brunatnej zgnilizny drewna w redukcji jonów srebra do postaci metalicznej i formowania nanocząstek.

Analiza właściwości fizykochemicznych zsyntetyzowanych AgNPs wykazała, że zastosowane różnice w warunkach przeprowadzania procesu biosyntezy wpływały na zmienność takich cech jak potencjał zeta czy rozmiar powstałych nanocząstek. Zastosowane warunki wpływały także na wydajność samego procesu syntezy nanocząstek srebra. Uzyskane rezultaty potwierdziły zatem możliwość uzyskania produktu o zaplanowanych właściwościach fizykochemicznych, często determinujących właściwości biologiczne nanocząstek srebra, poprzez odpowiednie dobranie warunków procesu biosyntezy.

Badania aktywności przeciwdrobnoustrojowej zsyntetyzowanych mikrobiologicznie nanocząstek srebra dowiodły, że uzyskane nanomateriały wykazały zróżnicowaną skuteczność wobec wybranych szczepów drobnoustrojów, a różnice te zależały od zastosowanych warunków syntezy AgNPs oraz od rodzaju organizmu, na który oddziaływały. Na podstawie wyznaczonych wartości MIC stwierdzono, że zsyntetyzowane AgNPs były bardziej aktywne wobec wybranych szczepów grzybowych w porównaniu ze szczepami bakteryjnymi wykorzystanymi w badaniu, jednak aktywność przeciwgrzybowa miała charakter fungistatyczny, a całkowity efekt bójczy nie został osiągnięty w zastosowanym zakresie stężeń nanocząstek. Nie wykryto wyraźnie wyższej skuteczności jednego rodzaju AgNPs wobec wszystkich badanych szczepów drobnoustrojów, a różnice w aktywności, zależne od warunków syntezy, widoczne były jedynie w obrębie pojedynczych modeli badawczych – w przypadku bakterii *S. epidermidis* ATCC 12228, *S. aureus* ATCC 29213, *S. aureus* ATCC 43300 i *S. aureus* ATCC 6538, grzybów strzępkowych *A. flavus* ATCC 9643 i *A. fumigatus* ATCC 204305 oraz drożdży *C. albicans* ATCC 10231. Uzyskane wyniki uwidoczniły natomiast wyraźnie wyższą wrażliwość

szczepów bakterii gram-ujemnych w porównaniu do gram-dodatnich oraz bakterii tlenowych w porównaniu do beztlenowych na działanie nanocząstek srebra oraz wyższą wrażliwość drożdży w porównaniu do pozostałych testowanych szczepów grzybowych.

Najbardziej podatnym na działanie zsyntetyzowanych AgNPs spośród badanych szczepów bakteryjnych był gram-ujemny *P. aeruginosa* ATCC 27853, dla którego wykazano całkowite zahamowanie wzrostu komórek bakterii przy zastosowaniu AgNPs w stężeniu równym 1,56 µg/ml, niezależnie od rodzaju nanomateriału. Podane stężenie nanocząstek hamowało także wytwarzanie biofilmu bakteryjnego przez wskazany szczep. Dodatkowo zaobserwowano, że zastosowanie stężenia o połowę niższego, które ograniczało, ale nie skutkowało całkowitym zahamowaniem wzrostu bakterii, powodowało stymulację aktywności biofilmotwórczej *P. aeruginosa*, co może być przypisane mechanizmowi odpowiedzi bakterii na niekorzystne warunki wzrostu wywołane obecnością nanocząstek srebra. Spośród szczepów grzybowych najbardziej podatnym na działanie uzyskanych nanocząstek srebra był szczep drożdży *M. furfur*, którego wzrost został zahamowany w obecności wszystkich rodzajów nanocząstek w stężeniu 0,39 µg/ml. Dodatkowo potwierdzono, że obecność nanocząstek srebra pochodzenia mikrobiologicznego wpływa na właściwości błony komórkowej drożdży i grzybów strzępkowych w kierunku jej zwiększonej płynności. Jednak mechanizm odpowiedzi na działanie nanocząstek może różnić się między szczepami i zależeć zarówno od zmian w zawartości i kompozycji fosfolipidów błonowych, jak i od innych związków organicznych budujących błony komórkowe.

Badania przeprowadzone w ramach oceny potencjału toksycznego zsyntetyzowanych AgNPs udowodniły, że nanocząstki pochodzenia mikrobiologicznego stosowane w stężeniach aktywnych wobec drobnoustrojów chorobotwórczych mogą stanowić zagrożenie dla komórek ludzkich. Badane nanocząstki srebra wykazywały efekt hemolityczny na poziomie 60% w stężeniu 12,5 µg/ml, natomiast w niższych stężeniach, aktywnych m.in. wobec bakterii *P. aeruginosa* i *E. coli* lub drożdży *M. furfur* efekt ten nie był obserwowany. Działanie cytotoksyczne nanocząstek wobec badanej linii komórkowej było silniejsze, a spośród wszystkich uzyskanych rodzajów nanocząstek najniższą aktywnością wobec fibroblastów ludzkich wykazywały się nanomateriały syntetyzowane w 4°C, również w stężeniach aktywnych wobec *P. aeruginosa*, *C. albicans* czy *M. furfur*. Uzyskane rezultaty potwierdziły, że warunki syntezy mogą determinować biologiczną aktywność nanocząstek srebra zarówno w kontekście działania pożądanego, jak i zagrażającego komórkom niebędącym ich celem.

Przeprowadzona analiza potencjału ekotoksycznego zsyntetyzowanych mikrobiologicznie AgNPs pozwoliła stwierdzić, że nanomateriały te mogą stanowić zagrożenie dla organizmów różnych poziomów troficznych ekosystemów najbardziej narażonych na ich zwiększoną ekspozycję. Stwierdzono, że najbardziej podatnym spośród wszystkich przebadanych organizmów gatunkiem były skorupiaki słodkowodne *D. magna*. Organizmy te wykazały ponad 400-krotnie wyższą wrażliwość na obecność nanocząstek srebra w środowisku w porównaniu do badanych skorupiaków słonowodnych. Udowodniono również, że warunki biosyntezy nanocząstek mogą determinować ich efekt ekotoksyczny. W tym kontekście szczególnie istotna okazała się ocena oddziaływania nanocząstek srebra na roślinę słodkowodną *S. polyrhiza*, która wykazała, że najbardziej toksyczne wobec badanego gatunku organizmu były nanocząstki syntetyzowane w 4°C bez napowietrzania. Jednakże, wskazany typ nanocząstek w stężeniach aktywnych wobec bakterii *P. aeruginosa* i drożdży *C. albicans* nie wykazywał toksyczności wobec badanych gatunków grzybów glebowych, bakterii środowisk wodnych, skorupiaków słonowodnych oraz roślin uprawnych. Ponadto, ten sam rodzaj nanocząstek w stężeniu aktywnym wobec drożdży *M. furfur* nie wykazywał potencjału ekotoksycznego wobec badanych organizmów z wyjątkiem skorupiaków słodkowodnych *D. magna.*

Badania zrealizowane w ramach pracy doktorskiej pozwoliły stwierdzić, że nanocząstki srebra zsyntetyzowane w warunkach 4°C bez napowietrzania były najbardziej atrakcyjnym pod względem aktywności przeciwdrobnoustrojowej i potencjału toksycznego wariantem nanomateriałów uzyskanych podczas przeprowadzonej biosyntezy. Analiza aktywności synergistycznej tych nanocząstek z konwencjonalnie stosowanymi antybiotykami wobec szczepów bakterii chorobotwórczych dla człowieka miała na celu weryfikację, czy możliwe będzie obniżenie stosowanych dawek nanocząstek srebra i zminimalizowanie ich potencjału toksycznego, przy jednoczesnym zachowaniu efektywności względem drobnoustrojów.

Stwierdzono, że jednoczesne zastosowanie ciprofloksacyny lub gentamycyny z 4ns AgNPs wzmocniło aktywność przeciwbakteryjną i przeciwbiofilmową badanych środków przeciwdrobnoustrojowych wobec bakterii *S. aureus* ATCC 29213. Dla bakterii *P. aeruginosa* wzmocnienie aktywności przeciwbakteryjnej odnotowano jedynie podczas jednoczesnego zastosowania nanocząstek srebra i gentamycyny. W badaniach aktywności przeciwbiofilmowej nie zaobserwowano wzmocnienia działania nanocząstek srebra w połączeniu z zastosowanymi antybiotykami w przypadku *P. aeruginosa*. Przeprowadzone analizy umożliwiły także wykrycie odmiennej odpowiedzi badanych szczepów

na występowanie czynnika przeciwbakteryjnego, polegającej na zmniejszeniu przepuszczalności błony komórkowej przez gram-dodatnie bakterie *S. aureus* lub odwrotnej tendencji w przypadku gram-ujemnych bakterii *P. aeruginosa*.

Na podstawie zgromadzonych danych można stwierdzić, że postawiona podczas realizacji niniejszej pracy doktorskiej hipoteza badawcza dotycząca zdolności *G. striatum* do skutecznej syntezy nanocząstek srebra została potwierdzona. Dodatkowo, potwierdzono także hipotezę dotyczącą wpływu warunków syntezy nanocząstek srebra pochodzenia mikrobiologicznego na ich aktywność biologiczną i potencjał toksyczny. Uzyskane wyniki dowiodły, że zarówno aktywność przeciwdrobnoustrojowa, jak i działanie cytotoksyczne czy ekotoksyczne mogą różnić się w zależności od zastosowanej podczas syntezy temperatury lub napowietrzania. Fakt ten potwierdzają dodatkowo wykryte różnice we właściwościach fizykochemicznych, wyraźnie zależne od warunków przeprowadzania procesu, które mogą determinować aktywność biologiczną zsyntetyzowanych biologicznie nanocząstek srebra.

Trzecia z hipotez badawczych niniejszej pracy doktorskiej dotyczyła możliwości uzyskania nanocząstek srebra o wysokiej skuteczności przy jednoczesnej niskiej toksyczności i nieznacznej toksyczności środowiskowej poprzez zastosowanie różnych warunków syntezy nanocząstek. Rezultaty przeprowadzonych badań dowiodły, że nanocząstki syntetyzowane w 4°C bez napowietrzania najbardziej odpowiadały wymienionym w hipotezie kryteriom. Wskazany wariant nanocząstek srebra wykazywał skuteczność wobec bakterii gram-ujemnych *P. aeruginosa* oraz drożdży *C. albicans* i *M. furfur* w stężeniach, które jednocześnie nie powodowały efektu hemolitycznego oraz wywierały najniższy efekt cytotoksyczny wobec badanych komórek ludzkich. 4ns AgNPs wykazywały także niewielki potencjał ekotoksyczny, który ograniczał się jedynie do bakterii glebowych i organizmów słodkowodnych w przypadku stężenia nanocząstek aktywnego wobec *P. aeruginosa* i *C. albicans*, oraz tylko do skorupiaków słodkowodnych *D. magna* w przypadku stężenia aktywnego wobec *M. furfur*. Ponadto udowodniono, że wskazany typ nanocząstek srebra wykazywał aktywność synergistyczną w połączeniu z antybiotykiem gentamycyną, co umożliwiło obniżenie aktywnej dawki nanocząstek wobec bakterii *P. aeruginosa* do stężenia 0,39 μg/ml, korespondującego z wartością MIC wyznaczoną dla drożdży *M. furfur*.

Na podstawie uzyskanych rezultatów można wnioskować, że odpowiednia optymalizacja warunków procesu syntezy nanocząstek srebra z wykorzystaniem grzybów strzępkowych może służyć uzyskaniu produktów o określonych właściwościach,

warunkujących aktywność przeciwko konkretnemu szczepowi drobnoustroju. Rezultat ten wskazuje na dalszą konieczność opracowywania nowych metod syntezy biologicznej nanocząstek srebra i podkreśla, jak ważnym kierunkiem badań jest nie tylko analiza potencjału przeciwdrobnoustrojowego nowo zsyntetyzowanych nanomateriałów, ale także kompleksowa ewaluacja ich potencjału toksycznego. Ponadto, udowodniona aktywność synergistyczna wykazywana przez uzyskane AgNPs w połączeniu z konwencjonalnie stosowanymi antybiotykami wskazuje na możliwość obniżenia stosowanych dawek nanocząstek srebra przy zachowaniu ich pożądanej aktywności przeciwdrobnoustrojowej, tym samym ograniczając ryzyko związane z potencjałem toksycznym i ekotoksycznym nanocząstek.

## Wnioski i stwierdzenia końcowe

## IV. Wnioski i stwierdzenia końcowe

Na podstawie rezultatów uzyskanych podczas realizacji niniejszej pracy doktorskiej udowodniono, że:

1) Grzyb strzępkowy brunatnej zgnilizny drewna *Gloeophyllum striatum* DSM 9592 może zostać wykorzystany do przeprowadzenia wydajnej, mikrobiologicznej biosyntezy nanocząstek srebra metodą zewnątrzkomórkową;
2) Warunki przeprowadzania procesu syntezy, takie jak temperatura lub napowietrzanie, determinują właściwości fizykochemiczne i biologiczne uzyskanych nanomateriałów, a także wydajność procesu ich syntezy;
3) Nanocząstki srebra uzyskane przy udziale *G. striatum* charakteryzują się zróżnicowaną aktywnością przeciwdrobnoustrojową wobec badanych szczepów mikroorganizmów i są bardziej aktywne wobec badanych szczepów grzybów w porównaniu ze szczepami bakteryjnymi oraz wobec bakterii gram-ujemnych w porównaniu do gram-dodatnich i bakterii tlenowych w porównaniu do beztlenowych.
4) Najbardziej wrażliwym na działanie wszystkich wariantów AgNPs szczepem bakteryjnym był *Pseudomonas aeruginosa* ATCC 27853 (MIC = 1,56 µg/ml), a szczepem grzybowym – drożdże *Malassezia furfur* DSM 6170 (MIC = 0,39 µg/ml).
5) Zsyntetyzowane AgNPs mogą wykazywać potencjał hemolityczny i cytotoksyczny, przy czym działanie cytotoksyczne było silniejsze i było determinowane przez zastosowane warunki biosyntezy nanocząstek. Najsłabszym działaniem cytotoksycznym charakteryzowały się rodzaje nanocząstek syntetyzowane w temperaturze 4°C.
6) Uzyskane nanocząstki srebra mogą wykazywać zróżnicowany potencjał ekotoksyczny w zależności od zastosowanych warunków biosyntezy oraz rodzaju organizmu, na który oddziałują. Najbardziej podatnymi na obecność wszystkich rodzajów nanocząstek srebra w środowisku były skorupiaki słodkowodne *Daphnia magna*, a najmniej – gatunki roślin uprawnych *Sorgho saccharatum*, *Lepidium sativum* i *Sinapis alba.*
7) Nanocząstki srebra syntetyzowane w 4°C bez napowietrzania (4ns AgNPs) są najbardziej obiecujące pod względem aktywności przeciwdrobnoustrojowej i potencjału toksycznego. W stężeniach aktywnych wobec *Pseudomons aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231 i *M. furfur* DSM 6170 nie powodują efektu hemolitycznego, wykazują najniższą aktywność cytotoksyczną oraz niewielki potencjał ekotoksyczny.

8) 4ns AgNPs wykazują aktywność synergistyczną z antybiotykami wobec badanych bakterii chorobotwórczych, dzięki czemu możliwe jest obniżenie dawki nanocząstek skutecznej wobec drobnoustrojów i zminimalizowanie ich potencjału toksycznego.

## V. Streszczenie w języku polskim

Nanocząstki srebra są jednymi z najbardziej skomercjalizowanych nanomateriałów. Wszechstronne właściwości predysponują je do wykorzystania w różnorodnych dziedzinach, od elektroniki, przez przemysł tekstylny, kosmetyczny, aż do zastosowań biomedycznych. Największe znaczenie dla zastosowań nanocząstek srebra ma ich aktywność przeciwdrobnoustrojowa, uznawana za najsilniejszą wśród przebadanych pod tym kątem nanomateriałów.

Rosnące zapotrzebowanie na nanocząstki srebra wiąże się z potrzebą opracowania metod syntezy, które, poza aspektem ekonomicznym, będą proste do przeprowadzenia, szybkie i przyjazne środowisku. Kryteria te spełnia synteza biologiczna, znacznie wyróżniająca się na tle metod konwencjonalnych, wymagających użycia dużych nakładów energii lub toksycznych środków chemicznych. Grzyby strzępkowe, dzięki cechom takim jak łatwość hodowli, szybki wzrost i wydajna produkcja biomasy, synteza znacznych ilości metabolitów wtórnych, a także oporność na stres mechaniczny i obecność metali w środowisku wzrostu, są często wybierane jako potencjalny producent nanocząstek srebra. Co istotne, gatunek grzyba i produkowane metabolity mogą wpływać na właściwości wytwarzanych nanocząstek, tym samym determinując możliwości ich wykorzystania.

Potencjalna cytotoksyczność nanocząstek srebra wobec komórek wzbudza obawy o bezpieczeństwo ich zastosowania. Dodatkowo, nanocząstki srebra uwalniane do środowiska naturalnego mogą stanowić zagrożenie dla organizmów różnych poziomów troficznych. Konieczne jest więc przeprowadzanie kompleksowych analiz potencjału toksycznego nowo syntetyzowanych nanocząstek srebra, a także poszukiwanie metod obniżania skutecznych dawek nanocząstek, skutkujących ograniczeniem ich zużycia.

Celem niniejszej pracy doktorskiej była mikrobiologiczna synteza nanocząstek srebra przy udziale grzyba strzępkowego brunatnej zgnilizny drewna *Gloeophyllum striatum* DSM 9592 w różnych warunkach procesu, a także szeroka ocena ich aktywności biologicznej, obejmująca potencjał przeciwdrobnoustrojowy, cytotoksyczny, ekotoksyczny oraz aktywność synergistyczną z powszechnie stosowanymi antybiotykami.

Na podstawie uzyskanych rezultatów stwierdzono, że grzyb brunatnej zgnilizny drewna *G. striatum* może być wykorzystywany do wydajnej biosyntezy nanocząstek srebra. Wykazano także, że warunki przeprowadzania procesu syntezy, takie jak temperatura lub napowietrzanie, mogą determinować właściwości fizykochemiczne i biologiczne uzyskanych nanomateriałów, a także wydajność procesu ich syntezy.

Analiza potencjału przeciwdrobnoustrojowego wykazała, że zsyntetyzowane nanocząstki srebra były aktywne wobec badanych mikroorganizmów, a ich skuteczność zależała od warunków syntezy i rodzaju organizmu, na który oddziaływały. Nie zaobserwowano wyraźnej tendencji jednego z rodzajów nanocząstek srebra do silniejszej aktywności wobec wszystkich badanych szczepów drobnoustrojów. Spośród badanych szczepów drobnoustrojów grzyby były bardziej wrażliwe na działanie nanocząstek srebra w porównaniu do badanych bakterii. Najbardziej wrażliwymi organizmami wśród szczepów grzybowych były drożdże *Malassezia furfur* DSM 6170, a wśród szczepów bakteryjnych bakterie gram-ujemne *Pseudomonas aeruginosa* ATCC 27853.

Ocena potencjału toksycznego nanocząstek srebra udowodniła, że uzyskane nanomateriały wykazywały zróżnicowany efekt cytotoksyczny lub ekotoksyczny wobec testowanych modeli badawczych. Wykazano, że efekt cytotoksyczny wobec linii komórkowej fibroblastów ludzkich wywołany przez uzyskane nanocząstki był silniejszy niż ich działanie hemolityczne i determinowany był przez zastosowane warunki biosyntezy. Najsłabszą aktywnością cytotoksyczną spośród badanych typów nanomateriałów charakteryzowały się nanocząstki syntetyzowane w 4°C, również w stężeniach aktywnych wobec najbardziej wrażliwych szczepów. Badanie potencjału ekotoksycznego nanocząstek srebra wykazało, że zarówno badane szczepy bakterii glebowych, wodnych, gatunki skorupiaków słodkowodnych i słonowodnych oraz rośliny wodne wykazywały wrażliwość na obecność nanocząstek w środowisku. Nanocząstki były najbardziej toksyczne wobec gatunku skorupiaków słodkowodnych *Daphnia magna*, z kolei najmniejszy efekt toksyczny wykazano w przypadku roślin uprawnych *Sorgho saccharatum*, *Lepidium sativum* i *Sinapis alba*.

Na podstawie rezultatów badań stwierdzono, że nanocząstki srebra syntetyzowane w 4°C bez napowietrzania (4ns AgNPs) były najbardziej obiecujące pod względem aktywności przeciwdrobnoustrojowej i potencjału toksycznego. W stężeniach aktywnych wobec *P. aeruginosa*, *C. albicans* ATCC 10231 i *M. furfur* nie powodowały efektu hemolitycznego oraz wykazywały najniższą aktywność cytotoksyczną i niewielki potencjał ekotoksyczny. Ponadto, 4ns AgNPs wykazują aktywność synergistyczną z antybiotykami wobec badanych bakterii chorobotwórczych, dzięki czemu możliwe jest obniżenie dawki nanocząstek skutecznej wobec drobnoustrojów i zminimalizowanie ich potencjału toksycznego.

Uzyskane w trakcie realizacji niniejszej pracy doktorskiej wyniki badań wykazały, że poprzez optymalizację warunków procesu biosyntezy nanocząstek srebra z użyciem grzybów strzępkowych możliwe jest modyfikowanie właściwości produkowanych nanomateriałów co może prowadzić do uzyskania produktu o wysokiej aktywności przeciwdrobnoustrojowej z zachowaniem niewielkiego potencjału toksycznego.

## VI. Streszczenie w języku angielskim

Silver nanoparticles (AgNPs) are among the most commercialized nanomaterials. Their versatile properties enable their applications in a wide range of fields, from electronics, through the textile and cosmetic industries, to biomedicine. The antimicrobial activity of AgNPs, recognized as the strongest among nanomaterials investigated to date, constitutes their primary attribute of interest for practical applications.

The growing demand for silver nanoparticles is associated with the need to develop synthesis methods that are not only economically viable but also simple, rapid, and environmentally sustainable. Biological synthesis meets these criteria and stands out against conventional methods, which typically require high energy input or involve toxic reagents. Filamentous fungi are particularly attractive candidates for AgNP biosynthesis due to their ease of cultivation, rapid growth, efficient biomass production, ability to generate large quantities of secondary metabolites, and resilience to mechanical stress and metal exposure. Crucially, both the fungal species employed, and the metabolites produced can influence the characteristics of the resulting nanoparticles, and consequently – their potential applications.

The potential cytotoxicity of silver nanoparticles toward cells raises concerns about the safety of their use. Additionally, silver nanoparticles released into the natural environment may pose a threat to organisms at different trophic levels. Therefore, it is necessary to conduct comprehensive analyses of the toxic potential of newly synthesized silver nanoparticles, as well as to search for methods to reduce their effective doses, thereby limiting their consumption.

The objective of this doctoral dissertation was the microbiological synthesis of silver nanoparticles with the participation of the brown-rot wood fungus *Gloeophyllum striatum* DSM 9592 under various process conditions, as well as a broad evaluation of their biological activity, including antimicrobial, cytotoxic, ecotoxic potential, and synergistic activity with commonly used antibiotics.

Obtained results demonstrated that the brown-rot wood fungus *G. striatum* can be used for the efficient biosynthesis of silver nanoparticles. It was also proven that the conditions of the synthesis process, such as temperature or shaking, may determine the physicochemical and biological properties of the obtained nanomaterials, as well as the efficiency of their synthesis.

Analysis of the antimicrobial potential showed that the synthesized silver nanoparticles were active against the tested microorganisms, and their effectiveness

depended on the synthesis conditions and the target species. No clear tendency was observed for one type of silver nanoparticle to show stronger activity against all tested microbial strains. Among the tested microorganisms, fungi were more sensitive to the action of silver nanoparticles compared to bacteria. The most sensitive organisms among the fungal strains were the yeasts *Malassezia furfur* DSM 6170, while among the bacterial strains the most sensitive were the gram-negative bacteria *Pseudomonas aeruginosa* ATCC 27853.

Evaluation of the toxic potential of silver nanoparticles demonstrated that the obtained nanomaterials exhibited varying cytotoxic or ecotoxic effects on the tested models. It was shown that the cytotoxic effect on the human fibroblast cell line induced by the nanoparticles was stronger than their hemolytic activity and was determined by the applied biosynthesis conditions. The lowest cytotoxic activity among the tested nanomaterials was exhibited by nanoparticles synthesized at 4°C, even at concentrations active against the most sensitive strains. The study of the ecotoxic potential of silver nanoparticles revealed that the tested soil and aquatic bacterial strains, freshwater and marine crustacean species, as well as aquatic plants, all showed sensitivity to the presence of nanoparticles in the environment. The nanoparticles were most toxic to the freshwater crustacean species *Daphnia magna*, while the weakest toxic effect was observed in the case of the cultivated plants *Sorgho saccharatum*, *Lepidium sativum*, and *Sinapis alba*.

Based on the research results, it was found that silver nanoparticles synthesized at 4°C without shaking (4ns AgNPs) were the most promising in terms of antimicrobial activity and toxic potential. At concentrations active against *P. aeruginosa*, *C. albicans* ATCC 10231, and *M. furfur*, they did not cause hemolytic effects, exhibited the lowest cytotoxic activity and minor ecotoxic potential. Furthermore, 4ns AgNPs showed synergistic activity with antibiotics against the tested pathogenic bacteria, making it possible to reduce the dose of nanoparticles effective against microorganisms and minimize their toxic potential.

The obtained results demonstrated that by optimizing the conditions of the biosynthesis process of silver nanoparticles using filamentous fungi, it is possible to modify the properties of the produced nanomaterials, which may lead to obtaining a product with high antimicrobial activity while maintaining low toxic potential.

## Dorobek naukowy

## VII. Dorobek naukowy

### VII.1. Sumaryczny dorobek naukowy

- Publikacje – 5; $IF_{\sum} = 22{,}4$; $h = 2$; MEiN = 700; liczba cytowań[1] = 4
- Doniesienia konferencyjne – 8
- Szkolenia i kursy – 12
- Organizacja konferencji – 5

### VII.2. Spis publikacji niewchodzących w skład rozprawy doktorskiej

- Marta Nowak-Lange, Katarzyna Niedziałkowska, **Aleksandra Tończyk**, Carola Parolin, Beatrice Vitali, Katarzyna Lisowska. 2025. *Antibacterial and antibiofilm properties of postbiotics derived from Lactiplantibacillus pentosus B1*. International Journal of Molecular Sciences, 26(17): 8169; DOI: 10.3390/ijms26178169; $IF_{2025} = 4{,}9$; $IF_{5\text{-letni}} = 5{,}7$; MEiN = 140

### VII.3. Doniesienia konferencyjne

- *Evaluation of the activity of neomycin combined with biogenic silver nanoparticles*; **Aleksandra Tończyk**, Katarzyna Niedziałkowska, Katarzyna Lisowska; 2022; 8th Central European Congress of Life Sciences EUROBIOTECH, Kraków;
- *Ocena aktywności przeciwdrobnoustrojowej neomycyny w obecności nanocząstek srebra pochodzenia mikrobiologicznego*; **Aleksandra Tończyk**, Katarzyna Niedziałkowska, Katarzyna Lisowska; 2022; VI Ogólnopolska Konferencja Naukowa *Nanotechnologia wobec oczekiwań XXI wieku*, on-line;
- *Antimicrobial activity assessment of silver nanoparticles synthesized by the microbiological method*; **Aleksandra Tończyk**, Aleksander Nowak, Katarzyna Niedziałkowska, Katarzyna Lisowska; 2023; NanoTech Poland 2023, Poznań;
- *Ocena toksyczności nanocząstek srebra pochodzenia mikrobiologicznego wobec wybranych drobnoustrojów glebowych*; **Aleksandra Tończyk**, Katarzyna Niedziałkowska, Katarzyna Lisowska; 2023; I Krajowa Konferencja EkoBioTox, Łódź;

[1] Bez autocytowań; dane z dnia 26.09.2025 r. wg bazy Web of Science.

- *Ocena aktywności przeciwdrobnoustrojowej nanocząstek srebra syntetyzowanych na drodze mikrobiologicznej*; **Aleksandra Tończyk**, Katarzyna Niedziałkowska, Katarzyna Lisowska; 2024; VII Sesja Młodych Mikrobiologów Środowiska Łódzkiego, Łódź;
- *Mycogenic silver nanoparticles – promising solution or hidden danger?*; **Aleksandra Tończyk**, Katarzyna Niedziałkowska, Katarzyna Lisowska; 2024; 12th International Mycological Congress, Maastricht, Holandia;
- *Mycogenic silver nanoparticles – insights into antimicrobial activity, toxicity and environmental safety*; **Aleksandra Tończyk**, Katarzyna Niedziałkowska, Katarzyna Lisowska; 2025; 10th International Doctoral Students' Conference in Life Sciences BioOpen, Łódź;
- *Ecotoxicity of preservatives (MIT and PCMX): an in vitro assessment of risks toward soil bacteria*; Marta Nowak-Lange, Katarzyna Niedziałkowska, **Aleksandra Tończyk**, Przemysław Bernat, Katarzyna Lisowska; 2025; OBSIDIAN2025, Toruń.

### VII.4. Kursy i szkolenia

- *Publikowanie artykułu naukowego w prestiżowym czasopiśmie: wybór czasopisma i komunikacja*; Fundacja Science Watch Polska, 8.11.2022; on-line;
- *Seminarium internetowe „Pomiar gęstości – adjustacja, obsługa, wskazówki, porady*"; Mettler-Toledo Sp. z o. o., 15.02.2023; on-line;
- *Literatura naukowa na platformie ScienceDirect – nie tylko pdf*; Elsevier Researcher Academy, 28.02.2023; on-line;
- *Zarządzanie czasem*; Biuro Karier Uniwersytetu Łódzkiego, 13.03.2023; on-line;
- *Warsztat umiejętności trenerskich i prezentacyjnych*; Biuro Karier Uniwersytetu Łódzkiego, 16.03.2023; Łódź;
- *Praktyczna strona wystąpień publicznych*; Biuro Karier Uniwersytetu Łódzkiego, 17.03.2023; Łódź;
- *Mammalian Cell Culture*; BioGrad Examination Centre at Liverpool Science Park Limited, 26-30.06.2023; Liverpool, Wielka Brytania;
- *How to avoid predatory journals – Author Workshop Camp*; Elsevier Researcher Academy, 23.11.2023; on-line;
- *How to make your published article more popular*?; Elsevier Researcher Academy, 24.11.2023; on-line;

- *Przez lupę Studenta: Jak pytać, żeby Student chciał odpowiadać?*; Centrum Rekrutacji i Doskonałości Dydaktycznej UŁ; 18.04.2024; on-line;
- *Podstawy spektrometrii mas sprzężonej z chromatografią cieczową (LC-MS/MS)*; LabExperts, 28-30.10.2024; Łódź;
- *Transmission Electron Microscopy in Life Sciences*; Institute of Molecular Genetics of the Czech Academy of Sciences, 25-29.11.2024; Praga, Czechy.

### VII.5. Działalność organizacyjna

#### VII.5.1. Organizacja konferencji naukowych

- IX Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen; Łódź, 11-12.04.2024;
- Konferencja „50 lat Katedry Mikrobiologii Przemysłowej i Biotechnologii UŁ – Lata minione – Dzień dzisiejszy – Perspektywy"; Łódź, 17.05.2024;
- 6$^{th}$ MycoRise Up! Youth in Mycology; Spała, 12-13.04.2025;
- 10$^{th}$ International Doctoral Students' Conference in Life Sciences BioOpen; Łódź, 15-16.05.2025;
- VIII Sesja Młodych Mikrobiologów Środowiska Łódzkiego; Łódź, 13.06.2025;
- III Ogólnopolska Konferencja EkoBioTox; Łódź, 22-24.04.2026.

### VII.6. Działalność popularyzatorska

#### VII.6.1. Spis artykułów popularnonaukowych

- *Nanocząstki srebra – do rany przyłóż?*; Aleksandra Tończyk; 2024; Biotechnologia.pl Kwartalnik Portalu Internetowego; ISSN 2354-0028.

