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POLSKIEJ AKADEMII NAUK

Wiktoria Maj

ROLA WŁAŚCIWOŚCI METABOLICZNYCH,
MORFOLOGICZNYCH I GENETYCZNYCH GRZYBÓW
NEOSARTORYA SPP. (*ASPERGILLUS* SPP.) W
KSZTAŁTOWANIU ICH ODPORNOŚCI NA ZWIĄZKI
KONSERWUJĄCE, CHEMICZNE ORAZ NATURALNE
EKSTRAKTY ROŚLINNE

*The role of the metabolic, morphological and genetic properties of
Neosartorya spp. (Aspergillus spp.) fungi in shaping their resistance to
preservatives, chemicals and natural
plant extracts*

Rozprawa doktorska
Doctoral thesis

Rozprawa doktorska przygotowana pod kierunkiem
Promotora: prof. dr hab. Magdaleny Frąc
oraz
Promotora pomocniczego: dr Giorgii Pertile

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OŚWIADCZENIE PROMOTORA ROZPRAWY

Oświadczam, że niniejsza rozprawa doktorska została przygotowana pod moim kierunkiem i stwierdzam, że spełnia ona warunki do przedstawienia jej w postępowaniu o nadanie stopnia naukowego.

13.06.2025

data

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12.06.2025

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Wiktoria Skoj

podpis autorki rozprawy

*Niniejszą pracę pragnę zadedykować mojej rodzinie,
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KSZTAŁTOWANIU ICH ODPORNOŚCI NA ZWIĄZKI KONSERWUJĄCE,
CHEMICZNE ORAZ NATURALNE EKSTRAKTY ROŚLINNE*



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Streszczenie

Polska należy do czołowych producentów owoców w Unii Europejskiej, odgrywając szczególnie istotną rolę w uprawie truskawek oraz innych owoców miękkich. Ze względu na wysokie walory smakowe, bogactwo składników odżywczych oraz szerokie zastosowanie w przetwórstwie, owoce te stanowią kluczowy element krajowego sektora rolno-spożywczego. Jednocześnie ich znaczna podatność na zakażenia mikrobiologiczne stanowi poważne wyzwanie i zagrożenie zarówno dla producentów, jak i konsumentów. Szczególne ryzyko w tym kontekście stwarzają grzyby z rodzaju *Neosartorya*, charakteryzujące się wyjątkową odpornością na niekorzystne czynniki środowiskowe, w tym wysoką temperaturę oraz obecność środków konserwujących. Gatunki tego rodzaju, będące formami teleomorficznymi grzybów z rodzaju *Aspergillus*, są zdolne do przetrwania procesów pasteryzacji, co może prowadzić do wtórnego skażenia produktów spożywczych, skutkując zarówno stratami ekonomicznymi, jak i zagrożeniem dla zdrowia konsumentów z powodu wytwarzania niebezpiecznych mykotoksyn.

Badania zawarte w niniejszej rozprawie podejmują temat charakterystyki właściwości morfologicznych, metabolicznych i genetycznych grzybów *Neosartorya* spp., które wpływają na mechanizmy przetrwania, aktywności oraz wrażliwości na substancje przeciwdrobnoustrojowe. W pracy istotna była także ocena wpływu tych substancji na mikrobiom i potencjał metaboliczny truskawek sztucznie zakażonych tymi grzybami w rzeczywistych warunkach przechowywania produktów roślinnych. W ramach badań w pierwszej kolejności oceniono skuteczność zarówno naturalnych ekstraktów roślinnych, jak i syntetycznych środków konserwujących wobec 20 izolatów, wykorzystując testy inhibicji wzrostu metodą dyfuzyjno-krażkową. Następnie dla 10 izolatów, wybranych w ramach pierwszego etapu badań, dokonano oceny wrażliwości z użyciem mikropłytek MT2 systemu Biolog™. Uzyskane wyniki potwierdziły zróżnicowaną skuteczność badanych substancji oraz wykazały różnice wrażliwości testowanych izolatów grzybów, wskazując na wyodrębnienie 5 grup izolatów o rosnącej podatności na substancje o właściwościach antymikrobiologicznych, pomimo ich bliskiego pokrewieństwa. Wszystkie testowane ekstrakty naturalne wykazywały działanie hamujące wzrost *Neosartorya* spp., choć to ekstrakty z lawendy, drzewa herbacianego i nagietka wykazały najsilniejsze działanie przeciwgrzybicze. Pirosiarczyn sodu, sorbinian potasu, wodorosiarczyn sodu oraz kwas sorbowy również wykazały działanie hamujące wzrost grzybów, przy czym najbardziej skuteczny okazał się pirosiarczyn sodu.

W ramach badania wrażliwości chemicznej *Neosartorya* spp., przeanalizowano reakcje metaboliczne na 120 związków chemicznych dostępnych w mikropłytkach PM 21–25 z wykorzystaniem systemu Biolog™. Wykazano, że większość testowanych związków stymulowała wzrost grzybów, przy czym po 96-godzinnej inkubacji wszystkie izolaty wykształciły zdolność adaptacji do warunków stresowych. Faza równowagi wzrostu została osiągnięta po około 144 godzinach, co wskazuje na efektywną aklimatyzację do obecności związków toksycznych. Największy przyrost biomasy odnotowano w obecności antybiotyków, anionów oraz związków modyfikujących funkcjonowanie błon komórkowych. Z kolei wyraźne właściwości hamujące wykazały m.in. cyjanian sodu, dwuwodny chlorek miedzi (II) i cis-platyna. Uzyskane dane potwierdzają znaczący potencjał adaptacyjny *Neosartorya* spp. do

zróznicowanych stresorów chemicznych. Zdolność ta może stanowić czynnik umożliwiający kolonizację środowisk o wysokim stopniu przetworzenia i obecności substancji konserwujących, co ma istotne implikacje dla kontroli mikrobiologicznej w przemyśle.

W kolejnych etapach badań scharakteryzowano potencjał metaboliczny wybranych izolatów *Neosartorya* spp. w warunkach optymalnego wzrostu oraz po ekspozycji na wybrane ekstrakty roślinne i konserwanty żywności, wykorzystując test mikromacierzy fenotypowych z wykorzystaniem mikroplitek Biolog™ FF. Uzyskane profile metaboliczne wykazały wysoką plastyczność metaboliczną badanych izolatów oraz zdolność do wykorzystania różnorodnych źródeł węgla, w tym związków charakterystycznych dla substratów owocowych. Reakcja grzybów na stres w obecności ekstraktu z nagietka charakteryzowała się zmniejszonym zużyciem aminokwasów i węglowodanów oraz zwiększonym wykorzystaniem kwasów karboksylowych na płytkach FF. Po ekspozycji grzybów na konserwanty spożywcze, w porównaniu do kontroli, odnotowano istotne obniżenie wykorzystania wszystkich badanych grup związków organicznych. W badaniu 10 izolatów zaobserwowano również różnice w ich odporności, co pozwoliło wyróżnić trzy wyraźne grupy wrażliwości grzybów, w tym izolaty charakteryzujące się wysoką, średnią i niską wrażliwością na testowane ekstrakty i konserwanty.

Badania mikroskopowe, przeprowadzone z wykorzystaniem skaningowej mikroskopii elektronowej (SEM – Scanning Electron Microscopy) oraz konfokalnej mikroskopii fluorescencyjnej, wykazały, że zarówno kontakt z konserwantem, jak i z ekstraktem roślinnym powodował deformacje powierzchni strzępek. Obserwowane zmiany były zróznicowane w zależności od izolatu, co wskazuje na istotną rolę cech fenotypowych w determinowaniu odporności grzybów na czynniki stresowe. Uzupełniając, przeprowadzono niecelowaną analizę metabolomiczną metodą LC-QTOF MS w celu charakterystyki profili metabolicznych wybranych izolatów grzybów pod wpływem działania substancji o właściwościach antymikrobiologicznych. Dodatek do podłoża hodowlanego ekstraktu z nagietka lub pirosiarczynu sodu spowodował wyraźne zmiany metabolomiczne i podział widm metabolitów w zależności od warunków wzrostu. Każdy z izolatów posiadał unikalny metaboliczny „odcisk palca” i różnił się swoim profilem od pozostałych próbek, wskazując, że izolaty grzybów o różnej wrażliwości na ekstrakty roślinne i konserwanty posiadały odmienny profil metabolomiczny.

Analizy genomowe wybranych izolatów *Neosartorya* spp. przeprowadzono metodą sekwencjonowania całogenomowego (WGS – Whole Genome Sequencing). Uzyskane dane umożliwiły identyfikację genów potencjalnie związanych z termotolerancją, detoksykacją związków chemicznych oraz syntezą metabolitów wtórnych. Obecność licznych genów kodujących m.in. białka szoku cieplnego oraz enzymy antyoksydacyjne sugeruje złożone mechanizmy adaptacyjne tych grzybów do stresu środowiskowego i chemicznego.

Końcowy etap badań obejmował eksperyment z wykorzystaniem owoców truskawki kontaminowanych wybranymi izolatami *Neosartorya* spp. Owoce poddano działaniu konserwantu lub ekstraktu roślinnego, a następnie analizowano zmiany w składzie mikrobiomu metodą sekwencjonowania następnej generacji (NGS – Next Generation Sequencing) oraz aktywności metabolicznej przy użyciu mikroplitek EcoPlates (Biolog™). Sekwencjonowanie

amplikonów wykazało, że rodzaj *Pseudomonas* był dominujący wśród zbiorowisk bakteryjnych, a rząd Burkholderiales ulegał wzbogaceniu pod wpływem naturalnego ekstraktu, podczas gdy Microbacteriaceae redukcji w obecności środka konserwującego. W zbiorowiskach grzybowych, w próbach kontrolnych dobrze reprezentowane były zarówno Ascomycota, jak i Basidiomycota. Ekstrakt naturalny sprzyjał rozwojowi Basidiomycota, z kolei środek konserwujący utrzymywał wyższy udział Ascomycota, w tym rodzajów *Aspergillus* i *Botrytis*, które są związane z procesami psucia owoców. Oba stosowane zabiegi przeciwgrzybicze, obejmujące traktowanie owoców ekstraktem roślinnym lub konserwantem, istotnie wpływały na profil metaboliczny mikroorganizmów zasiedlających owoce, jednak efekty te były zróżnicowane w czasie i zależne od zastosowanego zabiegu.

Uzyskane wyniki wskazują na znaczne zróżnicowanie odpowiedzi izolatów *Neosartorya* spp. na substancje o właściwościach przeciwdrobnoustrojowych oraz potwierdzają ich zdolność do przetrwania w niekorzystnych warunkach środowiskowych. Zgromadzone dane wskazują na istotną rolę właściwości metabolicznych, morfologicznych i genetycznych grzybów *Neosartorya* spp. w kształtowaniu ich odporności na związki konserwujące, chemiczne oraz naturalne ekstrakty roślinne i mogą stanowić podstawę do opracowania skutecznych strategii ograniczania obecności tych grzybów w łańcuchu produkcji żywności oraz wspierać rozwój bardziej zrównoważonych i selektywnych metod utrwalania owoców miękkich.

Słowa kluczowe: grzyby termooporne, konserwanty, ekstrakty roślinne, aktywność metaboliczna, mikromacierze fenotypowe, mikroskopia elektronowa, sekwencjonowanie całogenomowe, mikrobiom.

Abstract

Poland is among the leading fruit producers in the European Union, playing a particularly important role in the cultivation of strawberries and other soft fruits. Due to their excellent taste, rich nutritional value, and wide range of applications in food processing, these fruits constitute a key element of the national agri-food sector. At the same time, their significant susceptibility to microbiological contamination poses a major challenge and a threat to both producers and consumers. Of particular concern in this context are fungi of the genus *Neosartorya*, characterised by exceptional resistance to unfavourable environmental conditions, including high temperatures and the presence of preservatives. Species of this genus which are the teleomorphic forms of *Aspergillus* fungi, are capable of surviving pasteurisation processes, potentially leading to secondary contamination of food products, resulting in both economic losses and a threat to consumer health due to the production of hazardous mycotoxins.

The research presented in this dissertation addresses the characterisation of the morphological, metabolic, and genetic properties of *Neosartorya* spp. fungi, which influence their survival mechanisms, activity, and susceptibility to antimicrobial substances. An important aspect of the study was also the evaluation of the effects of these substances on the microbiome and metabolic potential of strawberries artificially contaminated with these fungi under real storage conditions for plant products. Initially, the effectiveness of both natural plant extracts and synthetic preservatives was evaluated for 20 isolates using disc-diffusion inhibition tests. Then, for 10 isolates selected during the first stage of the study, sensitivity was assessed using Biolog™ MT2 microplates. The results confirmed the diverse efficacy of the tested substances and revealed differences in sensitivity, indicating the existence of five groups of isolates with increasing susceptibility to antimicrobial agents, despite their close genetic relatedness. All tested natural extracts inhibited the growth of *Neosartorya* spp., with lavender, tea tree, and marigold extracts showing the strongest antifungal effects. Sodium metabisulphite, potassium sorbate, sodium bisulphite, and sorbic acid also exhibited inhibitory effects, with sodium metabisulphite being the most effective.

To study the chemical resistance of *Neosartorya* spp., metabolic responses to 120 chemical compounds available in Biolog™ PM 21–25 microplates were analysed. It was demonstrated that the majority of substances stimulated fungal growth, and after 96 hours of incubation, all isolates developed the ability to adapt to stress conditions. The growth plateau phase was reached after approximately 144 hours, indicating effective acclimatization to the presence of toxic compounds. The greatest biomass increase was observed in the presence of antibiotics, anions, and compounds modifying cell membrane function. Sodium cyanate, copper (II) chloride dihydrate, and cisplatin showed pronounced inhibitory properties. Obtained data confirms the significant adaptive potential of *Neosartorya* spp. to various chemical stressors. This ability may be a factor enabling the colonisation of highly processed environments containing preservatives, which has important implications for microbiological control in the industry.

In subsequent stages of the study, the metabolic potential of selected *Neosartorya* spp. isolates was characterised under optimal growth conditions and after exposure to selected plant extracts and food preservatives, using phenotypic microarray tests with Biolog™ FF

microplates. The obtained metabolic profiles demonstrated high metabolic plasticity of the tested isolates and their ability to utilise a wide range of carbon sources, including compounds typical for fruit-based substrates. The fungal response to stress in the presence of marigold extract was characterised by reduced utilisation of amino acids and carbohydrates and increased use of carboxylic acids on FF plates. After exposure to food preservatives, a significant decrease in the utilisation of all tested groups of organic compounds was observed compared to the control. In the study of 10 isolates, differences in resistance were also observed, which allowed the identification of three distinct sensitivity groups, including isolates with high, medium, and low sensitivity to the tested extracts and preservatives.

Microscopic analyses, conducted using scanning electron microscopy (SEM) and confocal fluorescence microscopy, showed that both contact with a preservative and with a plant extract caused deformation of the hyphal surface. The observed changes varied depending on the isolate, indicating a significant role of phenotypic traits in determining fungal resistance to stress factors. Additionally, untargeted metabolomic analysis using LC-QTOF MS was conducted to characterise the metabolic profiles of selected fungal isolates under the influence of antimicrobial substances. The addition of marigold extract or sodium metabisulphite to the growth medium caused distinct metabolomic changes and separation of metabolite spectra depending on the growth conditions. Each isolate had a unique metabolic “fingerprint” and differed in its profile from the other samples, indicating that fungal isolates with varying sensitivity to plant extracts and preservatives had different metabolomic profiles.

Genomic analyses of selected *Neosartorya* spp. isolates were performed using whole genome sequencing (WGS). The obtained data enabled the identification of genes potentially involved in thermotolerance, detoxification of chemical compounds, and the synthesis of secondary metabolites. The presence of numerous genes encoding heat shock proteins and antioxidant enzymes suggests complex adaptive mechanisms of these fungi to environmental and chemical stress.