#### VII.6.2. Aktywność popularyzująca naukę

- Organizacja warsztatów naukowych dla uczniów Szkoły Podstawowej nr 199 im. Juliana Tuwima w Łodzi; 2.12.2022; 6.12.2024;
- Organizacja warsztatów naukowych dla młodzieży w ramach ogólnopolskiej Nocy Biologów; 13.01.2023; 10.01.2025;
- Organizacja warsztatów naukowych dla młodzieży w ramach Instytutu Kreatywnej Biologii 2022/2023; 24.02.2023;

- Udział w Drzwiach Otwartych Wydziału Biologii i Ochrony Środowiska UŁ; 25.03.2023;
- Organizacja warsztatów naukowych dla młodzieży w ramach Uniwersytetu Zawsze Otwartego; 21.04.2023; 7.02.2025; 13.02.2025;
- Organizacja warsztatów naukowych dla dzieci w ramach XXII Festiwalu Nauki, Techniki i Sztuki; 21.04.2023;
- Organizacja warsztatów naukowych dla młodzieży w ramach Instytutu Kreatywnej Biologii 2023/2024; 24.11.2023, 19.01.2024, 23.02.2024, 19.04.2024;
- Organizacja warsztatów naukowych dla dzieci w ramach XXIII Festiwalu Nauki, Techniki i Sztuki; 19.04.2024;
- Organizacja warsztatów naukowych dla dzieci Przedszkola Miejskiego nr 149 w Łodzi; 4.10.2024;
- Organizacja warsztatów naukowych dla młodzieży w ramach Instytutu Kreatywnej Biologii 2024/2025; 25.10.2024, 15.11.2024, 13.12.2024, 17.01.2025, 7.02.2025, 14.03.2025;
- Organizacja warsztatów dla uczniów Liceum Ogólnokształcącego im. Marii Konopnickiej w Poddębicach; 10.04.2025;
- Członek i opiekun Studenckiego Koła Naukowego Biotechnologiczno-Mikrobiologicznego „Bio-Mik”; 2019 – do chwili obecnej.

## Literatura uzupełniająca

## VIII. Literatura uzupełniająca


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## Załączniki

# IX. Załączniki

## IX.1. Kopie publikacji wchodzących w skład rozprawy doktorskiej

Publikacja 1

## *Optimizing the microbial synthesis of silver nanoparticles using Gloeophyllum striatum and their antimicrobial potential evaluation*

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2023, Scientific Reports, 13: 21124; DOI: 10.1038/s41598-023-48414-9

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# Optimizing the microbial synthesis of silver nanoparticles using *Gloeophyllum striatum* and their antimicrobial potential evaluation

Aleksandra Tończyk[1,2], Katarzyna Niedziałkowska[1] & Katarzyna Lisowska[1]✉

**The search for new sources of silver nanoparticles (AgNPs) is highly relevant in many fields. Mycosynthesis seems to be advantageous for large-scale production, and using brown rot fungi might be a promising solution. In this study, AgNP synthesis using *Gloeophyllum striatum* DSM 9592 was performed under various process conditions. The resulting AgNPs were characterized using UV/Vis, FT-IR, SEM and NTA techniques and their biological activities were determined. It was found that different synthesis conditions changed the production efficiency, which was the highest in 28 s AgNPs. Moreover, temperature and shaking conditions slightly affected the activity of the resulting AgNP types. Gram-negative bacteria were generally more susceptible to the action of AgNPs with MIC values two- or three-fold lower compared to Gram-positive strains. *Pseudomonas aeruginosa* was the most sensitive among tested strains with a MIC value of 1.56 µg/ml. The research was additionally extended by the biofilm formation assay for this strain. It was found that AgNPs of all types led to a reduction in biofilm-forming capability of *P. aeruginosa* over the tested concentration range. Haemolytic and cytotoxic activity assays showed that synthesis conditions also affected AgNP toxicity. For instance, 4 ns AgNPs were the least cytotoxic and cause less than 50% reduction of fibroblast viability in the concentration that inhibits the growth of *P. aeruginosa* completely. These results highlight the possible utility of mycogenic silver nanoparticles as an antibacterial agent in antiseptics or other external treatments.**

In recent years, nanotechnology has received extensive attention as a rapidly developing, multidisciplinary field impacting the industry, agriculture, and pharmacology areas[1–3]. Nanomaterials can exhibit novel physical, chemical, and mechanical properties due to the 'nano' dimension. That property makes them more versatile than their initial bulk materials[4,5]. Thus, it is considered that nanomaterials as products of nanotechnology will determine the future of science[3].

There are three strategies of metal nanoparticle (NP) synthesis, namely, physical, chemical, and biological. Physical and chemical routes are considered environmentally harmful, time-consuming, and expensive because of the necessity to use hazardous chemicals or provide special conditions[3,5,6]. Biological methods, also described as 'green synthesis', constitute the opposite characteristics as they are more cost-efficient, eco-friendly, and minimalise the use of toxic chemicals. Thus, biosynthesis is promising as a potential tool for producing NPs that are appropriate for medical purposes[3,7]. Green synthesis can be performed with the use of different organisms such as algae, plants, bacteria, and filamentous fungi. The capability to grow under various pHs, temperatures, and pressure conditions in addition to fast growth rates and easy cultivation methods make microorganisms advantageous for NP synthesis[3,8].

Silver nanoparticles (AgNPs) are widely used in various industrial fields, including textile, cosmetics, packaging, and coatings, catalysis, or water and environmental contamination control. AgNPs possess efficient antimicrobial action against a broad spectrum of microbial species, which is regarded as the strongest efficiency among known synthesized metal NPs[7,9,10]. Hence, AgNPs are considered novel biomedicine tools for combatting infectious diseases, including ones caused by drug-resistant strains[11,12].

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Among all organisms used in NP biosynthesis, filamentous fungi seem to be most appropriate for AgNP production[6]. Fungi show high metal tolerance[12] and produce a variety of bioactive metabolites that serve as reducing and capping agents for newly synthesized NPs. Moreover, fungal biomass is easy to cultivate under laboratory conditions and provides sufficient volume for the process in relatively short periods of time[5,8]. Mycosynthesis can be performed both intracellularly and extracellularly using extracted bioactive compounds originating from the biomass. The extracellular route is preferred for large-scale production because of the simplicity of downstream processes after synthesis[8,12,13].

A possible use of the filamentous fungi of *Trichoderma*, *Aspergillus*, *Penicillium*, and *Fusarium* species for AgNP synthesis has been described. However, very little information is available about the use of wood decay fungi, which possess degradative capabilities[14]. White rot fungi are able to degrade lignin and plant cell wall carbohydrates using an extracellular enzyme complex. This complex is also known to be capable of degrading a variety of xenobiotics. Moreover, it has been stated that white rot fungi are capable of performing metal–NP biosynthesis. For instance, *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Pleurotus sajorcaju* have been found to lead to a successful reduction of silver nitrate to silver NPs. Brown rot fungi possess an efficient lignocellulose decay system consisting of oxidases[14–16]. Information about the brown rot fungi NP synthesis is scarce. It is reported, however, that *Gloeophyllum striatum* DSM 10335 is capable of synthesizing silver NPs[14]. This topic is therefore in urgent need of further investigation.

In this paper, the antimicrobial activity and toxicity of AgNPs produced by the brown rot fungus *Gloeophyllum striatum* DSM 9592 in various process conditions have been described. Newly synthesized nanoparticles were characterized using ultraviolet/visible (UV/Vis) spectroscopy and Fourier transform infrared (FT-IR) spectroscopy, nanoparticle tracking analysis (NTA), and scanning electron microscopy (SEM). The antimicrobial activities of AgNPs were examined against a broad spectrum of bacterial strains: *Escherichia coli* ATCC 25922, *Proteus hauseri* ATCC 15442, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, *S. aureus* ATCC 43300, *S. aureus* ATCC 6538, *S. aureus* ATCC 780699, *Campylobacter jejuni* ATCC 33560, and *Listeria monocytogenes* ATCC 19115. The toxicity evaluation procedure involved a haemolytic activity test and cytotoxicity assessment of a human fibroblast cell line.

## Results

### Biosynthesis of AgNPs using *G. striatum* and physicochemical characterization of obtained nanoparticles

Biosynthesis of AgNPs was carried out extracellularly using the *G. striatum* DSM 9592 strain under four different synthesis conditions: 28 °C without shaking (28 ns AgNPs), 28 °C with shaking (28n AgNPs), 4 °C without shaking (4 ns AgNPs) and 4 °C with shaking (4 s AgNPs). The pH value of the post-culture liquid of *G. striatum* was 4.98. The successful synthesis of AgNPs under all tested conditions was proven by obtaining UV/Vis spectra of the fungal post-culture liquid supplemented with a nanoparticles precursor and checked after the process (Fig. 1a). In all cases, the maximum absorbance was detected at $\lambda = 430$ nm, which can be considered characteristic for silver NP surface plasmon resonance (SPR). The yields of AgNP production varied depending on the synthesis conditions. The highest efficiency was detected in 28 s AgNPs from which the resulting concentration of particles per milliliter was $6.46 \times 10^{12}$. The yield of AgNP synthesis expressed as the number of particles per milliliter was then distributed as described: $2.17 \times 10^{12}$ for 28 ns AgNPs, $3.96 \times 10^{12}$ for 4 ns AgNPs, and $2.58 \times 10^{12}$ for 4 s AgNPs (Table 1). FT-IR analyses showed a distinct band of 1644 $cm^{-1}$ in all AgNP types, which differed in intensity of transmittance (Fig. 1b).

SEM and nanoparticle tracking analysis (NTA) allowed estimations of the sizes of the synthesized AgNPs. These methods proved that all of the resulting AgNPs were polydispersed and varied in size among the AgNP types (Fig. 2). The diameters of the most numerous NPs of every type were 80–87, 121, 67, and 117 nm for 28 ns AgNPs, 28 s AgNPs, 4 ns AgNPs, and 4 s AgNPs, respectively, (Fig. 3).

### Determination of AgNP antibacterial activity

The antibacterial activities of the resulting AgNPs (Figs. 4, 5) were tested in the concentration range of 0.098 to 25 µg/ml against the nine previously described bacterial strains. The results showed that Gram-negative bacterial strains were more sensitive compared to Gram-positive ones according to the obtained minimum inhibitory and minimum bactericidal concentrations (MIC and MBC, respectively) values (Table 2). The most sensitive strain was obligate aerobic *P. aeruginosa* with the MIC values reaching 1.56 µg/ml for all AgNP types although the MBC values were the highest in this case. The least susceptible Gram-negative strain was *P. hauserii* with MIC values equal to 6.25 µg/ml in all cases. Among Gram-positive bacteria strains, the most sensitive one was *S. epidermidis* with MIC values of 3.125 µg/ml for 28 ns AgNPs, 28 s AgNPs, and 4 s AgNPs and 6.25 µg/ml for 4 ns AgNPs. The MIC values associated with the *S. aureus* strains varied between 6.25 and 12.5 µg/ml depending on the NP type. The MBC values of all Gram-positive bacteria strains were comparable and exceeded the range of the tested concentration in almost all cases. The same phenomenon was detected in the strains cultivated under anaerobic conditions. The microaerophilic Gram-negative *C. jejuni* strain was more susceptible to the activity of all the tested AgNPs. MIC values were three-fold lower than those obtained for the Gram-positive facultative anaerobic *L. monocytogenes* and the MBC values were two- or three-fold lower depending on the NP type.

### Assessment of *P. aeruginosa* biofilm formation in the presence of AgNPs

The *P. aeruginosa* biofilm formation capability was tested in the presence of the resulting AgNPs in the concentration range of 0.098 to 25 µg/ml (Fig. 6). The optical density (OD) value of the biotic control was approximately 0.8648; therefore the tested strain was categorized as a moderate biofilm former[17]. It was established that biofilm formation was not affected by the presence of AgNPs in the concentrations of 0.098–0.39 µg/ml. All AgNP types

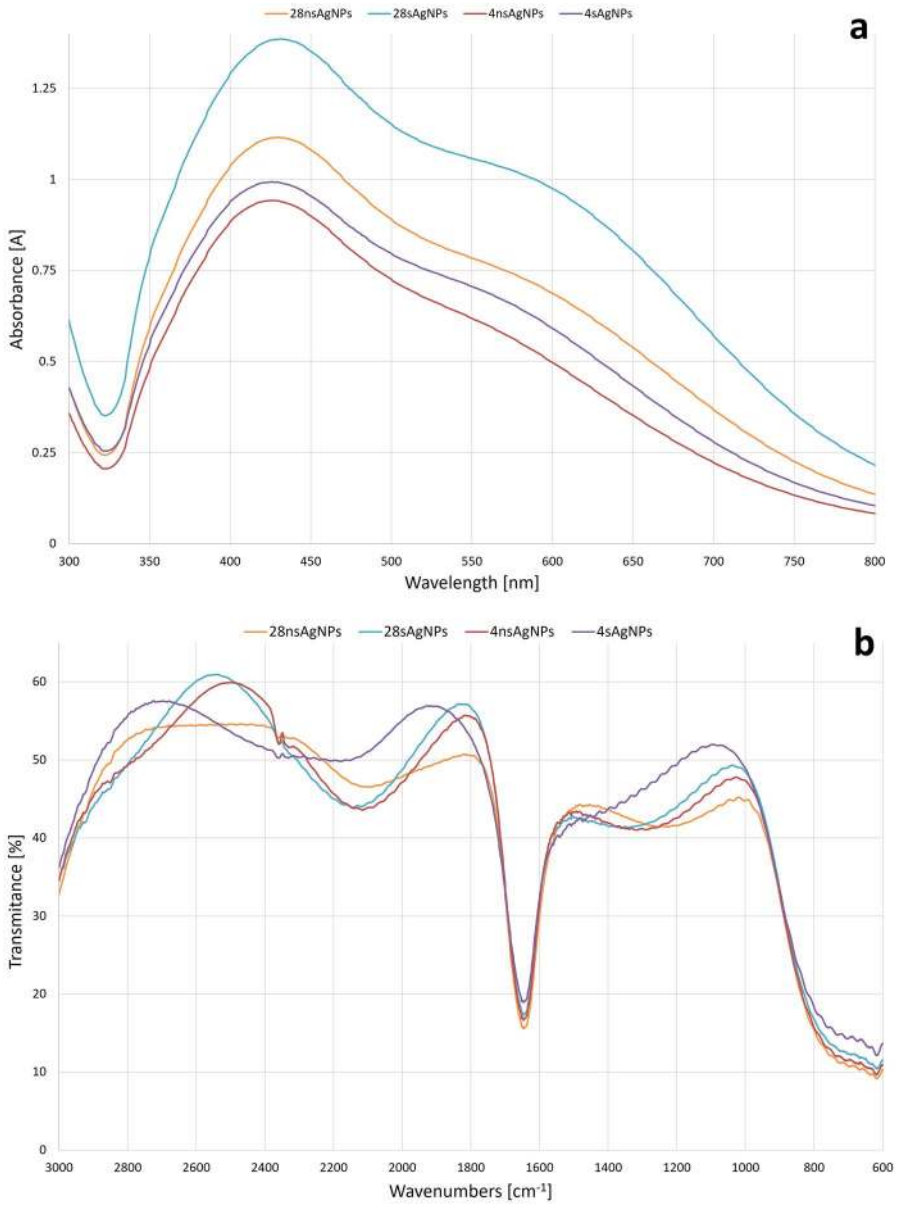


**Figure 1.** Physicochemical characteristics of the synthesized silver nanoparticles (AgNPs): (**a**) ultraviolet/visible (UV/Vis) analysis, (**b**) Fourier-transform infrared spectroscopy (FT-IR) analysis.

| | 28 ns AgNPs | 28 s AgNPs | 4 ns AgNPs | 4 s AgNPs |
|---|---|---|---|---|
| Diameter–mean [nm] | 146.9 ± 2.0 | 160.7 ± 4.5 | 103.0 ± 5.7 | 122.1 ± 17.3 |
| Diameter–mode [nm] | 146.9 ± 2.0 | 121.4 ± 15.0 | 66.9 ± 6.9 | 94.0 ± 29.3 |
| Concentration [particles/ml] | $2.17 \times 10^{12} \pm 5.33 \times 10^{10}$ | $6.46 \times 10^{12} \pm 4.12 \times 10^{11}$ | $3.96 \times 10^{12} \pm 1.52 \times 10^{11}$ | $2.58 \times 10^{12} \pm 4.58 \times 10^{11}$ |
| Zeta potential [mV] | − 19.42 ± 0.9399 | − 20.26 ± 2.504 | − 24.21 ± 1.801 | − 25.19 ± 1.065 |
| Conductivity [mS/cm] | 0.1158 | 0.1137 | 0.1171 | 0.1139 |

**Table 1.** The results of the nanoparticle tracking analysis (NTA).

caused an increase in the intensity of the biofilm formation process at a concentration of 0.78 µg/ml, reaching 150–200% of the biotic control. Higher concentrations of all AgNP types had a strong inhibitory effect on *P. aeruginosa* biofilm formation.

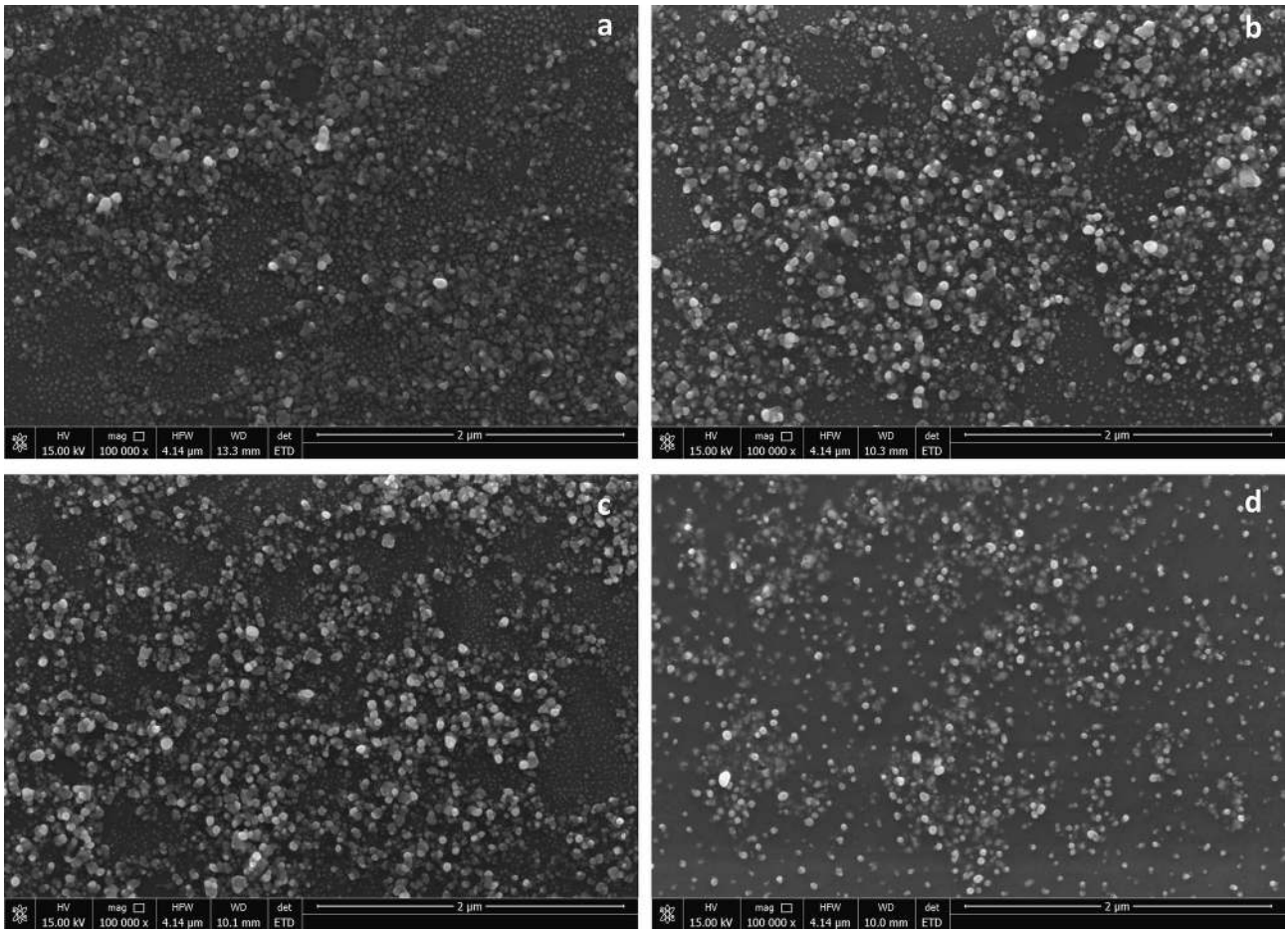


**Figure 2.** Scanning electron microscopy (SEM) images of the synthesized AgNPs: (**a**) 28 ns AgNPs, (**b**) 28 s AgNPs, (**c**) 4 ns AgNPs, and (**d**) 4 s AgNPs.

### Assessment of haemolytic activity of AgNPs

The haemolytic activities of all types of biosynthesized AgNPs were tested in the concentration range of 0.098–25 µg/ml, corresponding to the concentrations used in the antibacterial activity assay (Fig. 7). After 24 h of incubation, the amount of released haemoglobin was measured spectrophotometrically. The results were calculated as a percentage of positive control, which represented 100% haemolysis. None of the resulting AgNP types cause a haemolytic effect at concentrations from 0.098 to 1.56 µg/ml. A 50% haemolityc effect was detected in samples with the addition of 28 s AgNPs and 4 ns AgNPs in the concentration of 6.25 µg/ml. For higher tested concentrations, the addition of all types of the obtained silver nanoparticles caused haemolysis on the level of 60% or higher.

### Evaluation of the cytotoxic potential of AgNPs

The cytotoxic potential of all types of the resulting AgNPs was checked over the concentration range of 0.098 to 25 µg/ml, which corresponded to the concentrations used in the antibacterial activity assay (Fig. 8). The results of the cell viability assay of human fibroblasts indicated that at a concentration of 1.56 µg/ml, almost all of the tested AgNPs caused an > 50% decrease in cell viability. 4 ns AgNPs constituted an exception and were actually less cytotoxic at this concentration. At the concentration of 3.125 µg/ml, 28 ns AgNPs and 4 ns AgNPs caused a 100% decrease in cell viability. At higher concentrations, this effect was observed for all AgNP types.

## Discussion

Nanomaterials exhibit unique properties that make them a versatile tool for medicine, agriculture, and industry. Among them, AgNPs are the most thoroughly investigated. They are exploited in the production of textiles, cosmetics, sensors, and coatings, in addition to food packaging, plasmonics, and optoelectronics. They possess strong antimicrobial, anti-inflammatory, and anticancer potentials, characteristics that enable AgNPs to be used in the biomedical and pharmaceutical industries[5,7,18]. Biosynthesis of AgNPs using various organisms and filamentous fungi are of great scientific interest and relevance in terms of 'green' processes. They are preferable for large-scale synthesis because they can produce stable NPs. They are also easy to grow under laboratory conditions, secrete high amounts of bioactive compounds, and develop resistance to the presence of metal ions. Fungal synthesis

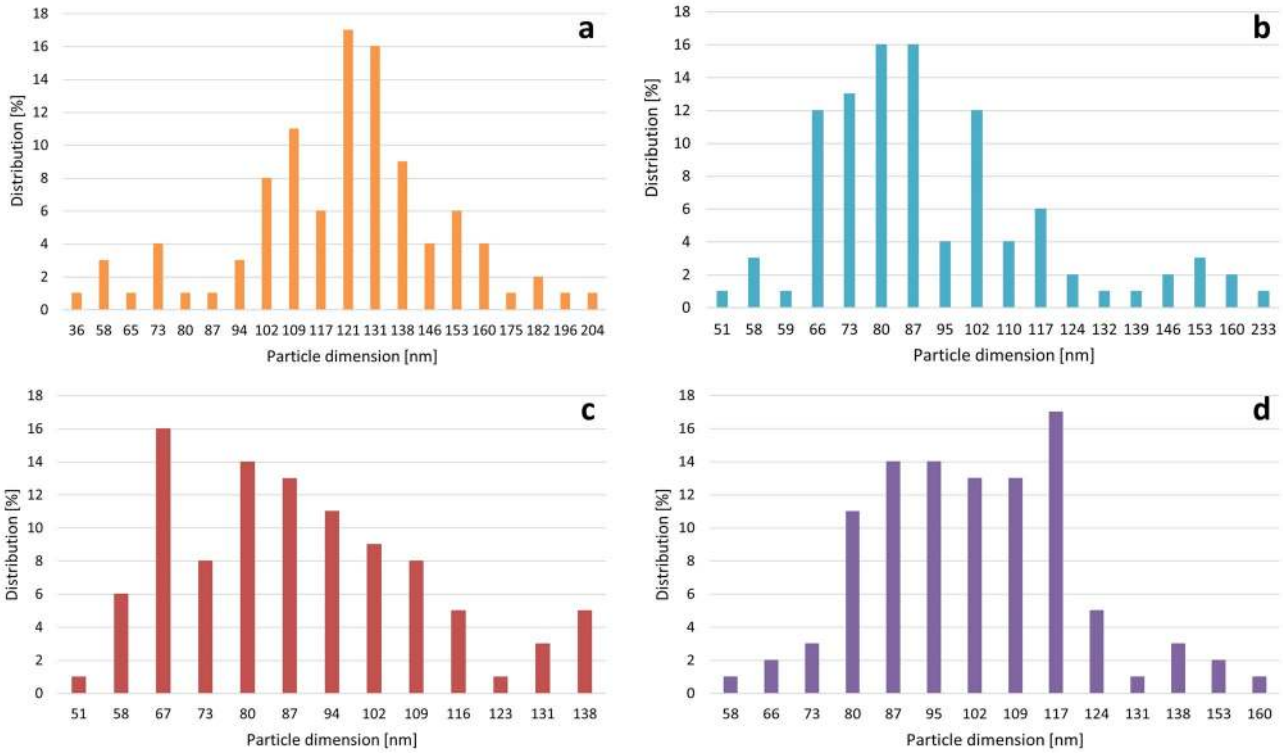


**Figure 3.** Distribution of diameter sizes of synthesized AgNPs: (**a**) 28n AgNPs, (**b**) 28 s AgNPs, (**c**) 4 ns AgNPs, and (**d**) 4 s AgNPs. Diameter sizes are given in nanometers.

can occur intracellularly or extracellularly; however, the latter is preferable for NP synthesis because it enhances the post-production processes[5,6,8,12,13].

Several fungal species are known to be capable of successfully synthesizing functional AgNPs[12]. Due to their enzymatic activities, wood decay fungi are considered promising candidates NP production. White rot fungi are known to reduce silver nitrate to AgNPs[15]. However, information about AgNP synthesis using brown rot fungi is scarce. According to Zawadzka et al.[14], the brown rot fungus *G. striatum* DSM 10335 is able to synthesize silver nanoparticles. In the present study, *G. striatum* DSM 9592, known to be capable of degrading fluoroquinolone antibiotics[19] was selected for further investigation.

The properties of newly synthesized NPs depend on the synthesis conditions. Size distribution, shape, and surface charge seem to be crucial factors, affecting the effectiveness of NP-associated antimicrobial activity. Therefore, it is vital that the synthesis conditions are adjusted to obtain the desired product[20]. In this study, two variables affecting the process of synthesis were taken into consideration: temperature and shaking occurrence during AgNP production. It was found that both of these factors could cause differences in the resulting NPs. Maintaining high temperatures during the production process led to the synthesis of AgNPs with relatively large diameter sizes. The same tendency was observed in the case of shaking occurrence in which AgNPs synthesized under shaking conditions during the process were larger in comparison to the ones produced without shaking. The activity of synthesized AgNPs was also affected by the selected conditions. For example, AgNPs synthesized at 28 °C were more efficient against *S. aureus* strains than the ones produced at lower temperatures. In contrast, AgNPs synthesized at 4 °C without shaking were slightly less cytotoxic than the other AgNPs.

As physicochemical properties are the main factors determining the potential utility of NPs, post-synthesis characterization of the resulting AgNPs is necessary. UV/Vis analysis was chosen to confirm whether the reduction of silver nitrate in the presence of fungal post-culture liquid occurred and AgNPs were indeed synthesized. The peak of maximum absorption was found at a wavelength of 430 nm for every type of NP synthesized by *G. striatum* under different reaction conditions. Zawadzka et al.[14] observed the peak at 425 nm in the case of silver nanoparticles synthesized by a different *G. striatum* strain. For other fungal species, the peaks in the AgNP UV/Vis spectrum were found in the range of 405–420 nm[12,13]. The peak value of 405 nm corresponded to AgNPs of plant origin[7]. SEM and NTA analyses were used to establish AgNPs size. It was found that all of the synthesized AgNPs were polydispersed. The diameter ranges of the nanoparticles in each type of AgNPs were 51–233, 36–204, 51–138, and 58–160 nm for 28 ns AgNPs, 28 s AgNPs, 4 ns AgNPs, and 4 s AgNPs, respectively. In comparison, the *G. striatum* DSM 10,335 strain had the capability of synthesizing AgNPs with an average diameter of approximately 20 nm under the synthesis conditions of 28 °C with shaking[14]. Smaller-sized AgNPs were obtained also with other fungal species, such as *Chaetomium thermophillum* and *Penicillium radiotolobatum*[12,13] or strawberry seed extracts[7]. FT-IR spectroscopy was used to obtain insight into the chemical structure of synthesized AgNPs. It was found that every type of resulting AgNP exhibited a band of 1644 $cm^{-1}$. This result can be considered

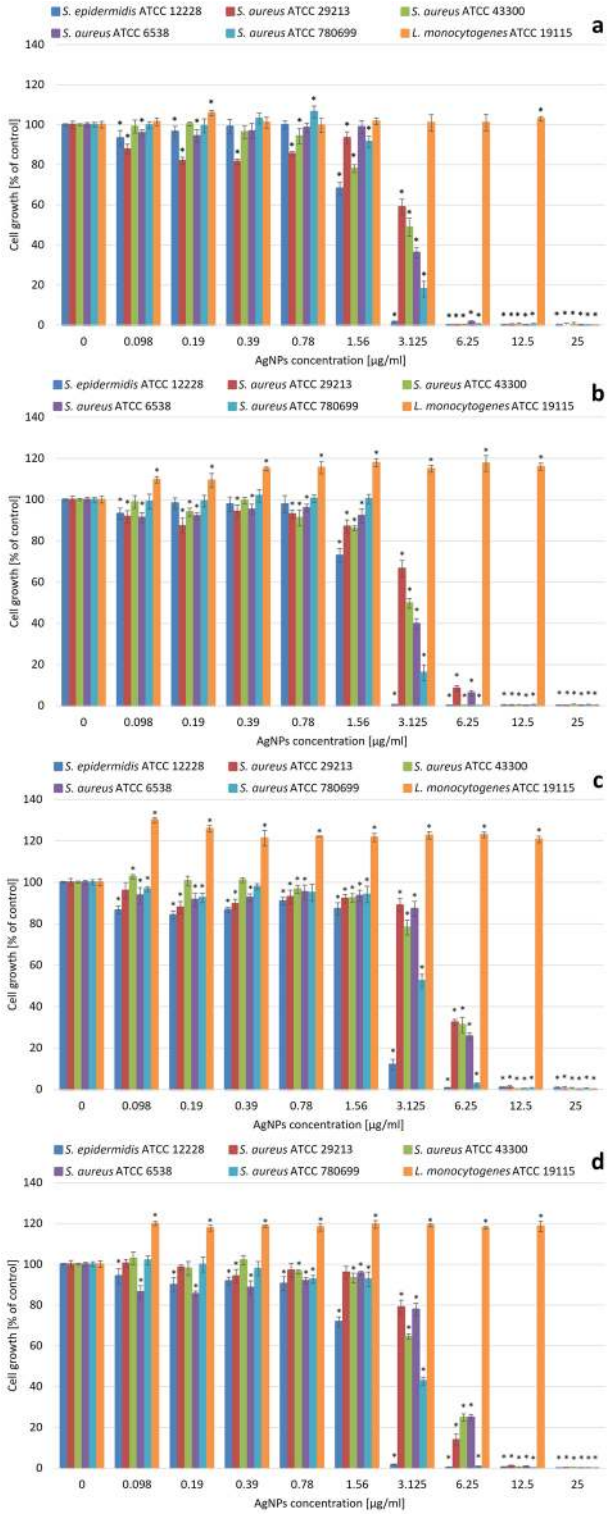


**Figure 4.** Antibacterial activity of obtained AgNPs against Gram-positive bacteria strains: (**a**) 28 ns AgNPs, (**b**) 28 s AgNPs, (**c**) 4 ns AgNPs, and (**d**) 4 s AgNPs. The results are shown as average percentage values with standard deviations of optical density (OD) of the biotic control. The statistical significance was estimated using a one-way analysis of variance (ANOVA) test with * $p < 0.05$ and is shown by an asterisk.

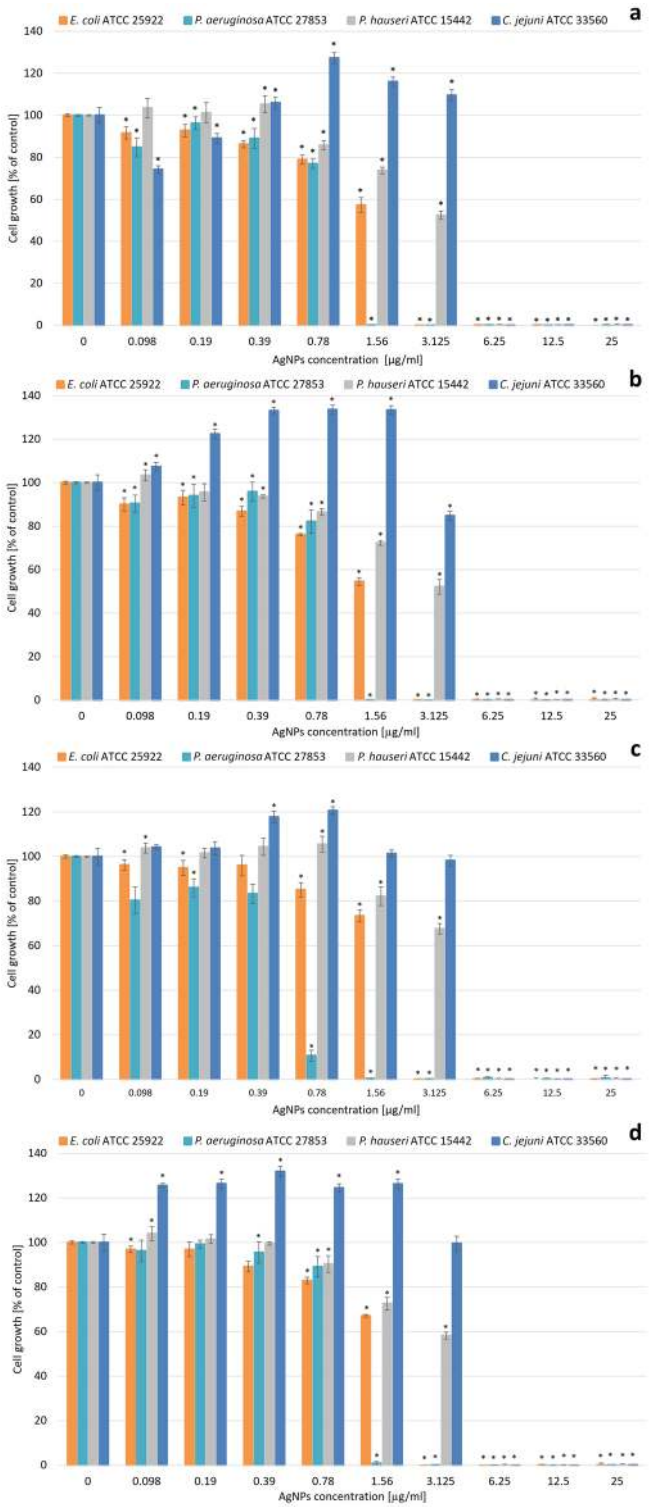


**Figure 5.** Antibacterial activity of synthesized AgNPs against Gram-negative bacteria strains: (**a**) 28 ns AgNPs, (**b**) 28 s AgNPs, (**c**) 4 ns AgNPs, and (**d**) 4 s AgNPs. The results are shown as average percentage values of optical density (OD) of the biotic control with standard deviations. The statistical significance was estimated using a one-way ANOVA test with * $p < 0.05$ and is shown as an asterisk.

| | MIC | | | | MBC | | | |
|---|---|---|---|---|---|---|---|---|
| | 28 ns | 28 s | 4 ns | 4 s | 28 ns | 28 s | 4 ns | 4 s |
| *E. coli* ATCC 25922 | 3.125 | 3.125 | 3.125 | 3.125 | 25 | 3.125 | 6.25 | 3.125 |
| *P. hauseri* ATCC 15442 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | > 25 | 12.5 |
| *P. aeruginosa* ATCC 27853 | 1.56 | 1.56 | 1.56 | 1.56 | 12.5 | 12.5 | > 25 | > 25 |
| *C. jejuni* ATCC 33560 | 6.25 | 6.25 | 6.25 | 6.25 | 12.5 | 12.5 | 6.25 | 12.5 |
| *S. epidermidis* ATCC 12228 | 3.125 | 3.125 | 6.25 | 3.125 | > 25 | 25 | > 25 | > 25 |
| *S. aureus* ATCC 29213 | 6.25 | 12.5 | 12.5 | 12.5 | > 25 | > 25 | > 25 | > 25 |
| *S. aureus* ATCC 43300 | 6.25 | 6.25 | 12.5 | 12.5 | > 25 | > 25 | > 25 | > 25 |
| *S. aureus* ATCC 6538 | 6.25 | 12.5 | 12.5 | 12.5 | > 25 | > 25 | > 25 | > 25 |
| *S. aureus* ATCC 780699 | 6.25 | 6.25 | 6.25 | 6.25 | 25 | > 25 | > 25 | 25 |
| *L. monocytogenes* ATCC 19115 | 25 | 25 | 25 | 25 | > 25 | > 25 | 25 | > 25 |

**Table 2.** Minimum inhibitory and minimal bactericidal concentrations (MIC and MBC, respectively, μg/ml) values of synthesized AgNPs against selected bacterial strains.

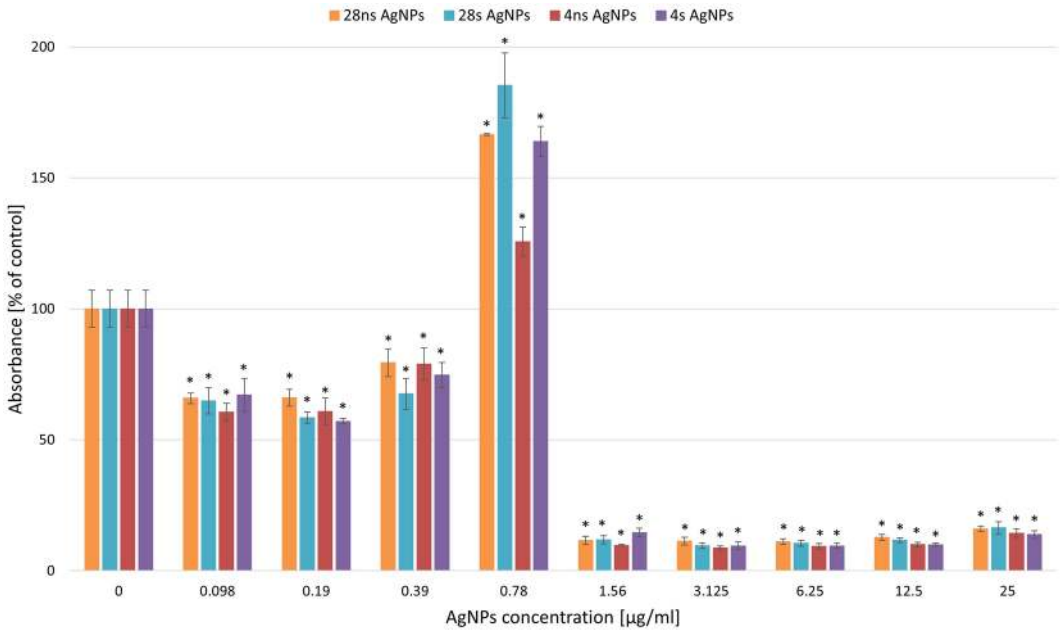


**Figure 6.** Biofilm forming ability of *Pseudomonas aeriginosa* treated with synthesized AgNPs. The results are shown as average percentage values with standard deviations of absorbance in biotic controls. The statistical significance was estimated using a one-way ANOVA test with * $p < 0.05$ and is shown as an asterisk.

characteristic of the C–N and C=O bonds of the amide I band. These results correspond with those obtained by Zawadzka et al.[14] as reported in the previous study.

AgNPs have become an alternative to classic antimicrobial agents, and the bactericidal effect of AgNPs is stronger in comparison to other nanomaterials. The AgNPs develop a complex mode of action based on concurrent damage to cells at both the extracellular and intracellular levels. This phenomenon makes AgNPs effective against different groups of microorganisms, including drug-resistant strains[21–23]. Therefore, it is necessary to develop an effective method of AgNP synthesis to satisfy the need for them in various applications.

Our study showed that all types of synthesized AgNPs using the *G. striatum* DSM 9592 strain were active against the tested bacterial strains. The differences in activity levels were visible depending on the synthesis conditions; however, no specific tendency for any type of AgNP to be more effective was found. Generally, the Gram-negative strains were more susceptible with MIC values two- or three-fold lower than Gram-positive ones. The exception was the Gram-negative strain *P. hauserii* with an MIC value similar to those observed in most of the tested Gram-positive strains. In contrast, Naveen et al.[13] found that the effectiveness of the AgNPs synthesized with *P. radiatolobatum* against anaerobic *L. monocytogenes* and *S. enterica* strains was comparable to that observed in the case of aerobic bacteria tested in our study. Moreover, their results indicated that the Gram-negative bacterium *E. coli* was less susceptible to AgNP action than Gram-positive *S. aureus*. The results from Barapatre et al.'s study on silver nanoparticles synthesized by *Emericella nidulans* confirmed that tendency. The MBC values of the resulting AgNPs in this investigation were lower for Gram-positive *S. aureus* than for the Gram-negative strains tested, which proved that the Gram-positive bacterial strains are more sensitive to AgNP

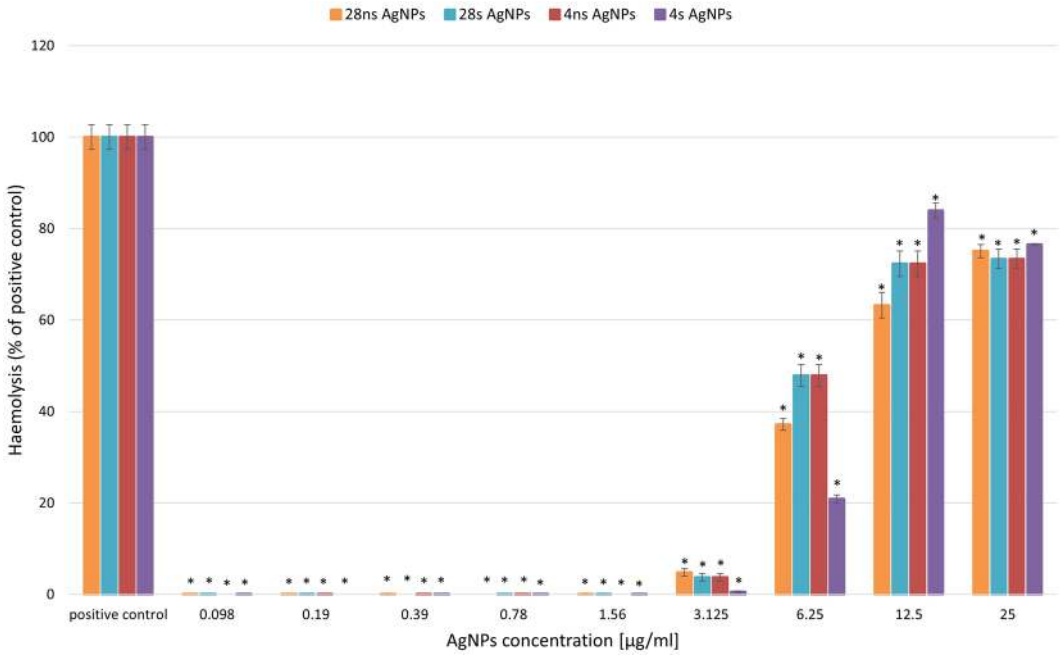


**Figure 7.** Haemolytic activity of synthesized AgNPs. The results are shown as average percentage values of absorbance with standard deviations in positive control. The statistical significance was estimated using a one-way ANOVA test with * $p < 0.05$ and is shown as an asterisk.

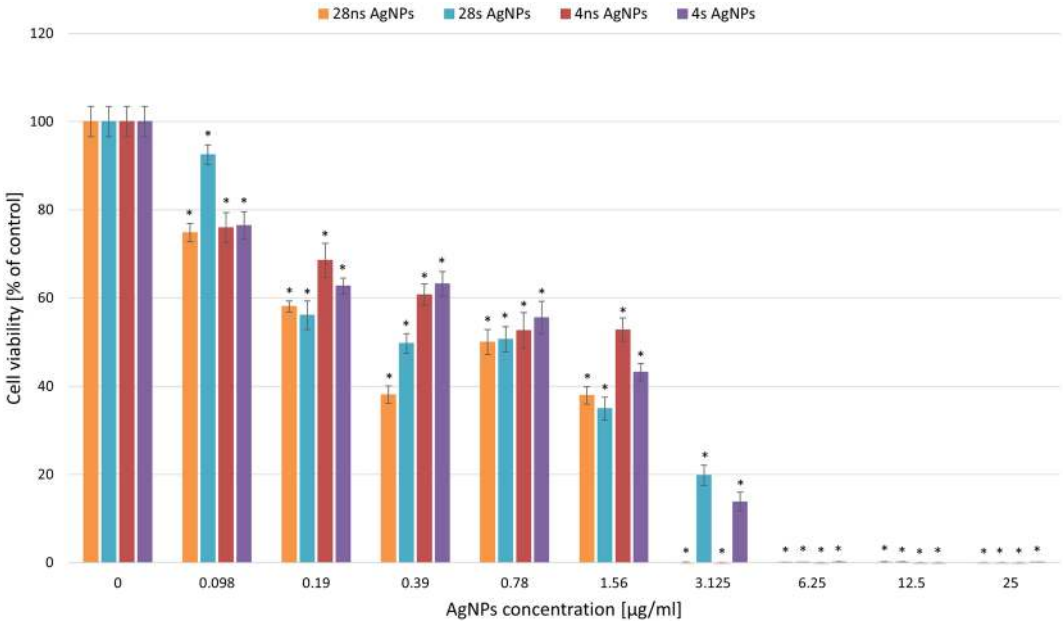


**Figure 8.** Cytotoxic activity of synthesized AgNPs. Cell viability is shown as average percentage values of absorbance with standard deviations in biotic control. The statistical significance was estimated using one-way ANOVA with * $p < 0.05$ and is shown as an asterisk.

activity[9]. These findings are in contrast to the results of our research. On the other hand, Hu et al.[24] revealed that Gram-negative *E. coli* and *P. aeruginosa* were more sensitive than Gram-positive *S. aureus*, which confirms our results. Moreover, they established that *P. aeruginosa* was the most affected by silver nanoparticles synthesized with the use of the fungus from the genus *Talaromyces purpureo*.

Among all strains tested in our study, *P. aeruginosa* ATCC 27853 was the most susceptible to the activity of all types of synthesized NPs. Therefore, our research was expanded by focusing on the interactions between *P. aeruginosa* and the resulting AgNPs. *P. aeruginosa* is an opportunistic bacterium that infects patients with

comorbid health problems, such as burns, cystic fibrosis, or suppressed immunity. Moreover, this specific bacterium is the cause of urinary tract infections associated with catheter implementation. A serious threat posed by *P. aeruginosa* results from its capability to form biofilms, which are defined as groups of bacteria of the same or different species that are capable of growing on several surfaces. Bacterial cells that have formed biofilms obtain properties different from those exhibited by single bacterial cells, which makes the bacteria within the biofilms very difficult to treat. Using AgNPs as surface coating agents can potentially prevent the development of *P. aeruginosa* biofilm[25,26]. In this study, the capability of *P. aeruginosa* to form biofilms was tested in the presence of our synthesized AgNPs. The results indicated that biofilm formation was strongly reduced at concentrations of 1.56 µg/ml and higher in the case of all AgNP variants. This value corresponded with the resulting MIC values. Interestingly, a strong enhancement in biofilm formation was visible in the AgNP concentration of 0.78 µg/ml even reaching up to 180% of biotic control in 28 s AgNPs. This may suggest that an increased biofilm-forming activity is a defense mechanism *P. aeruginosa* induced by the presence of AgNPs at the concentrations closest to the MIC values. LewisOscar et al.[25] found that AgNPs synthesized with the use of *Spirulina platensis* methanolic extract also inhibited the biofilm formation by *P. aeruginosa*. In their research, the biofilm formation assay was performed in two ways: on polystyrene plates as a standard method and on the surface of latex urinary catheters (modified method). In both cases, the highest inhibition of biofilm formation was obtained with the use of the AgNP concentration of 100 µg/ml and reached 85% for the standard assay and 77% for the modified method. The effectiveness of AgNPs against *P. aeruginosa* biofilm formation has been confirmed in other studies. Shah et al.[27] revealed that AgNPs of plant origins inhibited biofilm development at the level of 78% with the use of 8 µg/ml AgNPs. The efficacy of AgNPs against biofilm formation was confirmed also in *E. coli* and *C. albicans*[28,29].

The possibilities for using AgNPs in different areas are increasing. Massive consumption of AgNPs in various industries has become a source of concern about the safety of AgNP exploitation[30]. It has been proven that AgNPs can cause damage to mammalian cells at several levels. Their cytotoxicity mechanisms are not fully known, but it has been reported that AgNPs can lead to an increase in reactive oxygen species production, which is believed to cause damage to DNA and other cellular compartments[10,31]. Considering the risk of AgNP-induced adverse effects on human health and the scarcity of appropriate research, further investigation into AgNP-induced cytotoxic effects is necessary.

In this study, the toxicity of synthesized AgNPs toward human red blood cells and the human fibroblast cell line was tested. The haemolytic assay revealed that all synthesized types of NPs caused at least a 60% haemolysis at the AgNP concentration of 12.5 µg/ml. At lower concentrations, haemolytic activities varied depending on the nanoparticle type. Interestingly, in the concentration of 6.25 µg/ml, 4 s AgNPs showed haemolytic activity at the level of 20%, while 28 s and 4 ns AgNPs caused almost 50% haemolysis. AgNPs obtained with the use of *G. striatum* DSM 10335 showed a lower haemolytic activity in which 66% haemolysis was induced by AgNPs at a concentration of 40 µM[14]. In the case of AgNPs of plant origin, haemolytic activity was found to be less severe. For example, AgNPs synthesized with the use of *Azadirachta indica* extract caused over 50% haemolysis at the concentration of 125 µg/ml[32], and AgNPs obtained with *C. sativa* root extract exhibited maximal haemolytic activity at the level of 6.5% using 200 µg/ml AgNPs[33].

The cytotoxic activity study of AgNPs synthesized by *G. striatum* revealed that three out of the four resulting NP types caused a 50% cell viability reduction at the concentration of 1.56 µg/ml in comparison to the biotic control. 4 ns AgNPs were found to be less toxic at this concentration. In the previous study, Zawadzka et al.[14] had established that the $IC_{50}$ value of silver nanoparticles derived from *G. striatum* DSM 10335 in murine fibroblasts was 28.76 µM, indicating that they were less cytotoxic than AgNPs used in this study. Alves et al.[12] found that AgNPs synthesized by the fungus *C. thermophillum* were less cytotoxic in comparison to *G. striatum*-derived AgNPs. In their study, the $IC_{50}$ value for murine fibroblast cells was obtained at an AgNP concentration of approximately 119 µg/ml, which is in contrast to the results of our research in which AgNP-associated cytotoxic effects were visible at lower concentrations. For instance, AgNPs synthesized biologically by *Bacillus* sp. showed cytotoxic effects at a concentration of 5 µg/ml thus achieving the $LC_{50}$ value in human fibroblasts[34].

Our research led us to draw the conclusion that modifying the conditions of the synthesis process could affect the properties of AgNPs. It was revealed that biological activity varied depending on the synthesis variant, but no specific tendency of one type of AgNP to be more bactericidal against the strains tested has been found. Gram-negative bacteria strains were generally more susceptible to the action of all tested AgNPs and *P. aeruginosa* was confirmed to be the most affected. It was also proved that the resulting AgNPs were capable of inhibiting the biofilm formation activity of this strain. Considering the high cytotoxic and haemolytic potential of the resulting AgNPs, it was confirmed that AgNPs could produce similar effects on various biological systems. The effectiveness against microorganisms is a desirable AgNP property, but the potential adverse effects of AgNPs must be considered. However, the slightly lower cytotoxic potential of 4 ns AgNPs in terms of antimicrobial activity maintained at a level comparable to that observed for the other synthesized AgNPs proves that optimisation of the synthesis process might lead to the production of effective AgNPs with reduced cytotoxicity.

## Methods

### Materials

The tested fungal strain, *Gloeophyllum striatum* DSM 9592, was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (Germany). The human fibroblast BJ ATCC CRL-2522 cell line was purchased from the American Type Culture Collection (ATCC; USA). Erythrocytes used in the haemolysis assay were obtained from the Regional Center of Blood Donation and Blood Treatment in Lodz (Poland). Sabouraud dextrose broth (Difco™) and Mueller–Hinton broth (BBL™) were obtained from Becton Dickinson (Poland). Silver nitrate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Merck (Poland). Dimethyl sulfoxide (DMSO) and phosphate-buffered saline (PBS) were obtained from BioShop

(Canada). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from BioWest (France). Crystal violet and acetic acid were obtained from Chempur (Poland).

## Biosynthesis of AgNPs

*G. striatum* DSM 9592 was grown on potato dextrose agar slants at 28 °C. After 18 days of cultivation, a fungal inoculum was prepared in Sabouraud Dextrose Broth supplemented with 2% glucose. The resulting *G. striatum* inoculum was incubated for 120 h at 28 °C on a rotary shaker at 120 rpm.

After incubation, fungal biomass was filtered through sterile filter paper and transferred to sterile deionized water with a volume that was equivalent to the amount of Sabouraud medium used previously. The *G. striatum* biomass was incubated for 120 h under the same conditions as previously described. Again, the mycelium was filtered through sterile filter paper to obtain post-culture fluid. The fungal filtrate was divided into four parts of equal volume. All samples were supplemented with a stock solution of silver nitrate prepared earlier on sterile deionized water. The final concentration of silver ions in each sample was 5 mM. The samples were incubated in the dark for 24 h under specific conditions: 28 °C with shaking, 28 °C without shaking, 4 °C with shaking, and 4 °C without shaking. For shaking, a magnetic stirrer was used. All samples were then stored for seven days at 4 °C.

## Characterization of synthesized AgNPs

AgNP production was checked according to the changes in the UV/Vis spectrum during silver ion reduction. Monitoring of AgNP production was conducted with the use of the UV/Vis UV/Vis spectrophotometer Specord 200 (Analytik Jena, Jena, Germany) in the absorbance mode at wavelengths from 300 to 800 nm at a resolution of 2 nm.

Biosynthesized AgNPs were examined using FT-IR spectroscopy, NTA, and SEM.

FT-IR analyses were carried out with a Nicolet™ iS50 FT-IR Spectrometer (Thermo Scientific, Waltham, MA, USA) in transmittance mode at a resolution of 0.25 $cm^{-1}$ and spectral range of 3000–600 $cm^{-1}$.

For NTA analyses of AgNPs, the NanoSight NS300 (Malvern Panalytical Ltd., Malvern, UK) equipped with green laser at 532 nm and NTA 3.4 Build 3.4.4 software were used. The samples were diluted 10,000 times in deionized water. In this study, zeta potential and conductivity were also determined using the Zetasizer Ultra (Malvern Panalytical Ltd., Malvern, UK).

SEM analyses were conducted with a scanning electron microscope from Quanta 250 FEG (FEI, USA). The samples were prepared by spreading AgNPs on silicon wafers, which were dried in the dark at room temperature. The images were taken in immersion mode using the Everhart–Thornley detector (ETD) at 15,00 kV acceleration at a magnification of 100,000x. The size distribution was established by measuring AgNP diameters from the SEM images with the use of the ImageJ software. The diameter was calculated based on an average of 100 NPs.

## Evaluation of antibacterial properties of synthesized silver nanoparticles

The effectiveness of synthesized AgNPs against bacteria was tested using the microdilution method according to the Clinical and Standard Laboratory Institute (CSLI) guidelines M07 ($11^{th}$ Edition) concerning aerobic bacterial strains and M11 ($9^{th}$ Edition) for anaerobic bacteria. The antimicrobial assay was performed with the *S. aureus* ATCC 29213, *S. aureus* ATCC 43300, *S. aureus* ATCC 6538, *S. aureus* ATCC 780699, *S. epidermidis* ATCC 12228, *E. coli* ATCC 25922, *P. hauseri* ATCC 15442, *P. aeruginosa* ATCC 27853, *C. jejuni* ATCC 33560, and *L. monocytogenes* ATCC 19115 strains. The differences in growth of all tested bacteria strains with and without AgNPs were evaluated using 96-well cell culture plates in Mueller–Hinton Broth (MHB) and Tryptic Soy Broth (TSB) for the aerobic and anaerobic bacteria, respectively. The tested AgNPs were diluted in appropriate growth medium, and the final evaluated concentrations ranged from 0.098 to 25 µg/ml in all cases. A bacterial inoculum prepared in MHB or TSB medium was added to each well to achieve a final density of $5 \times 10^5$ colony-forming units (CFU)/ml and $1 \times 10^6$ CFU/ml for the aerobic and anaerobic strains, respectively. Aerobic bacterial samples and adequate biotic and abiotic controls were incubated for 24 h at 37 °C. For anaerobic strains, the 96-well plates including samples, biotic and abiotic controls were placed in jars to maintain the anaerobic conditions formed using Anoxomat Mark II CTS (Mart Microbiology, B.V., the Netherlands) and then incubated for 48 h at 37 °C. After the required incubation period, the OD was measured at a wavelength of 630 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Pudong, Shanghai, China), and the MIC was established for each tested variant as the lowest concentration of AgNPs at which no growth of microorganisms was observed. The agar and TSB agar plates were then inoculated with 100 µl bacterial suspension taken from the wells in which no growth was observed. The agar plates with the aerobic strains were incubated for 24 h at 37 °C, and the TSB plates with the anaerobic strains were incubated under anaerobic conditions for 48 h at 37 °C to establish the MBC. The MBC was defined as the lowest concentration of AgNPs that caused a complete reduction in the viability of the tested microorganisms. Both MIC and MBC values were expressed in µg/ml.

## Assessment of the *P. aeruginosa* biofilm formation in the presence of AgNPs

The assay was carried out on 96-well plates using a scheme that was similar to one used in antimicrobial activity tests. After incubation, the media and unattached cells were removed from the wells. Each well was then washed twice with 0.85% NaCl after which 96% ethylic alcohol was added. The prepared plates were incubated for 20 min. After incubation, ethanol was removed, and the plates were left to dry. Next, a 0.1% crystal violet solution was added to all wells, and the plates were incubated for 30 min at room temperature. The stain was removed after which the wells were washed three times with 0.85% NaCl. Last, a 33% acetic acid solution was added to the dry wells, and the plates were incubated for 10 min on a rotary shaker. The absorbance was then measured at a wavelength of 600 nm using a FLUOstar Omega microplate reader.

### Evaluation of the haemolytic properties of synthesized AgNPs

The red blood cells were washed five times with phosphate-buffered saline (PBS) and then suspended in PBS in a 1:1 ratio. AgNP solutions were prepared in PBS and added to the erythrocyte samples to obtain the final AgNP concentrations ranging from 0.098 to 25 µg/ml. Moreover, negative (red blood cells in PBS without AgNPs) and positive (erythrocytes suspended in deionized water without tested NPs) controls were prepared. All samples were incubated in the dark for 24 h at 37 °C. After incubation, all samples were centrifuged for 5 min at 5000 x*g* and 4 °C. The absorbance of the resulting supernatants was measured spectrophotometrically at wavelength λ = 540 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Pudong, Shanghai, China). The haemolytic activities of the AgNPs was calculated based on the quantity of haemoglobin released from erythrocytes into the supernatants using the formula: % Haemolysis $= \frac{A_{AgNPs}}{A_{PC}} \times 100\%$ in which $A_{AgNPs}$ represents the absorbance of samples incubated with AgNPs, and $A_{PC}$ is the absorbance of positive controls, which corresponds to 100% haemolysis.

### Evaluation of the cytotoxic activity of synthesized AgNPs

The potential cytotoxic activities of the resulting fungal AgNPs were examined using the human fibroblast BJ ATCC CRL-2522 cell line. Fibroblasts at a final density of $1 \times 10^4$ cells/well were cultivated in 96-well microplates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 UI penicillin and 100 µg/ml streptomycin). The cells were incubated under conditions of 5% $CO_2$ and 37 °C for 24 h. After incubation, the medium was removed, and each well was refilled with 100 µl of the fresh DMEM medium supplemented with AgNPs in the concentration range of 0.098–25 µg/ml. Corresponding biotic and abiotic controls were also prepared and incubated under the same conditions for 24 h. The medium was then removed, and 100 µl of fresh growth medium and 10 µl of 500 µg/ml MTT were added to each well. The microplates were again incubated under the same parameters for 5 h. After the final incubation, 80 µl of the solution was removed from each well after which 50 µl dimethyl sulphoxide (DMSO) was added to all wells, and the plates were left for 20 min at 28 °C to allow formazan crystals to dissolve. Spectrophotometric measurements of the cultures were obtained at λ = 595 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Pudong, Shanghai, China). Cell viability was calculated based on the capability to reduce MTT by the cells incubated with AgNPs compared to biotic controls.

### Statistical analysis

Each experiment was conducted in four replicates (n = 4). All results were analyzed with the use of a one-way ANOVA test with * $p < 0.05$ to estimate the statistical significance. The estimation and all needed calculations were carried out by using Excel, Microsoft® Office 2021 (Microsoft Corporation, Redmont, WA, USA). The results shown in the figures are expressed as the average values with the standard deviation (SD).

## Data availability

The data presented in this study are available on request from the corresponding author.

Received: 15 September 2023; Accepted: 26 November 2023
Published online: 30 November 2023

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## Author contributions

Conceptualization, A.T., K.N.; methodology, K.N., A.T.; investigation, A.T., K.N.; manuscript—original draft preparation, A.T.; manuscript—review and editing, K.N., K.L.

## Competing interests

The authors declare no competing interests.

## Additional information

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Publikacja 2

## *Mycogenic silver nanoparticles: promising antimicrobials with fungistatic properties*

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2025, International Journal of Molecular Sciences, 26: 6639; DOI: 10.3390/ijms26146639

International Journal of
*Molecular Sciences*

MDPI

*Article*

# Mycogenic Silver Nanoparticles: Promising Antimicrobials with Fungistatic Properties

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**Abstract**

The antimicrobial activity of silver nanoparticles (AgNPs) makes them a valuable tool in various industries. Recently, biosynthesis has become the preferred method for nanoparticle synthesis, and among organisms that can be used as AgNP producers, filamentous fungi have attracted the greatest interest. In particular, wood decay fungi are considered promising candidates for AgNP biosynthesis. Biogenic AgNPs have been proven to have strong antibacterial potential and antifungal activity. The aim of this study was to evaluate the antifungal potential of AgNPs synthesized using the brown-rot decay fungus *Gloeophyllum striatum* DSM 9592 against four pathogenic fungal strains: *Candida albicans*, *Malassezia furfur*, *Aspergillus flavus* and *Aspergillus fumigatus*. Moreover, changes in the tested strains' lipidome and cell membrane properties induced by the presence of AgNPs were investigated. The results revealed that the obtained AgNPs exerted fungistatic activity against all the strains tested. *M. furfur*, with a MIC value of 0.39 µg/mL obtained for all AgNP types, was found to be the most susceptible to the action of AgNPs. The lipidomic analysis revealed that the presence of AgNPs caused an increase in cell membrane fluidity in both *A. flavus* and *C. albicans*, and the mechanisms of response to AgNPs differed between the tested strains.

**Keywords:** silver nanoparticles; biogenic; antifungal; cell membrane; fluidity; permeability; lipidome



Academic Editor: Christian Celia

Received: 2 June 2025
Revised: 2 July 2025
Accepted: 9 July 2025
Published: 10 July 2025

**Citation:** Tończyk, A.; Niedziałkowska, K.; Nowak-Lange, M.; Bernat, P.; Lisowska, K. Mycogenic Silver Nanoparticles: Promising Antimicrobials with Fungistatic Properties. *Int. J. Mol. Sci.* **2025**, *26*, 6639. https://doi.org/10.3390/ijms26146639



## 1. Introduction

Silver has been recognized as an effective antimicrobial agent for centuries. Silver nanoparticles (AgNPs), a product of the modern field of science called nanotechnology, are proven to possess similar potential. In general, nanoparticles have a more extensive range of possible applications compared to their bulk materials. This broader applicability also applies to AgNPs, whose scope of application includes medicine, pharmaceuticals and cosmetics, as well as engineering and other technologies [1,2].