The final stage of the study included an experiment using strawberries artificially contaminated with selected *Neosartorya* spp. isolates. The fruits were treated with a preservative or a plant extract and subsequently analysed for changes in microbiome composition using next-generation sequencing (NGS) and metabolic activity using EcoPlates (Biolog™). Amplicon sequencing showed that the genus *Pseudomonas* was dominant among the bacterial communities, and the order Burkholderiales increased under the influence of the natural extract, whereas Microbacteriaceae decreased in the presence of the preservative. In fungal communities, both Ascomycota and Basidiomycota were well represented in control samples. The natural extract promoted the development of Basidiomycota, while the preservative maintained a higher share of Ascomycota, including *Aspergillus* and *Botrytis*, which are associated with fruit spoilage. Both antifungal treatments, involving exposure to either a plant extract or a preservative, significantly affected the metabolic profile of microorganisms colonising the fruit, although these effects varied over time and depended on the treatment applied.

The obtained results indicate considerable variation in the response of *Neosartorya* spp. isolates to antimicrobial substances and confirm their ability to survive under adverse

environmental conditions. The collected data point to the important role of metabolic, morphological, and genetic characteristics of *Neosartorya* spp. fungi in shaping their resistance to chemical preservatives and natural plant extracts. These findings may serve as a basis for developing effective strategies to reduce the presence of these fungi in the food production chain and support the development of more sustainable and selective preservation methods for soft fruits.

Keywords: heat-resistant fungi, preservatives, plant extracts, metabolic activity, phenotypic microarrays, electron microscopy, whole genome sequencing, microbiome.

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Lista publikacji stanowiących podstawę rozprawy doktorskiej

Niniejsza rozprawa doktorska oparta jest na cyklu trzech publikacji powiązanych z tematem:

„Rola właściwości metabolicznych, morfologicznych i genetycznych grzybów *Neosartorya* spp. (*Aspergillus* spp.) w kształtowaniu ich odporności na związki konserwujące, chemiczne oraz naturalne ekstrakty roślinne”.

P.1: **Maj, W.**, Pertile, G., Frąc, M. **2023**. Soil-Borne *Neosartorya* spp.: A Heat-Resistant Fungal Threat to Horticulture and Food Production –An Important Component of the Root-Associated Microbial Community. *International Journal of Molecular Sciences*, 24(2), 1543. <https://doi.org/10.3390/ijms24021543>

Czasopismo umieszczone w bazie ISI Journal Citation Reports

Impact Factor₂₀₂₃: 4.9

5-letni Impact Factor₂₀₂₃: 5.6

punktacja MNiSzW: 140 punktów

P.2: **Maj, W.**, Pertile, G., Różalska, S. Skic, K., Frąc, M. **2024**. Comprehensive antifungal investigation of natural plant extracts against *Neosartorya* spp. (*Aspergillus* spp.) of agriculturally significant microbiological contaminants and shaping their metabolic profile. *Scientific Reports*, 14, 8399. <https://doi.org/10.1038/s41598-024-58791-4>

Czasopismo umieszczone w bazie ISI Journal Citation Reports

Impact Factor₂₀₂₃: 3,8

5-letni Impact Factor₂₀₂₃: 4.3

punktacja MNiSzW: 140 punktów

P.3: **Maj, W.**, Pertile, G., Różalska, S., Skic, K., Frąc, M. **2024**. The role of food preservatives in shaping metabolic profile and chemical sensitivity of fungi-an extensive study on crucial mycological food contaminants from the genus *Neosartorya* (*Aspergillus* spp.). *Food Chemistry*, 453, 139583. <https://doi.org/10.1016/j.foodchem.2024.139583>

Czasopismo umieszczone w bazie ISI Journal Citation Reports

Impact Factor₂₀₂₃: 8.5

5-letni Impact Factor₂₀₂₃: 8.3

punktacja MNiSzW: 200 punktów

Uzupełnieniem wyników badań przedstawionych w wyżej wymienionych publikacjach jest zastosowanie metody sekwencjonowania całogenomowego Illumina® na platformie MiSeq do scharakteryzowania genomów blisko spokrewnionych izolatów *Neosartorya* spp. wykazujących różnice we wrażliwości na naturalne ekstrakty roślinne i konserwanty żywności. Dodatkowo, chromatografia cieczowa sprzężona z kwadрупolowym spektrometrem masowym czasu przelotu (LC-QTOF MS) została zastosowana do analizy zmian w profilu metabolicznym, skaningowa mikroskopia elektronowa (SEM) umożliwiła ocenę adaptacji morfologicznych, a mikroskopia konfokalna fluorescencyjna pozwoliła na określenie żywotności komórek grzybów poddanych ekspozycji na ekstrakt z nagietka i pirośniarczyny sodu. Wyniki tych badań zostały przygotowane do publikacji w manuskrypcie zatytułowanym:

P4: **Maj, W.**, Pertile, G., Panek, J., Różalska, S., Skic, K., Kozub-Pędrak, A., Wielgosz, A., Fornal, E., Frąc, M., Genome-Wide Analysis, Metabolomic Profiling and Microscopic Evaluation of *Neosartorya* spp. (*Aspergillus* spp.) Diversity and Adaptations to Natural and Synthetic Antimicrobials.

Kolejnym uzupełnieniem wyników badań przedstawionych w wyżej wymienionych publikacjach jest analiza wpływu naturalnego ekstraktu oraz konserwantu spożywczego na

mikrobiom owoców truskawki kontaminowanych izolatami *Neosartorya* spp., o różnej wrażliwości na ekstrakty roślinne i konserwanty. Sekwencjonowanie przez syntezę markerów molekularnych (v4 16S rRNA oraz ITS2) przeprowadzone na platformie Illumina® MiSeq oraz analiza metataksonomiczna uzyskanych wyników pozwoliły na określenie składu taksonomicznego bakteryjnych i grzybowych społeczności mikrobiologicznych oraz wskaźników bioróżnorodności, a także predykcję cech funkcjonalnych mikrobiomu bakteryjnego i grup troficznych mykobiomu. Dodatkowo, testy Biolog™ EcoPlates umożliwiły ocenę aktywności metabolicznej mikrobiomów owoców truskawki w odpowiedzi na zastosowane czynniki antymikrobiologiczne. Wyniki tych badań zostały przygotowane do publikacji w manuskrypcie zatytułowanym:

P5: **Maj, W.**, Pertile, G., Panek, J., Frąc, M., Antifungal Agents Alter Strawberry Microbial and Metabolic Profiles During *Neosartorya* spp. (*Aspergillus* spp.) Contamination.

1. Wprowadzenie

1.1 Produkcja owoców w Polsce i Unii Europejskiej – skala, trendy i regulacje środowiskowe

Produkcja płodów rolnych odgrywa kluczową rolę w gospodarce Polski, stanowiąc istotne źródło surowców zarówno dla rynku krajowego, jak i międzynarodowego (Łakomiak & Zhichkin, 2020). Polska, jako jeden z czołowych producentów żywności w Unii Europejskiej, może konkurować z takimi krajami jak Francja i Niemcy pod względem popularności swoich produktów na rynku amerykańskim (Bajan et al., 2021). Dynamiczny rozwój agrobiznesu, w tym sektora przetwórstwa, jest ściśle związany z rosnącą intensyfikacją handlu zagranicznego (Mickiewicz et al., 2021). Polska jest także piątym krajem w Unii Europejskiej pod względem powierzchni użytków rolnych i pierwszym pod względem liczby ludności zatrudnionej w rolnictwie, co podkreśla znaczenie tego sektora dla krajowej gospodarki i rynku pracy. (Klikocka et al., 2022). Dodatkowo, Polska jest jednym z czołowych producentów owoców w Europie, przodując w uprawie m.in. jabłek (3.08 milionów ton w 2019, Muder et al., 2022), truskawek (167.3 tysięcy ton w 2020, Pawlak & Wróblewska, 2022), wiśni (200 tysięcy ton w 2018, Sokół-Łętowska et al., 2020) czy malin (121.7 tysięcy ton w 2020, Pawlak & Wróblewska, 2022). Celem dalszego rozwoju gospodarczego, nieustannie potrzebne są badania przyczyniające się do zwiększenia innowacyjności procesów produkcyjnych. Wysoka jakość surowców oraz ich bezpieczeństwo mikrobiologiczne są niezbędne do utrzymania konkurencyjności na rynku, szczególnie w kontekście zaostrzających się regulacji oraz rosnących oczekiwań konsumentów.

W ostatnich latach produkcja ekologicznych owoców w Unii Europejskiej dynamicznie wzrasta, odzwierciedlając ogólny trend wzrostu rolnictwa ekologicznego (Trávníček et al., 2021; Siegieda et al., 2024). W latach 2012–2020 udział gruntów rolnych prowadzonych zgodnie z zasadami rolnictwa ekologicznego zwiększył się o ponad 50%, a sektor owoców,

oliwek i winorośli stanowił 11% tego obszaru (Malusà & Neri, 2023). Przejście z konwencjonalnej na ekologiczną produkcję owoców wymaga intensywnych badań oraz efektywnego transferu wiedzy, aby sprostać rosnącym wymaganiom rynkowym i regulacyjnym. W tym kontekście, główne znaczenie ma implementacja systemu rolnictwa zrównoważonego, co jest jednym z głównych założeń *Europejskiego Zielonego Ładu* (Fetting, 2020; Boix-Fayos & de Vente, 2023). Jego celem jest osiągnięcie neutralności klimatycznej Europy do 2050 roku, a także ochrona różnorodności biologicznej i łagodzenie skutków zmian klimatu (Peeters et al., 2020). W ramach tych działań Komisja Europejska zaleca wdrażanie strategii „*od pola do stołu*”, która ma na celu zwiększenie produkcji i dystrybucji żywności ekologicznej (Santos et al., 2023). Najważniejszymi założeniami strategii są: ograniczenie negatywnego wpływu sektora rolno-spożywczego na środowisko, poprawa bezpieczeństwa żywności, oraz promowanie metod upraw i ochrony roślin, które zmniejszają wykorzystanie środków chemicznych (Silva et al., 2022). Chociaż wokół Europejskiego Zielonego Ładu w ostatnim czasie toczą się różnorodne dyskusje, to niewątpliwie produkcja zdrowej, wysokiej jakości żywności, przy dbałości o zdrowie i równowagę agroekosystemów, jest kluczowa dla rozwoju rolnictwa przyszłości odpornego na zmiany klimatu (Frąc et al., 2022). W związku z tym wzrasta zapotrzebowanie na alternatywne metody ochrony owoców i żywności przetworzonej przed zakażeniami mikrobiologicznymi, obejmujące m.in. zastosowanie naturalnych substancji konserwujących oraz biologicznych środków ochrony roślin, zgodnych z założeniami polityki Unii Europejskiej (Tataridas et al., 2022). Skuteczność tych strategii jest szczególnie istotna w kontekście zagrożeń wynikających z zanieczyszczeń mikrobiologicznych, które mogą prowadzić do strat środowiskowych i ekonomicznych na różnych etapach produkcji i dystrybucji owoców (Maj et al., 2023).

1.2 Zagrożenia wynikające z mikrobiologicznego skażenia owoców

Mikroorganizmy, w tym grzyby, mogą powodować psucie się żywności, zmniejszając jej wartość handlową i prowadząc do konieczności wycofywania partii produktów (Frąc et al., 2015; Karanth et al., 2023). Szacuje się, że za sprawą psucia powodowanego czynnikami mikrobiologicznymi tracona jest około jedna trzecia całej żywności produkowanej na świecie (Guleria, 2025). Oblicze strat spowodowanych przez mikroorganizmy wynika z różnych czynników, począwszy od łatwości kolonizacji i pogorszenia parametrów jakościowych produktów, związanych z ich strukturą i zawartością składników odżywczych (Bevilacqua et al., 2024). Ważne są także czynniki środowiskowe sprzyjające rozwojowi patogenów, w tym wilgotność, pH, temperatura czy dostępność tlenu (Alegbeleye et al., 2022). Istotna jest także obecność uszkodzeń mechanicznych owoców i brak, lub niewystarczająca skuteczność metod konserwacji i ochrony plonów (Adams et al., 2024).

Choć jakość owoców i warzyw zaczyna obniżać się tuż po ich odcięciu od naturalnego źródła składników odżywczych, okres ich największej podatności na zakażenia mikrobiologiczne przypada na czas dojrzewania i starzenia się komórek (James & Zikankuba, 2017). Mechanizm tworzenia się zakażenia jest niezwykle prosty, ponieważ mikroorganizmy powodujące psucie się żywności mogą przenosić się na plony poprzez kontakt z glebą i otoczeniem rolniczym, zarówno w fazie wzrostu, zbiorów, a także w trakcie przechowywania, przetwarzania czy transportu (Snyder & Worobo, 2018).

Samo psucie się żywności jest konsekwencją biochemicznych procesów zachodzących pod wpływem mikroorganizmów, które rozkładają składniki ścian komórkowych owoców i przekształcają składniki pokarmowe prowadząc do zmian sensorycznych, takich jak pogorszenie smaku, tekstury, barwy oraz zapachu (Rawat, 2015; Cybulska et al., 2022). Wśród głównych mechanizmów psucia wyróżnia się proteolizę, lipolizę, fermentację, zakwaszenie oraz produkcję gazów (Kapoor et al., 2023). Proteoliza, czyli rozkład białek do peptydów i

aminokwasów, jest szczególnie istotnym mechanizmem psucia w produktach bogatych w białko, takich jak mięso, nabiał czy ryby. Proces ten jest katalizowany przez enzymy bakteryjne i grzybowe, a jego skutkiem jest degradacja tkanek oraz nieprzyjemny zapach produktów (Singh & Benjakul, 2018). Ponadto, rozkład białek może prowadzić do powstawania amin biogennych, takich jak histamina czy putrescyna, które stanowią zagrożenie dla zdrowia konsumentów (Durak-Dados et al., 2020). Dla grzybów z rodzaju *Aspergillus*, proteoliza to narzędzie ułatwiające kolonizację struktur owoców poprzez wykorzystanie enzymów proteolitycznych do wnikania w tkanki roślinne (Asis et al., 2009). Z kolei lipoliza, to hydroliza tłuszczów do wolnych kwasów tłuszczowych, aldehydów i ketonów (Edwards & Mohiuddin, 2020). Grzyby z rodzaju *Aspergillus* rozkładają tłuszcze w takich produktach jak ziarna kakao i olej, generując krótkołańcuchowe kwasy tłuszczowe, które nadają żywności nieprzyjemny smak i zmniejszają jej wartość odżywczą poprzez modyfikację składu tłuszczowego (Pérez et al., 2021; Stéphanie et al., 2023). Fermentacja, polegająca na metabolizowaniu cukrów, również może prowadzić do psucia produktów spożywczych (Pitt & Hocking, 2022a). Komercyjnie, grzyby z rodzaju *Aspergillus*, a zwłaszcza *Aspergillus oryzae*, wykorzystywane są do celowej fermentacji żywności celem jej obróbki i uzyskania pożądanych właściwości reologicznych (Daba et al., 2021). Jednak w sprzyjających im warunkach, *Aspergillus* spp. mogą dokonywać niekontrolowanej fermentacji powodując zmiany żywności, co skutkuje jej zdyskwalifikowaniem z obrotu handlowego. Powiązana z wymienionymi mechanizmami jest produkcja gazów na skutek procesów gnilnych. Grzyby z rodzaju *Aspergillus* wytwarzają szeroki zakres lotnych związków organicznych (VOCs – Volatile Organic Compounds) zarówno w procesach metabolizmu pierwotnego, jak i wtórnego, co może mieć istotne konsekwencje dla jakości i bezpieczeństwa żywności (Schnürer et al., 1999). W zamkniętych opakowaniach objawia się to ich pęcznieniem, stanowiącym jednoznaczny sygnał zepsucia produktu (Ogwu & Ogunsola, 2024). W trakcie wzrostu kolonii tych grzybów dochodzi do

emisji lotnych alkoholi, ketonów, estrów i znacznej ilości dwutlenku węgla, co w dużej mierze zmienia sensoryczne właściwości produktów spożywczych (Zhai et al., 2015; Josselin et al., 2021).