Recently, biosynthesis has become the preferred method for nanoparticle synthesis. Biosynthesis provides nanoparticles with the desired properties while simultaneously being more efficient, cost-effective and eco-friendly compared to conventional chemical and physical production methods. In the case of AgNPs, various species have been proven to

be able to reduce silver ions, which leads to the formation of nanoparticles [3]. Filamentous fungi are considered the best option for metallic nanoparticle biosynthesis. Their beneficial properties include ease of cultivation and the ability to secrete numerous proteins that act as reducing, capping and stabilizing agents during the production process [4,5]. The number of investigated fungal strains for AgNP synthesis continues to increase [6]. Fungi from the *Aspergillus*, *Penicillium*, *Fusarium* and *Trichoderma* genera are recognized for their ability to synthesize AgNPs [7], and recent studies have proven that other fungal sources, such as *Alternaria* sp. [8], *Rhizoctonia solani*, *Cladosporium cladosporoides* [9] and *Talaromyces funiculosus* [10], can be successfully used in green methods of AgNP production. Species from the wood decay fungi group are interesting yet poorly investigated candidates for AgNP producers. This is mostly due to their ability to produce large amounts of different biologically active compounds [11] that can serve as crucial agents during AgNP synthesis. Moreover, fungi belonging to this group exhibit other properties that could benefit production processes, such as a fast growth rate, resulting in high biomass production, and ease of cultivation [12]. Some reports show that white-rot fungi, e.g., *Trametes* sp., *Ganoderma* sp. and *Phanorochatete* sp., can be used as AgNP sources [13,14]. On the contrary, brown-rot fungi have rarely been researched for this purpose. Given their proven abilities in other biotechnological processes, such as decolorization of dyes [15] or degradation of xenobiotics [16], brown-rot fungi have emerged as another promising option for nanoparticle production. The search for new sources of AgNPs is still of great importance, as biosynthesis processes can vary depending on the fungal species, which produce distinct metabolites affecting the properties of the final products [17]. Our previous work revealed that the brown-rot fungus *Gloeophyllum striatum* is able to reduce silver ions, resulting in AgNP production [18,19].

AgNPs of biological origin are known to possess strong antibacterial potential [7]. It is also proven that AgNPs can work effectively against fungal pathogens, such as *Aspergillus* sp., *Fusarium* sp. and *Candida albicans*. The importance of this property is emphasized by the fact that AgNPs can exert stronger antifungal effects against both human and plant pathogens compared to conventional fungicidal agents [20,21]. The antifungal potential of AgNPs is a result of simultaneous processes caused by exposure to nanoparticles, such as accumulating reactive oxygen species, inducing potassium ion efflux and decreasing the activity of cellular enzymes. AgNPs and silver ions released from the surface of nanoparticles can alter the transcriptome, epigenome and metabolome of fungal cells, negatively influencing their vital functions [22]. Given that pathogenic fungal strains have been reported to develop resistance to commonly used agents [23,24], the complex action of AgNPs against fungi has been gaining great significance.

As fungal infections are one of the most commonly occurring threats to skin health globally [25], evaluating the antifungal activity of *G. striatum*-derived AgNPs was deemed necessary, and four different pathogenic fungal strains were selected for tests. *Candida albicans* is a yeast strain that can be found on mucosal surfaces in healthy humans. However, as an opportunistic pathogen, it can become a cause of infections that affect mucosa, e.g., oral, oropharyngeal or vulvovaginal candidiasis. Moreover, infections caused by *C. albicans* can cross mucosal barriers [26]. *Malassezia furfur* is a yeast-like, commensal microorganism colonizing human skin and can cause a variety of skin diseases, such as dandruff, alopecia or atopic dermatitis. It has also been reported that invasive infections caused by this pathogen are occurring more frequently [27,28]. *Aspergillus fumigatus* and *Aspergillus flavus* belong to a large group of commonly encountered saprophytic filamentous fungi that can be isolated from the air, the soil or materials of plant origin. *Aspergillus* species can be the causal agents of certain superficial and cutaneous mycoses, including onychomycosis and primary cutaneous aspergillosis. Skin infections caused by Aspergilli

are most often developed during hospitalization, commonly in infants and patients with immunodeficiencies. Such infections are also a risk factor in surgeries, burns and catheter usage in patients [29,30]. Conventional antifungal drugs, such as clotrimazole, ketoconazole and itraconazole, are commonly provided in topical formulations and administered via spreading or rubbing. The topical delivery of antifungal agents enables their direct access to the target, increases the efficacy of treatment and reduces the risk of developing systemic toxicity [25]. AgNPs with antimicrobial properties can be used in "on-skin" formulations, e.g., in wound care products such as dressings or creams [31]. Consequently, using AgNPs with strong antifungal properties in a topical formulation can be considered beneficial, as both the efficient activity of the agent and the advantages of the topical delivery route would be maintained.

The aim of this study was to evaluate the antifungal activity of AgNPs synthesized using the *G. striatum* DSM 9592 strain against four pathogenic fungal strains, namely, *Candida albicans* ATCC 10231, *Malassezia furfur* DSM 6170, *Aspergillus flavus* ATCC 9643 and *Aspergillus fumigatus* ATCC 204305, in order to determine their possible application potential in combating fungal infections. Moreover, the possible mechanisms of action of AgNPs were explored by performing a lipidomic analysis to identify changes in phospholipid profiles and evaluating changes in cell membrane fluidity and permeability in the presence of AgNPs. To the best of our knowledge, this is the first investigation of the antifungal properties exhibited by mycogenic AgNPs synthesized using a brown-rot fungus.

## 2. Results

### *2.1. Evaluation of the Antifungal Activity of Mycogenic AgNPs*

The antifungal activity of *G. striatum*-derived AgNPs was determined at nanoparticle concentrations ranging from 0.098 to 25 µg/mL against four different human pathogenic fungal strains: the yeasts *C. albicans* ATCC 10231 and *M. furfur* DSM 6170 and the filamentous fungi *A. flavus* ATCC 9643 and *A. fumigatus* ATCC 204305. It was confirmed that, compared to the filamentous fungi, the tested yeast strains were more susceptible to the action of AgNPs (Figure 1).

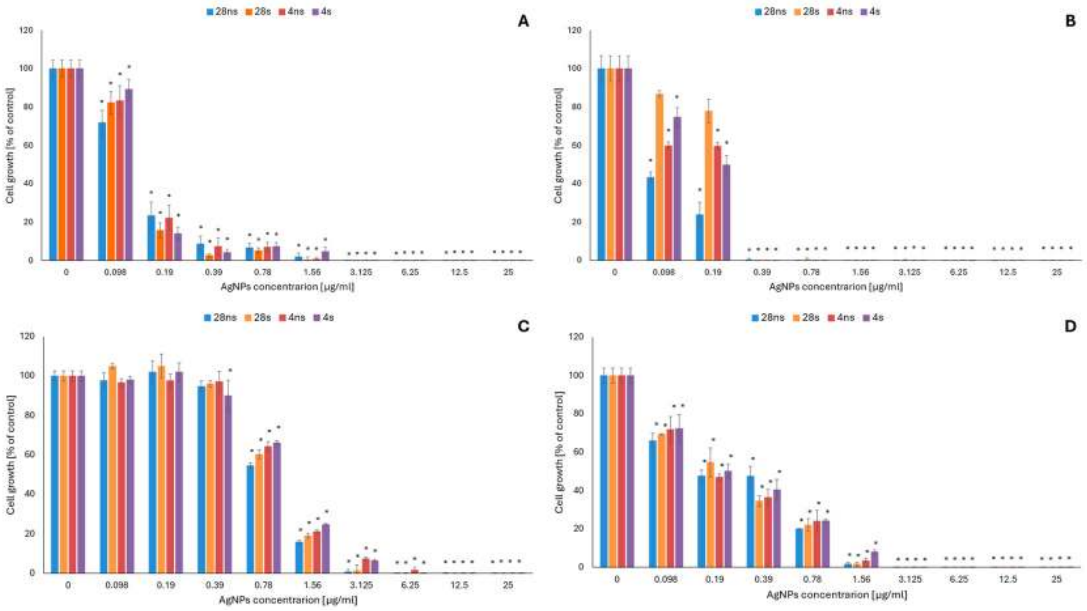


**Figure 1.** Antifungal activity of *G. striatum*-derived silver nanoparticles (AgNPs) against *C. albicans* (**A**), *M. furfur* (**B**), *A. flavus* (**C**) and *A. fumigatus* (**D**). The results are shown as average percentage values with standard deviations of the optical density (OD) of the biotic control. Statistical significance ($p < 0.05$) is shown by an asterisk (*).

Among all tested organisms, the *M. furfur* strain, with a minimal inhibitory concentration (MIC) of 0.39 µg/mL for all tested AgNP types, was found to be the most sensitive (Table 1). In *C. albicans*, growth inhibition reached 85% or higher using all tested AgNP types at a concentration of 0.39 µg/mL. However, according to the obtained MIC values (Table 1), the 4 s AgNP type was the least active against the tested microorganism—total growth inhibition was observed at a concentration of 3.125 µg/mL, while the same effect for the rest of the AgNPs tested was obtained using a two-fold lower concentration. Among the tested filamentous fungal strains, *A. fumigatus* was the most susceptible to the action of mycogenic AgNPs, with a 50% or higher inhibitory effect on cell growth obtained using all tested AgNP types at a concentration of 0.39 µg/mL (Figure 1). The MIC values obtained for this strain were 1.56 µg/mL for 28 ns and 28 s AgNPs and 3.125 µg/mL for 4 ns and 4 s AgNPs (Table 1). *A. flavus* was the least sensitive to the AgNPs. For this strain, the obtained MIC values were 3.125, 3.125, 6.25 and 12.5 µg/mL using 28 ns, 28 s, 4 s and 4 ns AgNPs, respectively (Table 1). Minimal fungicidal concentration (MFC) values (Table 1) were also determined in the current study and reached 25 µg/mL or exceeded the tested range of AgNP concentrations.

**Table 1.** Minimum inhibitory and minimum fungicidal concentration (MIC and MFC, respectively, µg/mL) values of mycogenic AgNPs against selected fungal strains.

| | **MIC** | | | | **MFC** | | | |
|---|---|---|---|---|---|---|---|---|
| | **28 ns** | **28 s** | **4 ns** | **4 s** | **28 ns** | **28 s** | **4 ns** | **4 s** |
| *A. fumigatus* ATCC 204305 | 1.56 | 1.56 | 3.125 | 3.125 | >25 | >25 | >25 | >25 |
| *A. flavus* ATCC 9643 | 3.125 | 3.125 | 6.25 | 12.5 | >25 | >25 | >25 | >25 |
| *C. albicans* ATCC 10231 | 1.56 | 1.56 | 1.56 | 3.125 | >25 | >25 | >25 | 25 |
| *M. furfur* DSM 6170 | 0.39 | 0.39 | 0.39 | 0.39 | >25 | >25 | >25 | >25 |

### 2.2. Changes in the Phospholipid Profiles of Fungal Cells in the Presence of Mycogenic AgNPs

To further characterize the antifungal activity of mycogenic AgNPs, changes in the phospholipid profiles of *C. albicans* and *A. flavus* were evaluated. To accomplish this task, the following experimental variations were selected based on the AgNP antifungal activity determined in the previous experiments—28 ns AgNPs and 4 s AgNPs at a concentration of 0.19 µg/mL for *C. albicans* and 1.56 µg/mL for *A. flavus*. As a result, three phospholipid classes were detected in each of the tested fungal strains—phosphatidylcholines (PCs), phosphatidylethanolamines (PEs) and phosphatidylinositols (PIs). In *C. albicans* (Figure 2B), visible changes were noted in the content of PCs and PEs. The levels of PCs increased and those of PEs slightly decreased in the presence of both tested AgNP types compared to the growth control. The PI content was barely affected by the AgNPs. In the case of *A. flavus* (Figure 2A), changes in the content of the same phospholipid classes were observed. The presence of 28 ns and 4 s AgNPs caused about a two-fold increase in the content of PCs compared to the growth control. In parallel, a significant decrease in PEs was observed—the content of this phospholipid class was reduced by more than 50%. The content of PIs was only slightly influenced by the tested AgNPs.

The PC/PE ratios and Double Bond Indexes (DBIs) were calculated and are shown in Table 2. In both *A. flavus* and *C. albicans*, the PC/PE ratio increased in the presence of the tested AgNPs, and 4 s AgNPs had a slightly stronger effect. Changes in the DBI induced by the presence of AgNPs were observed only in the case of *A. flavus*, in which the DBI increased. In the case of *C. albicans*, no changes were detected.

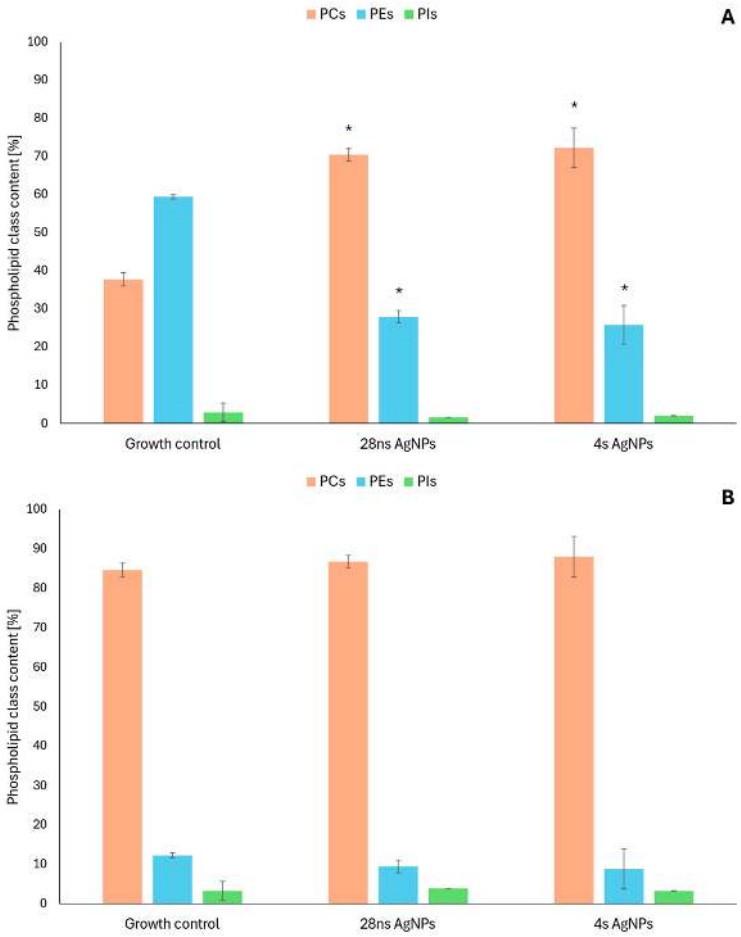


**Figure 2.** Changes in phospholipid content in the presence of 28 ns AgNPs and 4 s AgNPs: *A. flavus* (**A**) and *C. albicans* (**B**). The results are shown as average values with standard deviations. The statistical significance ($p < 0.05$) is shown by an asterisk (*).

**Table 2.** PC/PE ratios and DBIs established for *A. flavus* and *C. albicans* with and without treatment with 28 ns AgNPs and 4 s AgNPs.

| | ***A. flavus* ATCC 9643** | | | ***C. albicans* ATCC 10231** | | |
|---|---|---|---|---|---|---|
| | **Growth Control** | **28 ns AgNPs** | **4 s AgNPs** | **Growth Control** | **28 ns AgNPs** | **4 s AgNPs** |
| PC/PE | 0.64 | 2.52 | 2.87 | 6.92 | 9.24 | 10.09 |
| DBI | 0.95 | 1.20 | 1.17 | 1.08 | 1.10 | 1.07 |

Specific results for certain phospholipid species detected in this study are shown in Tables S1 and S2 in the Supplementary Materials. In *C. albicans*, the most visible changes were observed in PC 16:0 18:2, PC 16:0 18:1 PC 18:3 18:2, PC 18:3 18:1, PC 18:0 18:2, PE 16:0 18:2 and PE 16:0 18:1. The contents of the mentioned PC and PE species decreased in the presence of the tested mycogenic AgNPs. In *A. flavus*, the most visible changes were detected in PC 16:0 18:2, PC 16:0 18:1, PC 18:2 18:2, PC 18:2 18:1, PC 18:1 18:1, PC 18:0 18:2, PE 16:0 18:2, PE 16:0 18:1, PE 18:2 18:1 and PE 18:1 18:1. Here, the content of PC and PE species with one saturated 16C acyl chain decreased in the presence of both tested AgNP types, by as much as 4 times in the case of PE 16:0 18:2 compared to the growth control. The levels of PCs and PEs with two unsaturated acyl chains increased in the same conditions, and the most significant change was observed in the case of PC 18:2 18:1 and PC 18:1 18:1, with a 6-fold or higher increase in the content of certain phospholipid species compared to the untreated samples.

### 2.3. Changes in the Cell Membrane Properties of C. albicans in the Presence of Mycogenic AgNPs

#### 2.3.1. Cell Membrane Fluidity

Changes in the cell membrane fluidity of *C. albicans* were determined in the presence of 28 ns and 4 s AgNPs at a concentration of 0.19 µg/mL using the Laurdan fluorescence spectroscopy method. The calculated generalized polarization (GP) parameter of Laurdan (Figure 3) confirmed that the presence of both tested AgNP types caused an increase in *C. albicans* cell membrane fluidity compared to the growth control. Moreover, it was observed that 4 s AgNPs at the tested concentration had a more severe effect.

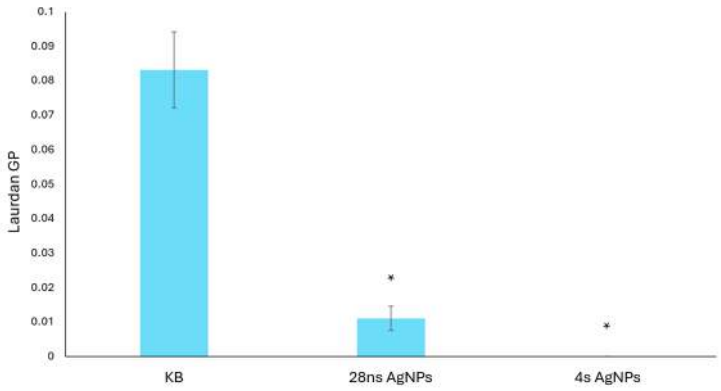


**Figure 3.** Changes in the cell membrane fluidity of *C. albicans* caused by the presence of AgNPs. The results are shown as average values with standard deviations of the calculated Laurdan GP parameter. The statistical significance ($p < 0.05$) is shown by an asterisk (*).

#### 2.3.2. Cell Membrane Permeability

Confocal microscopy imaging of untreated *C. albicans* cells and those treated with 0.19 µg/mL 28 ns AgNPs was performed using propidium iodide staining. The analysis of the obtained images (Figure 4) revealed that the presence of the tested AgNP type caused a reduction in the growth and number of *C. albicans* cells compared to the untreated sample. Moreover, about 30% of the cells treated with AgNPs showed an increase in cell membrane permeability.

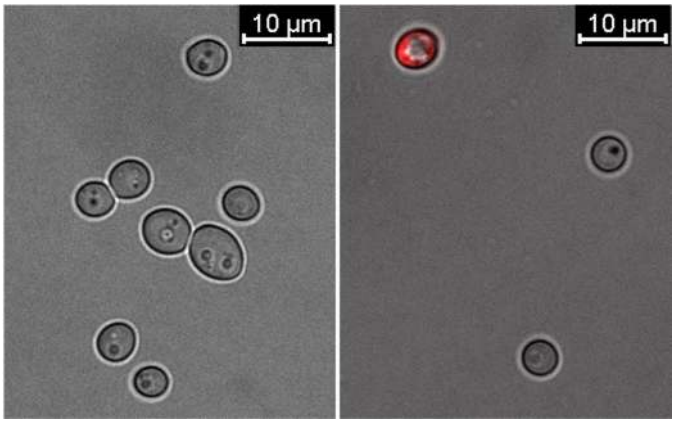


**Figure 4.** Changes in the cell membrane permeability of *C. albicans* in the presence of 0.19 µg/mL 28 ns AgNPs. The left image shows biotic control cells not treated with AgNPs. The right image shows cells treated with 28 ns AgNPs.

## 3. Discussion

Filamentous fungi have emerged as attractive candidates for AgNP synthesis. This is mostly due to their ease of cultivation, high metal tolerance and ability to secrete large

amounts of extracellular agents that play a key role in silver ion reduction [32]. Fungi-derived AgNPs are proven to possess antibacterial properties. For instance, AgNPs synthesized using the endophytic fungus *Penicillium radiatolobatum* showed concentration-dependent activity against Gram-positive and Gram-negative bacterial strains [33]. In our previous study, AgNPs synthesized using *G. striatum* DSM 9592 strains in different synthesis conditions were found to be active against various bacterial strains [18]. Our current results prove that all obtained AgNP types also exhibit antifungal potential.

The antifungal activity of the synthesized AgNPs was tested against four fungal strains: *C. albicans*, *M. furfur*, *A. flavus* and *A. fumigatus*. The effectiveness of the AgNPs varied depending on the synthesis conditions. As stated previously, changes in the temperature and shaking conditions during the production process determined the physicochemical properties of the obtained AgNP types [18]. It is known that the antifungal performance of AgNPs is highly dependent on their characteristics, e.g., particle size [34]. Here, both types of AgNPs synthesized at 28 °C were more effective against almost all tested fungal strains compared to the other nanoparticles. Generally, yeast strains were more sensitive to the action of AgNPs compared to the filamentous fungal strains tested, and *C. albicans* was found to be more resistant than the other yeast strains, with 4-fold or 8-fold higher MIC values depending on the AgNP type. Barabadi et al. found that AgNPs synthesized using *Penicillium fimorum* were effective against *C. albicans* ATCC 10231, reaching a MIC value of 4 µg/mL [34], which indicated that *G. striatum*-derived AgNPs were more effective against that strain. A similar conclusion can be made for *M. furfur* investigated in another study. Sathishkumar et al. showed that biogenic AgNPs of plant origin were active against the microorganism, with a calculated MIC value of 25 µg/mL [35]. Compared to these results, the mycogenic AgNPs synthesized in our study were up to 64 times more active against *M. furfur* than those originating from plants. Among filamentous fungi, *A. flavus* was less sensitive to the action of all obtained AgNPs compared to the second tested strain. The opposite phenomenon was observed for AgNPs of bacterial origin synthesized using *Bacillus thuringiensis*. Here, *A. fumigatus* was observed to be more resistant to the action of AgNPs—the MIC value of the AgNPs against this strain was 62.5 µg/mL, while in the case of *A. flavus*, it reached 15.6 µg/mL [36]. Both values were higher than those revealed in our study, again indicating that the obtained AgNPs have stronger activity against the tested fungal strains. Our findings regarding the antifungal activity of mycogenic AgNPs are especially important in light of the results of a cytotoxic potential assessment of *G. striatum*-derived AgNPs obtained in our previous study [18]. According to these results, the obtained AgNPs are active against some of the tested fungal species at concentrations that caused less than a 50% decrease in the viability of a human fibroblast cell line. It can thus be concluded that AgNPs synthesized using *G. striatum* are active against the chosen pathogenic strains and simultaneously have no severe cytotoxic effects.

The MIC and MFC values obtained in our study suggest that *G. striatum*-derived AgNPs exhibited fungistatic rather than fungicidal properties. In another study, AgNPs obtained using green nanotechnology employing *Aspergillus terreus* acted similarly against *Aspergillus niger* strain. Here, the MFC value were 10 µg/mL while the obtained MIC value for this strain reached 0.312 µg/mL [37]. The same phenomenon was observed using plant-derived AgNPs and *C. albicans*, where the MFC values were at least 2-fold higher than the MICs obtained for nanoparticle types varying in size and reached 124.33 ± 4.04 µg/mL or more [38]. On the other hand, Różalska et al. found that AgNPs synthesized using *Metharizium robertsii* waste biomass showed stronger fungicidal activity against *C. albicans* ATCC 10231, with obtained MIC and MFC values of 1.56 µg/mL [39]. Our study indicates that, in terms of the calculated MIC values, all tested types of *G. striatum*-derived AgNPs were effective against *M. furfur*. Another study focusing on the efficacy of chemically

synthesized AgNPs against *M. furfur* isolates showed that they had almost three-fold lower fungistatic activity against the tested strain compared to those tested in our study. However, the MFC values obtained in that study ranged between 0.5 and 2 mg/L, indicating that chemically produced AgNPs were more effective [40]. *M. furfur* is a lipid-dependent species, lacking the ability to perform de novo synthesis of fatty acids [41]. This fact could affect its ability to adapt its cell membrane properties to an inhospitable growth environment caused by the presence of AgNPs and might explain the increased susceptibility of this strain to the action of AgNPs compared to the other fungal species tested.

The antifungal potential of AgNPs has been attributed to their interference with fungal cell membrane integrity [42,43]. Phospholipids (PLs) constitute important structural and functional components of cell membranes. The exact composition of PLs can determine cell membrane properties, such as stability and fluidity. Thus, changes in the PL content can be considered a major indicator of perturbations to cell membrane functioning [44]. In our study, three PL classes were detected in both tested fungal species: PCs, PEs and PIs. It has been established that, in fungi, PCs and PEs are critical for the vegetative growth of mycelia [45]. PCs contribute to the formation and stabilization of the bilayer cell membrane form, while PEs are non-bilayer lipids forming single-layer phases, thus destabilizing the membrane structure [46]. In our study, an increase in the PC/PE ratio was observed in both tested microorganisms treated with AgNPs compared to the biotic control. This may indicate that the abiotic stress caused by the presence of AgNPs triggers phospholipid composition changes in the cell membrane, thus increasing its fluidity. One of the indicators that can be useful for evaluating changes in cell membrane properties is the Double Bond Index (DBI), which provides insights into membrane fluidity [47]. Generally, the occurrence of saturated fatty acids in PLs, consequently leading to the formation of straight acyl chains, decreases membrane fluidity due to the tight packing of these PLs in the membrane layers [48]. In our study, the DBI established for *A. flavus* increased in the presence of both types of AgNPs. It can be assumed that the presence of AgNPs altered the properties of the fungal cell membrane of *A. flavus*, increasing its fluidity. On the other hand, no differences in DBIs were obtained for untreated *C. albicans* cells or those treated with either of the tested AgNP types. However, the results of Laurdan fluorescence spectroscopy showed enhanced cell membrane fluidity in *C. albicans* cells in the presence of AgNPs, and the obtained effect was stronger when using 4 s AgNPs. The phenomenon was additionally confirmed by propidium iodide staining, where *C. albicans* cells showed increased cell membrane permeability in the presence of AgNPs. It cannot be excluded that AgNPs harm cells through multiple pathways, including a reduction in PE content, which was observed in *Pleurotus ostreatus* mycelia treated with cadmium ions [49]. Although the increase in the PC/PE ratio may suggest a more organized cell membrane state, it cannot be ruled out that other important components of the fungal cell membrane—sterols, sphingolipids and membrane proteins—contribute to the increased membrane fluidity observed. It is believed, based on many studies involving different species of yeast, that ergosterol plays a key role in regulating fungal membrane fluidity. Therefore, many antifungal drugs, including azoles, target this sterol [50].

Based on the obtained data, it can be concluded that AgNPs affect the structure of microbial biological membranes. However, further studies are required to explain the exact processes taking place.

## 4. Materials and Methods

### 4.1. Materials

The *C. albicans*, *A. flavus* and *A. fumigatus* strains were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). The *M. furfur* strain was acquired

from the German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). RPMI 1640 broth was purchased from Merck (Darmstadt, Germany). Sabouraud dextrose broth was obtained from Becton Dickinson (Warsaw, Poland). Glycerol and methanol came from Chempur (Piekary Slaskie, Poland). Chloroform and methanol of chromatographic purity, as well as propidium iodide and Laurdan stains, were obtained from Merck (Darmstadt, Germany).

AgNP Synthesis Using *Gloeophyllum striatum* DSM 9592

The AgNPs of mycological origin tested in this study were obtained using *Gloeophyllum striatum* DSM 9592 post-culture liquid; the synthesis process has been described previously. Four different nanoparticle variants were synthesized by using different temperatures and shaking conditions during the process, namely, 28 °C without shaking (28 ns AgNPs), 28 °C with shaking (28 s AgNPs), 4 °C without shaking (4 ns AgNPs) and 4 °C with shaking (4 s AgNPs). The obtained AgNPs were then separated from the *G. striatum* post-culture liquid by centrifugation and dispersed in sterile deionized water. Then, the synthesized AgNPs were analyzed in order to evaluate differences in their physicochemical properties depending on the synthesis scheme. All the synthesized AgNP types were confirmed to be polydispersed, and those produced without shaking were smaller than the other types when comparing the diameter sizes of the most numerous nanoparticle fractions. Moreover, other nanoparticle properties also differed, such as zeta-potential, which was lower in both nanoparticle types synthesized at 4 °C compared to those synthesized at 28 °C [18]. The prepared AgNPs were then used in further experiments.

### *4.2. Methods*

#### 4.2.1. Evaluation of the Antifungal Activity of Mycogenic AgNPs

The antifungal potential of *G. striatum*-derived AgNPs was tested using the microdilution method in accordance with the Clinical and Standard Laboratory Institute (CSLI) guidelines M27 (4th Edition) for yeast strains and M38 (3rd Edition) for filamentous fungal strains. The antifungal activity was evaluated against four fungal strains, namely, *Candida albicans* ATCC 10231, *Malassezia furfur* DSM 6170, *Aspergillus flavus* ATCC 9643 and *Aspergillus fumigatus* ATCC 204305. The evaluation was performed in 96-well cell culture plates using different liquid media—RPMI 1640 broth for *C. albicans*, RPMI 1640 broth supplemented with glycerol for *M. furfur* and Sabouraud dextrose broth for filamentous fungal strains. The tested AgNPs were diluted in appropriate media to reach the final concentration range of 0.098–25 μg/mL. The inocula of the tested microorganisms were prepared in appropriate media, reaching a final density of $2.5 \times 10^5$ spores/mL for filamentous fungi and $1 \times 10^5$ colony-forming units (CFU)/mL for yeasts. Then, 96-well plates containing the samples and accordingly prepared abiotic and biotic controls were incubated for 48 h at 37 °C. After incubation, the optical density (OD) was measured at a wavelength of 630 nm using a Multiscan$^{TM}$ FC Microplate Photometer (ThermoFisher Scientific, Pudong, Shanghai, China), and MIC values were determined for all experimental variants, with MIC defined as the lowest concentration of AgNPs at which no microorganism growth was observed in the plate wells. Then, the ZT plates were inoculated with 100 μL of fungal suspension samples with AgNP concentrations equal to the MIC value or higher in order to determine MFC values. The plates were then incubated for 48 h at 37 °C. MFC values were defined as the lowest concentration of AgNPs that reduced the viability of the tested fungal strains to zero, with no growth of colonies observed. Both MIC and MFC values were expressed in μg/mL.

#### 4.2.2. Changes in the Phospholipid Profiles of Fungal Cells in the Presence of Mycogenic AgNPs

Changes in the phospholipid profiles were analyzed in selected experimental variants based on the obtained results of antifungal activity. Specifically, 28 ns AgNPs and 4 s AgNPs were selected, and the evaluation was performed using two fungal strains: *A. flavus* and *C. albicans*. The tested concentrations of AgNPs varied depending on the tested strain and were 1.56 µg/mL and 0.19 µg/mL, respectively. Fungal cultures, including biotic and abiotic controls, were cultivated in the conditions described in Section 4.2.1. in a volume of 20 mL per sample. After incubation, the samples were centrifuged at 20 °C/5 min/8000 rpm. Then, supernatants were removed, and 8 mL of methanol was added to each sample. The prepared samples were homogenized using the ultrasound method. After homogenization, 16 mL of chloroform of chromatographic purity and 1 mL of 0.85% NaCl were added to each sample for the extraction process. Next, the organic phases were collected, evaporated and dissolved in methanol of chromatographic purity. The samples were then subjected to analysis.

The phospholipid analysis procedure was conducted as in Bernat et al. using an ExionLC AC UHPLC system (Sciex, Framingham, Massachusetts, USA) and a 4500 Q-TRAP mass spectrometer (Sciex, Framingham, Massachusetts, USA) with an ESI source at a spray voltage of −4.500 V and a temperature of 600 °C. A 10 µL volume of the lipid extract was injected onto a Kinetex C18 column (50 mm × 2.1 mm, particle size: 5 µm; Phenomenex, Torrance, California, USA) with a flow rate of 500 µL $min^{-1}$ at 40 °C. The mobile phases, water (A) and methanol (B), contained 5 mM ammonium formate. The solvent gradient was initiated at 70% B, increased to 95% B over 1.25 min, and maintained at 95% B for 5 min before returning to the initial solvent composition over 2 min [51].