Przedstawione mechanizmy obrazują złożoność procesów degradacyjnych zachodzących przy udziale grzybów z rodzaju *Aspergillus*, które wykorzystują szeroki zakres narzędzi metabolicznych prowadzących do psucia żywności. Ponadto, w wielu przypadkach procesy te nie przebiegają w izolacji, lecz są wynikiem synergistycznych interakcji między różnymi grupami mikroorganizmów. Na przykład bakterie mogą zakwaszać środowisko sprzyjając rozwojowi pleśni i drożdży, co przyspiesza procesy psucia się żywności (Guleria, 2025). Zrozumienie podłoża tych mechanizmów, w tym wrażliwości na środki ochrony plonów, a także właściwości genetycznych, morfologicznych oraz metabolicznych grzybów z rodzaju *Neosartorya*, jest kluczowe dla opracowania skutecznych metod zabezpieczania żywności przed psuciem, a tym samym dla ograniczenia strat ekonomicznych i zmniejszenia ilości odpadów spożywczych (Maj et al., 2023).

1.3 Metody ochrony owoców przed psuciem mikrobiologicznym – skuteczność i kierunki badań

Obecnie, w celu ochrony plonów stosuje się przeważnie zabiegi termiczne (np. gorąca woda, para wodna, gorące powietrze), które zwalczają patogeny roślinne poprzez wywoływanie umiarkowanych warunków stresowych (Moradinezhad & Ranjbar, 2023). Takie zabiegi mogą jednak negatywnie wpływać na jakość odżywczą i sensoryczną produktów (Aghajanzadeh et al., 2023). Dodatkowo, pasteryzacja żywności podczas przetwórstwa nie jest adekwatną metodą ochrony produktów przed mikroorganizmami termoodpornymi (Ishara & Gunasena, 2021; Zhao et al., 2024). W rolnictwie konwencjonalnym popularne jest za to wykorzystanie chemicznych pestycydów do kontroli zanieczyszczeń mikrobiologicznych. Jednakże poprzez zbyt intensywną chemizację rolnictwa, wiele patogenów wykształciło odporność na często

używane środki ochrony roślin (Frąc et al., 2016; Paul & Mandal, 2019). Dodatkowo, chemiczne środki ochrony plonów i roślin są negatywnie postrzegane przez społeczeństwo, ponieważ często ich nadmierne użycie przyczynia się do pogorszenia stanu ekosystemów naturalnych oraz zdrowia konsumentów (Kumar et al., 2023; Soudani et al., 2020). W związku z tym, stosowanie fungicydów na owoce po zbiorach w celu zapobiegania gniciu jest coraz bardziej ograniczane. W wielu krajach wprowadzane są odgórne limity wykorzystania substancji chemicznych w rolnictwie (de O. Gomes et al., 2020). Z tego względu poszukuje się pestycydów alternatywnych, czyli substancji o właściwościach antymikrobiologicznych lub insektobójczych, które mogą być wykorzystywane w rolnictwie zrównoważonym (Vlaiculescu & Varrone, 2022). Jednymi z bardziej popularnych kierunków nauki eksplorujących tę tematykę, są badania dotyczące organizmów antagonistycznych, opracowania formuł biopreparatów, czy wykorzystania substancji organicznych lub naturalnych do kontroli wzrostu patogenów (Pylak et al., 2019; Mącik et al., 2020; Coban, 2020; Guglielmi et al., 2020; Zhang et al., 2020), a także mikrobiologicznych zanieczyszczeń żywności, w tym grzybów termoopornych (Frąc et al. 2015; Panek et al., 2016). Wśród tych ostatnich, prym wiodą substancje biobójcze pozyskane z roślin, w tym pojedyncze kategorie związków takich jak związki polifenolowe, lub całe ich mieszaniny zawarte w olejkach eterycznych czy suchych ekstraktach (Bouarab Chibane et al., 2019; Othman et al., 2020). Warto wspomnieć, że substancje pochodzenia naturalnego były z powodzeniem wykorzystywane w rolnictwie na długo przed popularyzacją chemicznej ochrony roślin, a także, iż wiele badań donosi o ich komparatywnym działaniu w stosunku do syntetycznych pestycydów (Oguh et al., 2019) czy środków konserwujących żywność, zapobiegając przed rozwojem mikrobiologicznych zanieczyszczeń żywności (Oleszek et al., 2019). Jednakże, pomimo dynamicznego rozwoju badań nad nowoczesnym i ekologicznym rolnictwem, wciąż brakuje kompleksowego zrozumienia mechanizmów odpowiedzialnych za skuteczność substancji naturalnych, co

warunkuje dalszą eksplorację naukową. Wiele badań koncentruje się na sprawdzaniu efektywności ekstraktów roślinnych w warunkach *in vitro*, często na pojedynczych szczepach danego mikroorganizmu (Seepe et al., 2020; Šernaitė et al., 2020; Kursa et al., 2022). Tego typu eksperymenty stanowią istotną podstawę w ocenie wrażliwości szczepów oraz potencjału badanych związków do hamowania wzrostu mikroorganizmów. Coraz częściej jednak podkreśla się potrzebę weryfikacji tych założeń w warunkach *in vivo* oraz w rzeczywistych systemach produkcji i przechowywania żywności (Boer, 2017; Ferrocino et al., 2023). Stosunkowo nowym, ale coraz popularniejszym podejściem są badania uwzględniające całą mikrobiotę żywności, co pozwala na pełniejszą ocenę interakcji między drobnoustrojami (Brown et al., 2019; Cybulska et al., 2022). Pomimo szerokiego zasięgu występowania grzybów z rodzaju *Neosartorya*, a także znając wyniki badań nad ich odpornością na szereg związków chemicznych (Panek et al., 2016), wciąż brakuje kompleksowych analiz ich wrażliwości na konserwanty żywności i ekstrakty roślinne, oraz badań nad zmianami w metabolizmie tych grzybów pod wpływem wymienionych substancji. Dodatkowo, w literaturze brakuje doniesień o skutkach stosowania naturalnych ekstraktów roślinnych wobec jakości plonów roślin zakażonych *Neosartorya* spp., zwłaszcza w odniesieniu do zmian w zbiorowiskach bakteryjnych i grzybowych naturalnie rezydujących w surowcach roślinnych i żywności. Zrozumienie tych zmian i mechanizmów może dostarczyć cennych informacji na temat adaptacyjnych strategii tych mikroorganizmów i ich potencjalnej oporności, co jest kluczowe dla opracowania skutecznych metod ich kontroli. Ma to szczególne znaczenie w obliczu rosnącej odporności na fungicydy, która stanowi wyzwanie dla obecnych strategii zapobiegania rozwojowi mikroorganizmów (Maj et al., 2023).

1.4 Grzyby z rodzaju *Neosartorya* (*Aspergillus*) jako czynnik psucia żywności

Grzyby z rodzaju *Neosartorya* należą do rodziny Trichocomaceae i historycznie bywały traktowane jako odrębny takson od rodzaju *Aspergillus* (Taylor et al., 2016). Obecnie, na

podstawie badań filogenetycznych, uznaje się, że *Neosartorya* spp. reprezentują jedynie formy płciowe (teleomorficzne) niektórych gatunków *Aspergillus*, szczególnie w obrębie sekcji *Fumigati* (Samson et al., 2014). Z ponad 450 odkrytych gatunków w rodzaju *Aspergillus*, tylko około 30 ma zdolność do wykształcania form teleomorficznych (Hong et al., 2008; Riedling et al., 2024). Kluczową cechą odróżniającą zatem *Neosartorya* spp. od *Aspergillus* spp. jest zdolność do tworzenia askospor, czyli zarodników workowych stadium płciowego, które są wyjątkowo odporne na warunki środowiskowe, w tym na wysokie temperatury stosowane w przetwórstwie żywności (Wyatt et al., 2015a). W badaniach skupiających się tylko na grzybach tworzących takie zarodniki, czasem wciąż zaleca się używanie nazwy *Neosartorya* spp. ze względu na wysoką zmienność fenotypową form płciowych (Samson & Varga, 2009).

Grzyby z rodzaju *Neosartorya* występują powszechnie w środowisku, zwłaszcza w glebie, wodzie morskiej, na roślinach oraz na rozkładających się materiałach organicznych (War May Zin et al., 2016; Fornal et al., 2017; Lin et al., 2024). Ich częste występowanie uzasadnione jest elastycznością co do warunków wzrostu. Grzyby te, mogą bowiem swobodnie wzrastać w temperaturze od 7°C do 52°C, zarówno przy niskiej (0.1%), jak i normalnej dostępności tlenu i w szerokim spektrum pH (3–8; Evelyn et al., 2016; Lane Paixão dos Santos et al., 2020). Mogą też kolonizować szeroki wachlarz produktów spożywczych, w tym surowe owoce, przeciery, puree, soki, dżemy czy żywność w puszkach (Gryta et al., 2015; Evelyn et al., 2016; Berni et al., 2017; Lane Paixão dos Santos et al., 2020; J. Santos et al., 2020). Grzyby *Neosartorya* spp. stanowią szczególne zagrożenie dla żywności przetwarzanej przez procesy ukierunkowane na ochronę termiczną, w tym pasteryzację, ponieważ ich askospory wykazują odporność na temperatury dochodzące do 85-95°C (Wyatt et al., 2015a; Berni et al., 2017). Uważa się, że spowodowane jest to zawartością takich czynników ochronnych jak trehaloza oraz poliole (np. mannitol) oraz oligosacharydy na bazie trehalozy, takie jak izobemizioza, neosartoza i fiszeroza (Wyatt et al., 2015a; Wyatt et al., 2015b). W konsekwencji askospory

mogą przetrwać standardowe procedury konserwacji i doprowadzać do późniejszego psucia produktów, często objawiającego się zmianą smaku, tekstury oraz obecnością niepożądanych metabolitów wtórnych (Pitt & Hocking, 2022b).

Jednym z głównych zagrożeń związanych z zakażeniami grzybami *Neosartorya* spp. jest ich zdolność do produkcji mykotoksyn, takich jak fumitremorgina C, verrukulogen, aflatoksyna czy gliotoksyna, które mogą wykazywać działanie immunosupresyjne i cytotoksyczne (Hubka et al., 2013; Liang et al., 2014; Fornal et al., 2017; Darsana & Chandrasehar, 2021). Spożywanie mykotoksyn wraz z żywnością może przyczynić się do zakłócania normalnego funkcjonowania układu nerwowego (Bridgeman et al., 2023). Co istotne, mykotoksyny *Neosartorya* spp. wykazują wysoką stabilność termiczną, co oznacza, że nawet po eliminacji strzępek grzyba w procesie przetwarzania żywności, te toksyczne metabolity mogą pozostawać w produkcie końcowym (Stadler et al., 2020). Dodatkowo, niektóre *Neosartorya* spp. wykazują się potencjałem do oportunistycznej patogenności, powodując infekcje układu oddechowego lub zakażenia skóry (Bilovol & Panaccione, 2016; Patel et al., 2024; Tatarinova et al., 2024). Właśnie dlatego identyfikacja, charakterystyka i monitoring *Neosartorya* spp., a w szczególności poznanie roli ich właściwości metabolicznych, morfologicznych i genetycznych w kształtowaniu odporności na różne związki, są kluczowe dla zrozumienia mechanizmów adaptacyjnych tych grzybów oraz zapewnienia bezpieczeństwa konsumentów i ograniczenia strat ekonomicznych (Maj et al., 2023).

1.5 Strategie badawcze w analizie odpowiedzi grzybów *Neosartorya* spp. na stres środowiskowy

Badania nad mechanizmami odporności grzybów z rodzaju *Neosartorya* na stres środowiskowy mają kluczowe znaczenie dla bezpieczeństwa żywności, zwłaszcza w kontekście przechowywania owoców i ich przetworów (Maj et al., 2023). Pomimo identyfikacji niektórych związków odpowiadających za tolerancję niekorzystnych warunków

środowiskowych przez *Neosartorya* spp., brakuje kompleksowych analiz łączących cechy morfologiczne, genetyczne i metaboliczne z opornością na stresory. Literatura uboga jest także w doniesienia ilustrujące wrażliwość *Neosartorya* spp. na związki naturalne i syntetyczne o potencjale antymikrobiologicznym, co uniemożliwia ocenę zmian w oporności szczepów w kontekście globalnego nabywania odporności przez mikroorganizmy.

Badania nad mechanizmami odporności grzybów na stres środowiskowy integrują metody molekularne, a także te służące do oceny fizjologii i morfologii, uwzględniając zarówno adaptacje strukturalne, metaboliczne, jak i genetyczne. Aby lepiej zrozumieć te zależności, w pierwszym etapie badań nad grzybami przede wszystkim dokonuje się identyfikacji izolatów na podstawie sekwencjonowania fragmentów regionu ITS (Internal Transcribed Spacer). Wykorzystywanie „*barcodingu*”, czyli ściśle określonych fragmentów DNA do identyfikacji gatunku jest powszechnym sposobem na dokonanie klasyfikacji izolatów (Lücking et al., 2020). Jednakże użycie pojedynczego markera często uniemożliwia rozgraniczenie gatunków, ponieważ między blisko spokrewnionymi taksonami na ogół brakuje znaczącej rozbieżności (Kausrud, 2023). Z tego powodu wykorzystuje się także sekwencjonowanie fragmentów kodujących białka, takich jak β -tubulina czy kalmodulina (Michalecka & Puławska, 2021; Yaguchi et al., 2012; Pertile et al., 2020). Pozwala to na bardziej precyzyjne określenie pokrewieństwa filogenetycznego i jest stosowane w badaniach taksonomicznych nad grzybami pleśniowymi, w tym z rodzaju *Aspergillus* (Tekpinar & Kalmer, 2019). To podejście pozwala także na odróżnienie form anamorficznych od teleomorficznych, co jest istotne w kontekście badań koncentrujących się na wrażliwości grzybów, ponieważ wykazują one odmienną odporność na warunki środowiskowe (Yaguchi, 2023). Do samego sekwencjonowania wykorzystuje się zazwyczaj metodę Sanger, która do dziś jest jedną z najbardziej efektywnych i niezawodnych sposobów analizy krótkich ciągów DNA (Salem-Bango et al., 2023). Ponadto, metoda dideoksy opisywana jest zazwyczaj jako dokładniejsza od sekwencjonowania następnej

generacji (NGS), a zatem pozwala na bardziej dogłębną analizę „barkodów” DNA (Al-Shuhaib & Hashim, 2023). Z kolei NGS, pozwala na wysoką przepustowość i poznanie długich sekwencji, w tym całych genomów (Nafea et al., 2024). Wykorzystanie tej technologii pozwala zatem na dokładną charakterystykę gatunkową patogenów i mikrobiologicznych zanieczyszczeń żywności, a także na identyfikację genów kodujących m.in. oporność na substancje antymikrobiologiczne w każdym badanym izolacie (Tsang et al., 2021).

Tradycyjnym podejściem do badań wrażliwości grzybów są testy inhibicji metodą dyfuzyjno-krażkową na podłożu agarowym. Polega ona na pomiarze stref zahamowania wzrostu powodowanych przez podaż czynnika wstrzymującego rozwój mikroorganizmu na krążki bibułowe (Farian & Wójcik-Fatla, 2022; Oszust and Frąc, 2020; Maj et al., 2024a; Maj et al., 2024b). Metoda ta znajduje zastosowanie w badaniach efektywności związków naturalnych w kontroli wzrostu patogenów roślinnych, a także do oceny oporności *Aspergillus* spp. na azole (Habisukan et al., 2021; Yerbanga et al., 2023). Istnieją jednak nowoczesne alternatywy tej metody, na przykład mikropłytki MT2 systemu Biolog™, które pozwalają na dokładną analizę wrażliwości mikroorganizmów na zadane związki (Frąc et al., 2016; Oleszek et al., 2019; Rafieenia et al., 2022). Mikropłytki MT2 nie zawierają źródeł węgla, co pozwala na ich dowolne dostosowanie poprzez uzupełnienie roztworem badanych substancji. Zmiana gęstości optycznej oceniana jest spektrofotometrycznie i dostarcza informacji o potencjale do wzrostu mikroorganizmów (Frąc et al., 2016; Ali et al., 2025). Oprócz płytek MT2, istnieje wiele różnych rodzajów mikropłytek systemu Biolog™. Na przykład mikropłytki FF to metoda dedykowana grzybom strzępkowym, która pozwala na analizę zdolności mikroorganizmów do metabolizowania 95 różnych źródeł węgla (Frąc, 2012; Chou et al., 2022; Maj et al., 2024a; Maj et al., 2024b). Mikropłytki FF bywały wykorzystywane do opisywania profilu metabolicznego grzybów termoopornych (Panek et al., 2016; Pertile et al., 2020), charakterystyki fitopatogenów i mikroorganizmów dobroczynnych (Oszust et al., 2020; Pylak

et al., 2020), a także różnorodności funkcjonalnej grzybów z próbek środowiskowych (Oszust et al., 2019; Masigol et al., 2021; Oszust & Frąc, 2021). Z kolei mikropłytki PM umożliwiają testy wrażliwości na szereg związków chemicznych (Panek et al., 2016; Hu et al., 2024; Maj et al., 2024b). Oprócz systemu Biolog™, analizy zdolności metabolicznych techniką spektrofotometrii można dokonać przy użyciu m.in. dioctanu fluoresceiny (FDA), który jest substratem dla esteraz komórkowych (Różalska et al., 2014; Maj et al., 2024b). Barwnik może funkcjonować jako wskaźnik żywotności i pozwala na ocenę zarówno aktywności enzymatycznej, niezbędnej do zainicjowania fluorescencji, jak i integralności błony komórkowej, kluczowej dla zatrzymania produktu fluorescencyjnego w komórce (Soboń et al., 2018).

Morfologia strzępek grzybów stanowi ważny element ich zróżnicowania gatunkowego. Analizy wyglądu strzępek dokonuje się metodami mikroskopowymi, ze szczególnym uwzględnieniem elektronowej mikroskopii skaningowej (SEM) pozwalającej na dokładną obserwację niewielkich struktur w powiększeniu osiągającym nawet 1000000x (Mohammed & Abdullah, 2018; Maj et al., 2024a). Metoda ta, bywa używana do obserwacji zmian w morfologii pod wpływem toksycznych substancji czy przy zarodkowaniu grzybów (Chen et al., 2017; Vujanovic et al., 2019). Obserwacje SEM pozwalają na połączenie zmian zaistniałych w niekorzystnych warunkach środowiskowych z adaptacją strzępek grzybów do tych warunków (Bowen et al., 2007). Oprócz weryfikacji zmian strukturalnych powierzchni komórek, istotne jest także ilustrowanie ich żywotności, na co pozwala fluorescencyjna mikroskopia konfokalna (Hickey et al., 2004). Jest to technika umożliwiająca wykonywanie zdjęć w wysokiej rozdzielczości, nawet przy dużej grubości badanego materiału (Elliott, 2020). W praktyce, fluorescencyjna mikroskopia konfokalna bywa używana między innymi do dynamicznej wizualizacji tworzenia się biofilmów grzybów strzępkowych na żywności i obserwacji ich wzrostu (Kulišová et al., 2023).

Innym podejściem do badania wrażliwości grzybów jest wykorzystanie metod metabolomiki czy proteomiki. Nauki omiczne przyczyniają się do poznania cyklu życiowego, mechanizmów infekcji, odporności i wirulencji grzybów (Gonzalez-Fernandez & Jorin-Novo, 2012). Dzięki ilościowej proteomice celowanej, w przeszłości zidentyfikowano m.in. białka związane z degradacją substancji toksycznych i neutralizacją antybiotyków, co wiąże się z adaptacją do środowiska i tolerancją na ciepło u *Neosartorya pseudofischeri* (Chen et al., 2022). Z kolei podejście metabolomiczne, na przykład z wykorzystaniem chromatografii cieczowej ze spektrometrią masową (LC-MS), pozwala na ocenę przemiany lub produkcji metabolitów (Isogai et al., 2021; Huang et al., 2023). Metabolomika dostarcza zatem cennych informacji o fizjologii grzybów i ich reakcji na stres środowiskowy (Pellissier et al., 2023). Ponadto, analizy metabolomiczne mogą być stosowane do badania interakcji międzygatunkowych grzybów, dostarczając informacji o zmianach metabolicznych zachodzących podczas konkurencji o terytorium i zasoby (Luo et al., 2017). Integracja danych z różnych platform omicznych, w tym metabolomiki i proteomiki, w połączeniu z zaawansowanymi metodami obliczeniowymi, umożliwia także tworzenie kompleksowych modeli interakcji gospodarz-grzyb i opracowywanie metod zwalczania infekcji grzybiczych (Carvalho & Goldman, 2017).

W ostatnich latach podkreśla się konieczność oceny interakcji między badanymi środkami konserwującymi a mikrobiomem w rzeczywistych systemach żywnościowych, zamiast wykonywania badań ściśle w warunkach laboratoryjnych (Ferrocino et al., 2023). W szczególności brakuje systematycznych analiz wpływu substancji mających zabezpieczać żywność przed patogenami i mikrobiologicznymi zanieczyszczeniami żywności na różnorodność mikrobiologiczną oraz aktywność metaboliczną zbiorowisk mikroorganizmów obecnych w surowcach rolniczych i produktach żywnościowych oraz w układach pokarmowych organizmów wyższych (Boer, 2017; Gultekin et al., 2020). Analiza mikrobiomu może być prowadzona dwiema głównymi metodami sekwencjonowania: metagenomiczną,

obejmującą całe genomy drobnoustrojów w podejściu „*shotgun*”, oraz metataksonomiczną, opartą na sekwencjonowaniu markerowych genów, takich jak gen rybosomalnego RNA (Kioroglou et al., 2019). W kontekście oceny interakcji między mikroorganizmami i substancjami konserwującymi szczególnie przydatne jest sekwencjonowanie metataksonomiczne z wykorzystaniem platformy Illumina® MiSeq. Pozwala ono między innymi na określenie dominujących taksonów w próbkach, co jest przydatne w ilustrowaniu zmian w składzie mikrobiomu przy zakażeniach czy pod wpływem stresorów środowiskowych (Alessandri et al., 2023; Ignacio et al., 2022; Siegieda et al., 2023; Siegieda et al., 2024). Uzupełnieniem tej metody mogą być dodatkowe analizy spektrofotometryczne, na przykład z wykorzystaniem mikroplacytek EcoPlates systemu Biolog™ umożliwiających profilowanie zmian w aktywności metabolicznej mikrobiomu z próbek środowiskowych (Frąc, 2012; Gryta et al., 2014). EcoPlates były z powodzeniem wykorzystywane w badaniach toksyczności ścieków, do monitorowania aktywności metabolicznej glebowych zbiorowisk mikroorganizmów i w ocenie różnorodności funkcjonalnej gleb (Frąc 2012; Gryta et al., 2014; Xu et al., 2015; Rutgers et al., 2016; Mącik et al., 2025), a także różnych nisz ekologicznych roślin (Pylak et al., 2023; Pylak et al., 2025).

Zastosowanie opisanych technik badawczych pozwala na wszechstronną analizę mechanizmów odpowiedzi grzybów na stres środowiskowy, obejmując zarówno aspekty morfologiczne, metaboliczne, jak i genetyczne. Połączenie metod mikroskopowych, omicznych oraz biologii funkcjonalnej umożliwia kompleksowe zrozumienie strategii przystosowawczych mikroorganizmów i ich interakcji z otoczeniem. Uzyskane w ten sposób dane mogą przyczynić się do opracowania skuteczniejszych strategii kontroli grzybów w środowisku żywnościowym oraz minimalizacji strat wynikających z ich odporności na czynniki konserwujące.

2. Hipotezy badawcze oraz cel rozprawy doktorskiej

2.1 Hipotezy badawcze

Główna hipoteza badawcza zakładała, że właściwości metaboliczne, morfologiczne i genetyczne grzybów z rodzaju *Neosartorya* mają wpływ na kształtowanie wrażliwości tych grzybów na naturalne ekstrakty roślinne i związki konserwujące, a adaptacja *Neosartorya* spp. do wykorzystania wielu substratów, nawet potencjalnie szkodliwych, wiąże się ze zmianami ich profilu metabolicznego i cech morfologicznych.

Na podstawie przeglądu literatury dotyczącego przeżywalności grzybów termofilnych w warunkach przechowalniczych, skuteczności naturalnych i syntetycznych środków konserwujących stosowanych w ochronie owoców, a także zmian zachodzących w grzybach pod wpływem czynników stresowych, postawiono następujące szczegółowe hipotezy badawcze:

1. Pomimo bliskiego pokrewieństwa izolaty *Neosartorya* spp. wykazują zróżnicowaną wrażliwość na zastosowane naturalne ekstrakty roślinne, konserwanty żywności i substancje chemiczne.
2. Konserwanty żywności i naturalne ekstrakty roślinne charakteryzują się podobną efektywnością co do właściwości grzybobójczych.
3. Ekspozycja *Neosartorya* spp. na działanie naturalnego ekstraktu roślinnego lub konserwantu żywności wpływa na morfologię tych grzybów.
4. Naturalne i syntetyczne środki konserwujące mogą indukować specyficzne strategie przystosowawcze *Neosartorya* spp. do warunków stresowych.
5. Zastosowanie naturalnego ekstraktu roślinnego lub konserwantu żywności prowadzi do zmian w profilu metabolicznym oraz metabolomicznym *Neosartorya* spp.
6. Czas inkubacji wraz z substancjami o właściwościach antymikrobiologicznych ma istotny wpływ na przeżywalność i aktywność metaboliczną izolatów *Neosartorya* spp.

7. Sekwencjonowanie całogenomowe (NGS Illumina®) daje możliwość identyfikacji mechanizmów obronnych *Neosartorya* spp., które mogą odpowiadać za ich wysoką odporność na czynniki stresowe.
8. Ekspozycja owoców truskawki na działanie naturalnego ekstraktu roślinnego lub konserwantu żywności powoduje ukierunkowane, specyficzne zmiany w składzie taksonomicznym społeczności drobnoustrojów i strukturze mikrobiomu owoców.
9. Różna wrażliwość izolatów *Neosartorya* spp. na antygrzybiczne działanie naturalnego ekstraktu roślinnego i konserwantu spożywczego wpływa na skład mikrobiomu oraz profil metaboliczny sztucznie zakażonych owoców truskawki.

2.2 Główny cel rozprawy doktorskiej

Głównym celem badawczym rozprawy doktorskiej było określenie roli właściwości metabolicznych, morfologicznych i genetycznych grzybów z rodzaju *Neosartorya* w kształtowaniu ich odporności na konserwanty, substancje chemiczne i naturalne ekstrakty roślinne, obejmując również testy *in vivo* nad wpływem tych czynników na profil metaboliczny i mikrobiom owoców truskawki.

2.3 Cele szczegółowe rozprawy doktorskiej

Cele szczegółowe pracy obejmowały:

1. Określenie wrażliwości izolatów *Neosartorya* spp. na naturalne ekstrakty roślinne i związki konserwujące.
2. Określenie wrażliwości chemicznej oraz właściwości metabolicznych i morfologicznych izolatów *Neosartorya* spp. o różnej wrażliwości na naturalne ekstrakty roślinne i konserwanty żywności.
3. Określenie wpływu ekstraktu z nagietka oraz pirosiarczynu sodu na fenotyp izolatów z rodzaju *Neosartorya*, o różnej wrażliwości na naturalne ekstrakty roślinne i

konserwanty żywności, wraz z określeniem zmian w metabolomie, uzdolnieniach metabolicznych oraz żywotności i morfologii tych grzybów.

4. Analizę genomu izolatów *Neosartorya* spp. o różnej wrażliwości na konserwanty i ekstrakty roślinne w celu zidentyfikowania różnic w sekwencjach genów, które mogą wpływać na oporność tych izolatów na badane czynniki antymikrobiologiczne.
5. Określenie odporności wybranych izolatów *Neosartorya* spp. na konserwanty i ekstrakty roślinne w warunkach rzeczywistych, tj. w owocach truskawki kontaminowanych tymi grzybami, poprzez analizę wpływu czynników konserwujących na profil metaboliczny i mikrobiom owoców przechowywanych w warunkach chłodniczych.

3. Materiały i metody

3.1 Identyfikacja badanych izolatów

Badania przeprowadzono na wybranych izolatach grzybów z rodzaju *Neosartorya*, pochodzących z kolekcji Laboratorium Mikrobiologii Molekularnej i Środowiskowej Instytutu Agrofizyki PAN w Lublinie. Grzyby te zostały wcześniej wyizolowane z powierzchni owoców pozornych truskawki (*Fragaria* × *ananassa* Duchesne) oraz gleby pobranej z plantacji truskawek zlokalizowanych na terenie Polski w ramach projektu realizowanego pod kierunkiem prof. dr hab. M. Frąć (SONATA 4, NCN, 2012/07/D/NZ9/03357). Szczepy przechowywano w kriobanku w temperaturze -80°C i przed rozpoczęciem doświadczeń poddano ożywieniu na podłożu stałym PDA (Potato Dextrose Agar, BioMaxima S. A., Lublin, Polska) w optymalnej temperaturze 30°C.

Po pięciodniowej inkubacji grzybnię przenoszono do probówek o pojemności 2 ml zawierających kulki szklane (250 mg o średnicy 1,45 mm oraz 500 mg o średnicy 3,15 mm) i bufor Lyse F (EURx®, Polska), a następnie homogenizowano za pomocą homogenizatora FastPrep-24 (MP Biomedicals, USA) przez 20 sekund przy prędkości 4 m s⁻¹. Izolację DNA przeprowadzono z wykorzystaniem zestawu GeneMATRIX Plant and Fungi DNA Purification Kit (EURx®, Polska; Malarczyk et al., 2020).

Identyfikację molekularną szczepów wykonano na podstawie amplifikacji fragmentów genów ITS1 (z użyciem starterów ITS1/ITS2; White et al., 1989) oraz β-tubuliny (startery specyficzne dla *Neosartorya* spp. N2F/N2R oraz *Aspergillus fumigatus* Af1F/R4; Yaguchi et al., 2012). Reakcje PCR prowadzono z wykorzystaniem mieszanki REDTaq® ReadyMix™ (Sigma-Aldrich, USA) oraz 10 μM starterów i 2 μl rozcieńczonego DNA. Produkty PCR analizowano na żelu agarozowym o stężeniu 2%, a następnie oczyszczano enzymatycznie (Exo-BAP, EURx) i poddawano sekwencjonowaniu metodą Sanger (Big Dye® Terminator

v1.1, Thermo Fisher Scientific, USA) z wykorzystaniem analizatora genetycznego Applied Biosystems 3130 Genetic Analyzer (Yaguchi et al., 2012).

W celu określenia pokrewieństwa filogenetycznego, uzyskane sekwencje porównano z danymi dostępnymi w bazie NCBI, włączając sekwencje reprezentatywnych gatunków z rodzaju *Neosartorya* oraz innych blisko spokrewnionych rodzajów grzybów. Analizy filogenetyczne wykonano w programie MEGA 11, stosując 3-parametrowy model Tamury z dyskretnym rozkładem gamma dla regionu ITS1 (Tamura et al., 2021) oraz 2-parametrowy model Kimury (Nishimaki & Sato, 2019) z dyskretnym rozkładem gamma dla sekwencji β -tubuliny. Wyniki identyfikacji pozwoliły na wybór do kolejnych etapów badań 20 izolatów przypisanych do rodzaju *Neosartorya*.