#### 4.2.3. Changes in *C. albicans* Cell Membrane Fluidity in the Presence of Mycogenic AgNPs

Changes in *C. albicans* cell membrane fluidity in the presence of mycogenic AgNPs were investigated with the use of the Laurdan fluorescence spectroscopy method. To accomplish this, *C. albicans* cells were cultivated in Sabouraud dextrose broth with and without 28 ns AgNPs and 4 ns AgNPs at a concentration of 0.19 µg/mL to reach an OD at a wavelength of 630 nm equal to 0.5. Next, suspensions of *C. albicans* cells cultivated with or without the supplementation of nanoparticles were transferred to different 2 mL reaction tubes. The Laurdan stain (6-Dodecanoyl-2-Dimethylaminonaphthalene) was then added to each tube to reach a final concentration of 10 µM. The prepared samples were incubated in the dark at 37 °C for 5 min. After incubation, the cells were washed four times with PBS and again adjusted to an $OD_{630}$ of 0.5 in a volume of 2 mL. Then, 500 µL was transferred from each sample to a new reaction sample and centrifuged to obtain the supernatant serving as Laurdan background fluorescence. Then, 150 µL of each cell suspension sample and fluorescence background sample were transferred to a black flat-bottom 96-well microtiter plate. Laurdan fluorescence was measured using a SpectraMax i3 Multimode Microplate Reader (Molecular Devices, San Jose, California, USA) at an excitation of 350 nm and two emission wavelengths: 435 nm and 500 nm. The results are shown as the values of Laurdan generalized polarization (*GP*) parameters calculated according to the following formula:

$$GP = \frac{I_{435} - I_{500}}{I_{435} + I_{500}}$$

#### 4.2.4. Confocal Microscopy Analysis

*C. albicans* cells were cultivated as described in Section 4.2.1. with and without the addition of 0.19 µg/mL 28 ns AgNPs. Then, 1600 µL of each treated or untreated cell suspension was transferred to a new reaction tube, washed three times using PBS and

stained with 3 mM propidium iodide. The samples were incubated in the dark for 15 min at room temperature. After incubation, the samples were again washed three times with PBS. Finally, 20 µL of each cell suspension was transferred to a microscopic slide and analyzed using a Leica TCS SP8 microscope equipped with plan achromatic objectives (Leica, Nußloch, Germany) with a magnification of 100× (oil immersion).

#### 4.2.5. Statistical Analysis

Every experiment presented in this study, excluding the confocal microscopy analysis, was performed in four replicates ($n$ = 4). The results of the experiments were analyzed using a one-way ANOVA test with * $p < 0.05$ in order to estimate the statistical significance. The estimations and calculations were carried out by using Excel, Microsoft® Office 2021 (Microsoft Corporation, Redmont, WA, USA). The results shown in the tables and figures are expressed as average values with standard deviations (SDs).

## 5. Conclusions

Our study revealed that AgNPs synthesized extracellularly using the post-culture liquid of *G. striatum* DSM 9592 have fungistatic potential. Among the tested fungal strains, *M. furfur* was found to be the most susceptible to the action of AgNPs. Changes in the phospholipid profiles of *C. albicans* and *A. flavus* caused by the presence of AgNPs were also analyzed in order to deduce a possible mechanism of nanoparticle action. It was found that in both tested species, the presence of AgNPs caused an increase in the PC/PE ratio. Based on the obtained DBIs, in *A. flavus*, the presence of AgNPs caused an increase in the content of unsaturated fatty acids, which may have been associated with enhanced cell membrane fluidity in response to AgNP treatment. In *C. albicans*, no changes in the DBI were observed. However, the results of Laurdan spectroscopy and confocal microscopy analyses confirmed that the cell membrane fluidity of *C. albicans* also increased in the presence of AgNPs. This finding may suggest that the properties of the cell membrane can be adjusted not only by changes in fatty acid saturation but also by other alterations in the membrane composition. Simultaneously, the versatility of mycogenic AgNPs originating from the brown-rot decay fungus was confirmed.

**Supplementary Materials:** The following supporting information can be downloaded: https://www.mdpi.com/article/10.3390/ijms26146639/s1. Table S1: The influence of 28 ns and 4 s AgNPs at a concentration of 1.56 µg/mL on the phospholipid profile of *A. flavus* ATCC 9643. The asterisk * ($p < 0.05$) indicates values that differ significantly from the control. Table S2: The influence of 28 ns and 4 s AgNPs at a concentration of 0.19 µg/mL on the phospholipid profile of *C. albicans* ATCC 10231. The asterisk * ($p < 0.05$) indicates values that differ significantly from the control.

**Author Contributions:** Conceptualization, A.T. and K.N.; methodology, K.N., P.B., A.T. and M.N.-L.; investigation, A.T., K.N., M.N.-L. and P.B.; manuscript—original draft preparation, A.T.; manuscript—review and editing, K.N., P.B. and K.L. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the University of Lodz, Department of Industrial Microbiology and Biotechnology internal funds (B2411000000036.01).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The raw data supporting the conclusions of this article will be made available by the authors on request.

**Acknowledgments:** Special thanks to the staff of the Laboratory of Microscopic Imaging and Specialized Biological Techniques, Faculty of Biology and Environmental Protection, University of Lodz for the help with confocal microscopy imaging.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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Publikacja 3

## *Ecotoxic effect of mycogenic silver nanoparticles in water and soil environment*

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2025, Scientific Reports, 15: 10815; DOI: 10.1038/s41598-025-95485-x

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# Ecotoxic effect of mycogenic silver nanoparticles in water and soil environment

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**Silver nanoparticles (AgNPs) are one of the most widely used nanomaterials due to their antimicrobial properties. Among the AgNPs synthesis methods, the biological route has become preferable because of its efficiency and eco-friendly character. Filamentous fungi can be successfully used in biosynthesis of AgNPs. The extensive application of AgNPs and their ever increasing production raise concerns about their environmental safety. AgNPs can be released during manufacturing processes or by leaching from AgNPs-supplemented products, and then enter the natural environment. Water and soil ecosystems are most exposed to the AgNPs presence. The present study aimed at evaluating the ecotoxicological potential of AgNPs derived from *Gloeophyllum striatum* fungus. The assessment was performed using organisms from water and soil ecosystems. Our results suggest that the presence of AgNPs can threaten the organisms inhabiting exposed ecosystems and the adverse effects of AgNPs differ depending on the organism species. Freshwater crustacean *Daphnia magna* was found to be the most sensitive among the tested species with $EC_{50}$ values ranging 0.026–0.027 µg/mL after 48 h exposure. Crop plants were the least affected by the presence of AgNPs with $EC_{50}$ values above tested AgNPs concentration range. Moreover, it was noted that ecotoxicological potential varied depending on the AgNPs synthesis scheme and these differences were the most visible in the case of *S. polyrhiza*.**

**Keywords** Silver nanoparticles, Biosynthesis, Mycogenic, Ecotoxicity, Soil ecosystems, Water ecosystems

Nanomaterials have gained broad utility due to their unique properties[1]. Among them, silver nanoparticles (AgNPs) seem to be the most recognizable, as they constitute 25% of all nanoparticles used in commercial products[2]. The antimicrobial potential of silver itself has been known since ancient times[3] and such a widespread use of AgNPs is mostly due to this property. They are exploited in various areas such as medicine, biotechnology, personal care and cosmetics, textile or food industry[1,4]. Moreover, AgNPs exhibit some physicochemical qualities making them useful in technical fields, e.g. engineering, microelectronics or environmental remediation[1,3].

The world annual production of AgNPs is expected to exceed 800 tons by the year 2025[5]. The extensive use of products containing AgNPs has led to the increased presence of these nanoparticles in the natural environment[6]. AgNPs can be accidentally released during the production process, distribution, use and disposal of the product. The most exposed ecosystems are water and terrestrial environments, where AgNPs are transferred mostly by wastewater and sewage sludge used in agriculture as fertilizer[2,3]. The presence of AgNPs in the environment raises concerns regarding their toxicity towards organisms that inhabit endangered ecosystems. Moreover, there is a risk of AgNPs entering parts of the food chain such as plants and animals, which can pose a serious threat to the human health. The knowledge of AgNPs toxicity mechanisms as well as short- and long-term exposure effects is scarce[1,2]. Therefore, research concerning the environmental impact of AgNPs is needed to enable designing efficient and safe antimicrobial agents.

AgNPs can be synthesized by three methods: chemical, physical and biological, also known as green synthesis. The last one is considered to be more environmentally friendly compared to the others, mostly because of the minimal toxic chemical use and reduced toxic waste emission[7,8]. Therefore, biological synthesis is preferable in terms of environmental protection. Unfortunately, AgNPs in general are known to impact some ecological processes, for example primary productivity, decomposition or nitrogen cycling. The presence of AgNPs in the soil can lead to the decrease of the quantity and activity of soil microbiota. AgNPs are also proved to be able to accumulate in plant tissues[9,10]. So, despite the green synthesis of AgNPs being an eco-friendly method, bio-manufactured nanoparticles can still pose a threat to the natural environment and their

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potential ecotoxic effect remains unknown[11]. In the case of biogenic AgNPs, the Among the organisms used in the biological method of AgNPs synthesis, filamentous fungi seem to be the most advantageous, as they are easy to cultivate, grow fast and secrete high amounts of extracellular compounds, serving as reducing and stabilizing agents. Moreover, filamentous fungi show high metal tolerance and the ability of metal bioaccumulation[12–14]. Various fungal species, such as *Fusarium*, *Aspergillus*, *Trichoderma*, *Cladosporium*, *Alternaria*, *Phytophora*, *Metarhizium*, *Beauveria*, *Isaria*, *Trametes*, *Phanerochaete*, *Ganoderma* and *Gloeophyllum* have been proven to successfully produce AgNPs with antimicrobial properties[13,15–21]. Wood decay fungi are especially worthy of extended attention in the terms of AgNPs synthesis due to their enzymatic abilities. Unlike white rot fungi, the information about brown rot fungal species used in AgNPs production are scarce. However, it was reported that *Gloeophyllum striatum* can perform the silver nitrate reduction[19]. Therefore, *G. striatum* DSM 9592 was chosen to perform the AgNPs synthesis.

In our previous study, the AgNPs production using *G. striatum* DSM 9592 was conducted in four different synthesis conditions – at 28 °C with shaking, 28 °C without shaking, 4 °C with shaking and 4 °C without shaking. Then, the antimicrobial potential of obtained nanoparticles was evaluated. It was found that all AgNPs were active against Gram-positive and Gram-negative bacterial strains and their potential varied depending on the synthesis scheme. Although no clear tendency of one synthesis scheme to be better than others was observed, it was noted that AgNPs synthesized at 4 °C without shaking showed the best efficacy towards *Pseudomonas aeruginosa* reaching the lowest from all established minimal inhibitory concentration (MIC) values[22]. Proven antimicrobial potential of obtained AgNPs and the green method of their synthesis make *G. striatum*-derived AgNPs good candidates to be used on the industrial scale. Therefore, there is an urgent need for further examination of their potential adverse effects on the natural environment.

This paper presents research aiming in a comprehensive investigation of the ecotoxicity of mycogenic AgNPs. The study focused on the assessment of AgNPs toxicity towards organisms from different ecosystems and different trophic levels, shedding a light on their potentially negative impact in a broad ecological context. The tested organisms included soil bacteria *Pseudomonas putida* and *Pseudomonas moorei*, soil fungi *Trichoderma virens* and *Trichoderma reesei*, water bacteria *Aliivibrio fischeri*, water crustaceans *Artemia franciscana* and *Daphnia magna* and plants: *Spirodela polyrhiza*, *Sorgho saccharatum*, *Lepidium sativum* and *Sinapis alba*. All the tested species are representative for their environments and the changes of their viability can be indicative for adverse effect of xenobiotics. Some of them are used in the evaluation of acute toxicity as a standard organisms. Therefore, the use of mentioned species in the evaluation of AgNPs ecotoxic potential is highly relevant. To our best knowledge, such broad evaluation of ecotoxicity including organisms from different ecosystems and trophic levels regarding AgNPs originated from brown root fungi was performed for the first time. Moreover, our research shed a light on changes in AgNPs toxic effect depending on the synthesis conditions.

## Results

### The assessment of AgNPs activity towards soil microorganisms

The evaluation of the *G. striatum*-derived AgNPs activity against soil bacterial (Fig. 1) and fungal (Fig. 2) strains proved that bacterial strains were more susceptible to AgNPs action than fungal strains. The minimal inhibitory concentration ($MIC_{95}$) for both tested bacterial strains reached 1.56 µg/mL. However, it was shown that *P. moorei* was more sensitive to 4ns AgNPs action at the concentration 0.78 µg/mL compared to the other bacterial strain tested.

Soil fungi occurred to be more resistant to AgNPs activity and the MIC values were higher than the tested concentration range. However, slight alternations of fungal growth in the presence of AgNPs were noticed. In the case of *T. virens*, all tested AgNPs caused up to 30% growth inhibition in concentrations 0.098–12.5 µg/mL. In the concentration of 25 µg/mL, the growth inhibition caused by 28ns AgNPs was even higher, reaching about 50%. *T. virens* was more tolerant to the presence of AgNPs in the growth environment. Only 28ns AgNPs and 4ns AgNPs had a negative influence causing about 20% growth inhibition in the concentration of 12.5 µg/mL. 50% growth inhibition was caused by 25 µg/mL 4ns AgNPs.

### AgNPs toxicity towards water organisms

*Aliivibrio fischeri*

The toxic effect of AgNPs on *A. fischeri* (Table 1) was established based on the changes in the bioluminescence intensity. It was proven that after 30 min of exposition, all types of AgNPs were active towards the tested bacterial strain with the $EC_{50}$ values of 8.191, 7.855, 7.052 and 1.096 µg/mL for 28ns AgNPs, 28s AgNPs, 4ns AgNPs and 4s AgNPs, respectively. Both nanoparticle types synthesized at 4 °C showed a stronger inhibitory effect on bioluminescence than the other analyzed AgNPs. It occurred that 28ns AgNPs were the least active against *A. fischeri* after the full time of incubation. The most severe effect on *A. fischeri* was caused by 4s AgNPs.

*Water crustaceans*

The ecotoxicity of mycogenic AgNPs towards water crustaceans (Table 2) was established with the use of freshwater *D. magna* and saline water *A. franciscana* (Fig. 3). The obtained results showed that *D. magna* was more susceptible to the presence of all tested AgNPs types in the growth environment compared to *A. franciscana*. After 24 h of incubation, the $EC_{50}$ values established for *D. magna* were 0.029, 0.028, 0.042 and 0.029 µg/mL for 28ns AgNPs, 28s AgNPs, 4ns AgNPs and 4s AgNPs, respectively. After 48 h of incubation, the $EC_{50}$ were 0.026, 0.027, 0.027 and 0.026 µg/mL in the order above. There were no significant differences in the toxic effect of AgNPs tested on *D. magna*. In *A. franciscana* the $EC_{50}$ values were higher: 67.112, 58.440, 56.584, 61.736 µg/mL after 24 h of incubation and 26.162, 10.965, 23.945, 19.867 µg/mL after 48 h of incubation for 28ns AgNPs, 28s AgNPs, 4ns AgNPs and 4s AgNPs, respectively. Here, the tendency of AgNPs synthesized with shaking to be more toxic towards *A. franciscana* was observed and the most severe effect was caused by 28s AgNPs.

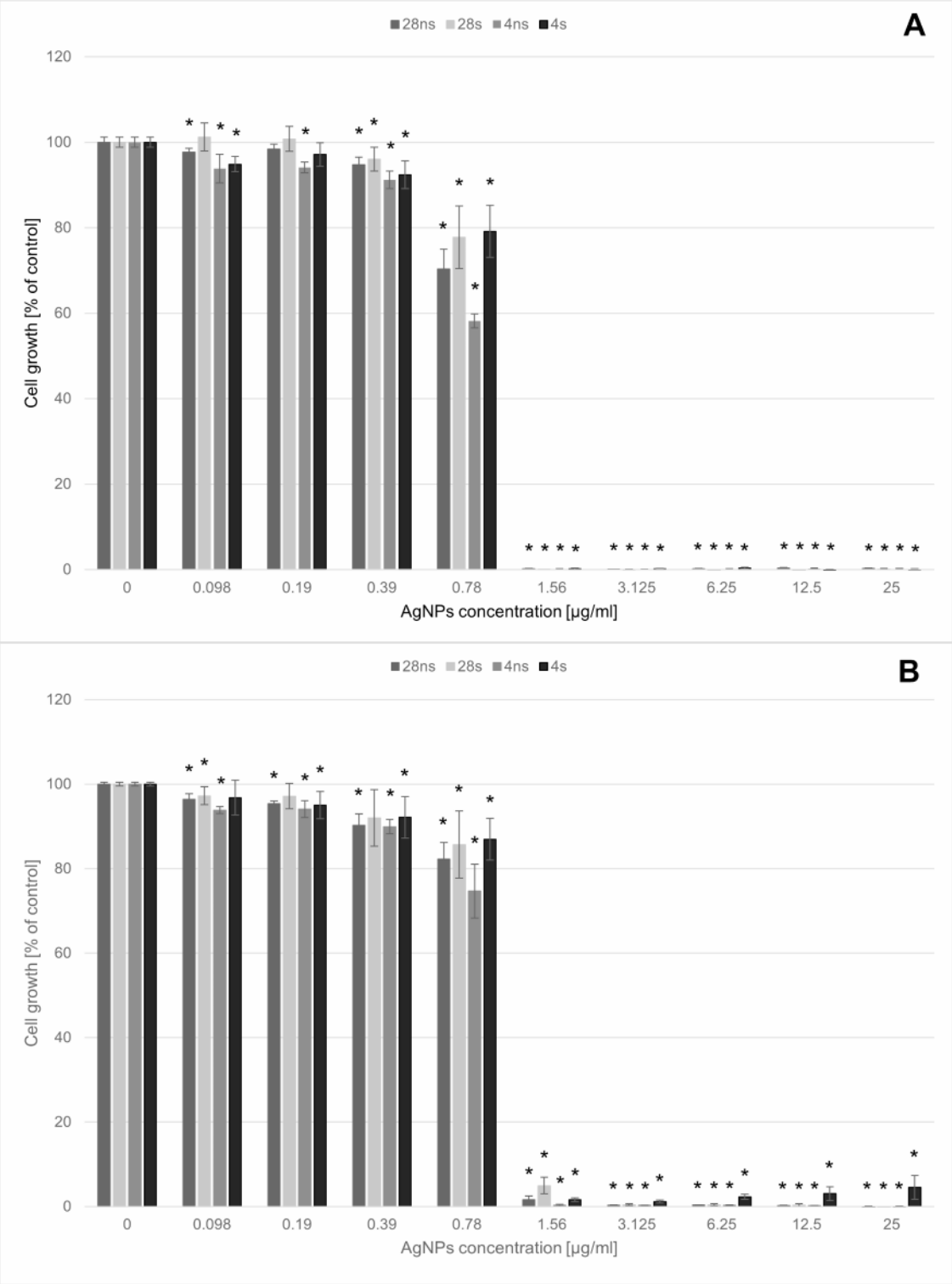


**Fig. 1**. The mycogenic AgNPs activity against soil bacteria strains. (**A**) *Pseudomonas moorei* DSM 12,647, (**B**) *Pseudomonas putida* DSM 291. The results are shown as average percent values with standard deviations of optical density (OD) of the biotic control. The statistical significance was estimated using a one-way analysis of variance (ANOVA) test with * $p < 0.05$ and is shown by an asterisk.

## AgNPs toxicity towards plants

*Spirodela polyrhiza*

The evaluation of the toxicity of. *G. striatum*-derived AgNPs towards *S. polyrhiza* (Fig. 4) indicated that all types of AgNPs affected the growth of the plant. After 72 h of incubation the $EC_{50}$ values (Table 3) established for 28ns,

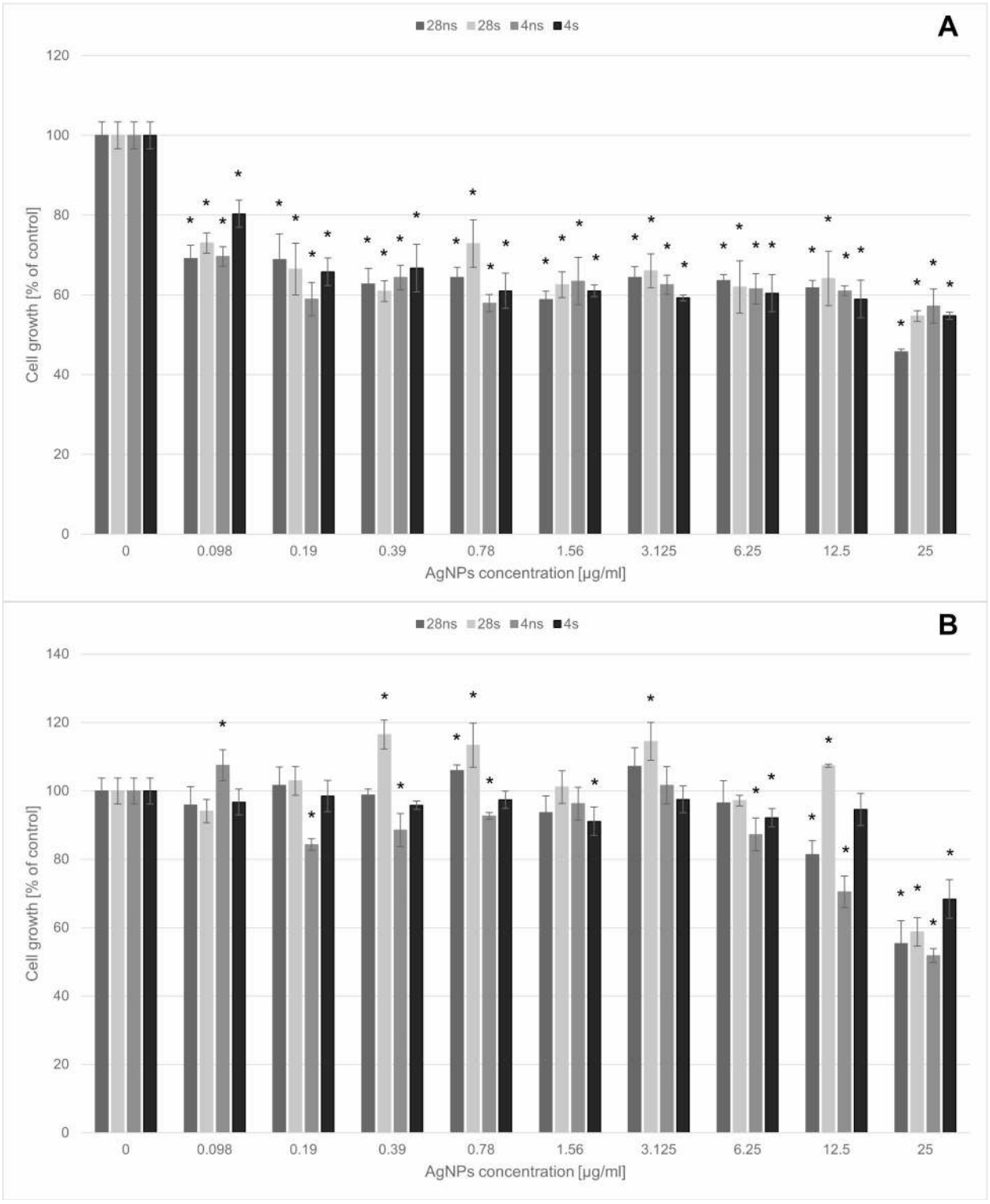


**Fig. 2**. The mycogenic AgNPs activity against soil fungal strains. (**A**) *Trichoderma reesi* QM 9414, (**B**) *Trichoderma virens* DSM 1963. The results are shown as average percent values with standard deviations of optical density (OD) of the biotic control. The statistical significance was estimated using a one-way analysis of variance (ANOVA) test with * $p < 0.05$ and is shown by an asterisk.

| | 28ns | 28s | 4ns | 4s |
|---|---|---|---|---|
| 0 min | 15.093 | 11.584 | 15.801 | 12.057 |
| 5 min | 11.638 | 9.544 | 11.191 | 9.507 |
| 15 min | 9.326 | 8.721 | 8.105 | 7.709 |
| 30 min | 8.191 | 7.855 | 7.052 | 7.096 |

**Table 1**. The toxic effect of AgNPs on water bacterium *Aliivibrio fischeri* DSM 7151. The values shown in the table represent $EC_{50}$ parameter corresponding to the concentration of AgNPs [µg/mL] causing the 50% inhibition of bioluminescence at 0, 5, 15 and 30 min of incubation.

| | | 28ns | 28s | 4ns | 4s |
|---|---|---|---|---|---|
| 24 h | *Daphnia magna* | 0.029 | 0.028 | 0.042 | 0.029 |
| | *Artemia franciscana* | 67.112 | 58.440 | 56.584 | 61.736 |
| 48 h | *Daphnia magna* | 0.026 | 0.027 | 0.027 | 0.026 |
| | *Artemia franciscana* | 26.162 | 10.965 | 23.945 | 19.867 |

**Table 2**. The toxic effect of AgNPs towards water crustaceans *Daphnia magna* and *Artemia franciscana*. The values shown in the table represent $EC_{50}$ parameter corresponding to the concentration of AgNPs [µg/mL] causing immobilization in 50% of tested individuals after 24 h and 48 h of incubation.

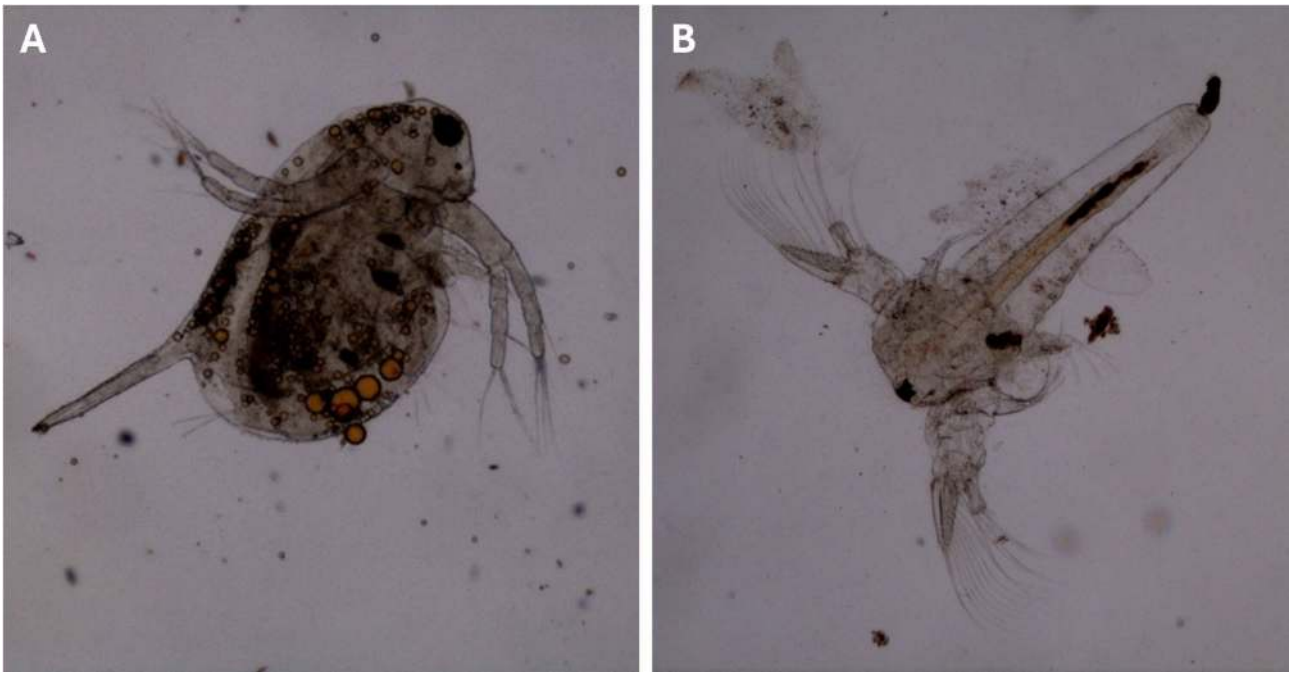


**Fig. 3**. Microscopic image (400x magnification) of tested crustaceans. (**A**) *Daphnia magna* after 48 h exposition to 12.5 µg/mL AgNPs synthesized in 4 °C without shaking and (**B**) *Artemia franciscana* after 48 h exposition to 25 µg/mL AgNPs synthesized in 4 °C without shaking.

28s, 4ns and 4s AgNPs were 1.329, 2.819, 0.671 and 2.736 µg/mL, respectively. The obtained results indicate that the AgNPs synthesis scheme affected their toxicity potential – the effect of AgNPs synthesized without shaking was the most severe, and the 4ns AgNPs was the most toxic among tested nanoparticle types.

*Sorgho saccharatum, Lepidium sativum, Sinapis alba*

The results of the phytotoxic effect investigation showed that crop plants used in the study were the least affected by the presence of mycogenic AgNPs among all organisms tested. It was established that for all tested plant species the $EC_{50}$ values (Table 3) were above 100 µg/mL, which exceeded the tested concentration range of AgNPs.

**Fig. 4**. The effect of AgNPs synthesized in 28 °C without shaking on *Spirodela polyrhiza* after 72 h of incubation. The AgNPs concentration was in the order from the left: 0, 0.78, 3.125, 12.5, 50 µg/mL.

| | 28ns | 28s | 4ns | 4s |
|---|---|---|---|---|
| *Spirodela polyrhiza* | 1.329 | 2.819 | 0.671 | 2.736 |
| *Sorgho saccharatum* | > 100 | > 100 | > 100 | > 100 |
| *Lepidium sativum* | > 100 | > 100 | > 100 | > 100 |
| *Sinapis alba* | > 100 | > 100 | > 100 | > 100 |

**Table 3**. The toxic effect of AgNPs towards tested plants *Spirodela polyrhiza*, *Sorgho saccharatum*, *Lepidium sativum* and *Sinapis alba*. The values shown in the table represent $EC_{50}$ parameter corresponding to the concentration of AgNPs [µg/mL] causing 50% of plant growth inhibition after 72 h of incubation. The inhibitory effect on plant growth was determined based on changes in the first frond surface in *S. polyrhiza* or changes in the root lengths in *S. saccharatum*, *L. sativum* and *S. alba*.

## Discussion

As it was stated earlier, the water and soil ecosystems are the most endangered by the AgNPs presence due to their release to the environment. The estimated concentrations of AgNPs in soil are in the range of 0.24–729.23 ng/kg AgNPs. It is predicted that these numbers will have grown up to 10 µg/kg by 2050[23]. The concentration of dissolved Ag in natural water ecosystems varies from 0.03 to 500 mg/L. AgNPs are being detected in surface waters in different amounts ranging between 0.3 and 320 ng/L and higher in European countries[24]. These numbers show that organisms inhabiting soil and water ecosystems are in fact endangered by the AgNPs presence.

The first part of this study involved evaluating the effects of the presence of mycogenic AgNPs on soil bacteria and fungi. Our results showed that soil bacterial strains *P. putida* and *P. moorei* were more susceptible to the action of all tested AgNPs types than the two tested fungal strains. This phenomenon is in accordance with available data[23]. The $MIC_{95}$ values established for both tested bacterial strains were 1.56 µg/mL for all AgNPs types. High susceptibility of *P. putida* to AgNPs both of biological and physical origin has been described in literature. Khan et al.[25] revealed that AgNPs synthesized via the physical dispersion method showed antibacterial effect on the level higher than 80% in the concentration of 0.1 µg/mL. Gupta et al.[26] investigated the effects of mycogenic AgNPs and proved that *P. putida* KT2440 was susceptible to their presence in the concentration of 0.4–0.8 µg/mL. In comparison, *G. striatum*-derived AgNPs synthesized in our study were less toxic. The adverse effects of AgNPs on whole microbial communities was also proven by Zhang et al.[27] They found that the microbial community richness was decreased in the presence of AgNPs in the cucumber-planted soil. However, the positive influence of the biogenic AgNPs presence on microbial community abundance in soil was also proven[28]. That shows the necessity of further investigations to fully understand the impact of AgNPs on soil bacterial communities. Our results revealed that fungal strains *T. virens* and *T. reesei* showed lower susceptibility to the AgNPs presence in the environment compared to the bacterial strains tested. The high tolerance of *Trichoderma* species to the AgNPs presence has been described in literature. Oktarina et al.[29] revealed that *T. virens* was able to grow in the AgNPs concentration of 300 mg/L and still reach about 80% of the biotic control growth level. In comparison, El-Ghany et al.[30] investigated the impact of AgNPs synthesized by silver-resistant actinomycetes on mycotoxigenic fungi. Their research showed that biogenic AgNPs originating from two actinomycetes species inhibited the growth of *Aspergillus flavus* and *Aspergillus ochraceus* in the concentrations between 29.15 and 37.35 µg/mL. Moreover, it was shown that the presence of those AgNPs in the concentrations

between 4.59 and 7.74 µg/mL inhibited the mycotoxin production completely. It indicates that the reaction to the AgNPs presence in the growth environment depends on the fungal species. In natural environments rhizosphere is colonized by multiple and diverse microbial communities. Bacteria can attach into the fungal surfaces forming fungal-bacterial biofilms that change the conditions in rhizosphere improving plant growth. Biofilm formation is one of the bacterial mechanisms of resistance to antimicrobial factors, including AgNPs. Consequently, it can be concluded that co-habiting the rhizosphere by both fungi and bacteria can indirectly enhance the protection of bacterial cells from AgNPs adverse effects[31,32].