3.2 Testy inhibicji metodą dyfuzyjno-krażkową

W celu oceny przeciwrzybiczego działania wybranych ekstraktów roślinnych oraz konserwantów żywności wobec 20 izolatów z rodzaju *Neosartorya* zastosowano metodę dyfuzyjno-krażkową (Jeannot et al., 2023). Badaniom poddano suche ekstrakty z nagietka (*Calendula officinalis*; Zrób Sobie Krem Kosmetyki Naturalne, Polska) i lawendy (*Lavandula angustifolia*; Greenvit Sp. z o.o., Polska), a także olejki eteryczne (Dr Beta®, FSZ Pollena-Aroma Sp. z o.o., Polska): eukaliptusowy (*Eucalyptus globulus*), lawendowy (*Lavandula angustifolia*), rozmarynowy (*Rosmarinus officinalis* L.), goździkowy (*Eugenia caryophyllus*) i drzewa herbacianego (*Melaleuca alternifolia*). Ekstrakty suche rozpuszczano w wodzie do stężenia 1000 $\mu\text{g mL}^{-1}$, natomiast olejki przygotowywano w postaci wodnych dyspersji o analogicznym stężeniu, celowo unikając stosowania rozpuszczalników organicznych, które mogłyby zakłócić naturalne właściwości lotnych związków czynnych (Pesis, 2005). Następnie wszystkie preparaty rozcieńczano do stężeń: 250, 150, 100, 50 i 5 $\mu\text{g mL}^{-1}$. Konserwanty chemiczne, takie jak pirosiarczyn sodu, sorbinian potasu, wodorosiarczyn sodu i kwas sorbowy (Sigma-Aldrich, USA), rozpuszczano w wodzie do stężeń 6.25 mg mL^{-1} , 12.5 mg mL^{-1} ,

25 mg mL⁻¹, 50 mg mL⁻¹, 75 mg mL⁻¹ i 100 mg mL⁻¹. Dodatkowo analizowano wpływ parametrów fizykochemicznych typowych dla przetworów owocowych, takich jak wartość pH (3,2 i 3,5) oraz zawartość cukru ogólnego wyrażona w stopniach Brix (°Bx) o wartościach 45°Bx, 43°Bx, 39°Bx i 35°Bx (Ermis et al., 2015; Park et al., 2017).

Do przeprowadzenia testów zastosowano podłoże PDA (Potato Dextrose Agar), które inokulowano zawiesiną zawierającą grzybnie i askospory pochodzące z 10-dniowych kultur grzybów z rodzaju *Neosartorya*, standaryzowaną do 75% transmitancji, co odpowiadało liczebności askospor na poziomie około $4,4 \times 10^4$ w 1 mL zwiawiesiny inokulacyjnej. Po wysianiu mikroorganizmów na podłoże, na powierzchnię agaru nanoszono sterylne krążki bibułowe o średnicy 5 mm (Whatman No. 1), na które aplikowano po 30 µL badanego ekstraktu roślinnego lub konserwantu żywności (Pertile & Frąc, 2023). Każdy wariant testowano w trzech powtórzeniach, umieszczając po trzy krążki, zawierające preparaty o tym samym stężeniu w jednej szalce Petriego. Płytki inkubowano w temperaturze 30°C przez 10 dni, a pomiary średnicy stref zahamowania wzrostu prowadzono codziennie przy użyciu cyfrowej suwmiarki.

Uzyskane wyniki umożliwiły ocenę wrażliwości poszczególnych izolatów na badane ekstrakty roślinne i konserwanty żywności oraz identyfikację grup o zróżnicowanym stopniu wrażliwości na analizowane ekstrakty i konserwanty. Na podstawie wyników wytypowano 10 izolatów reprezentujących różne grupy wrażliwości oraz wybrano czynniki konserwujące do dalszych testów inhibicji wzrostu: suche ekstrakty z nagietka i lawendy oraz olejki eteryczne z lawendy, rozmarynu, drzewa herbacianego i goździka, pirosiarczyn sodu, sorbinian potasu, wodorosiarczyn sodu i kwas sorbowy.

3.3 Testy inhibicji metodą mikropłytek MT2 Biolog™

W celu oceny wrażliwości oraz zdolności metabolicznej izolatów *Neosartorya* spp. w obecności wybranych naturalnych ekstraktów roślinnych oraz konserwantów żywności zastosowano testy na mikropłytkach MT2 (Biolog™, Hayward, CA, USA; Frąc et al., 2016).

Na podstawie badań przeprowadzonych w pierwszym etapie wybrano odpowiednie stężenia do testów inhibicji metodą mikropłytek MT2. Każdy ekstrakt roślinny testowano w czterech stężeniach: 1000, 150, 50 oraz 5 $\mu\text{g mL}^{-1}$, a konserwanty żywności badano w trzech stężeniach: 25, 50 oraz 100 mg mL^{-1} . Dodatkowo, badaniami objęto wpływ wodnych roztworów cukrowych o ustalonym pH, odpowiadających warunkom stosowanym w przetwórstwie owoców: 45°Bx przy pH 3,5, 43°Bx przy pH 3,2 oraz 39°Bx przy pH 3,2 (Ermis et al., 2015; Park et al., 2017). Do przygotowania inokulum, izolaty hodowano na podłożu PDA (BioMaxima S.A., Lublin, Polska) w temperaturze 30°C przez 10 dni. Po inkubacji zebrano askospory wraz z grzybnią, które zawieszono w sterylnym płynie inokulacyjnym FF IF (Biolog™, Hayward, CA, USA) i poddano homogenizacji. Zawiesinę standaryzowano do 75% transmitancji. Do każdego dołka płytki MT2 wprowadzano 100 μL roztworu ekstraktu oraz 50 μL zawiesiny grzybni z askosporami. Kontrolę stanowiła zawiesina grzybni z 100 μL wody, bez dodatku ekstraktu. Płytki inkubowano w temperaturze 30°C przez 10 dni, a pomiary wykonywano co 24 godziny przy długościach fali 490 nm (ocena wykorzystania substratów metabolicznych) oraz 750 nm (ocena przyrostu biomasy grzybni) za pomocą czytnika Biolog™ MicroStation (Biolog™, Hayward, CA, USA; Oszust et al., 2023).

Wyniki spektrofotometryczne korygowano poprzez odjęcie wartości tła uzyskanych dla próbek kontrolnych, w których ekstrakty rozcieńczono w płynie inokulacyjnym bez inokulum, aby wyeliminować zakłócenia związane z właściwościami optycznymi ekstraktów. Na podstawie skorygowanych danych obliczono wskaźniki: Średnią Intensywność Oddychania Grzybni (AFRI – *Average Fungal Respiration Intensity*) oraz Średnią Intensywność Wzrostu Grzybni (AFGI – *Average Fungal Growth Intensity*). Porównanie wartości AFRI i AFGI dla próbek z ekstraktami i kontroli pozwoliło na ocenę wpływu poszczególnych ekstraktów na metabolizm i rozwój grzybów (Pertile & Frąc, 2023).

3.4 Analiza profilu metabolicznego izolatów *Neosartorya* spp. z wykorzystaniem płytek Biolog™ FF

Zdolność 10 wybranych izolatów *Neosartorya* spp. do wykorzystania 95 różnych źródeł węgla oceniano za pomocą mikropłytek Biolog™ FF (*Filamentous Fungi MicroPlates*; Biolog™, USA), zawierających barwnik tetrazolowy, który umożliwia pomiar aktywności metabolicznej grzybów (Dobranic & Zak, 1999; Preston-Mafham et al., 2002; Oszust et al., 2018). Izolaty hodowano przez 10 dni w temperaturze 30°C na standardowym podłożu PDA (kontrola), PDA wzbogaconym o pirosiarczyn sodu w stężeniu 150 µg mL⁻¹ lub PDA wzbogaconym o ekstrakt naturalny z nagietka (*C. officinalis*) w stężeniu 150 µg mL⁻¹.

Po inkubacji askospory wraz z grzybnią zbierano i zawieszano w sterylnym płynie inokulacyjnym FF IF (Biolog™), standaryzując zawiesinę do 75% transmitancji (Pawlik et al., 2019). Do każdego dołka płytki dodawano 100 µL zawiesiny grzybni. Eksperyment prowadzono w dwóch powtórzeniach biologicznych i trzech powtórzeniach technicznych, wykonując pomiary w odstępach 24 godzinnych przez 10 dni, przy długościach fali 490 nm oraz 750 nm za pomocą czytnika Biolog™ MicroStation (Biolog™, Hayward, CA, USA).

Wyniki korygowano przez odjęcie wartości tła uzyskanych dla kontroli (zawiesina wody bez substratów). Na podstawie skorygowanych danych obliczano wskaźniki: Średnią Intensywność Oddychania Grzybni (AFRI) oraz Średnią Intensywność Wzrostu Grzybni (AFGI). Różnorodność funkcjonalną określano jako bogactwo substratów (*Substrate Richness*, R), czyli liczbę różnych źródeł węgla wykorzystywanych przez dany szczep, przyjmując próg absorbancji OD > 0,25. Analizowano wykorzystanie substratów z sześciu grup związków organicznych: amin i amidów, węglowodanów, polimerów, aminokwasów, kwasów karboksylowych oraz innych związków (Frąc, 2012; Pinzari et al., 2016).

3.5 Analiza wrażliwości chemicznej izolatów *Neosartorya* spp. z wykorzystaniem płytek Biolog™ PM

Do oceny wrażliwości chemicznej 10 izolatów *Neosartorya* spp. wykorzystano panele Biolog™ PM21-PM25 (Biolog™, USA), zawierające 120 różnych związków chemicznych (Panek et al., 2016). Izolaty grzybów hodowano przez 10 dni na podłożu PDA w temperaturze 30°C, a następnie askospory z grzybnią zawieszano w płynie inokulacyjnym FF IF, standaryzując inokulum do 62% transmitancji. Po 100 µL przygotowanej zawiesiny mikrobiologicznej nanoszono do każdego dołka płytki.

Inkubację prowadzono przez 9 dni w temperaturze 30°C, wykonując codzienne pomiary przy długościach fali 490 nm i 750 nm za pomocą czytnika Biolog™ MicroStation. Wyniki korygowano o tło z kontroli, a na ich podstawie obliczano wskaźniki intensywności oddychania (AFRI) i wzrostu grzybni (AFGI). Linie bazowe ustalano na podstawie kontroli bez dodatku substancji chemicznych.

3.6 Analiza aktywności metabolicznej izolatów *Neosartorya* spp. metodą fluorescencyjną z wykorzystaniem dioctanu fluoresceiny (FDA)

Aktywność metaboliczną pięciu wybranych izolatów *Neosartorya* spp. o różnym stopniu wrażliwości na ekstrakty roślinne i konserwanty oceniano po 10 i 30 dniach inkubacji w temperaturze 30°C na trzech wariantach podłoża PDA: standardowym, z dodatkiem pirosiarczynu sodu (150 µg mL⁻¹) lub ekstraktu z nagietka (150 µg mL⁻¹). Do oceny metabolicznej zastosowano zmodyfikowaną metodę fluorescencji dioctanu fluoresceiny (FDA; Różalska et al., 2014). Po zakończeniu hodowli askospory wraz z grzybnią delikatnie zeszkrobywano z powierzchni podłoża i zawieszano w wodzie, standaryzując zawiesinę do 10% transmitancji. Następnie 1 mL zawiesiny przenoszono do dołków płytki 24-dołkowej (Biologix®, USA). Roztwór FDA (0,7 mM) przygotowywano przez rozpuszczenie proszku FDA (Sigma-Aldrich, USA) w acetonie, a następnie mieszano z buforem fosforanowym o pH

6,4 w stosunku 1:9. Do każdego dołka dodawano 1 mL mieszanki roztworu FDA i buforu. Inkubację prowadzono w ciemności w temperaturze pokojowej, a pomiary fluorescencji wykonywano po 0, 30 i 60 minutach za pomocą czytnika Infinite® 200 Pro (Tecan, Szwajcaria) przy długości fali wzbudzenia 485 nm i emisji 520 nm. Wyniki wyrażano jako intensywność fluorescencji w jednostkach arbitralnych (AU), co pozwalało na ocenę metabolicznej aktywności komórek grzybów (Soboń et al., 2018).

3.7 Analizy mikroskopowe: skaningowa mikroskopia elektronowa (SEM)

Pięć izolatów *Neosartorya* spp., wybranych ze względu na różny poziom wrażliwości na ekstrakty roślinne, hodowano przez 10 i 30 dni na podłożu PDA: standardowym, z dodatkiem pirosiarczynu sodu ($150 \mu\text{g mL}^{-1}$) lub ekstraktu z nagietka ($150 \mu\text{g mL}^{-1}$). Następnie próbki przygotowano do analizy skaningowym mikroskopem elektronowym Phenom ProX (Thermo Fisher Scientific, USA). Materiał umieszczano na aluminiowych stelażach za pomocą przewodzącej taśmy węglowej, suszono w obecności żelu krzemionkowego, a następnie pokrywano warstwą złota o grubości 5 nm (Sputter coater CCU-010 LV, Safematic GmbH, Szwajcaria). Obrazy rejestrowano przy napięciu przyspieszającym 10 kV, z powiększeniami od $200\times$ do $10\,000\times$, wykorzystując detektor BS (Beer-Lech et al., 2022).

3.8 Analizy mikroskopowe: mikroskopia konfokalna fluorescencyjna

Izolaty *Neosartorya* spp. badano również za pomocą konfokalnego mikroskopu fluorescencyjnego Leica TCS SP8 (Wetzlar, Niemcy; Agier et al., 2020). Po 10 i 30 dniach inkubacji na trzech wariantach podłoża PDA (standardowe PDA, PDA z dodatkiem ekstraktu z nagietka w stężeniu $150 \mu\text{g mL}^{-1}$ oraz PDA z pirosiarczynem sodu w stężeniu $150 \mu\text{g mL}^{-1}$) zbierano askospory wraz z grzybnią. Materiał zawieszano w buforze (2% D-glukoza i Na-HEPES), a następnie po wirowaniu ($10\,000\times g$, 5 min) zawiesinę barwiono barwnikiem FUN-1 (10 mM w DMSO, Thermo Fisher Scientific, USA). Inkubację prowadzono w ciemności w temperaturze 30°C przez 5 minut.

Zabarwiony materiał наносzono na szkiełko podstawowe, przykrywano szkiełkiem nakrywkowym i obrazowano przy długości fali wzbudzenia 488 nm oraz emisji 530 nm (Walaszczyk et al., 2024). Dodatkowo wykonywano zdjęcia w świetle widzialnym w celu wizualizacji różnic w produkcji zarodników. Analizę żywotności przeprowadzano, wyznaczając stosunek intensywności fluorescencji czerwonej do zielono-żółtej w jedenastu obszarach o wymiarach $20 \times 20 \mu\text{m}$ na trzech powtórzeniach dla każdego wariantu hodowli (Lee & Bae, 2018). Otrzymany stosunek mnożono przez 100, aby wyrazić procentową wartość żywotności komórek.

3.9 Analiza metabolomiczna metodą LC-QTOF MS

Profilowanie metabolomiczne izolatów *Neosartorya* spp. wykonano przy użyciu chromatografii cieczowej (LC) sprzężonej z kwadropolowym spektrometrem mas czasu przelotu (QTOF MS) – Agilent Infinity 1290 oraz Agilent 6550 iFunnel QTOF (Agilent Technologies, USA) z jonizacją elektrosprejową (ESI; Fornal et al., 2013). Materiał biologiczny stanowiła grzybnia z 10- i 30-dniowych kultur *Neosartorya* spp. na podłożu PDA, hodowanych z i bez dodatku pirosiarczynu sodu lub ekstraktu z nagietka. Z każdej próbki odważano 20 mg grzybni, a rozdrabnianie tkanek przeprowadzono za pomocą kul ceramicznych (2,8 mm) w homogenizatorze Bead Mill MAX (VWR) w czterech cyklach po 10 sekund przy prędkości 6 m s^{-1} . Do zhomogenizowanej grzybni dodano 1 mL schłodzonego roztworu ekstrakcyjnego (metanol:chloroform:woda, stosunek objętościowy 2,5:1:0,5), mieszano przez 10 sekund i inkubowano na lodzie przez 8 minut. Po wirowaniu ($14\,000 \times g$, 4 min, 4°C) oddzielono supernatant zawierający metabolity od osadu. Dodano 500 μL wody Milli-Q, wymieszano i ponownie odwirowano w celu rozdzielenia faz polarnych i niepolarnych. Fazę polarną przeniesiono do nowej probówki i suszono w warunkach próżniowych (miVac Duo, Genevac). Próbkę rozpuszczano w 100 μL mieszaniny metanol:etanol (1:1) dla frakcji niepolarnej lub metanol:woda z 0,1% dodatkiem kwasu

mrówkowego (1:1) dla frakcji polarnej, mieszając przez 5 minut. Separację chromatograficzną przeprowadzono na kolumnie Agilent ZORBAX RRHD Extend-C18 (2,1 × 100 mm, 1,8 μm) z fazą ruchomą: 0,1% kwas mrówkowy w wodzie (faza A) oraz 0,1% kwasu mrówkowego w acetonitrylu (faza B), przy przepływie 0,4 mL min⁻¹ i gradiencie 3–95% fazy B w ciągu 25 minut. Temperatura kolumny wynosiła 45°C, a objętość wtrysku 10 μL. Analizy prowadzono w trybie jonizacji dodatniej (ESI+), z parametrami: temperatura źródła jonów 225°C, przepływ azotu 12 L min⁻¹, ciśnienie nebulizatora 50 psi, temperatura gazu osłonowego 275°C, napięcie kapilarne 3500 V, napięcie dyszy 1000 V oraz fragmentatora 275 V. Spektrometr pracował z szybkością skanowania 1,2 spektrum s⁻¹. Kalibrację wewnętrzną zapewniono za pomocą dwóch mas referencyjnych (121,0509 i 922,0098 m/z). Dane rejestrowano i analizowano przy użyciu oprogramowania Agilent Mass Hunter (wersje B.09.00 i B.10.00).

3.10 Sekwencjonowanie całogenomowe (WGS) izolatów *Neosartorya* spp.

Do analizy różnic genetycznych między pięcioma izolatami *Neosartorya* spp. zastosowano technikę sekwencjonowania następnej generacji (NGS - Next Generation Sequencing). Materiał genetyczny izolowano z homogenizowanych próbek grzybni przy użyciu zestawu GeneMATRIX Plant & Fungi DNA Purification Kit (EURx, Polska; Malarczyk et al., 2020). Jakość i ilość DNA oceniono spektrofotometrycznie (NanoDrop 2000) oraz fluorometrycznie (QuantusFluor® dsDNA, Promega). DNA rozcieńczono do stężeń 100–500 ng w objętości 30 μl. Biblioteki do sekwencjonowania przygotowano za pomocą zestawu Illumina DNA Prep Kit (Price et al., 2021). Jakość bibliotek weryfikowano za pomocą systemu TapeStation 4150 oraz fluorometru Quantus. Biblioteki normalizowano do stężenia 0,02 nmol L⁻¹, łączono i denaturowano przed sekwencjonowaniem na platformie Illumina MiSeq w trybie 300 par odczytów (paired-end). Dane surowe poddano obróbce bioinformatycznej. Porównania sekwencji i wyrównania wykonano za pomocą narzędzi BLAST (Basic Local Alignment

Search Tool, Altschul et al., 1990; Camacho et al., 2009) oraz zasobów NCBI (National Center for Biotechnology Information, Sayers et al., 2022).

3.11 Analiza aktywności metabolicznej mikrobiomu owoców truskawki metodą Biolog™ EcoPlates

Do oceny aktywności metabolicznej społeczności mikrobiologicznych owoców truskawki zakażonych *Neosartorya* spp. poddanych różnym zabiegom zastosowano system Biolog™ z wykorzystaniem mikroplatek EcoPlates (Biolog™, USA; Pylak et al. 2023). Z każdej próbki tworzono homogenizat (Smasher®, AES Laboratoire, Francja), z którego 1 g dodawano do 99 mL jałowej soli peptonowej. Zawiesinę poddawano 20-minutowemu wstrząsaniu w temperaturze pokojowej, a następnie inkubowano przez 30 minut w 4°C. Po inkubacji do każdego dołka płytek EcoPlates, zawierających 31 różnych źródeł węgla przeznaczonych do profilowania metabolicznego mikrobiomu, наносono 120 µL przygotowanego inokulum (Mącik et al., 2023). Płytki inkubowano w temperaturze 30°C przez 216 godzin, dokonując pomiarów gęstości optycznej przy długości fali 590 nm w odstępach 24-godzinnych za pomocą czytnika MicroStation (Biolog™, Hayward, CA, USA).

3.12 Sekwencjonowanie metataksonomiczne (Illumina MiSeq)

Z 0,25 g homogenatu każdej próbki owoców truskawki zanieczyszczonych *Neosartorya* spp. izolowano całkowite DNA za pomocą zestawu Fast DNA Spin Kit for Feces (MP Biomedical, USA) zgodnie z protokołem producenta, wzbogaconym o dodatkowy etap homogenizacji kulkami w homogenizatorze FastPrep-24 (MP Biomedical, USA) przez 40 sekund przy prędkości 6,0 m s⁻¹, co zwiększało wydajność izolacji DNA grzybów. Jakość i ilość DNA oceniano spektrofotometrycznie (NanoDrop 2000/2000c) oraz fluorometrycznie (Quantus z odczytnikami QuantiFluor® ONE, Promega).

Do profilowania społeczności mikrobiologicznych amplifikowano fragmenty genu 16S rRNA (region V4) dla bakterii przy użyciu starterów 515F–806R (Apprill et al., 2015) oraz

region ITS2 dla grzybów za pomocą starterów ITS3/ITS4 (Op De Beeck et al., 2014). Przygotowano biblioteki do sekwencjonowania (Siegieda et al., 2023). W tym celu amplifikację prowadzono z użyciem polimerazy KAPA HiFi HotStart ReadyMix (Kapa Biosystems) w warunkach: denaturacja 95°C przez 3 minuty, 35 cykli (95°C 30 s, 55°C 30 s, 72°C 60 s) oraz elongacja końcowa 72°C przez 5 minut. Amplikony oczyszczano za pomocą kulek magnetycznych CleanNGS (CleanNA).

Każda próbka została zindeksowana unikalnym adapterem Illumina. Po oczyszczeniu i pomiarze stężenia DNA (Quantus, Promega) oraz ocenie jakości i rozmiaru fragmentów (D1000 DNA ScreenTape, TapeStation 4150, Agilent), próbki rozcieńczano do jednolitego stężenia i łączono w pulę o końcowej koncentracji 8 pM. Sekwencjonowanie przeprowadzono na platformie Illumina MiSeq w trybie 2×300 par odczytów, z dodatkiem 2.5% PhiX.

4. Omówienie wyników publikacji oraz badań uzupełniających

W publikacjach P.1, P.2, P.3 oraz w badaniach uzupełniających przedstawionych w manuskryptach publikacji P.4 i P.5 przeanalizowano wpływ właściwości metabolicznych, morfologicznych i genetycznych grzybów *Neosartorya* spp. (*Aspergillus* spp.) na ich odporność wobec związków konserwujących, substancji chemicznych oraz naturalnych ekstraktów roślinnych. Szczególną uwagę poświęcono zmianom w fizjologii i morfologii, zachodzącym pod wpływem wyżej wymienionych czynników stresowych. Ponadto, istotną część badań stanowiła analiza mikrobiomu owoców truskawki kontaminowanych izolatami *Neosartorya* spp., w tym analiza metaboliczna oraz metataksonomiczna, pozwalające na ocenę interakcji między grzybami, ekstraktem roślinnym lub konserwantem, a środowiskiem żywnościowym.

4.1 Publikacja P.1: Soil-Borne *Neosartorya* spp.: A Heat-Resistant Fungal Threat to Horticulture and Food Production –An Important Component of the Root-Associated Microbial Community

Bibliografia: Maj, W., Pertile, G., Frąc, M. 2023. Soil-Borne *Neosartorya* spp.: A Heat-Resistant Fungal Threat to Horticulture and Food Production—An Important Component of the Root-Associated Microbial Community. *International Journal of Molecular Sciences*, 24(2), 1543. <https://doi.org/10.3390/ijms24021543>

Celem publikacji P.1 było zebranie i usystematyzowanie aktualnej wiedzy na temat mechanizmów molekularnych, biochemicznych i strukturalnych warunkujących szeroką odporność na niekorzystne warunki środowiskowe u grzybów z rodzaju *Neosartorya*. Grzyby te, zostały przedstawione jako istotny czynnik zanieczyszczający produkty roślinne i owocowe, podkreślając ich negatywny wpływ na jakość produktów spożywczych. W pracy przedstawiono także mechanizmy adaptacyjne grzybów, które zwiększają ich odporność na niekorzystne warunki środowiskowe. Przedstawiono również potencjalne kierunki wykorzystania tej wiedzy

w celu opracowania metod przedłużania trwałości produktów spożywczych oraz przeciwdziałania zakażeniom w kontekście zrównoważonego rolnictwa.

Unia Europejska promuje zrównoważone rolnictwo oraz ograniczenie stosowania syntetycznych środków chemicznych, kładąc nacisk na naturalne rozwiązania wspierające zdrowie roślin i jakość żywności. W tym kontekście istotne są grzyby termoodporne, takie jak niektóre gatunki z rodzaju *Neosartorya*, które kolonizują ryzosferę i mogą obniżać jakość produktów przetworzonych. Grzyby te są w stanie przetrwać procesy pasteryzacji i powodować psucie się owoców. Wysoka odporność chemiczna dodatkowo utrudnia ich zwalczanie. Choć wybrane konserwanty i kwasy organiczne wykazują częściową skuteczność, obserwuje się wzrost oporności *Neosartorya* spp., m.in. na azole. Publikacja przeglądowa systematyzuje wiedzę o *Neosartorya* spp. w kontekście jakości żywności i zgodności z europejskimi strategiami ekologicznymi.

W pracy przedstawiono charakterystykę morfologii, cyklu życiowego oraz bioróżnorodności gatunków rodzaju *Neosartorya*. *Neosartorya* spp. to grzyby należące do rodziny Aspergillaceae, będące teleomorficzną formą *Aspergillus* spp., zdolne do wytwarzania wysoce termoodpornych askospor. Ich struktury rozrodcze, takie jak kleistotecja i gymnotecja, oraz ornamentyka powierzchni askospor stanowią cechy taksonomiczne, umożliwiające rozróżnianie gatunków. Mimo morfologicznych podobieństw do *Aspergillus fumigatus*, gatunki takie jak *N. fischeri*, *N. pseudofischeri*, czy *N. glabra* wykazują większą różnorodność genetyczną oraz odmienność w tempie wzrostu oraz morfologii i strukturze kolonii. Sekcja Fumigati obejmuje 23 potwierdzone gatunki *Neosartorya*, z których niektóre – jak *N. spinosa*, *N. laciniosa*, *N. hiratsukae* – były izolowane z owoców i gleby w Polsce i odpowiadają za psucie żywności poddawanej obróbce cieplnej.

Następnie opisano zagrożenia oraz korzyści związane z występowaniem grzybów z rodzaju *Neosartorya*. To wyjątkowo termoodporne grzyby, zdolne do wzrostu i sporulacji nawet

po obróbce cieplnej żywności, szczególnie w produktach kwaśnych o niskim progu tolerancji temperaturowej. Skutkiem ich obecności może być produkcja mykotoksyn, takich jak aflatoksyny, fumitremorginy, werrukulogen i gliotoksyna, które stanowią zagrożenie dla jakości roślin oraz zdrowia ludzi i zwierząt. *Neosartorya* spp. może być też patogenem ludzi, często błędnie identyfikowanym jako *A. fumigatus*, co utrudnia diagnostykę i leczenie. Należy jednak podkreślić, że chociaż grzyby te syntetyzują trwałe metabolity wtórne, które mogą być szkodliwe, to część z nich znajduje również zastosowanie w przemyśle, co czyni je przydatnymi np. procesach bioremediacji, zwalczaniu szkodliwych, często fitopatogenicznych drobnoustrojów dzięki właściwościom przeciwbakteryjnym, a także degradacji różnych niepożądanych i toksycznych związków ze względu na syntezę enzymów. Grzyby z rodzaju *Neosartorya* mogą być zatem zarówno zagrożeniem, jak i stanowić potencjalne narzędzie biotechnologiczne.

W pracy przedstawiono także mechanizmy odpowiedzialne za wysoką odporność *Neosartorya* spp. na niekorzystne warunki otoczenia. Zdefiniowano też wytrzymałość grzybów należących do tego rodzaju na takie czynniki jak wysoka temperatura, pH, czy zawartości cukru. Termoodporność *Neosartorya* spp. powodowana jest m.in. obecnością białek szoku cieplnego, struktur wielokomórkowych i przetrwalników. Białka szoku cieplnego (HSP), takie jak Hsp90, Hsp70 i Hsp40, odgrywają kluczową rolę w odporności grzybów na stres, wspomagając procesy takie jak fałdowanie białek, transkrypcję i konidiację, co umożliwia przetrwanie w warunkach podwyższonej temperatury. Trehaloza oraz oligosacharydy na bazie trehalozy (izobemizioza, neosartoza i fiszeroza), nagromadzone w komórkach grzybów pod wpływem stresu cieplnego, działają jako substancje ochronne, stabilizując biomolekuły komórkowe, co przyczynia się do termotolerancji. Mannitol, współdziałając z trehalozą, zmniejsza stres oksydacyjny i wspomaga przetrwanie grzybów w trudnych warunkach termicznych. Grzyby z rodzaju *Neosartorya* produkują także różne metabolity odporne na

wysokie temperatury, takie jak egzopoligalakturonaza, które podtrzymują aktywność metaboliczną w warunkach o podwyższonej temperaturze. Produkcja enzymów termofilnych, takich jak glukoamylaza czy glikozydaza, także pozwala grzybom na przetrwanie i wzrost w wysokotemperaturowych środowiskach, zapewniając im odporność na stres cieplny.

W pracy opisano również interakcje grzybów z rodzaju *Neosartorya* z roślinami, zwracając uwagę na ich powszechne występowanie w glebie, co ułatwia ich przenoszenie na rośliny i wpływa na jakość owoców po zbiorach. Grzyby te mogą zarówno szkodzić roślinom, przyspieszając ich gnicie, jak i tworzyć symbiozę, promując ich wzrost. Wskazano również, że *Neosartorya* spp. mogą powodować straty plonów, co stanowi zagrożenie dla sektora rolnego. W obliczu rosnącej potrzeby zrównoważonego rozwoju w rolnictwie, konieczne staje się prowadzenie badań nad odpornością *Neosartorya* spp. na środki zwalczania grzybów i opracowanie nowych metod ochrony plonów.

W pracy podkreślono także znaczenie Polski w produkcji owoców. Kraj ten stał się liderem w produkcji owoców, takich jak jabłka, wiśnie czy truskawki, co ma ogromne znaczenie dla gospodarki, zwłaszcza w kontekście eksportu. Zwrócono uwagę na zagrożenia związane z grzybami termoodpornymi, które powodują straty po zbiorach i podkreślono potrzebę dalszych badań nad nowymi, ekologicznymi metodami ochrony plonów, w tym nad wykorzystaniem naturalnych fungicydów, roślinnych ekstraktów czy olejków eterycznych.

W niniejszej pracy dokonano kompleksowego przeglądu mechanizmów adaptacyjnych grzybów *Neosartorya* spp. do warunków stresowych, szczególnie pod kątem ich odporności na metody ochrony plonów i roślin. Przedstawiono także analizę potencjalnych zagrożeń związanych z obecnością tych grzybów w produktach roślinnych i owocowych, zwracając uwagę na ich wpływ na jakość żywności. Wskazano na potrzebę dalszych badań w celu opracowania nowych, ekologicznych metod ochrony plonów, które będą mogły skutecznie

przeciwdziałać zakażeniom przez grzyby termoodporne, jednocześnie wspierając rozwój zrównoważonego rolnictwa.