The second part of the present study involved investigating the impact of biogenic AgNPs on aquatic organisms. Water ecosystems are at risk of increased exposition to AgNPs not only due to the industrial wastewater efflux that may contain AgNPs from manufacturing-connected processes, but also because of AgNPs leaching from available consumer products[33]. Therefore, research on the possible negative effects of the presence of biogenic AgNPs in aquatic environment is necessary.

The investigation of the changes in bioluminescence intensity revealed that the aquatic bacterium *A. fischeri* was sensitive to the presence of tested AgNPs types. The $EC_{50}$ values established after 30 min of exposition to AgNPs fluctuated around 7–8 µg/mL. In comparison, Binaeian et al.[34] found that AgNPs synthesized by *E. coli* were more harmful towards *Vibrio fischeri* PTCC 1693 with the $EC_{50}$ value after 30 min of exposition equal 34,550 ± 5,670 ppm. Interestingly, the research proved that the effect of biologically synthesized AgNPs was more severe compared to the effect of AgNPs of chemical origin. Different results were obtained by Gagné[35], who investigated the effect of commercially available AgNPs on *V. fischeri* and estimated that the $EC_{20}$ value after 30 min was above 500 µg/mL. Kyzioł-Komosińska et al.[36] attempted at evaluating the toxicity of AgNPs released from textiles. They found that *V. fischeri* was less sensitive to the AgNPs leaching from the examined textiles than *D. magna* with $EC_{50}$ values up to 46 times higher.

Our results concerning the toxicity of mycogenic AgNPs towards water crustaceans revealed that freshwater *D. magna* was more affected by the presence of all AgNPs types than saline *A. franciscana*. These results are in accordance with the findings of Zawadzka et al.[19] investigating the effect of AgNPs derived from *G. striatum* DSM 10,335. High susceptibility of *D. magna* to the AgNPs influence was also confirmed by Ivask et al.[37] In this research the size-dependent effect of AgNPs was established and calculated $EC_{50}$ values ranged from 0.001 ± 0.014 mg Ag/L to 0.218 ± 0 mg Ag/L for 10 nm and 80 nm AgNPs, respectively. In contrast, Parkashi et al.[38] proved that AgNPs in the concentration of 0.5 µg/l caused about 30% mortality in *D. magna* after 5 days of exposition. Similarly, a less severe effect of AgNPs on *D. magna* was observed by Aksakal et al.[39], who compared the action of AgNPs of chemical and biological origin towards freshwater crustaceans. It was found that 48 h exposition to 50 ppb of chemical and biological AgNPs caused 19.3% and 31.8% mortality, respectively. These results highlight the possibility of biogenic AgNPs being more dangerous due to the penetration ability enhanced by the presence of biological compounds. Lower susceptibility of crustaceans from *Artemia* species was also described in the literature. Lish et al.[40] found that after 48 h exposition of *A. salina* to commercially available AgNPs, the $EC_{50}$ value was 50 mg/L. Bhakya et al.[41] evaluated the effect of biogenic AgNPs on *A. salina*. It was revealed that the adverse effect of AgNPs was dose dependent, and after 48 h of exposition 50% of mortality was observed only at 150 and 200 µg/mL concentrations. It was noted, however, that after prolonging the exposition time, mortality was increasing. These results suggest that the time of exposition may be one of the important factors to be considered in ecotoxicological research concerning AgNPs. In another research study, the susceptibility of *A. salina* to AgNPs was notably higher, as the $EC_{50}$ value established after 72 h was 10.70 ± 1.3 mg/L[42]. In the case of *Artemia* sp. the toxicity of AgNPs can be connected with their interaction with the cuticle of crustacean and modifying chitin structure by silver ions. Moreover, aggregates of AgNPs can be found in the crustaceans' gut causing interferences in food uptake and leading to the death of starvation[43,44]. In daphnids, the bioaccumulation of AgNPs inside the organisms is caused mainly by ingestion, and nanoparticles can be found agglomerated in the gut. Moreover, AgNPs can accumulate under the carapace, in the brood chamber and can be attached to the external parts of the body[45,46]. As mechanisms of action of AgNPs on tested crustacean species do not differ significantly, the most convincing explanation for *Artemia* sp. being more resistant to AgNPs action is differences in external parameters of organisms' environment. It was shown that increasing water salinity significantly reduces mortality of *Oncorhynchus mykiss* fry exposed to colloidal AgNPs[47]. The same phenomenon was also proven in the case of invertebrate *Saccostrea cucullata*, where the decrease of water salinity intensified the toxic effect of AgNPs on tested species[48]. This effect can be attributed to the reduced ion release from the surface of nanoparticles at higher water salinity[49]. Given above examples it can be concluded that the level of water salinity is the factor differentiating the toxic effect of mycogenic AgNPs on tested crustaceans.

The last part of the present research was the investigation of the AgNPs impact on plants. Here, the water plant *S. polyrhiza* and crop plants *S. saccharatum*, *L. sativum* and *S. alba* were taken into consideration. This kind of research is of particular value, because plants as primary producers may play a role of a transport pathway for AgNPs to the organisms of higher trophic levels[50].

Our results showed that the growth of *S. polyrhiza* was inhibited by all tested AgNPs in a concentration-dependent manner. The established $EC_{50}$ values varied between 0.671 and 2.819 µg/mL. The same tendency was proven by Radić et al.[6], who investigated the effect of commercially available AgNPs on different species of duckweed, *Lemma minor*. It was observed that 0.5 and 1 mg/L AgNPs caused *L. minor* growth inhibition between 32 and 37% and 52–59%, respectively. Jang et al.[51] found that gum-arabic coated AgNPs also caused growth inhibition of *S. polyrhiza* in a concentration-dependent manner. The tested AgNPs had a less severe effect on *S. polyrhiza* than mycogenic AgNPs in our research with the $EC_{50}$ value of 13.39 ± 1.06 mg Ag/L. A less serious influence of chemical AgNPs on duckweed species *Landolita punctata* was also proven by Lalau et al.[4], who found the $EC_{50}$ value of tested AgNPs to be 6.84 ppm.

According to our research, the effect of mycogenic AgNPs on plant growth was the least severe compared to the other performed tests. The $EC_{50}$ values for three tested plant species were above the tested concentration

range, and the effect of AgNPs on the plant growth was not visible. The benign influence of chemical AgNPs on sorghum and white mustard growth was confirmed by Matras et al.[50] They found that three different types of chemically synthesized AgNPs did not cause significant inhibitions either in root growth or seeds germination compared to the controls. Tomaszewska-Sowa et al.[52] observed that *S. alba* roots growth was even stimulated by the presence of AgNPs. However, AgNPs showed adverse effects inhibiting the growth of *S. alba* hypocotyls in the highest concentration tested. The influence of AgNPs on plants is thought to be both species- and dose-dependent, as a different amount of AgNPs can promote or inhibit plant growth. It has been proven, however, that AgNPs inhibit root growth of other plant species, e.g. *Arabidopsis thaliana* roots growth was inhibited in the presence of 3 mg/L AgNPs[52–54]. It is worth noticing that AgNPs present in the aquatic or soil environment can have indirect effects on plant growth. As it was stated earlier, AgNPs can accidentally enter the water and terrestrial environments[2]. In the soil ecosystems, increased presence of AgNPs can lead to lowering the quality of the soil and negatively affect growth and abundance of microbial communities, included microorganisms supporting plant growth and nutrient cycling[27,55]. These alternations can influence plants development. Moreover, AgNPs in soil can themselves enter the plant organisms via roots. After crossing cellular covers, AgNPs can be transported symplasticaly or apoplasticaly to the inner parts of the plant and then accumulate for example in root meristem or epidermis. AgNPs can have adverse effects on seeds germination and growth of the roots. They can negatively influence processes that are crucial for the plant development, such as gaseous exchange, photosynthesis or transpiration rate. On the molecular level, the presence of AgNPs leads to enhanced reactive oxygen species production in plant cells. This can end in processes with potentially lethal outcomes, for example oxidation of the proteins, lipid peroxidation, changing the properties of cell membranes, impairing the activity of the enzymes, destroying nucleic acids or initiating programmed cell death[56]. As the phytotoxicity of AgNPs is mainly connected with oxidative stress, some antioxidant defense mechanisms can be developed in plant cells. These mechanisms involve the activity of enzymes of antioxidative properties, for example superoxide dismutase, catalase or glutathione reductase. Moreover, non-enzymatic antioxidants like ascorbate, glutathione or ascorbic acid and carotenoids can play a role in the protection of the cell against adverse AgNPs effects[57]. It can be concluded, that activated antioxidant defence mechanisms were the reason for the lack of sensitivity of tested crop plants to AgNPs action. Although studies of AgNPs impact on plants mostly suggest their adverse effects, a few confirmations of nanoparticles stimulatory influence can be found. Plant response to AgNPs can strictly depend on their dosage causing enhancement or inhibition of plant growth. While exposing on low or high concentrations of AgNPs has negative effects, exposure to specific, proper concentrations can improve plant growth, seed germination, chlorophyll content and fertilizer or water efficiency in plants[53,56].

The ecotoxic effect of AgNPs may be altered depending on the stability of nanoparticles, as it constitutes one of the main factors determining their activity. Some environmental factors, such as pH, temperature, light exposure, presence of reactive species, dissolved oxygen content, dissolved organic matter content or presence of cations and ions can all affect the AgNPs stability, which can lead to changes in the behavior of nanoparticles as a xenobiotic in different ecosystems[19,58–60]. Therefore, it should be highlighted that further investigation of AgNPs ecotoxicological potential is highly needed and should include environmental matrices for better understanding the fate of nanoparticles and changes in their activity.

## Conclusion

In our previous study we described the synthesis of AgNPs by brown root fungus *G. striatum* DSM 9592 for the first time. It was proven that obtained nanoparticles possessed antibacterial activity that can vary depending on the synthesis scheme. This research provided a broad evaluation of the ecotoxicity potential of these mycogenic AgNPs towards organisms from different ecosystems and trophic levels. Among these organisms were soil fungi species, which made the analysis of AgNPs impact on soil microbiota more complete. It was proved that AgNPs can pose a threat to the organisms living in water and soil environments, but the sensitivity to their presence varied between the tested species. *D. magna* was the most affected among all tested organisms – here, the $EC_{50}$ values for all AgNPs types tested were below 0.1 µg/mL. *S. saccharatum*, *L. sativum*, *S. alba* were the least sensitive to the presence of all AgNPs showing no adverse effects in the concentration of 100 µg/mL. It was also noted that the ecotoxic potential of biogenic AgNPs could vary depending on the synthesis scheme, e.g. in the case of *S. polyrhiza*. Our results confirmed the necessity of conducting ecotoxicological research regarding potential adverse effects of AgNPs on endangered ecosystems. In the era of the widespread use of AgNPs and ongoing search for new methods of their synthesis, the ecological safety of their utilization should become one of the most fundamental priorities. The future directions of this kind of research should cover the exact mechanisms of AgNPs impact on different organisms and evaluating how different environmental factors such as salinity, pH, temperature, as well as AgNPs properties like stability can affect the toxicological effect of nanoparticles.

## Methods

### Materials

All toxkits used in the presented study, namely: Daphtoxkit F magna (MicroBioTests Inc.) for the evaluation of AgNPs toxicity towards *D. magna*, Artroxkit M (MicroBioTests Inc.) for the determination of AgNPs toxic effect towards *A. franciscana*, Duckweed toxkit F (MicroBioTests Inc.) for the investigation of toxicological impact of AgNPs on *S. polyrhiza* and Phytotoxkit (MicroBioTests Inc.) for evaluating the toxic potential of AgNPs towards crop plants were obtained from Tigret Sp. z o.o. (Poland). During conducting the experiments with the use of toxktis, the following organism species were used: *D. magna*, *A. franciscana*, *S. polyrhiza*, *S. saccharatum*, *L. sativum*, *S. alba*. All mentioned species are commonly used in the evaluation of the acute toxicity and are prescribed in the manufacturer's protocols. The *A. franciscana* cysts, *D. magna* ephippia, *S. polyrhiza* turions and *S. saccharatum*, *L. sativum*, *S. alba* seeds came as a part of the purchased toxkits (Tigret Sp. z o.o., Poland).

The tested strains *P. putida* DSM 291, *P. moorei* DSM 12,647, *A. fischeri* DSM 7151 and *T. virens* DSM 1963 were acquired from the German Collection of Microorganisms and Cell Cultures GmbH (Germany) as well as fungal strain *G. striatum* DSM 9592 used for AgNPs synthesis. The fungal strain *T. reesei* QM 9414 was obtained from the American Type Culture Collection (ATCC). *P. putida*, *P. moorei*, *T. virens* and *T. reesei* are the ubiquitously present microorganism in the soil environment, therefore choosing these strains for the evaluation of AgNPs impact on soil microbiota seemed appropriate. *A. fischeri* is commonly used as a standard organism in the evaluation of the toxicity towards aquatic ecosystems. Silver nitrate (Sigma-Aldrich) was purchased from Merck (Poland). Sabouraud dextrose broth (Difco) and Mueller-Hinton broth (BBL) were bought from Becton Dickinson (Poland).

## Silver nanoparticles synthesis

Silver nanoparticles were synthesized according to the previously described method[22]. The process was conducted with the use of *G. striatum* DSM 9592 supernatant and $AgNO_3$ as a nanoparticle precursor. *G. stratium* DSM 9592 was cultivated in the Sabouraud dextrose broth supplemented with 2% glucose in 28 °C on a rotary shaker at 120 rpm for 120 h. After incubation period, fungal biomass was filtered through sterile filter paper and suspended in sterile deionized water. Prepared sample was again incubated in the same conditions for 120 h. Then, the biomass was filtered to obtain supernatant. The prepared fungal filtrate was divided into four parts. Each part was supplemented with $AgNO_3$ stock which reached the final concentration of 5 mM. Prepared samples was incubated in the dark at four different process conditions – at 28 °C with shaking (28s AgNPs), 28 °C without shaking (28ns AgNPs), 4 °C with shaking (4s AgNPs) and 4 °C without shaking (4ns AgNPs).

## AgNPs activity assessment against soil microorganisms

*Soil bacteria*

The antibacterial potential of fungi-derived AgNPs against *P. putida* DSM 291 and *P. moorei* DSM 12,647 was determined using the protocol described by Nowak-Lange et al.[61] with modifications. Both tested strains were cultivated in Mueller-Hinton broth medium. The tested AgNPs concentrations were 0.098, 0.19, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5 and 25 µg/mL and the dilutions were made in the appropriate medium. Biotic and abiotic controls were also prepared. The bacterial susceptibility to the investigated AgNPs was determined based on the optical density (OD) measurement at a wavelength of 630 nm carried out with a Multiskan™ FC Microplate Photometer (Thermo Fischer Scientific, Pudong, China). The minimal inhibitory concentration ($MIC_{95}$) values were also determined as the lowest concentration of AgNPs that inhibited bacterial growth. $MIC_{95}$ values were expressed in µg/mL.

*Soil fungi*

The antifungal potential of investigated AgNPs was determined by the microdilution method in accordance with the Clinical and Standard Laboratory Institute (CSLI) M38 (3rd Edition) guideline dedicated to filamentous fungi with some modifications. The evaluation was performed in 96-well cell culture plates. Inocula of both tested strains – *T. virens* DSM 1963 and *T. reesei* QM 9414 were prepared in Sabouraud dextrose broth and reached the final density of $8 \times 10^6$ spores/mL. Dilutions of AgNPs were prepared in the same medium and reached the concentration range of 0.098–25 µg/mL. Biotic and abiotic controls were also prepared. After 48 h of incubation at 28 °C the OD was measured in the same way as described in point 4.3.1. $MIC_{95}$ values were also established.

## AgNPs ecotoxicity assessment against water organisms

*Aliivibrio fischeri*

Toxicity of AgNPs towards *A. fischeri* DSM 7151 was evaluated according to the ISO 11348-1:2007(E) standard. A single colony of *A. fischeri* was transferred into dedicated liquid medium and cultivated at 20 °C/180 rpm for 22 h. After cultivation, the competent bacterial cells were diluted to the turbidance of 2500 FAU in the fresh protective medium prepared earlier according to the mentioned standard. Obtained bacterial suspension was divided into 100 µL stocks and stored at -80 °C. Before the procedure, the competent bacterial cells stored in stocks were refrozen and diluted in dedicated medium chilled to 15 °C. Next, 12 mL of medium was added to each 100 µL of bacterial suspension. The evaluation was performed in dedicated 96-well plates with black frames and white wells and the concentration range of the tested AgNPs was 0.098–25 µg/mL. 20% NaCl was used as a dilutant. Changes in bioluminescence intensity indicating changes in the bacterial cells viability in the presence of AgNPs were measured on a SpectraMax i3 Multimode Microplate Reader (Molecular Devices, USA) after 0, 5, 15 and 15 min of incubation. Between measurements, the tested plates were stored at 15 °C in darkness. Half-maximal effective response ($EC_{50}$) values for each measurement point were established as 50% inhibition of bioluminescence compared to the biotic control.

*Artemia franciscana*

The evaluation of AgNPs toxicity towards saline crustaceans *A. franciscana* was performed using the Arthroxkit M test, which was carried out in accordance with the producer's protocol and ISO/TS 20787:2017 standard. The cysts of *A. franciscana* were incubated in saline water at 25 °C and constant lightness of 3000 lx for 48 h. After hatching, the larvae were transferred to 24-well plates, where AgNPs dilution was prepared in the same medium reaching the concentration range of 6.25–100 µg/mL. The plates were incubated for 48 h at 25 °C in darkness. After the incubation period, $EC_{50}$ values were established based on the number of immobile *A. franciscana* individuals. The untreated larvae were used as a biotic control.

*Daphnia magna*

The assessment of AgNPs toxicity towards freshwater crustaceans *D. magna* was performed with the use of Daphtoxkit F magna according to the producer's protocol and ISO 6341:2012 standard. The ephippia of *D. magna* were incubated in fresh water at 20 °C and constant lightness of 6000 lx for 72 h. After incubation, the motile individuals were transferred to 6-well plates with AgNPs diluted in the same medium to the concentration range of 0.005–0.1 µg/mL. The plates were incubated for 48 h at 20 °C in darkness. After incubation, $EC_{50}$ values were determined based on the number of immobile larvae. The untreated individuals were used as a biotic control.

## AgNPs ecotoxicity assessment against plants

*Spirodela polyrhiza*

The investigation of AgNPs toxicity towards *S. polyrhiza* was conducted using the Duckweed toxkit F according to the producer's protocol and ISO 20079:2005(E) standard. The turions of *S. polyrhiza* were incubated in the dedicated Stainberg medium at 25 °C and constant lightness of 6000 lx for 72 h. After incubation, germinated turions were transferred to 48-well plates with diluted AgNPs in the same medium to the concentration range of 0.78–25 µg/mL. The plates were incubated for 72 h at 25 °C and constant lightness of 6000 lx. $EC_{50}$ values were established based on the changes in the first fonds surface size before and after incubation with AgNPs. Germinated turions cultivated in the medium without AgNPs were used as a biotic control. Differences in fonds size were determined using the ImageJ software (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation, University of Wisconsin, USA).

*Sorgho saccharatum, Lepidium sativum, Sinapis alba*

The phytotoxic activity of mycogenic AgNPs was investigated using the Phytotoxkit according to the producer's protocol. In this test, the effect of AgNPs in the concentrations of 50 and 100 µg/mL towards three plant species, namely *S. saccharatum*, *L. sativum* and *S. alba*, was checked. AgNPs diluted in sterile deionized water were transferred to the plates with a layer of sterile filter paper. Then, the seeds of each plant were soaked in sterilized deionized water and placed on the test plates. The prepared plates were closed with the transparent covers and incubated in the vertical position for 72 h at 25 °C in darkness. After incubation, the root lengths were measured using the ImageJ software (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation, University of Wisconsin, USA). $EC_{50}$ values were established based on the differences in roots lengths between plants cultivated with and without AgNPs addition.

## Results analysis

*Statistical analysis*

Each experiment was conducted in four replicates ($n = 4$). The results of AgNPs activity against soil microorganisms were analyzed with the use of a one-way ANOVA test with * $p < 0.05$ to estimate the statistical significance. The estimation and all needed calculations were carried out by using Excel, Microsoft Office 2021 (Microsoft Corporation, Redmont, WA, USA). The results shown in the figures are expressed as the average values with the standard deviation (SD).

*Half-maximal effective response ($EC_{50}$) estimation*

The $EC_{50}$ values were established based on the calibration curves equations obtained by using Excel, Microsoft® Office 2021 (Microsoft Corporation, Redmont, WA, USA) and meant the concentration of AgNPs causing the 50% of biological response in the tested organisms.

## Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Received: 28 January 2025; Accepted: 21 March 2025
Published online: 28 March 2025

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## Acknowledgements

The authors extend thanks to Mariusz Krupiński for sharing *A. fischeri* bacteria strain and to Marta Nowak-Lange for the support during the AgNPs phytotoxic effect investigation.

## Author contributions

Conceptualization, A.T., K.N.; methodology, K.N., A.T.; investigation, A.T., K.N.; manuscript – original draft preparation, A.T.; manuscript – review and editing, K.N., K.L.

## Declarations

## Competing interests

The authors declare no competing interests.

## Additional information

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Publikacja 4

## *Synergistic activity of Gloeophyllum striatum-derived AgNPs with ciprofloxacin and gentamicin against human pathogenic bacteria*

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2025, International Journal of Molecular Sciences, 26: 3529; DOI: 10.3390/ijms26083529

International Journal of
*Molecular Sciences*

MDPI

*Article*

# Synergistic Activity of *Gloeophyllum striatum*-Derived AgNPs with Ciprofloxacin and Gentamicin Against Human Pathogenic Bacteria

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**Abstract:** Silver nanoparticles (AgNPs) are used in a variety of different fields due to their excellent antimicrobial potential. Despite clear advantages, concerns about their toxicity have arisen, also concerning biogenic nanoparticles. Simultaneously, global healthcare is facing a problem of spreading antimicrobial resistance towards existing antibiotics. Using combined therapies involving AgNPs and antibiotics seems to be a promising solution to the above problems. The aim of this study was to evaluate the enhancement of the effectiveness of AgNPs, ciprofloxacin, and gentamicin against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The research involved the assessment of antimicrobial and antibiofilm-forming activities and the analysis of phospholipid and fatty acid profiles. Our results showed that combining the tested antimicrobials can enhance their activity against the tested bacterial strains. However, no effect was observed while mixing AgNPs with ciprofloxacin against *P. aeruginosa*. The most significant effect was obtained by combining 3.125 µg/mL of AgNPs with 0.125 µg/mL of gentamicin against *S. aureus*. It was also shown that the tested antimicrobials applied in combination exhibited an increased inhibitory activity towards bacterial biofilm formation by *S. aureus*. Lipidomic analysis revealed that under the influence of the tested antimicrobials, the properties of the cell membrane were altered in different ways depending on the bacterial strain.

**Keywords:** silver nanoparticles; mycogenic; synergism; ciprofloxacin; gentamicin; lipid and fatty acid profiles; antibiofilm



Academic Editor: Antonella Piozzi

Received: 4 March 2025
Revised: 3 April 2025
Accepted: 7 April 2025
Published: 9 April 2025

**Citation:** Tończyk, A.; Niedziałkowska, K.; Bernat, P.; Lisowska, K. Synergistic Activity of *Gloeophyllum striatum*-Derived AgNPs with Ciprofloxacin and Gentamicin Against Human Pathogenic Bacteria. *Int. J. Mol. Sci.* **2025**, *26*, 3529. https://doi.org/10.3390/ijms26083529



## 1. Introduction

Silver nanoparticles (AgNPs) are one of the most commercialized and broadly used nanomaterials. This is because their high antimicrobial potential is considered as the strongest among metallic nanoparticles [1,2]. The complex mechanism of action involving a simultaneous impact on several cellular compartments and processes makes AgNPs effective against a broad spectrum of microorganisms. This property is especially interesting from the perspective of combating polymicrobial infections. It has also been noted that AgNPs are able to inhibit the ability of bacteria to adhere to surfaces and form biofilm [3,4]. All these features make it possible to utilize AgNPs in various industries, such as the food, textile, and cosmetics industries [5,6]. Moreover, AgNPs are used in biomedicine as medical device coatings or additives in medical formulations [7]. Despite the clear advantages of AgNPs, there are concerns regarding their potential cytotoxicity, especially in the case

of long-term exposure to active concentrations [4]. It has been proven that AgNPs of biological origin show cytotoxic effects towards healthy mammalian cells [8–10]. Therefore, the common use of AgNPs as an antimicrobial agent has become questionable and the search for methods of lowering active doses is ongoing. One of the possible solutions is to combine two or more antimicrobial agents, acting in a synergistic way, in order to reduce dosages and minimize the toxicological effect of the mixture's components [4].

At the same time, global healthcare is facing the problem of constantly growing antimicrobial resistance, resulting in both a high death rate among patients and the incrased expense of hospitalization caused by prolonged treatment [2]. With the still ongoing overuse of antibiotics not only in the field of medicine, but also in other branches, such as genetic engineering, animal breeding, and crop cultivation, the problem of the constant release of antibiotic residues into the natural environment has become inevitable. Concentrations of such compounds found in soil or water ecosystems vary between a few and hundreds of nanograms per liter or kilogram of soil. Additionally, despite the continuous introduction of antibiotics into the environment, their high persistence can enhance the threat posed [11,12]. Environmental antibiotic presence can force bacterial strains inhabiting polluted niches to develop resistance mechanisms, therefore promoting the occurrence of antimicrobial resistance [13]. The decreasing susceptibility of microorganisms to the existing conventional antibiotics and the emergence of multidrug-resistant (MDR) strains highlight the necessity of developing new, effective ways of treatment. Combined therapies using two or more agents with antimicrobial potential are a promising solution. It was found that AgNPs used simultaneously with antibiotics enhance the activity of various drugs, such as amoxicillin, vancomycin, tetracycline, neomycin, and chloramphenicol [1,3,4]. Moreover, this approach can lead to decreases in the quantity of the antibiotics required, as mixing AgNPs with antibiotics possibly reduces their required active dose [14], which, alongside the developing methods of antibiotic residue degradation [12], could reduce the threat posed to the natural environment by antibiotic pollution.

*Pseudomonas aeruginosa* and *Staphylococcus aureus* belong to the group of the most widespread human pathogens exhibiting antimicrobial resistance. These strains are among six constituting the ESKAPE group, associated with nosocomial infections, and are known to possess the ability of biofilm formation [15–18]. Bacterial biofilms can cover the surfaces of medical devices, such as catheters, and cause persistent infections, as cells forming biofilms can exhibit as much as 1000 times higher resistance to conventional antimicrobials compared to planktonic forms [19]. AgNPs have been proven to prevent biofilm formation and to successfully damage the developed biofilms [20].

Our previous study showed that *Gloeophyllum striatum*-derived AgNPs were active towards a broad spectrum of bacterial strains, including *S. aureus* and *P. aeruginosa*. It was also proven that they were able to inhibit biofilm formation by *P. aeruginosa*. However, the effective concentrations of the tested AgNPs showed increased toxicity towards the human fibroblast cell line [8]. These results emphasized the need for further research to seek possible ways of lowering active concentrations of AgNPs and maintaining their effectiveness.

The aim of this research was to evaluate the synergistic activity of AgNPs of mycological origin and two conventional antibiotics (ciprofloxacin and gentamicin) towards the Gram-positive bacterial strain *Staphylococcus aureus* ATCC 29213 and the Gram-negative bacterial strain *Pseudomonas aeruginosa* ATCC 27853. Both selected antibiotics are commonly used for the treatment of bacterial infections. Ciprofloxacin is a quinolone antibiotic displaying broad-spectrum activity based on the inhibition of DNA gyrase in bacterial cells leading to interference in the formation of double-stranded DNA. Ciprofloxacin is known to affect several Gram-positive bacterial strains, for example

*Staphylococcus*, as well as Gram-negative strains, including *P. aeruginosa*. Gentamicin belongs to aminoglycoside family of antibiotics and is active against Gram-negative bacteria by inhibiting the synthesis of bacterial proteins. In both cases, the increasing resistance of bacterial strains, including *S. aureus* and *P. aeruginosa*, to antibiotic action has been reported [21,22]. Consequently, an investigation concerning the synergistic effect of these two antibiotics with AgNPs can shed light on possible solutions to the still-developing antimicrobial resistance problem. The assessment included the examination of antibacterial activity, the evaluation of the inhibitory effect of the tested antimicrobials on biofilm formation, and the detection of changes in the phospholipid and fatty acid compositions caused by AgNPs and antibiotics applied in selected concentrations. The lipidomic analysis is vital in the investigation of AgNPs' influence on bacterial cells, as the first step of the antimicrobial action of AgNPs is the interaction with cell envelope's adhesion to the cell wall and/or cell membrane. This can lead to the destabilization of the cell membrane structure and its properties, such as permeability and fluidity [23,24]. The changes detected in lipid profiles in bacterial cells treated with AgNPs or a mixture of nanoparticles and selected antibiotics can provide information about possible shifts in AgNPs' action when combined with antibiotics with known modes of antibacterial activity.

## 2. Results

### *2.1. Antimicrobial Activity of AgNPs, Antibiotics, or a Combination of the Agents*

Our results showed that the simultaneous use of *G. striatum*-derived AgNPs (from now on described as 'AgNPs' throughout this section) and the tested antibiotics enhanced the activity of the nanoparticles against *S. aureus* and *P. aeruginosa*. In *S. aureus* (Figure 1), the AgNPs used alone at the concentrations of 0.195 µg/mL, 0.78 µg/mL, and 3.125 µg/mL caused about 5%, 20%, and 40% inhibition of bacterial growth compared to growth control samples, respectively. Ciprofloxacin used alone against *S. aureus* caused about 40% bacterial growth inhibition at the concentrations of 0.0313 µg/mL and 0.0625 µg/mL and 55% growth inhibition at the concentration of 0.125 µg/mL. Mixing AgNPs in all concentrations with 0.125 µg/mL of ciprofloxacin resulted in an over two-fold enhancement of the AgNPs' activity, which reached a 95% growth-inhibiting effect in the case of the highest concentration of the nanoparticles tested. Gentamicin used alone was less harmful to *S. aureus* compared to fluoroquinolone. However, combining gentamicin at a concentration of 0.125 µg/mL with 3.125 µg/mL of AgNPs caused a complete inhibitory effect on the growth of the tested bacterial strain and an about 90% inhibitory effect when the concentration of the antibiotic was four times lower.

In *P. aeruginosa* (Figure 2), AgNPs in the concentration range of 0.049–0.78 µg/mL caused a slight inhibition of bacterial growth. Ciprofloxacin used alone at the concentrations of 0.0156 µg/mL and 0.0313 µg/mL inhibited bacterial growth by about 20% and by about 50% when applied at the highest concentration tested. Mixing each concentration of AgNPs with 0.0156 µg/mL of ciprofloxacin resulted in a slight enhancement of the AgNPs' activity against *P. aeruginosa*. When combined with higher antibiotic concentrations, the AgNPs' activity was enhanced and caused bacterial growth inhibition by up to 40%. However, there were no vivid differences between the effectiveness of the tested AgNP concentrations. Gentamicin used alone was less effective against *P. aeruginosa* compared to the other antibiotics, and no inhibitory effect was observed for all tested concentrations. Mixing 0.195 µg/mL or 0.390 µg/mL of AgNPs with each tested concentration of gentamicin enhanced the effectiveness of AgNPs compared to when AgNPs were used alone. When 0.39 µg/mL of AgNPs and the highest gentamicin concentration were used, the effectiveness increased by 75%. Interestingly, the effect

of increasing the effectiveness of antimicrobials applied simultaneously was weaker in the case of the highest concentration of AgNPs tested mixed with each variant of gentamicin used.