4.2 Publikacja P.2: Comprehensive antifungal investigation of natural plant extracts against *Neosartorya* spp. (*Aspergillus* spp.) of agriculturally significant microbiological contaminants and shaping their metabolic profile

Bibliografia: Maj, W., Pertile, G., Różalska, S. Skic, K., Frąc, M. 2024. *Comprehensive antifungal investigation of natural plant extracts against Neosartorya spp. (Aspergillus spp.) of agriculturally significant microbiological contaminants and shaping their metabolic profile. Scientific Reports 14, 8399. <https://doi.org/10.1038/s41598-024-58791-4>*

Celem przeprowadzonych i opisanych w publikacji P.2 badań była ocena wrażliwości izolatów grzybów *Neosartorya* spp. na naturalne ekstrakty roślinne o udowodnionych w literaturze właściwościach antymikrobiologicznych. Dodatkowo, zbadano i porównano efektywność właściwości przeciwgrzybiczych testowanych naturalnych ekstraktów roślinnych oraz przeprowadzono profilowanie metaboliczne wybranych izolatów, aby określić zmiany w zdolnościach metabolicznych pod wpływem działania ekstraktów roślinnych.

W ostatnich latach, w wyniku zmian klimatycznych oraz częstszych zakażeń patogenami, obserwuje się nowe wyzwania ekonomiczne dla sektora rolnictwa, szczególnie w zakresie produkcji owoców i przechowywania produktów pożywnych. Grzyby odporne na wysokie temperatury (HRF - Heat-Resistant Fungi) są istotnym problemem w łańcuchu żywnościowym, zwłaszcza w produktach spożywczych bazujących w swoim składzie na owocach, ponieważ zarodniki grzybów są zdolne do przetrwania procesów pasteryzacji i sterylizacji, co znacząco utrudnia skuteczną konserwację. Na szczególną uwagę zasługują grzyby z rodzaju *Neosartorya*, teleomorficzne formy *Aspergillus* spp., które należą do najczęściej występujących grzybów w produkcji spożywczej. Odporność grzybów z rodzaju *Neosartorya* na wysokie temperatury, azole i szereg związków chemicznych, a także ich zdolność do produkcji

mykotoksyn powodują, że stanowią one istotny przedmiot badań nad alternatywnymi metodami kontroli i ograniczania ich występowania.

Pierwszy etap opisywanych badań obejmował wyselekcjonowanie 20 izolatów ze zbioru Laboratorium Mikrobiologii Molekularnej i Środowiskowej Instytutu Agrofizyki Polskiej Akademii Nauk (Lublin, Polska), stworzonej w ramach wcześniej realizowanego, pod kierunkiem prof. dr hab. M. Frąć, projektu SONATA 4 (2012/07/D/NZ9/03357), poprzez sekwencjonowanie markera ITS1 oraz fragmentu funkcjonalnego genu β -tubuliny specyficznego dla tego rodzaju grzybów. Następnie, do oceny wpływu ekstraktów roślinnych na wzrost 20 izolatów *Neosartorya* spp. zastosowano test dyfuzyjno-krażkowy. W eksperymencie wykorzystano suszony ekstrakt z lawendy i nagietka oraz olejki eteryczne: lawendowy, herbaciany, goździkowy i rozmarynowy, w różnych stężeniach (1000, 250, 150, 100, 50 i 5 $\mu\text{g mL}^{-1}$). Inokulum zawierające askospory *Neosartorya* spp. naniesiono na podłoże agarowe w płytkach Petriego, na którym umieszczono jałowe krążki papierowe (bibuła Whatman No. 1) nasączone badanymi ekstraktami. Płytki inkubowano w temperaturze 30°C przez 10 dni, codziennie obserwując wzrost grzybów oraz mierząc średnicę stref zahamowania wzrostu. Na podstawie wyników, 20 izolatów *Neosartorya* spp. podzielono na 5 grup o odmiennej wrażliwości na ekstrakty roślinne. Izolat G150/14 okazał się najbardziej wrażliwy, a G135/14 i G160/14 najodporniejsze na ogólny wpływ ekstraktów roślinnych. Najsilniejszy efekt hamujący wzrost grzybów zaobserwowano w szczególności dla olejku goździkowego w stężeniu 1000 $\mu\text{g mL}^{-1}$.

W celu jeszcze dokładniejszej oceny wrażliwości *Neosartorya* spp. na różne stężenia wyżej wymienionych ekstraktów roślinnych przeprowadzono testy mikropłytkowe MT2 (Biolog™). Czyste kultury grzybów hodowano na podłożu PDA, a następnie przygotowane z kultur płynne inokulum poddano analizie z zastosowaniem ekstraktów w różnych stężeniach w dołkach mikropłytek. Inkubację prowadzono w temperaturze 30°C przez 10 dni, a rozwój grzybów

monitorowano za pomocą spektrofotometrii przy długościach fal 490 nm i 750 nm. Wyniki porównano z kontrolą, w której grzyby nie były ekspozowane na działanie ekstraktów, co pozwoliło na ocenę wpływu badanych substancji na wzrost i metabolizm grzybów. Analiza testów mikropłytkowych MT2 pozwoliła na wyróżnienie trzech głównych grup izolatów *Neosartorya* spp., charakteryzujących się różną wrażliwością na ekstrakty roślinne: wysokiej (G150/14), średniej (G126/14, G127/14, G129/14, G130/14, G132/14, G151/14, G154/14) i niskiej (G135/14, G160/14). Większość ekstraktów wykazała działanie hamujące wzrost grzybów (AFGI), z wyjątkiem olejków lawendowego i rozmarynowego ($1000 \mu\text{g mL}^{-1}$) oraz goździkowego ($50 \mu\text{g mL}^{-1}$), które były nieskuteczne. Najsilniejsze właściwości przeciwgrzybicze zaobserwowano dla suszonego ekstraktu z lawendy ($5 \mu\text{g mL}^{-1}$), ekstraktu z nagietka (5 i $150 \mu\text{g mL}^{-1}$), olejków lawendowego i rozmarynowego ($150 \mu\text{g mL}^{-1}$) oraz olejku z drzewa herbacianego ($5 \mu\text{g mL}^{-1}$). Ponadto, różnice w stosunku AFRI/AFGI wskazały na metaboliczny stres wywołany przez niektóre substancje, szczególnie ekstrakt z nagietka oraz olejki lawendowy i rozmarynowy.

Aby zbadać zmiany metaboliczne *Neosartorya* spp. po preinkubacji z ekstraktem z nagietka, przeprowadzono test mikropłytkowy FF (Biolog™) na 10 wybranych izolatach. Grupa badawcza była hodowana na podłożu PDA wzbogaconym ekstraktem z nagietka ($150 \mu\text{g mL}^{-1}$), natomiast grupa kontrolna na podłożu bez dodatku ekstraktu. Po inkubacji zebrano askospory i grzybnię, które zostały wykorzystane celem stworzenia inokulum z płynem FF. Gęstość optyczną mierzono co 24 godziny przy długościach fal 490 nm i 750 nm. Mikropłytki FF zawierają sześć grup źródeł węgla, a zatem różnorodność funkcjonalną określono na podstawie liczby wykorzystywanych substratów w grupach, wyrażając ją jako bogactwo substratów (R). Dodatkowo różnorodność oceniono za pomocą średniej intensywności oddychania grzybów (Average Fungal Respiration Intensity, AFRI) oraz wzrostu grzybów (Average Fungal Growth Intensity, AFGI), przy czym wartości te skorygowano względem

kontroli wodnej. Preinkubacja izolatów *Neosartorya* spp. z ekstraktem z nagietka spowodowała odmienne odpowiedzi metaboliczne wśród izolatów. Większość wykazała utratę bogactwa substratów, podczas gdy trzy izolaty (G129/14, G135/14, G154/14), charakteryzujące się średnią i niską wrażliwością na działanie ekstraktów roślinnych, zachowały podobne właściwości metaboliczne do kontroli, co wskazuje na ich większą odporność na testowany ekstrakt z nagietka. Ekstrakt z nagietka na ogół powodował zmniejszenie zdolności metabolizowania amin, amidów i węglowodanów. Izolat G150/14, najbardziej wrażliwy na ekstrakty roślinne, wykazywał ograniczoną zdolność adaptacji do stresu metabolicznego.

W publikacji zwizualizowano również morfologię pięciu izolatów o różnych poziomach wrażliwości na testowane ekstrakty. Izolaty były w tym celu kultywowane w optymalnych warunkach i poddane wizualizacji technikami elektronowej mikroskopii skaningowej oraz fluorescencyjnej mikroskopii konfokalnej. Wyniki wykazały, że już po 10 dniach inkubacji wszystkie izolaty grzybów wytworzyły dobrze rozwinięte askospory.

W ramach przedstawionych badań zidentyfikowano trzy główne grupy izolatów o różnej wrażliwości na ekstrakty roślinne. Zaobserwowano także związek między preferencjami metabolicznymi (np. wykorzystaniem związków azotowych lub kwasów karboksylowych) a odpornością na naturalne ekstrakty roślinne. Wyniki podkreśliły skuteczność roślinnych ekstraktów naturalnych w hamowaniu wzrostu *Neosartorya* spp. oraz znaczenie tych badań dla opracowania nowych metod kontroli mikrobiologicznych zanieczyszczeń żywności i zrównoważonego rolnictwa.

4.3 Publikacja P.3: The role of food preservatives in shaping metabolic profile and chemical sensitivity of fungi-an extensive study on crucial mycological food contaminants from the genus *Neosartorya* (*Aspergillus* spp.)

Bibliografia: Maj, W., Pertile, G., Różalska, S., Skic, K., Frąc, M. 2024. The role of food preservatives in shaping metabolic profile and chemical sensitivity of fungi-an extensive study

on crucial mycological food contaminants from the genus *Neosartorya* (*Aspergillus* spp.). *Food Chemistry*, 453, 139583. <https://doi.org/10.1016/j.foodchem.2024.139583>

Celem badań zaprezentowanych w pracy P.3 było określenie poziomu wrażliwości izolatów *Neosartorya* spp. na wybrane konserwanty żywności i substancje chemiczne. Zakładano, że podobnie jak w przypadku ekstraktów naturalnych, blisko spokrewnione izolaty różnią się pod względem wrażliwości, co może wynikać z ich profilu metabolicznego oraz stopnia adaptacji.

Jak wcześniej wspomniano, grzyby z rodzaju *Neosartorya* są powszechnymi zanieczyszczeniami produktów rolnych i spożywczych, wykazującymi wysoką odporność na temperaturę oraz zdolność do przetrwania procesów pasteryzacji. Ich termooporność i oporność na środki konserwujące wiąże się z obecnością askospor o specjalnych właściwościach ochronnych oraz adaptacjami metabolicznymi. Skuteczność konserwantów, zarówno naturalnych, jak i syntetycznych, zależy od wielu czynników, w tym składu produktu (pH, zawartość cukrów) oraz mechanizmów oporności rozwiniętych przez mikroorganizmy. Ze względu na wzrastającą odporność grzybów na konwencjonalne środki grzybobójcze, konieczne jest monitorowanie ich wrażliwości oraz badanie skutecznych alternatyw. W niniejszej pracy oceniono wrażliwość izolatów *Neosartorya* spp. na różne substancje chemiczne i konserwanty oraz określono związek między ich profilem metabolicznym a poziomem oporności na związki konserwujące.

W publikacji P.3 dwadzieścia izolatów z rodzaju *Neosartorya*, które zostały wcześniej przebadane pod kątem wrażliwości na naturalne ekstrakty roślinne, zostało w podobny sposób przetestowanych w kontekście oporności na konserwanty żywności. W pierwszym etapie prezentowanych badań testowano zatem działanie pirosiarczynu sodu, sorbinianu potasu, wodorosiarczynu sodu i kwasu sorbowego, a także różnych kombinacji °Bx/pH na wzrost grzybów z rodzaju *Neosartorya*. W tym celu, przygotowano zawiesiny zarodników i grzybni każdego z izolatów, którymi zaszczepiono płytki z podłożem PDA, a następnie na które

nanoszono po trzy krążki filtracyjne nasączone roztworami substancji chemicznych w czterech stężeniach (25 mg mL⁻¹, 50 mg mL⁻¹, 75 mg mL⁻¹, 100 mg mL⁻¹). Dodatkowo analizowano roztwory o zróżnicowanym °Bx i pH, odpowiadające warunkom w sokach owocowych (45°Bx, 43°Bx, 39°Bx, 35°Bx, każdy o pH 3.2 i 3.5). Wszystkie testowane konserwanty chemiczne – pirosiarczyny sodu, sorbinian potasu, wodorosiarczyny sodu oraz kwas sorbowy – wykazały zdolność hamowania wzrostu 20 izolatów *Neosartorya* spp., choć ich skuteczność była zróżnicowana w zależności od testowanego związku konserwującego i izolatu grzyba. Najsilniejsze działanie zaobserwowano dla pirosiarczynu sodu oraz wodorosiarczynu sodu, natomiast kwas sorbowy był ogólnie najmniej skuteczny wobec testowanych izolatów grzybów. Kombinacje °Bx/pH również powodowały zahamowanie wzrostu grzybni, jednak efekt ten był słabszy i bardziej zbliżony między izolatami. Analiza wyników wykazała także dużą zmienność wrażliwości grzybów na testowane związki konserwujące, pomiędzy poszczególnymi izolatami, pomimo bliskiego stopnia pokrewieństwa.

W opisywanych badaniach, oprócz wykorzystania metody dyfuzyjno-krążkowej, przeprowadzono także testy na mikropłytkach MT2 (Biolog™) dla 10 izolatów wybranych na podstawie wyników pomiarów stref inhibicji. Zawiesiny grzybowe rozcieńczano do 75% transmitancji i nanoszono na mikropłytki zawierające wyżej wymienione chemiczne środki konserwujące lub roztwory cukru o określonym pH. Odczyty prowadzono codziennie przez 10 dni, a mikropłytki inkubowano w temperaturze 30°C. Na podstawie wartości absorbancji uzyskanych przy długościach fali 490 i 750 nm, obliczono wskaźniki AFRI i AFGI, które odzwierciedlają intensywność oddychania i wzrostu grzybów. Wyniki analizy przy użyciu mikropłyt Biolog™ MT2 wykazały, że wszystkie konserwanty hamowały wzrost *Neosartorya* spp., lecz w różnym stopniu i mniej konsekwentnie niż w klasycznym teście inhibicji. Najsilniejsze działanie przeciwdrobnoustrojowe wykazywały wodorosiarczyny sodu i sorbinian potasu w stężeniu 50 mg mL⁻¹, podczas gdy kwas sorbowy przy tym samym stężeniu

powodował wzrost biomasy grzybowej. Kombinacje °Bx/pH wykazywały ograniczoną skuteczność, a istotne zahamowanie wzrostu grzybni zaobserwowano tylko dla niektórych izolatów. Na podstawie obu testów wybrano pirosiarczyn sodu w niskim stężeniu ($150 \mu\text{g mL}^{-1}$), który był efektywnym czynnikiem kontroli grzybów, jednakże umożliwiającym wystarczający niezbędny wzrost kolonii grzybów, jako dodatek do dalszych analiz metabolicznych.