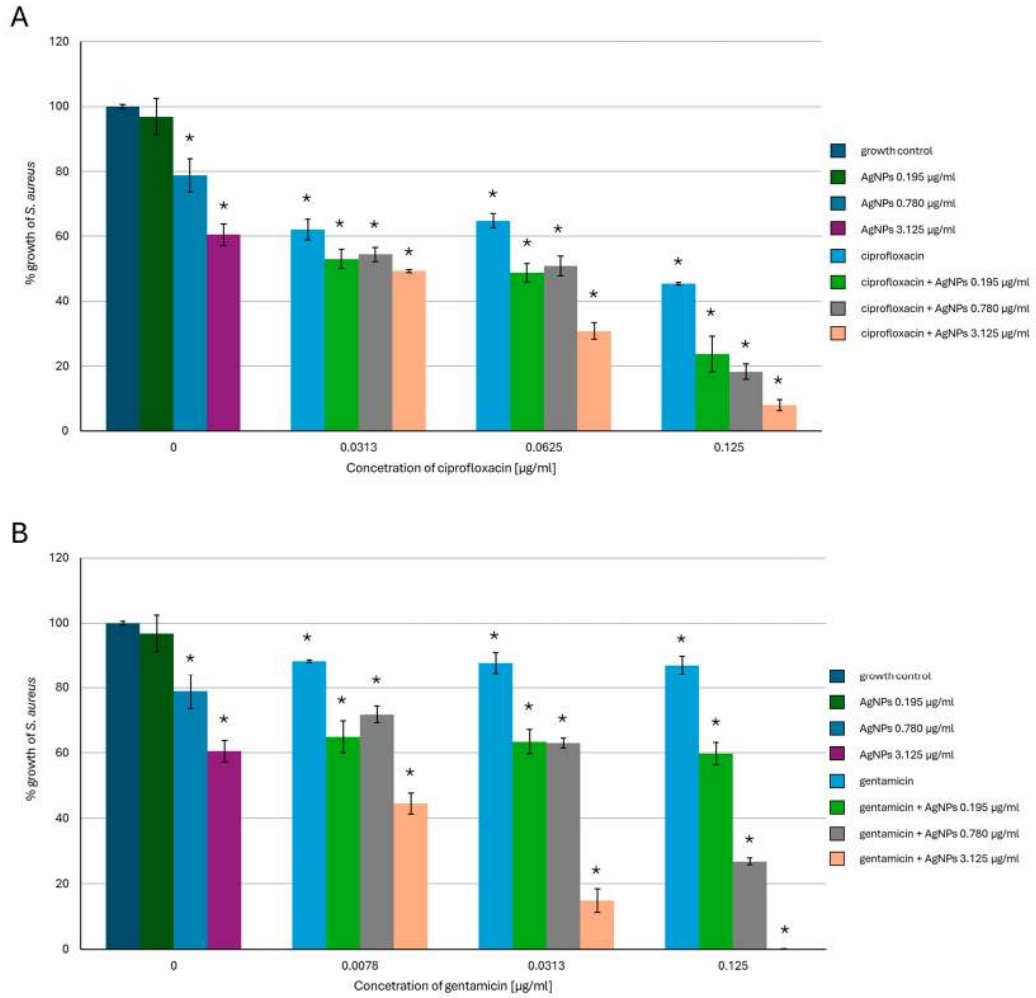


**Figure 1.** Antibacterial activity of mycogenic silver nanoparticles (AgNPs), ciprofloxacin (**A**), and gentamicin (**B**) against *S. aureus*. The results are shown as average percentage values with standard deviations of optical density (OD) of the biotic control. The statistical significance is shown by an asterisk (* $p < 0.05$).

### *2.2. Antibiofilm-Forming Activity of AgNPs, the Antibiotics and a Combination of the Agents*

The results of the evaluation of the antibiofilm-forming activity revealed that combining AgNPs with ciprofloxacin or gentamicin enhanced the inhibitory effect of the tested antimicrobials towards bacterial biofilm. It was shown that, in the case of *S. aureus* (Figure 3), mixing the lowest tested concentration of AgNPs with 0.0313 µg/mL of ciprofloxacin enhanced the antibiofilm activity of AgNPs by 50%. The same concentration of the antibiotic combined with 3.125 µg/mL of AgNPs caused a reduction in biofilm formation of over 20% compared to the biotic control, while a two-fold higher ciprofloxacin concentration combined with the same AgNPs variant caused over 90% inhibition of *S. aureus* biofilm-forming activity. Additionally, 0.125 µg/mL of ciprofloxacin alone or combined with all tested AgNP concentrations completely inhibited biofilm formation. In the case of gentamicin, mixing each antibiotic variant with the highest AgNP concentration enhanced the antibiofilm activity. Furthermore, 3.125 µg/mL of AgNPs combined with 0.0078 µg/mL of gentamicin caused about 70% inhibition of *S. aureus* biofilm formation and over 90% inhibition when combined with the antibiotic at a

concentration of 0.0313 µg/mL. Mixing 0.125 µg/mL of gentamicin with 0.78 µg/mL of AgNPs reduced bacterial biofilm formation by about 90%, while the same concentration of the antibiotic combined with 3.125 µg/mL of AgNPs caused a complete inhibition of biofilm formation.

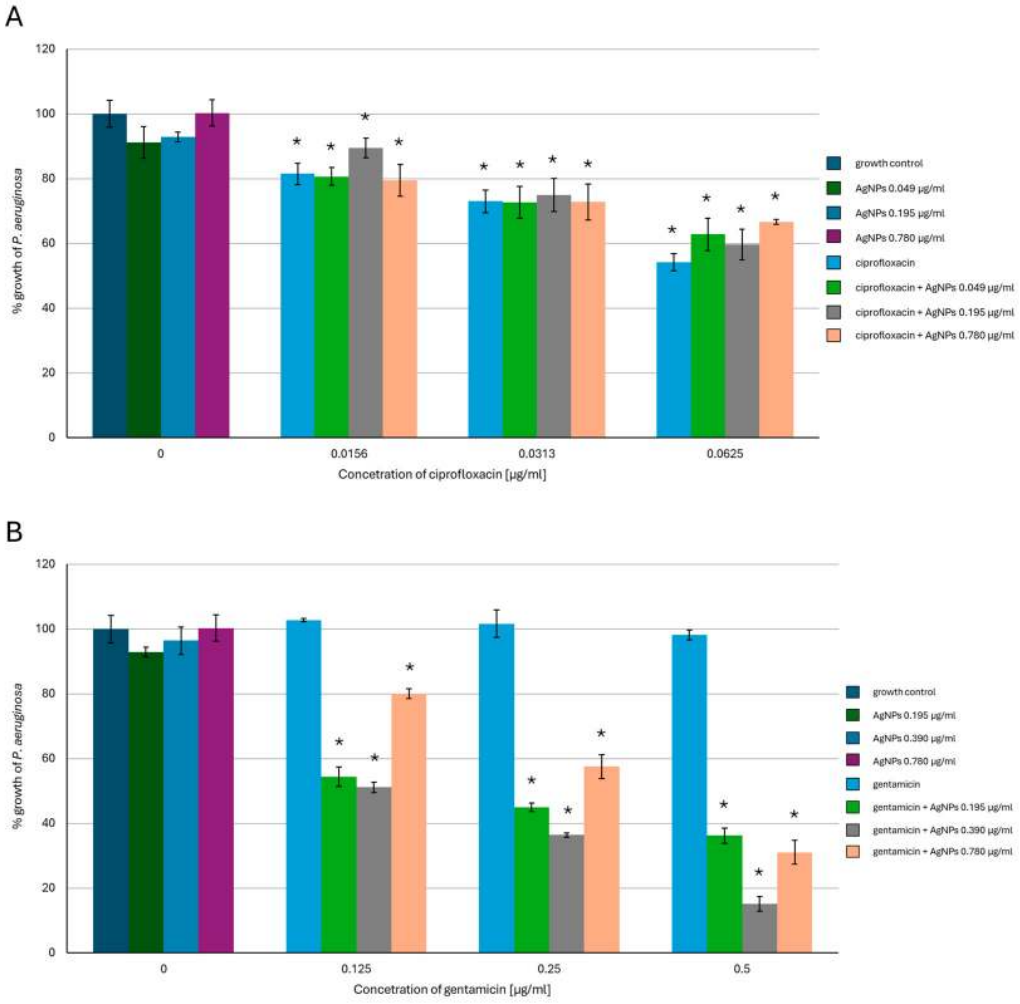


**Figure 2.** Antibacterial activity of mycogenic AgNPs, ciprofloxacin (**A**), and gentamicin (**B**) against *P. aeruginosa*. The results are shown as average percentage values with standard deviations of optical density (OD) of the biotic control. The statistical significance is shown by an asterisk (* $p < 0.05$).

An inhibitory effect on *P. aeruginosa* biofilm formation (Figure 4) of the tested antimicrobials was also detected; however, the addition of AgNPs alone caused an increase in the biofilm-forming activity of this bacterial strain. The best inhibitory result was observed in the case of mixing 0.049 µg/mL of AgNPs with 0.0625 µg/mL of ciprofloxacin. Here, the growth of bacterial biofilm was reduced to less than 40% of the biotic control. When AgNPs were combined with gentamicin, the stimulation of *P. aeruginosa* biofilm-forming activity was observed. This phenomenon reached the highest value of more than 200% of the biotic control in the cases of 0.39 µg/mL and 0.78 µg/mL of AgNPs combined with 0.125 µg/mL and 0.25 µg/mL of gentamicin, respectively. There was no inhibitory effect on the biofilm-forming activity compared to the biotic control in this variant of the experiment.

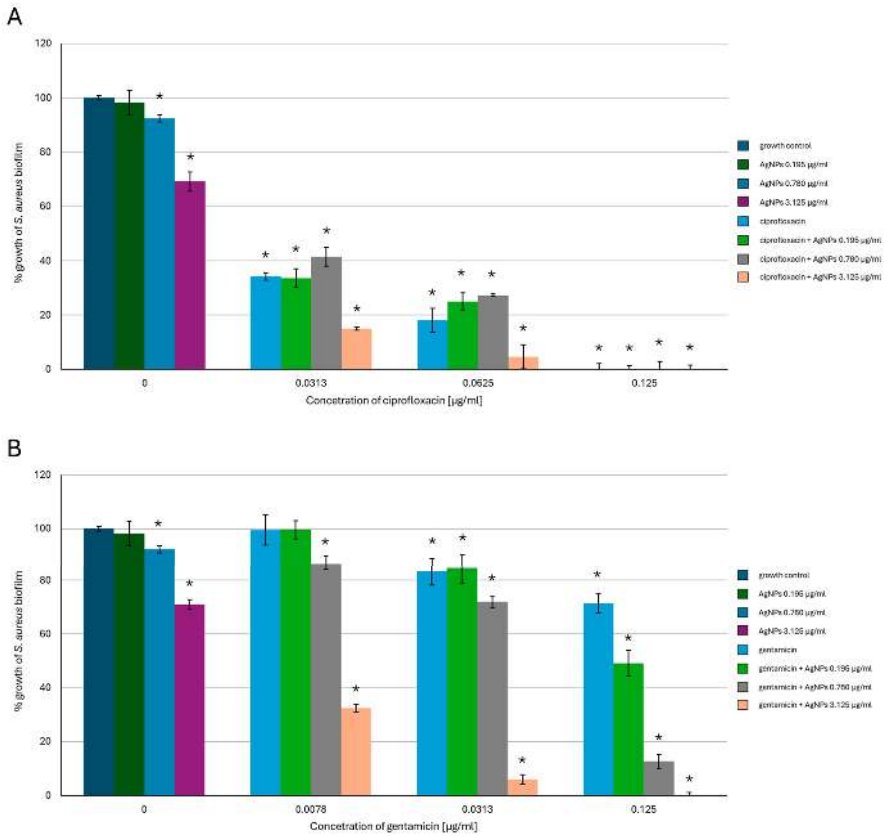


**Figure 3.** Antibiofilm-forming activity of mycogenic AgNPs, ciprofloxacin (**A**), and gentamicin (**B**) against *S. aureus*. The results are shown as average percentage values with standard deviations of optical density (OD) of the biotic control. The statistical significance is shown by an asterisk (* $p < 0.05$).

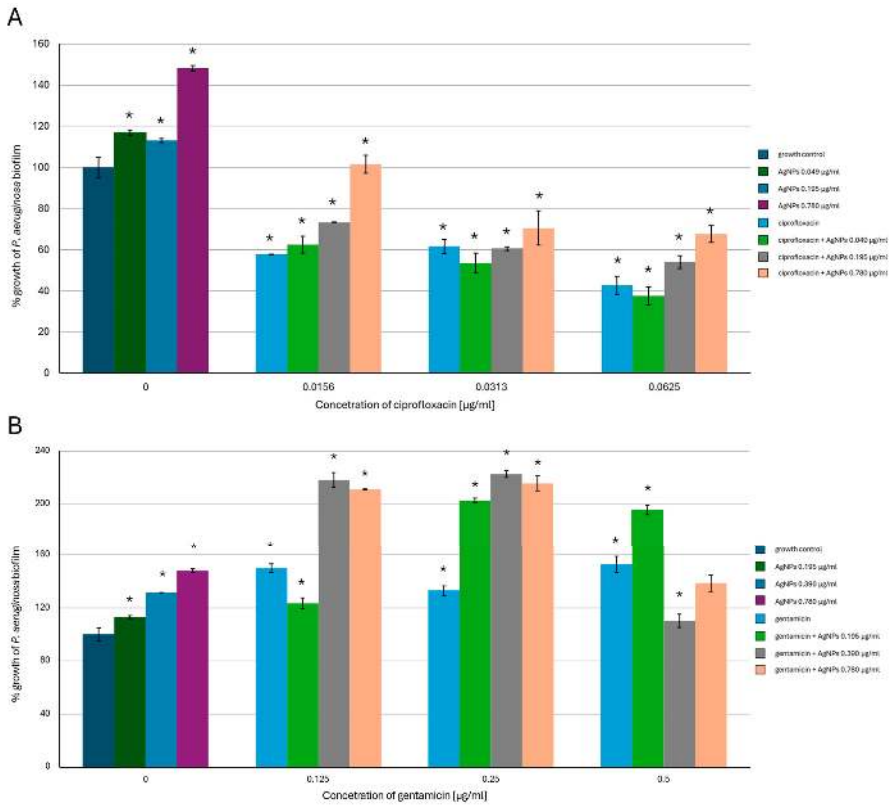


**Figure 4.** Antibiofilm-forming activity of mycogenic AgNPs, ciprofloxacin (**A**), and gentamicin (**B**) against *P. aeruginosa*. The results are shown as average percentage values with standard deviations of optical density (OD) of the biotic control. The statistical significance is shown by an asterisk (* $p < 0.05$).

### 2.3. Changes in the Phospholipid Profiles of the Tested Bacteria in the Presence of AgNPs, the Antibiotics or a Combination of Agents

In our research, four different species of phospholipids (PL) were detected in the tested bacterial species, namely phosphatidylethanolamines (PE), phosphatidylglycerols (PG), and lysyl-phosphatidylglycerols (L-PG) in *S. aureus* (Table 1), and phosphatidylcholines (PC), PE, and PG in *P. aeruginosa* (Table 2). In *S. aureus*, the most numerous class of phospholipids was L-PG, reaching 67.45% of the total phospholipid content in the growth control. The content distribution of the remaining PL species in biotic samples comprised 31.77% and 0.79% for PG and PE, respectively. The addition of the tested antimicrobials caused a decrease in the amount of PE, and the most significant change was observed in the case of 3.125 μg/mL of AgNPs and 0.0078 μg/mL of gentamicin mixture. In the case of PG, the addition of AgNPs, gentamicin, and AgNPs mixed with ciprofloxacin caused decreases in the phospholipid contents. Here, the % amount of PG in the presence of AgNPs was two times lower compared to the biotic control. The presence of gentamicin caused a decrease in the content of PG by about 20%, while the mixture of nanoparticles and ciprofloxacin caused a 30% decrease in PG content. On the other hand, the content of L-PG increased in the presence of the same antimicrobials. The most visible change was caused by the presence of AgNPs alone—the content of L-PG increased to 83.4%. In *P. aeruginosa*, the most numerous phospholipids species was PG, with a content of 71.18% in the biotic controls. The contents of the remaining phospholipid fractions were 26.47% for PE and 2.35% for PC. The addition of 0.78 μg/mL of AgNPs alone did not influence the content of PC, but it caused a slight decrease in PG and an increase in PE quantities. Both antibiotics alone caused a decrease in the amount of PC by about 30%, while the addition of the AgNPs and gentamicin mixture lowered the PC content twofold. The same effect of AgNPs combined with gentamicin was observed in PG, as the content of this phospholipid species decreased to 65.18%. The content of PE was increased by the presence of AgNPs and ciprofloxacin alone and by AgNPs combined with gentamicin, where the change was the most visible with the PE content, which increased by 30% compared to the growth control.

**Table 1.** The influence of the tested antimicrobials dosed alone and in combination on the phospholipid profile of *S. aureus* ATCC 29213. Antimicrobials were used at the following concentrations: 3.125 μg/mL of silver nanoparticles (AgNPs), 0.0625 μg/mL of ciprofloxacin, and 0.0078 μg/mL of gentamicin. Asterisk * ($p < 0.05$) indicates values that differ significantly from the control.

| Phospholipid Species | Growth Control | AgNPs | Ciprofloxacin | Gentamicin | AgNPs + C | AgNPs + G |
|---|---|---|---|---|---|---|
| PE 28:0 | 0.03 ± 0.028 | 0.01 ± 0.000 | 0.03 ± 0.007 | 0.02 ± 0.007 | 0.02 ± 0.007 | 0.01 ± 0.000 |
| PE 32:2 | 0.24 ± 0.014 | 0.11 ± 0.007 * | 0.36 ± 0.092 | 0.23 ± 0.134 | 0.3 ± 0.000 * | 0.16 ± 0.198 |
| PE 28:0 | 0.03 ± 0.028 | 0.01 ± 0.000 | 0.03 ± 0.007 | 0.02 ± 0.007 | 0.02 ± 0.007 | 0.01 ± 0.000 |
| PE 30:0 | 0.02 ± 0.007 | 0.04 ± 0.035 | 0.02 ± 0.007 | 0.04 ± 0.035 | 0.01 ± 0.014 | 0.01 ± 0.007 |
| PE 30:1 | 0.01 ± 0.000 | 0.01 ± 0.007 | 0.02 ± 0.000 | 0.01 ± 0.007 | 0.01 ± 0.007 | 0.03 ± 0.007 |
| PE 31:0 | 0.03 ± 0.000 | 0.02 ± 0.007 | 0.04 ± 0.021 | 0.02 ± 0.007 | 0.04 ± 0.021 | 0.01 ± 0.000 |
| PE 31:1 | 0.02 ± 0.007 | 0.02 ± 0.028 | 0.01 ± 0.000 | 0.02 ± 0.007 | 0.03 ± 0.014 | 0.02 ± 0.014 |
| PE 32:0 | 0.15 ± 0.106 | 0.20 ± 0.226 | 0.06 ± 0.014 | 0.11 ± 0.042 | 0.05 ± 0.035 | 0.10 ± 0.049 |
| PE 32:1 | 0.17 ± 0.007 | 0.10 ± 0.085 | 0.04 ± 0.028 * | 0.02 ± 0.000 * | 0.04 ± 0.021 * | 0.03 ± 0.000 * |
| PE 32:2 | 0.24 ± 0.014 | 0.11 ± 0.007 * | 0.36 ± 0.092 | 0.23 ± 0.134 | 0.3 ± 0.000 * | 0.16 ± 0.198 |
| PE 33:2 | 0.13 ± 0.007 | 0.09 ± 0.099 | 0.02 ± 0.007 * | 0.02 ± 0.007 * | 0.03 ± 0.007 * | 0.00 ± 0.000 * |
| PG 30:1 | 4.76 ± 0.06 | 1.93 ± 0.219 * | 5.37 ± 0.516 | 2.80 ± 0.184 * | 3.20 ± 1.195 | 3.85 ± 2.171 |

**Table 1.** *Cont.*

| Phospholipid Species | Growth Control | AgNPs | Ciprofloxacin | Gentamicin | AgNPs + C | AgNPs + G |
|---|---|---|---|---|---|---|
| PG 31:1 | 17.57 ± 2.638 | 8.48 ± 0.976 | 19.43 ± 2.857 | 13.02 ± 3.330 | 11.15 ± 1.853 | 18.34 ± 6.824 |
| PG 32:0 | 6.36 ± 1.513 | 3.59 ± 0.042 | 5.90 ± 1.584 | 6.36 ± 2.305 | 4.66 ± 0.530 | 6.79 ± 0.552 |
| PG 32:1 | 0.33 ± 0.028 | 0.22 ± 0.057 | 0.33 ± 0.085 | 0.25 ± 0.049 | 0.28 ± 0.198 | 0.33 ± 0.148 |
| PG 33:1 | 0.40 ± 0.085 | 0.15 ± 0.014 | 0.29 ± 0.177 | 0.31 ± 0.106 | 0.31 ± 0.028 | 0.32 ± 0.134 |
| PG 32:2 | 0.06 ± 0.042 | 0.03 ± 0.014 | 0.08 ± 0.064 | 0.04 ± 0.049 | 0.04 ± 0.021 | 0.05 ± 0.007 |
| PG 34:1 | 2.30 ± 0.417 | 1.62 ± 0.580 | 2.55 ± 0.834 | 2.71 ± 0.502 | 1.49 ± 0.156 | 2.09 ± 0.078 |
| LysylPG 31:0 | 18.99 ± 2.058 | 14.31 ± 4.045 * | 22.96 ± 1.846 | 20.76 ± 2.927 | 26.86 ± 8.577 | 18.44 ± 1.669 |
| LysylPG 32:0 | 27.15 ± 6.314 | 22.96 ± 8.061 | 15.09 ± 1.032 | 22.01 ± 3.366 | 9.73 ± 1.768 | 28.00 ± 10.628 |
| LysylPG 32:1 | 21.32 ± 3.776 | 46.13 ± 1.930 | 27.47 ± 8.902 | 31.31 ± 5.148 | 41.82 ± 14.121 | 21.47 ± 1.103 |

**Table 2.** The influence of the tested antimicrobials dosed alone and in combination on the phospholipid profile of *P. aeruginosa* ATCC 27853. Antimicrobials were used at the following concentrations: 0.78 μg/mL of AgNPs, 0.0313 μg/mL of ciprofloxacin, and 0.125 μg/mL of gentamicin. Asterisk * ($p < 0.05$) indicates values that differ significantly from the control.

| Phospholipid Species | Growth Control | AgNPs | Ciprofloxacin | Gentamicin | AgNPs + C | AgNPs + G |
|---|---|---|---|---|---|---|
| PC 34:0 | 0.47 ± 0.059 | 0.50 ± 0.003 | 0.27 ± 0.0176 | 0.27 ± 0.004 | 0.45 ± 0.076 | 0.27 ± 0.092 |
| PC 34:1 | 1.55 ± 0.178 | 1.61 ± 0.010 | 1.28 ± 0.001 | 1.39 ± 1.588 | 1.46 ± 0.267 | 0.74 ± 0.272 |
| PC 34:1 | 0.23 ± 0.022 | 0.10 ± 0.000 * | 0.12 ± 0.000 * | 0.09 ± 0.001 * | 0.16 ± 0.055 | 0.11 ± 0.024 * |
| PC 36:2 | 0.10 ± 0.014 | 0.06 ± 5.034 | 0.04 ± 0.004 | 0.09 ± 0.000 | 0.07 ± 0.016 | 0.07 ± 0.010 |
| PE 28:0 | 0.01 ± 0.010 | 0.02 ± 4.155 | 0.01 ± 2.663 | 0.01 ± 2.510 | 0.01 ± 0.002 | 0.02 ± 0.001 |
| PE 30:0 | 0.37 ± 0.017 | 0.38 ± 0.002 | 0.34 ± 6.527 | 0.37 ± 0.000 | 0.38 ± 0.019 | 0.42 ± 0.007 |
| PE 30:1 | 0.07 ± 0.009 | 0.08 ± 0.001 | 0.05 ± 4.326 | 0.07 ± 7.344 | 0.07 ± 0.011 | 0.09 ± 0.004 |
| PE 31:0 | 0.06 ± 0.001 | 0.07 ± 1.933 | 0.07 ± 0.000 | 0.07 ± 7.763 | 0.06 ± 0.003 | 0.06 ± 4.997 |
| PE 31:1 | 0.02 ± 0.004 | 0.02 ± 0.000 | 0.01 ± 2.730 | 0.02 ± 5.417 | 0.02 ± 0.001 | 0.02 ± 0.003 |
| PE 32:0 | 4.93 ± 0.047 | 4.70 ± 0.204 | 5.05 ± 0.104 | 5.07 ± 0.195 | 4.66 ± 0.191 | 5.23 ± 0.022 * |
| PE 32:1 | 4.51 ± 0.068 | 4.28 ± 0.810 | 4.30 ± 0.215 | 4.66 ± 0.005 | 4.73 ± 0.376 | 5.54 ± 0.305 * |
| PE 32:2 | 0.47 ± 0.009 | 0.50 ± 0.016 | 0.33 ± 8.678 * | 0.41 ± 0.000 | 0.48 ± 0.061 | 0.46 ± 0.007 |
| PE 33:1 | 0.13 ± 0.004 | 0.13 ± 1.358 | 0.14 ± 0.000 | 0.13 ± 2.713 | 0.13 ± 0.012 | 0.13 ± 0.011 |
| PE 33:2 | 0.18 ± 0.002 | 0.18 ± 0.000 | 0.181 ± 0.000 | 0.17 ± 1.125 | 0.23 ± 0.022 | 0.16 ± 0.000 * |
| PE 34:1 | 41.41 ± 0.681 | 39.54 ± 0.500 | 44.36 ± 2.384 | 40.62 ± 0.886 | 40.73 ± 1.352 | 35.64 ± 0.914 * |
| PE 34:2 | 16.01 ± 0.934 | 15.47 ± 0.010 | 15.58 ± 0.024 | 15.64 ± 0.000 | 16.27 ± 0.089 | 14.97 ± 0.849 |
| PE 35:1 | 0.16 ± 0.009 | 0.15 ± 2.215 | 0.15 ± 0.000 | 0.16 ± 4.219 | 0.11 ± 0.046 | 0.13 ± 0.013 |
| PE 35:2 | 1.02 ± 0.086 | 0.98 ± 0.061 | 1.19 ± 2.239 | 1.05 ± 0.000 | 0.97 ± 0.094 | 0.78 ± 0.026 |
| PE 36:2 | 1.81 ± 0.101 | 1.67 ± 0.008 | 1.74 ± 0.031 | 1.69 ± 0.004 | 1.82 ± 0.011 | 1.53 ± 0.145 |
| PG 31:1 | 0.01 ± 0.003 | 0.01 ± 3.656 | 0.01 ± 1.0389 | 0.01 ± 1.228 | 0.01 ± 0.000 | 0.01 ± 0.004 |
| PG 32:0 | 2.10 ± 0.047 | 2.18 ± 0.001 | 2.07 ± 0.012 | 2.43 ± 0.037 | 2.06 ± 0.005 | 3.19 ± 0.053 * |
| PG 32:1 | 2.21 ± 0.050 | 2.26 ± 0.188 | 1.86 ± 0.039 | 2.30 ± 0.015 | 2.23 ± 0.186 | 3.32 ± 0.296 * |
| PG 32:2 | 0.27 ± 0.007 | 0.24 ± 0.002 | 0.19 ± 0.004 | 0.23 ± 6.767 | 0.24 ± 0.043 * | 0.27 ± 0.037 |
| PG 33:1 | 0.07 ± 0.004 | 0.09 ± 9.740 | 0.07 ± 2.156 | 0.07 ± 1.903 | 0.07 ± 0.007 | 0.09 ± 0.002 * |

**Table 2.** *Cont.*

| Phospholipid Species | Growth Control | AgNPs | Ciprofloxacin | Gentamicin | AgNPs + C | AgNPs + G |
|---|---|---|---|---|---|---|
| PG 34:1 | 14.09 ± 0.792 | 16.32 ± 6.014 | 14.10 ± 0.392 | 15.66 ± 1.571 | 14.69 ± 0.834 | 17.50 ± 1.027 |
| PG 34:2 | 6.95 ± 0.495 | 7.61 ± 0.051 | 5.75 ± 0.003 | 6.53 ± 0.036 | 7.06 ± 0.078 | 8.37 ± 1.129 |
| PG 36:2 | 0.75 ± 0.012 | 0.86 ± 3.481 | 0.75 ± 0.000 | 0.81 ± 4.572 | 0.83 ± 0.054 | 0.87 ± 0.093 |

### *2.4. Changes in the Fatty Acid Content in the Tested Bacterial Strains in the Presence of AgNPs, the Antibiotics or a Combination of the Agents*

The results obtained for *S. aureus* (Table 3) revealed the presence of 14 types of fatty acids in the samples. The most numerous fractions in the biotic control were a-15:0 and 18:0 with the contents of 40.98% and 15.68%, respectively. The least numerous fractions were 15:0 and 14:0, where the percentage content was below 1%. The most visible changes in the content of fatty acids caused by the presence of the tested antimicrobials were observed in the case of 18:0. Here, the presence of AgNPs and antibiotics alone or in combination caused an increase in the quantity of this fatty acid type and gentamicin used alone showed the most significant effect. On the other hand, the tested antimicrobials caused a decrease in the content of a-15:0 fatty acid, and again gentamicin exhibited the strongest effect.

The investigation of the fatty acid profile of *P. aeruginosa* resulted in the detection of nine fatty acid species (Table 4). Here, the most numerous classes were 16:0 and 18:1, with percentage contents of 40.84% and 36.19%, respectively. The least numerous groups were the i-12:0 and 8:0 fatty acids. The most visible effect of the tested antimicrobials on the content of fatty acids was observed in the 16:1 species. Here, in the presence of AgNPs combined with gentamicin, the content of fatty acids increased by about 17% compared to the biotic control.

**Table 3.** The influence of tested antimicrobials dosed alone and in combination on fatty acid profile of *S. aureus* ATCC 29213. Antimicrobials were used in following concentrations: 3.125 µg/mL of AgNPs, 0.0625 µg/mL of ciprofloxacin, and 0.0078 µg/mL of gentamicin. Asterisk * ($p < 0.05$) indicates values that differ significantly from the control.

| Fatty Acid Species | Growth Control | AgNPs | Ciprofloxacin | Gentamicin | AgNPs + C | AgNPs + G |
|---|---|---|---|---|---|---|
| i-14:0 | 1.13 ± 0.003 | 1.22 ± 0.007 | 0.60 ± 0.138 | 1.41 ± 0.820 | 1.31 ± 0.108 | 1.23 ± 0.091 |
| 14:0 | 0.94 ± 0.016 | 1.59 ± 0.374 | 1.29 ± 0.400 | 1.99 ± 0.398 * | 1.53 ± 0.086 * | 1.33 ± 0.338 |
| i-15:0 | 8.760.301 | 8.16 ± 0.206 | 7.66 ± 0.921 | 7.68 ± 0.198 | 7.89 ± 0.003 | 8.80 ± 1.218 |
| a-15:0 | 40.98 ± 2.523 | 35.51 ± 1.320 | 37.66 ± 1.482 | 34.27 ± 4.277 * | 36.81 ± 0.543 | 37.34 ± 3.455 |
| 15:0 | 0.82 ± 0.003 | 0.78 ± 0.057 | 0.85 ± 0.020 | 0.92 ± 0.006 | 0.86 ± 0.019 | 0.83 ± 0.031 |
| 16:0 | 1.08 ± 0.007 | 1.21 ± 0.081 | 1.08 ± 0.077 | 1.12 ± 0.014 | 1.15 ± 0.093 | 1.15 ± 0.021 |
| i-17:0 | 3.44 ± 0.064 | 3.42 ± 0.357 | 2.98 ± 0.186 | 2.92 ± 0.029 | 3.14 ± 0.180 | 3.67 ± 0.494 |
| a-17:0 | 9.80 ± 0.434 | 8.83 ± 0.433 | 8.99 ± 0.113 | 8.29 ± 0.554 | 8.67 ± 0.679 | 9.58 ± 0.880 |
| 17:0 | 2.08 ± 0.001 | 1.97 ± 0.261 | 2.06 ± 0.204 | 2.08 ± 0.021 | 2.00 ± 0.010 | 1.95 ± 0.065 |
| 18:0 | 15.68 ± 7.019 | 25.78 ± 0.903 * | 22.66 ± 0.100 | 28.44 ± 5.714 * | 24.15 ± 1.237 | 21.10 ± 6.358 |
| i-19:0 | 2.18 ± 0.004 | 1.82 ± 0.361 | 1.88 ± 0.275 | 1.58 ± 0.186 * | 1.87 ± 0.175 | 2.08 ± 0.189 |
| a-19:0 | 4.95 ± 1.998 | 3.85 ± 0.609 | 4.53 ± 0.848 | 3.63 ± 0.482 * | 4.01 ± 0.359 | 4.34 ± 0.300 |
| 19:0 | 4.13 ± 0.032 | 3.07 ± 0.478 | 3.90 ± 0.793 | 2.89 ± 0.696 * | 3.45 ± 0.278 | 3.28 ± 0.095 |
| 20:0 | 4.05 ± 0.044 | 2.78 ± 0.289 * | 3.87 ± 1.089 | 2.76 ± 0.523 * | 3.16 ± 0.391 | 3.34 ± 0.168 |

**Table 4.** The influence of the tested antimicrobials dosed alone and in combination on the fatty acid profile of *P. aeruginosa* ATCC 27853. Antimicrobials were used at the following concentrations: 0.78 µg/mL of AgNPs, 0.0313 µg/mL of ciprofloxacin, and 0.125 µg/mL of gentamicin. Asterisk * ($p < 0.05$) indicates values that differ significantly from the control.

| Fatty Acid Species | Growth Control | AgNPs | Ciprofloxacin | Gentamicin | AgNPs + C | AgNPs + G |
|---|---|---|---|---|---|---|
| 8:0 | 0.24 ± 0.093 | 0.17 ± 0.007 | 0.35 ± 0.058 | 0.20 ± 0.036 | 0.219 ± 4.276 | 0.24 ± 0.048 |
| i-12:0 | 0,00 ± 0.000 | 0.06 ± 0.088 | 0.14 ± 0.191 | 0.16 ± 0.027 * | 0.17 ± 0.003 * | 0.00 ± 0.000 |
| 14:0 | 1.43 ± 0.217 | 1.23 ± 0.191 | 1.27 ± 0.160 | 1.12 ± 0.044 | 1.24 ± 0.122 | 1.34 ± 0.033 |
| i-15:0 | 0.46 ± 0.014 | 0.46 ± 0.033 | 0.41 ± 0.015 | 0.41 ± 0.025 | 0.43 ± 0.033 | 0.45 ± 0.012 |
| 16:0 | 40.84 ± 0.0460 | 40.26 ± 1.345 | 42.40 ± 1.046 | 41.65 ± 0.586 | 40.27 ± 0.496 | 40.16 ± 0.570 |
| 16:1 | 8.83 ± 0.273 | 9.62 ± 1.071 | 7.78 ± 0.861 | 8.87 ± 0.604 | 9.25 ± 0.410 | 10.32 ± 0.508 |
| 17:0 | 0.61 ± 0.143 | 0.52 ± 0.006 | 0.61 ± 0.081 | 0.48 ± 0.014 | 0.55 ± 0.021 | 0.57 ± 0.029 |
| 18:0 | 11.39 ± 2.360 | 7.10 ± 0.414 | 11.48 ± 2.075 | 7.82 ± 1.3120 | 9.02 ± 0.374 | 8.87 ± 0.573 |
| 18:1 | 36.19 ± 2.600 | 39.69 ± 0.274 | 35.57 ± 2.765 | 39.29 ± 1.390 | 38.86 ± 0.466 | 38.06 ± 0.757 |

## 3. Discussion

AgNPs are one of the most investigated metallic nanoparticles and have gained significant attention not only in the field of biomedicine but also in other industries. One of the most crucial properties of AgNPs is their strong inhibitory activity against a broad spectrum of microorganisms. The use of AgNPs is, therefore, considered as a potentially promising substitute for conventional antibiotic therapy [6,7]. The latter option outweighs the conventional routes, for example by providing reducing, stabilizing, and capping agents in the form of metabolites characteristic for the organism species used in the process. Mycogenic AgNPs are proven to be coated with the protein layer which can positively affect the functionality of nanoparticles [25]. However, there are still unresolved doubts concerning the potential toxicity of AgNPs. It has been reported that AgNPs can damage mammalian cells via the impairment of mitochondrial functionality, increases in cell membrane permeability, or the generation of reactive oxygen species [4,26]. Additionally, the still ongoing emergence of antimicrobial resistance towards available therapeutics has become a worldwide challenge. One of the proposed solutions involves combining nanoparticles with drugs [27]. AgNPs have been proven to enhance the activity of various antibiotics against different groups of microorganisms either in a synergistic or additive manner. According to the above, it seems possible to reduce the need for high antibiotic concentrations while lowering the effective dosages of AgNPs [4,28].

In our previous study, the antimicrobial activity of mycogenic AgNPs synthesized using *G. striatum* post-culture liquid was evaluated. It was found that AgNPs synthesized in the conditions of 4 °C without shaking were the most suitable for further investigation. The established minimal inhibitory concentration (MIC) values of this particular AgNP type were 12.5 µg/mL and 1.56 µg/mL for *S. aureus* and *P. aeruginosa*, respectively. Interestingly, these AgNPs did not cause a severe cytotoxic effect on the tested human fibroblast cell line in the concentration corresponding to the MIC value in *P. aeruginosa* [8]. The present research showed that combining *G. striatum*-derived AgNPs with ciprofloxacin or gentamicin enhanced the activity of the tested antimicrobials against *S. aureus* and *P. aeruginosa* compared to their effectiveness when used alone. For example, 3.125 µg/mL of AgNPs combined with 0.125 µg/mL of gentamicin completely inhibited the growth of *S. aureus*, thus reducing the effective dose of AgNPs by four times. In *P. aeruginosa*, 0.39 µg/mL of AgNPs mixed with 0.5 µg/mL of gentamicin caused almost 90% inhibition of bacterial

growth compared to the biotic control. Wang et al. [29] showed that the activity of chemically synthesized AgNPs against *S. aureus* was also enhanced in the presence of gentamicin. They concluded that a possible explanation for this phenomenon could be the increased dissolution of AgNPs or the facilitated interactions of nanoparticles with the cell surface in the presence of the antibiotic. Combining AgNPs with ciprofloxacin was most effective in the case of *S. aureus*. Here, the activity of AgNPs at a concentration of 3.125 μg/mL was increased twofold when mixed with 0.125 μg/mL of antibiotic compared to the effectiveness of AgNPs used alone. The synergistic effect between biogenic AgNPs and ciprofloxacin against *S. aureus* was proven by Bhat et al. Using the disc diffusion method, they observed that combining a 5 μg/disc of the antibiotic and a 20 μg/disc of AgNPs caused the zones of growth inhibition to increase two times when compared to the effect caused by AgNPs acting alone [30]. When it came to *P. aeruginosa*, only a slight enhancement of AgNP activity in the presence of ciprofloxacin was observed, as 0.0313 μg/mL and 0.0625 μg/mL of the antibiotic mixed with each tested concentration of AgNPs enhanced their effectiveness by about 40% and no differences in the effectiveness of various AgNP concentrations were observed. Our findings are in opposition to the results of Nikparast and Saliani [31]. In their study, the potential synergistic effect of plant-derived AgNPs originating from *Amaranthus retroflexus* combined with ciprofloxacin was evaluated towards the human pathogenic bacteria *P. aeruginosa* and plant pathogenic bacteria *P. syringae*. They found that in the presence of 6.25, 12.5, and 25 μg/mL of AgNPs, the MIC value of ciprofloxacin was reduced from 0.125 μg/mL to 0.0625 μg/mL in the case of *P. aeruginosa* and from 0.25 μg/mL to 0.0625 μg/mL in *P. syringae*. Based on their results, it can be concluded that *G. striatum*-derived AgNPs used alone showed 16 times better efficacy against *P. aeruginosa* compared to nanoparticles synthesized using *A. retroflexus* leaf extract [8,31].

Microbial biofilms are communities of microorganisms that possess the ability to adhere to different surfaces. Bacteria forming biofilms are surrounded by an extracellular matrix consisting of self-secreted compounds. This envelope forms a hospitable environment and protects cells from external stresses, such as antimicrobial agents [32,33]. Antimicrobial resistance can be as much as 1000 times stronger in biofilm forms compared to in planktonic bacteria and, as such, is one of the major concerns in contemporary healthcare [34,35]. It has been reported that AgNPs are able to successfully suppress biofilm formation [36]. In our study, the antibiofilm activity of AgNPs, ciprofloxacin, gentamicin, or a combination of the agents was evaluated against *S. aureus* and *P. aeruginosa*. The *S. aureus* strain used in our study was classified as a weak biofilm former [37]. The *P. aeruginosa* strain had been previously described as a strong biofilm former [8]. It was found that combining AgNPs with both tested antibiotics was effective against *S. aureus* and that the inhibitory activity towards bacterial biofilm of the tested antimicrobials was enhanced. Our results revealed that, in the case of *P. aeruginosa*, the inhibitory effect on biofilm production caused by the tested antimicrobials was weaker. When mixed with 0.0625 μg/mL of ciprofloxacin, 0.049 μg/mL of AgNPs caused biofilm reduction of about 60% compared to the biotic control. In the case of mixing AgNPs with gentamicin, no inhibitory effect was observed. We discovered, however, that AgNPs in the tested concentrations caused a stimulatory effect on biofilm formation, and the same phenomenon was observed when AgNPs were used in combination with gentamicin. According to our previous study, the AgNP concentration of 0.78 μg/mL had a sublethal effect on *P. aeruginosa*, as the established MIC value was equal to 1.56 μg/mL [8]. Therefore, it can be concluded that the concentrations of AgNPs that do not inhibit bacterial growth completely can increase biofilm formation by *P. aeruginosa*. Similar results were obtained by Yu and Alvarez, who found that a mixed microbial culture from a wastewater treatment plant after exposition to sublethal concentrations of AgNPs showed enhancement in the biofilm formation of up to six times compared to

the biotic control. The same effect was observed in their study on the *P. aeruginosa* PAO1 strain. The authors suggested that the promotion of biofilm formation could be a bacterial defense mechanism against AgNPs' toxicity [38]. Moreover, in the study cinducted by Kumar et al. (2024) it was found that sub-MIC concentrations of gentamicin led to an increase in biofilm formation in two environmental isolates of *P. aeruginosa*. Their research showed that during antibiotic treatment, the contents of the biofilm-forming components, such as exoproteins, eDNA, exolipids, and exopolymeric substances, were higher than in the control samples without the addition of gentamicin. Therefore, it can be concluded that, under the stress of antimicrobial treatment, *P. aeruginosa* is able to develop additional defense mechanisms, leading to increased biofilm formation [39]. Contrastingly, it was also stated in the other study that the effect of sub-MIC concentrations of AgNPs enhancing biofilm-forming activity was not detected in *S. aureus* [40]. On the other hand, in another study, it was reported that the presence of ciprofloxacin could stimulate the production of bacterial biofilm in *S. aureus* [41]. Nevertheless, this phenomenon was not observed in our study, neither when ciprofloxacin was applied alone nor in combination with nanoparticles.

The bacterial cell membrane is mainly composed of phospholipids, whose major function is the formation of a semipermeable barrier. Phospholipids are also involved in some cellular processes. One of the most important tasks fulfilled by membrane phospholipids is adaptation to inhospitable environment conditions. Thus, changes in the membrane phospholipids' content and composition can be an important factor of environmental stress [42,43]. In *S. aureus*, polar phosphatidylglycerol (PG) and lysyl-phosphatidylglycerols (L-PG) are among the most numerous fractions of lipids forming cell membranes. An increased L-PG content shows a protective effect, as the modification of PG with lysine reduces the negative charge of the membrane. This effect leads to the increased electrostatic repulsion of positively charged extracellular compounds [42]. Our results showed that the amount of L-PG increased when AgNPs, gentamicin, or a combination of AgNPs and ciprofloxacin were used. Thus, the occurrence of the process of adaptation to suboptimal growth conditions in *S. aureus* was revealed. In *P. aeruginosa*, commonly present groups of phospholipids are PG and phosphatidylethanolamines (PE). PE and PG constitute the majority of phospholipids forming the cell membrane [44]. Our results regarding the composition of *P. aeruginosa* membrane phospholipids are in agreement with the above data. Fatty acids determine the biophysical properties of cell membranes. In *S. aureus*, bacterial membranes are formed by straight-chained and branch-chained saturated fatty acids [45,46]. This phenomenon was confirmed by our study, where all of the detected *S. aureus* fatty acids were saturated. Branch-chained acids accounted for about 71% of all fatty acids in growth controls, while straight-chained acids constituted the remaining 29%. Straight-chained acids form a thick bilayer with low permeability, while branch-chained fatty acids, including iso- and anteiso- methyl forms, enhance the fluidity of the membrane [46]. In *P. aeruginosa*, unsaturated and saturated straight-chained fatty acids were detected, alongside branch-chained ones. The difference between the Gram-positive and Gram-negative bacterial strains found in our study is in the agreement with the available literature data. Dubois-Brisonnet et al. revealed that in planktonic forms of *S. aureus*, the iso-branch-chained fatty acids and anteiso-branch-chained fatty acids classes constituted about 70% of all fatty acids detected. There were no unsaturated fatty acids found. These data agree with the results obtained in our study. In the same study, unsaturated fatty acids were detected in *P. aeruginosa*. However, these species constituted more than a half of the whole fatty acid content, which is in slight disagreement with our results [47].

Our research revealed that changes in the contents of fatty acid species in the presence of AgNPs and antibiotics were different among the tested bacterial strains. In *S. aureus*,the two most numerous fatty acid species, octadecanoic acid (18:0) and iso-pentadecanoic acid,

changed in the presence of all of the tested antimicrobials alone or in combination. The content of straight-chained acid increased by as much as 80% compared to the biotic control in the case of gentamicin used alone. Contrastingly, the amount of branch-chained fatty acid decreased by more than 15% in the same samples. Similar changes were also observed in less numerous fatty acid species. These results indicated that the presence of the tested antimicrobials promoted changes in the bacterial membrane composition, resulting in decreased permeability and fluidity. Indeed, changing the properties of the cell membrane into a more solid state is considered to be a resistance response of *S. aureus* to antibiotic stress [48]. The results regarding *P. aeruginosa* fatty acid composition showed that saturated acids constituted up to 55% of all species, while unsaturated and branch-chained acids accounted for the remaining amount, while the branch-chained acids were present only in trace quantities in the biotic controls. The addition of AgNPs, ciprofloxacin, gentamicin, or a combination of the antimicrobials caused changes in the amounts of the second and third most numerous fatty acid fractions, namely saturated octadecanoic acid (18:0) and unsaturated oleic acid (18:1), respectively. The content of saturated acids decreased in the presence of every antimicrobial used except for ciprofloxacin applied alone. Contrastingly, the amount of oleic acid increased in the same cases. An increased content of unsaturated fatty acids was also observed in the case of palmitoleic acid (16:1) in *P. aeruginosa* samples incubated with AgNPs alone, AgNPs mixed with ciprofloxacin and AgNPs mixed with gentamicin. The changes in the saturated and unsaturated fatty acid contents are a primary mechanism of Gram-negative bacteria to regulate cell membrane fluidity [48]. Based on our results, it can, therefore, be concluded that the tested antimicrobials enhanced fluidity of the *P. aeruginosa* cell membrane.

The fatty acid profile structure can be a factor impacting biofilm-forming activity. In general, bacterial cells forming biofilm contain higher amounts of saturated acids compared to planktonic cells [47,49]. It has been found that lower membrane fluidity enhances biofilm-forming activity, which can be considered as a stress response [50,51]. However, our results on *P. aeruginosa* showed an increase in cell membrane fluidity in the presence of AgNPs and the tested antibiotics. It is speculated that this phenomenon can negatively impact the development of biofilm by disturbing the stability of the biofilm form during the formation process [52].

## 4. Materials and Methods

### *4.1. Materials*

The tested bacterial strains were purchased from the American Type Culture Collection (ATCC, USA). AgNPs of mycological origin were obtained with the use of *Gloeophyllum striatum* DSM 9592 post-culture liquid in the manner described previously [8]. In this study, nanoparticles synthesized at 4 °C without shaking were used. The tested antibiotics, namely ciprofloxacin and gentamicin, were purchased from Merck (Darmstadt, Germany). The Mueller–Hinton broth (BBL$^{TM}$) medium was obtained from Becton Dickinson (Warsaw, Poland). Acetic acid and cristal violet came from Chempur (Piekary Slaskie, Poland).

### *4.2. Antimicrobial Activity of AgNPs, Antibiotics and Agents Combined*

The assessment of the antimicrobial activity of AgNPs, ciprofloxacin, gentamicin, or a combination of the agents was performed using the microdilution method according to the Clinical and Standard Laboratory Institute (CSLI) guidelines M07 (11th Edition) towards two reference bacterial strains, namely Gram-positive *S. aureus* ATCC 29213 and Gram-negative *P. aeruginosa* ATCC 27853. The differences in growth of the tested bacterial strains in the presence of selected antimicrobial agents were examined in 96-well cell culture plates in Mueller–Hinton broth medium. The final concentration range of the tested antibiotics

was 0–8 μg/mL in both experimental variants and the final concentration ranges of AgNPs were 0–12.5 μg/mL and 0–1.56 μg/mL for *S. aureus* and *P. aeruginosa*, respectively. A bacterial inoculum was prepared in Mueller–Hinton broth medium and added to each cell, where the final bacterial density reached 5 × 105 colony-forming units (CFU)/mL. Adequate abiotic controls were also prepared. All the samples and abiotic controls were then incubated for 24 h at 37 °C. After incubation, the OD was measured at a wavelength of 630 nm using a MultiskanTM FC Microplate Photometer (Thermo Fisher Scientific, Pudong, Shanghai, China). Based on the primary results, the selected variants were further analyzed. The selected concentrations of the tested antimicrobials were distributed as follows: 0.195, 0.78, and 3.125 μg/mL of AgNPs, 0.0313, 0.0625, and 0.125 μg/mL of ciprofloxacin and gentamicin in the case of *S. aureus*, and 0.049, 0.195, 0.39, and 0.78 μg/mL of AgNPs, 0.0156, 0.0313, and 0.0625 μg/mL of ciprofloxacin, and 0.125, 0.25, and 0.5 μg/mL of gentamicin in the case of *P. aeruginosa*.

### *4.3. Antibiofilm-Forming Activity of AgNPs, Antibiotics and Agents Combined*

The ability of the tested antimicrobials to inhibit bacterial biofilm formation by *S. aureus* and *P. aeruginosa* was determined in the same experiment schemes as described in the previous paragraph. The assessment of changes in the biofilm formation was performed with the use of crystal violet solution according to the method described in our previous research [8].

### *4.4. Changes in the Phospholipid Profiles and Fatty Acid Contents in the Tested Bacteria in the Presence of AgNPs, the Antibiotics, or a Combination of the Agents*

The phospholipid and fatty acid profile analysis was performed in the selected concentration of antibiotics and AgNPs for both tested bacterial strains. For *S. aureus*, the concentration of AgNPs was 3.125 μg/mL and the concentrations of antibiotics were 0.0625 μg/mL and 0.0078 μg/mL for ciprofloxacin and gentamicin, respectively. For *P. aeruginosa*, the tested AgNP concentration was equal to 0.78 μg/mL, while the concentrations of antibiotics were 0.0313 μg/mL and 0.125 μg/mL for ciprofloxacin and gentamicin, respectively. All tested samples and biotic and abiotic controls were prepared in Mueller–Hinton broth medium and incubated for 24 h at 37 °C. After the incubation period, the tested samples and biotic controls were transferred to 50 mL falcon tubes and centrifuged at 20 °C/8000 rpm/6 min. Then, the supernatant was removed, and 100 mg of obtained biomass from each sample was placed in 2 mL Eppendorf tubes. Every tube was then filled with 1 mL of methanol and glass pearls and homogenized on a Fast-Prep-24 h Instrument (MP Biomedicals, Eschwege, Germany). When the process ended, all the samples were again centrifuged for 4 min/7000 rpm at room temperature and the methanolic phases were transferred to new tubes.

Phospholipid analysis was carried out using LC-MS/MS (ExionLC AC UHPLC system (Sciex, Framingham, MA, USA)) with a 4500 Q-TRAP mass spectrometer (Sciex, USA). The obtained lipid extract was first fractionated using the UHPLC system. For this, 10 μL of the extract was injected into a Kinetex C18 column (50 mm × 2.1 mm, particle size: 5 μm; Phenomenex, Torrance, CA, USA) at a flow rate of 500 μL $min^{-1}$ [53]. The column temperature was maintained at 40 °C. The mobile phases used were water (A) and methanol (B), both of which were supplemented with 5 mM ammonium formate. The solvent elution started from 70% B and was then increased to 95% B over 1.25 min and maintained at 95% B for 6 min before returning to 70% B over 3 min. The mass spectrometric analysis was carried out using a mass spectrometer equipped with an electrospray ionization (ESI) source, under the following conditions: spray voltage −4500 V, curtain gas 25 psi, nebulizer gas 50 psi, turbo gas 60 psi, and ion source temperature 600 °C. Data analysis was performed using the Analyst™ v1.6.3 software (Sciex, USA). The qualitative and quantitative analyses were

carried out according to the methods described previously [53] with the use of multiple reaction monitoring (MRM) pairs.

Fatty acids were analyzed according to the method described earlier [54]. A lipid sample was diluted in 1.5 mL methanol and transferred to a screw-capped glass test tube. To this lipid solution, 0.2 mL toluene and 0.3 mL HCl solution (8.0%) were added [55]. The tube was vortexed and then incubated overnight at 45 °C. After cooling to room temperature, 1 mL hexane and 1 mL water (deionized) were added for the extraction of fatty acid methyl esters (FAMEs). The tube was vortexed, and 0.3 mL of the hexane layer was moved to the chromatographic vial. The FAMEs analysis was conducted with an Agilent Model 7890 gas chromatograph connected to a 5975C mass detector (Agilent, Santa Clara, CA, USA). Helium and a capillary column of HP 5 MS methyl polysiloxane (30 m × 0.25 mm i.d. × 0.25 mm ft) were applied. The temperature of the column was maintained at 60 °C for 3 min, then increased to 212 °C at a rate of 6 °C $min^{-1}$, followed by an increase to 245 °C at a rate of 2 °C $min^{-1}$, and, finally, to 280 °C at a rate of 20 °C $min^{-1}$, at which it was held for 10 min. Split injection of the injection port at 250 °C was employed. Fungal fatty acids were identified by comparison with authenticated reference standards (Sigma, Darmstadt, Germany, Supelco, Darmstadt, Germany).

### 4.5. Statistical Analysis

All experiments presented in this study were performed in four replicates ($n = 4$). The results of the examination of phospholipid and fatty acid contents were analyzed using a one-way ANOVA test with * $p < 0.05$ in order to estimate the statistical significance. During the analysis of the results of antimicrobial and antibiofilm activity, a one-way ANOVA test was applied in the case of an antimicrobial agent used alone, and a two-way ANOVA test was employed for the evaluation of the results obtained when antimicrobials were combined. Both tests were performed with * $p < 0.05$. The estimations and all calculations were carried out by using Excel, Microsoft® Office 2021 (Microsoft Corporation, Redmont, WA, USA). The results shown in the figures and tables are expressed as the average values with the standard deviation (SD).

## 5. Conclusions

Our research showed that the tested mycogenic AgNPs and the antibiotics ciprofloxacin and gentamicin can perform synergistic action towards two pathogenic bacterial strains, namely *S. aureus* and *P. aeruginosa*. It was also revealed that selected antimicrobials possessed antibiofilm-forming activity that was enhanced by a simultaneous application of AgNPs and an antibiotic in almost all variants of the experiment. However, a more significant increase in antimicrobial and antibiofilm-forming efficacy of the tested antimicrobials was detected in the case of *S. aureus*, where a total inhibition of bacterial growth and biofilm formation was reached. The analysis of phospholipid and fatty acid profiles revealed differences in the response to unhospitable growth conditions in the tested bacteria caused by the presence of AgNPs and antibiotics. More vivid changes in the cell membrane properties were observed again in *S. aureus*, alongside with the highest susceptibility to the tested antimicrobials. The observed effect of the tested antimicrobials towards *P. aeruginosa* did not lead to the complete inhibition of bacterial cell growth. Moreover, it was revealed that the sublethal concentrations of AgNPs and antibiotics stimulated bacterial biofilm formation by the tested strain. Additionally, the changes in the phospholipid and fatty acid contents in the case of *P. aeruginosa* only slightly indicated the enhancement of cell membrane fluidity. Given these facts, it can be concluded that in *P. aeruginosa*, an increased biofilm-forming activity is the first defense mechanisms to the action of the tested antimicrobials. Thus, combining AgNPs with the commonly used antibiotics ciprofloxacin and gentamicin can

be an effective way to enhance their antimicrobial action and to lower the sufficient doses of the antimicrobials. However, the effectiveness of this method can differ among various bacterial strains, where an increase in the antimicrobial activity does not correlate with a complete inhibitory effect on bacterial growth. Moreover, the revealed stimulatory effect of AgNPs and their combinations with antibiotics on *P. aeruginosa* biofilm formation should attract more attention, as the mentioned phenomenon can pose an additional threat in the field of healthcare.

**Author Contributions:** Conceptualization, A.T. and K.N.; methodology, K.N., P.B. and A.T.; investigation, A.T., K.N. and P.B.; manuscript—original draft preparation, A.T.; manuscript—review and editing, K.N. and K.L. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the University of Lodz, Department of Industrial Microbiology and Biotechnology internal funds (B2411000000036.01).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Dataset available on request from the authors.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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## IX.2. Oświadczenia współautorów o udziale w publikacjach

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**Tończyk Aleksandra**, Niedziałkowska Katarzyna, Lisowska Katarzyna. 2023. *Optimizing the microbial synthesis of silver nanoparticles using Gloeophyllum striatum and their antimicrobial potential evaluation*, Scientific Reports, 13: 21124; DOI: 10.1038/s41598-023-48414-9

*Oświadczam, że mój udział w ww. pracy wynosi 80%, który obejmował współudział w opracowaniu koncepcji badań, zaplanowanie i realizację doświadczeń dotyczących optymalizacji biosyntezy nanocząstek srebra, oceny aktywności przeciwbakteryjnej, przeciwbiofilmowej, hemolitycznej i cytotoksycznej uzyskanych nanocząstek srebra, analizę statystyczną uzyskanych wyników, opracowanie manuskryptu, w tym opis wstępu, materiałów i metod, wyników, dyskusji i wniosków końcowych, opracowanie graficzne wyników, zebranie danych literaturowych, edycja tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

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**Tończyk Aleksandra**, Niedziałkowska Katarzyna, Nowak-Lange Marta, Bernat Przemysław, Lisowska Katarzyna. 2025. *Mycogenic silver nanoparticles: promising antimicrobials with fungistatic properties.* International Journal of Molecular Sciences, 26: 6639; DOI: 10.3390/ijms26146639

*Oświadczam, że mój udział w ww. pracy wynosi 70%, który obejmował współudział w opracowaniu koncepcji badań, zaplanowanie i realizację doświadczeń dotyczących oceny aktywności przeciwgrzybowej uzyskanych nanocząstek srebra oraz oceny zmian w płynności i przepuszczalności błony komórkowej drożdży C. albicans, jakościowej oraz ilościowej analizie chromatograficznej, analizie i interpretacji otrzymanych wyników, analizę statystyczną uzyskanych wyników, opracowanie manuskryptu, w tym opis wstępu,*

*materiałów i metod, wyników, dyskusji i wniosków końcowych, opracowanie graficzne wyników, zebranie danych literaturowych, edycja tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

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**Tończyk Aleksandra**, Niedziałkowska Katarzyna, Lisowska Katarzyna. 2025. *Ecotoxic effect of mycogenicsilver nanoparticles in water and soil environment*. Scientific Reports, 15: 10815; DOI: 10.1038/s41598-025-95485-x

*Oświadczam, że mój udział w ww. pracy wynosi 80%, który obejmował współudział w opracowaniu koncepcji badań, zaplanowanie i realizację doświadczeń dotyczących oceny potencjału ekotoksycznego uzyskanych nanocząstek srebra, analizę statystyczną uzyskanych wyników, opracowanie manuskryptu, w tym opis wstępu, materiałów i metod, wyników, dyskusji i wniosków końcowych, opracowanie graficzne wyników i abstraktu graficznego, zebranie danych literaturowych, edycja tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

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**Tończyk Aleksandra**, Niedziałkowska Katarzyna, Bernat Przemysław, Lisowska Katarzyna. 2025. *Synergistic activity of Gloeophyllum striatum-derived AgNPs with ciprofloxacin and gentamicin against human pathogenic bacteria.* International Journal of Molecular Sciences, 26: 3529; DOI: 10.3390/ijms26083529

*Oświadczam, że mój udział w ww. pracy wynosi 75%, który obejmował współudział w opracowaniu koncepcji badań, zaplanowanie i realizację doświadczeń dotyczących oceny działania synergistycznego uzyskanych nanocząstek srebra z wybranymi antybiotykami, w tym aktywności przeciwbakteryjnej i przeciwbiofilmowej, jakościowej oraz ilościowej analizie chromatograficznej, analizie i interpretacji otrzymanych wyników, analizę*

*statystyczną uzyskanych wyników, opracowanie manuskryptu, w tym opis wstępu, materiałów i metod, wyników, dyskusji i wniosków końcowych, opracowanie graficzne wyników, zebranie danych literaturowych, edycja tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

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**Prof. dr hab. Katarzyna Lisowska**
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Tończyk Aleksandra, Niedziałkowska Katarzyna, **Lisowska Katarzyna**. 2023. *Optimizing the microbial synthesis of silver nanoparticles using Gloeophyllum striatum and their antimicrobial potential evaluation*, Scientific Reports, 13: 21124; DOI: 10.1038/s41598-023-48414-9

*Oświadczam, że mój udział w ww. pracy wynosi 10%, który obejmował współudział w opracowaniu koncepcji badań, ocenę postępów pracy, edycję tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

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Tończyk Aleksandra, Niedziałkowska Katarzyna, Nowak-Lange Marta, Bernat Przemysław, **Lisowska Katarzyna**. 2025. *Mycogenic silver nanoparticles: promising antimicrobials with fungistatic properties.* International Journal of Molecular Sciences, 26: 6639; DOI: 10.3390/ijms26146639

*Oświadczam, że mój udział w ww. pracy wynosi 10%, który obejmował współudział w opracowaniu koncepcji badań, ocenę postępów pracy, edycję tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

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Tończyk Aleksandra, Niedziałkowska Katarzyna, **Lisowska Katarzyna**. 2025. *Ecotoxic effect of mycogenicsilver nanoparticles in water and soil environment*. Scientific Reports, 15: 10815; DOI: 10.1038/s41598-025-95485-x

*Oświadczam, że mój udział w ww. pracy wynosi 10%, który obejmował współudział w opracowaniu koncepcji badań, ocenę postępów pracy, edycję tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

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Tończyk Aleksandra, Niedziałkowska Katarzyna, Bernat Przemysław, **Lisowska Katarzyna**. 2025. *Synergistic activity of Gloeophyllum striatum-derived AgNPs with ciprofloxacin and gentamicin against human pathogenic bacteria.* International Journal of Molecular Sciences, 26: 3529; DOI: 10.3390/ijms26083529

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…….

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*Oświadczam, że mój udział w ww. pracy wynosi 10%, który obejmował współudział w opracowaniu koncepcji badań, analizie i interpretacji otrzymanych wyników, edycję tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

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Tończyk Aleksandra, **Niedziałkowska Katarzyna**, Nowak-Lange Marta, Bernat Przemysław, Lisowska Katarzyna. 2025. *Mycogenic silver nanoparticles: promising antimicrobials with fungistatic properties.* International Journal of Molecular Sciences, 26: 6639; DOI: 10.3390/ijms26146639

*Oświadczam, że mój udział w ww. pracy wynosi 10%, który obejmował współudział w opracowaniu koncepcji badań, jakościowej oraz ilościowej analizie chromatograficznej, analizie i interpretacji otrzymanych wyników, edycję tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

…….

Tończyk Aleksandra, **Niedziałkowska Katarzyna**, Lisowska Katarzyna. 2025. *Ecotoxic effect of mycogenicsilver nanoparticles in water and soil environment*. Scientific Reports, 15: 10815; DOI: 10.1038/s41598-025-95485-x

*Oświadczam, że mój udział w ww. pracy wynosi 10%, który obejmował współudział w opracowaniu koncepcji badań, analizie i interpretacji otrzymanych wyników, edycję tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

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*Oświadczam, że mój udział w ww. pracy wynosi 10%, który obejmował współudział w opracowaniu koncepcji badań, jakościowej oraz ilościowej analizie chromatograficznej, analizie i interpretacji otrzymanych wyników, edycję tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

…….

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*Oświadczam, że mój udział w ww. pracy wynosi 5%, który obejmował współudział w jakościowej oraz ilościowej analizie chromatograficznej, analizie i interpretacji otrzymanych wyników oraz udział w przygotowaniu odpowiedzi do recenzentów.*

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Tończyk Aleksandra, Niedziałkowska Katarzyna, **Bernat Przemysław**, Lisowska Katarzyna. 2025. *Synergistic activity of Gloeophyllum striatum-derived AgNPs with ciprofloxacin and gentamicin against human pathogenic bacteria.* International Journal of Molecular Sciences, 26: 3529; DOI: 10.3390/ijms26083529

*Oświadczam, że mój udział w ww. pracy wynosi 5%, który obejmował współudział w jakościowej oraz ilościowej analizie chromatograficznej, analizie i interpretacji otrzymanych wyników oraz udział w przygotowaniu odpowiedzi do recenzentów.*

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Tończyk Aleksandra, Niedziałkowska Katarzyna, **Nowak-Lange Marta**, Bernat Przemysław, Lisowska Katarzyna. 2025. *Mycogenic silver nanoparticles: promising antimicrobials with fungistatic properties.* International Journal of Molecular Sciences, 26: 6639; DOI: 10.3390/ijms26146639

*Oświadczam, że mój udział w ww. pracy wynosi 5%, który obejmował współudział w jakościowej oraz ilościowej analizie chromatograficznej oraz analizie i interpretacji otrzymanych wyników.*

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