W celu określenia wrażliwości chemicznej 10 izolatów *Neosartorya* spp. zastosowano zestaw mikropłytek Biolog™ PM (PM21–PM25), umożliwiających analizę 120 substancji, m.in. anionów, kationów, antybiotyków, chelatorów oraz związków cyklicznych. Izolaty hodowano przez 10 dni na pożywce PDA w temperaturze 30°C, a następnie pobrano askospory z grzybnią, które zawieszono w sterylnym płynie inokulacyjnym i rozcieńczono do 62% transmitancji. Do każdej płytki wprowadzono 100 μL roztworu przygotowanego zgodnie z protokołem producenta, przy czym inkubowano przez 216 godzin w temperaturze 30°C, z odczytami co 24 godziny. Otrzymane wyniki skorygowano o wartości kontrolne, a następnie obliczono wskaźniki AFRI i AFGI dla optycznych gęstości przy 490 nm i 750 nm, ustalając linie odniesienia na podstawie wyników kontroli pozytywnej. Analiza wykazała, że większość z 120 testowanych substancji chemicznych nie hamowała wzrostu izolatów *Neosartorya* spp., a w wielu przypadkach go stymulowała. Największy przyrost biomasy zaobserwowano w obecności antybiotyków, a także anionów, chelatorów, związków cyklicznych, związków wpływających na błony komórkowe i niektórych związków organicznych. Silne działanie hamujące wykazywały m.in. cyjanian sodu, chlorek miedzi (II), Niaproof®, wodzian metylu oraz cis-platyna. Zauważono znaczną zmienność między izolatami – np. G127/14 cechował się szerokim zakresem możliwości wzrostu na różnych substancjach, podczas gdy G150/14 wykazywał większą odporność na toksyczne związki, ale słabsze zdolności metaboliczne. Wzorce wzrostu wskazują na istnienie izolatów wrażliwych i odpornych, różniących się

zarówno intensywnością wzrostu, jak i zakresem wykorzystania poszczególnych grup związków.

W publikacji przedstawiono także zdolność 10 izolatów *Neosartorya* spp. do wykorzystywania 95 źródeł węgla, którą oceniono przy użyciu płytek Biolog FF, zarówno po hodowli na standardowym podłożu PDA, jak i PDA z dodatkiem pirosiarczynu sodu. Na podstawie skorygowanych wartości OD obliczono wskaźniki AFRI i AFGI oraz określono różnorodność funkcjonalną jako liczbę wykorzystywanych substratów ($OD > 0,25$). Preinkubacja z pirosiarczynem sodu znacząco ograniczyła zdolność 10 izolatów *Neosartorya* spp. do wykorzystywania źródeł węgla, obniżając zarówno intensywność oddychania (AFRI), jak i produkcję biomasy (AFGI). Najsilniejszą wrażliwość na działanie konserwantu, podobnie jak w przypadku ekstraktów roślinnych, wykazał izolat G150/14, a największą odporność — G129/14 i G135/14, które zachowały wysoką aktywność metaboliczną mimo działania konserwantu. Spośród 95 substratów jedynie i-erytrytol był uniwersalnie wykorzystywany przez wszystkie izolaty, pozwalając zarówno na oddychanie, jak i wzrost grzybni po kontakcie z pirosiarczynem sodu. Mimo identycznych warunków preinkubacji, profile metaboliczne izolatów istotnie się różniły.

W pracy dokonano również oceny aktywności metabolicznej z wykorzystaniem metody fluorescencyjnej. W tym celu, wybrano pięć izolatów *Neosartorya* spp. o różnej wrażliwości na konserwanty, które hodowano przez 10 lub 30 dni w temperaturze 30°C na podłożu PDA z dodatkiem pirosiarczynu sodu ($150 \mu\text{g mL}^{-1}$) oraz na podłożu kontrolnym (PDA bez dodatku). Aktywność metaboliczną komórek oceniano metodą fluorescencji diocjanu fluoresceiny (FDA), która wskazuje żywotność komórek poprzez emisję żółto-zielonej fluorescencji. Po inkubacji z roztworem FDA, pomiary fluorescencji prowadzono po 0, 30 i 60 minutach przy użyciu czytnika mikropłytek z funkcją fluorymetru. Spadek intensywności fluorescencji po zastosowaniu konserwantu wskazywał na uszkodzenie struktur komórkowych i obniżoną

aktywność metaboliczną. Barwienie wykazało, że długotrwała ekspozycja na pirosiarczyn sodu na ogół obniża aktywność metaboliczną izolatów *Neosartorya* spp., podczas gdy krótsza ekspozycja nie powodowała istotnych zmian. Niemniej jednak, reakcja na konserwant była zróżnicowana pomiędzy indywidualnymi izolatami w obydwu terminach.

4.4 Badania uzupełniające – P.4: Genome-Wide Analysis, Metabolomic Profiling and Microscopic Evaluation of *Neosartorya* spp. (*Aspergillus* spp.) Diversity and Adaptations to Natural and Synthetic Antimicrobials

4.4.1 Wstęp

Przedstawiciele *Neosartorya* spp. (rodzina Trichocomaceae), stanowiący teleomorficzną formę grzybów z sekcji Fumigati rodzaju *Aspergillus*, obejmują gatunki charakteryzujące się wysoką termoopornością oraz znaczną różnorodnością metaboliczną (Poeaim, 2015; de Sá et al., 2022). Grzyby te wytwarzają zarówno askospory, jak i konidiospory, co sprzyja ich przystosowaniu do różnorodnych środowisk, takich jak gleba czy wody morskie (Eamvijarn et al., 2013). Grzyby z rodzaju *Neosartorya* należą do grupy pleśni termoopornych (HRM - Heat-Resistant Moulds), które są zdolne do przetrwania procesów pasteryzacji i stanowią poważne zagrożenie dla bezpieczeństwa żywności, szczególnie produktów owocowych o wysokiej kwasowości (Santos et al., 2020; Alam et al., 2024). Askospory tych grzybów mogą pozostać nieaktywne w trakcie procesów konserwacji, lecz wykazują zdolność do ponownego rozwoju w warunkach ograniczających wzrost innych drobnoustrojów (Racchi et al., 2020). Dotychczasowe środki konserwujące, takie jak sorbiniany, okazują się wobec nich jedynie częściowo skuteczne lub nieskuteczne, co wskazuje na konieczność pogłębienia wiedzy na temat ich mechanizmów oporności (Berni et al., 2017).

Choć obecnie prowadzone są badania genetyczne dotyczące rodzaju *Aspergillus/Neosartorya*, koncentrują się one głównie na aspektach medycznych lub biotechnologicznych (Hajdu et al., 2019; Daisuke et al., 2020). Sekwencjonowanie

całogenomowe (WGS) umożliwia kompleksową analizę zmienności genetycznej oraz identyfikację potencjalnych mechanizmów oporności (Mariana et al., 2017). W niniejszej pracy, WGS zastosowano do analizy niezmiennych genomów blisko spokrewnionych izolatów *Neosartorya* spp., wykazujących uprzednio różnice we wrażliwości na środki przeciwdrobnoustrojowe (Maj et al., 2024a, 2024b).

W celu zbadania odpowiedzi fenotypowej oraz zdolności adaptacyjnych wybranych izolatów *Neosartorya*, przeprowadzono ekspozycję na dwa rodzaje substancji: syntetyczny konserwant – pirosiarczyn sodu, oraz naturalny ekstrakt z nagietka (*Calendula officinalis*), rozważany jako potencjalna alternatywa dla chemicznych konserwantów, zgodna z ideą zrównoważonego rolnictwa (Di Lorenzo et al., 2013; Ahmadi et al., 2018; do Nascimento & Canteri, 2020; Wesseler, 2022). Następnie, w analizie zastosowano techniki mikroskopowe: elektronową mikroskopię skaningową (SEM), umożliwiającą obserwację struktur morfologicznych (Hong et al., 2006), oraz konfokalną mikroskopię fluorescencyjną, pozwalającą na ocenę integralności błon komórkowych i aktywności metabolicznej (Różalska et al., 2018; Walaszczyk et al., 2024). Uzupełnieniem analiz było profilowanie metaboliczne z wykorzystaniem chromatografii cieczowej sprzężonej z wysokorozdzielczą spektrometrią mas (LC-QTOF-MS), umożliwiające identyfikację metabolitów związanych z odpowiedzią na stres (Tiwari et al., 2016; Chen et al., 2022; Yurchenko et al., 2023).

Celem przeprowadzonych badań była analiza niezmiennych genomów blisko spokrewnionych izolatów *Neosartorya* spp. wykazujących odmienną wrażliwość na środki przeciwdrobnoustrojowe. Przeprowadzona została także ocena fenotypowej odpowiedzi izolatów *Neosartorya* spp. na wybrane środki przeciwgrybicze, w kontekście ich potencjalnych mechanizmów przystosowawczych i podatności na działanie ekstraktów roślinnych i konserwantów żywności, istotnych z punktu widzenia bezpieczeństwa żywności.

4.4.2 Materiały i metody

4.4.2.1 Izolaty grzybowe i kultywacja

Do badań wybrano pięć izolatów z kolekcji Laboratorium Mikrobiologii Molekularnej i Środowiskowej Instytutu Agrofizyki PAN w Lublinie. Izolaty te zostały po raz pierwszy wyizolowane i zidentyfikowane w ramach projektu SONATA4 (ID: 2012/07/D/NZ9/03357, kierownik prof. dr hab. M. Frąc), a ich przynależność do rodzaju *Neosartorya* potwierdzono na podstawie sekwencjonowania regionu ITS1 oraz genu β -tubuliny (Maj et al., 2024a, 2024b). Do dalszych analiz wybrano pięć izolatów różniących się wrażliwością na naturalne ekstrakty roślinne i konserwanty spożywcze, co wykazano we wcześniejszych testach inhibicji (Maj et al., 2024a, 2024b). W celu oceny zmian morfologicznych i metabolicznych izolaty hodowano przez 10 i 30 dni w temperaturze 30°C na trzech wariantach podłoża PDA: bez dodatków, z ekstraktem z nagietka lekarskiego (*Calendula officinalis*) (150 $\mu\text{g mL}^{-1}$) oraz z pirosiarczynem sodu (150 $\mu\text{g mL}^{-1}$). Substancje te rozcieńczano do stężenia 150 $\mu\text{g mL}^{-1}$, które wybrano ze względu na jego zdolność do ograniczania wzrostu grzybni przy jednoczesnym zachowaniu jej rozwoju, co umożliwiało obserwację zmian strukturalnych. Do analizy genomowej izolaty hodowano wyłącznie przez 10 dni w optymalnych warunkach, tj. na podłożu PDA bez dodatków, w celu zachowania ich naturalnego profilu genetycznego.

4.4.2.2 Sekwencjonowanie całogenomowe (WGS)

W celu analizy naturalnych różnic genetycznych pomiędzy pięcioma izolatami *Neosartorya* spp. zastosowano technikę sekwencjonowania całogenomowego następnej generacji (WGS). Materiał genetyczny wyizolowano z użyciem zestawu GeneMATRIX Plant & Fungi DNA Purification Kit (EURx, Polska), zgodnie z protokołem producenta. Jakość i ilość pozyskanego DNA oceniano za pomocą spektrofotometru NanoDrop 2000 (Thermo Scientific, USA) oraz fluorymetru Quantus z odczynnikami QuantusFluor®DNA dsDNA (Promega, USA; Malarczyk et al., 2020). DNA rozcieńczano do stężenia 100–500 ng w

objętości końcowej 30 µl. Biblioteki do sekwencjonowania przygotowano przy użyciu zestawu Illumina DNA Prep Kit, zgodnie z instrukcją producenta oraz z modyfikacjami według literatury (Price et al., 2021). Genomowe DNA poddano tagmentacji w 55°C, a następnie oczyszczono i amplifikowano w 8 cyklach PCR. Jakość bibliotek oceniano przy użyciu systemu TapeStation 4150 i D1000 ScreenTape (Agilent Technologies), a następnie normalizowano do stężenia 0,02 nmol L⁻¹. Sekwencjonowanie przeprowadzono na platformie Illumina MiSeq (tryb 301–301 bp paired-end, zestaw MiSeq v3). Uzyskane dane analizowano bioinformatycznie.

4.4.2.3 Chromatografia cieczowa sprzężona ze spektrometrią mas z analizatorem kwadрупolowym czasu przelotu (LC-QTOF MS)

Profilowanie metabolomiczne metodą LC-QTOF MS przeprowadzono z użyciem systemu Agilent Infinity 1290 UPLC połączonego ze spektrometrem mas Agilent 6550 iFunnel QTOF z jonizacją ESI (JetStream, Agilent Technologies, USA; Fornal 2013). Analizowano grzybnie 10- i 30-dniowych kolonii *Neosartorya* spp. hodowanych na podłożu PDA, w warunkach kontrolnych oraz po inkubacji z pirosiarczynem sodu lub ekstraktem z nagietka lekarskiego (*Calendula officinalis*). Rozdział chromatograficzny przeprowadzono na kolumnie ZORBAX RRHD Extend-C18 (2,1 × 100 mm, 1,8 µm) z fazą ruchomą A: 0,1% HCOOH w wodzie i B: 0,1% HCOOH w acetonitrylu, z przepływem 0,4 mL min⁻¹ i gradientem: 0–25 min (3–95% B), 25–30 min (95% B). Objętość próbki wynosiła 10 µL, a temperatura kolumny – 45°C. Analizy prowadzono w trybie ESI+ przy następujących parametrach: temperatura gazu – 225°C, przepływ azotu – 12 L min⁻¹, ciśnienie nebulizatora – 50 psi, temperatura gazu osłonowego – 275°C, napięcie kapilary – 3500 V, napięcie fragmentora – 275 V. Kalibrację wewnętrzną zapewniały dwie masy referencyjne (121,0509 i 922,0098 m/z). Dane analizowano w oprogramowaniu Agilent MassHunter (B.09.00 i B.10.00).

4.4.2.4 Analizy mikroskopowe

Pięć izolatów *Neosartorya* spp. o odmiennej wrażliwości na substancje o właściwościach antymikrobiologicznych poddano analizie skaningowej mikroskopii elektronowej (SEM) z użyciem mikroskopu Phenom ProX (Thermo Fisher Scientific, USA). Fragmenty kolonii przeniesiono na aluminiowe podstawki z przewodzącą taśmą węglową, osuszono przy użyciu żelu krzemionkowego, a następnie pokryto 5 nm warstwą złota (napylarka CCU-010 LV, Safematic, Szwajcaria). Obrazowanie wykonano detektorem BS przy napięciu 10 kV (Beer-Lech et al., 2022). Pomiarów dokonano w programie ImageJ 1.54g (NIH, USA).

Dodatkowo izolaty analizowano za pomocą konfokalnego mikroskopu laserowego Leica TCS SP8. Po 10 i 30 dniach hodowli na trzech podłożach PDA (kontrolnym, z ekstraktem z *C. officinalis* 150 $\mu\text{g mL}^{-1}$, lub z pirosiarczynem sodu 150 $\mu\text{g mL}^{-1}$), zarodniki i grzybnia zostały zawieszone w buforze (2% glukoza, Na-HEPES) i odwirowane (10 000 $\times$ g, 5 min). Osad zawieszono w buforze z 1 μL barwnika FUN-1 (10 mM w DMSO, Thermo Fisher), inkubowano w ciemności (30°C, 5 min), a następnie naniesiono na szkiełko podstawowe i obrazowano przy użyciu lasera o długości fali wzbudzenia 488 nm i emisji 530 nm (Walaszczyk et al., 2024). Żywotność komórek określono na podstawie fluorescencji: komórki żywe wykazywały emisję czerwonej barwy, a komórki martwe – rozproszoną zielono-żółtą fluorescencję. Intensywność fluorescencji mierzono w jedenastu obszarach (20 $\times$ 20 μm) dla każdego wariantu (3 powtórzenia), a stosunek czerwonej do zielonej fluorescencji przeliczano na procent komórek żywych.

4.4.2.5 Analiza statystyczna

Surowe dane LC-QTOF MS dla izolatów *Neosartorya* spp. hodowanych na 3 wariantach podłoża i w 2 punktach czasowych analizowano w Mass Hunter Qualitative B.10.00, a następnie w Mass Profiler Professional 15.1. W celu redukcji wymiarowości i oceny

klasteryzacji (izolaty, warunki inkubacji, interakcje) zastosowano analizę głównych składowych (PCA) z użyciem danych skalowanych metodą Pareto.

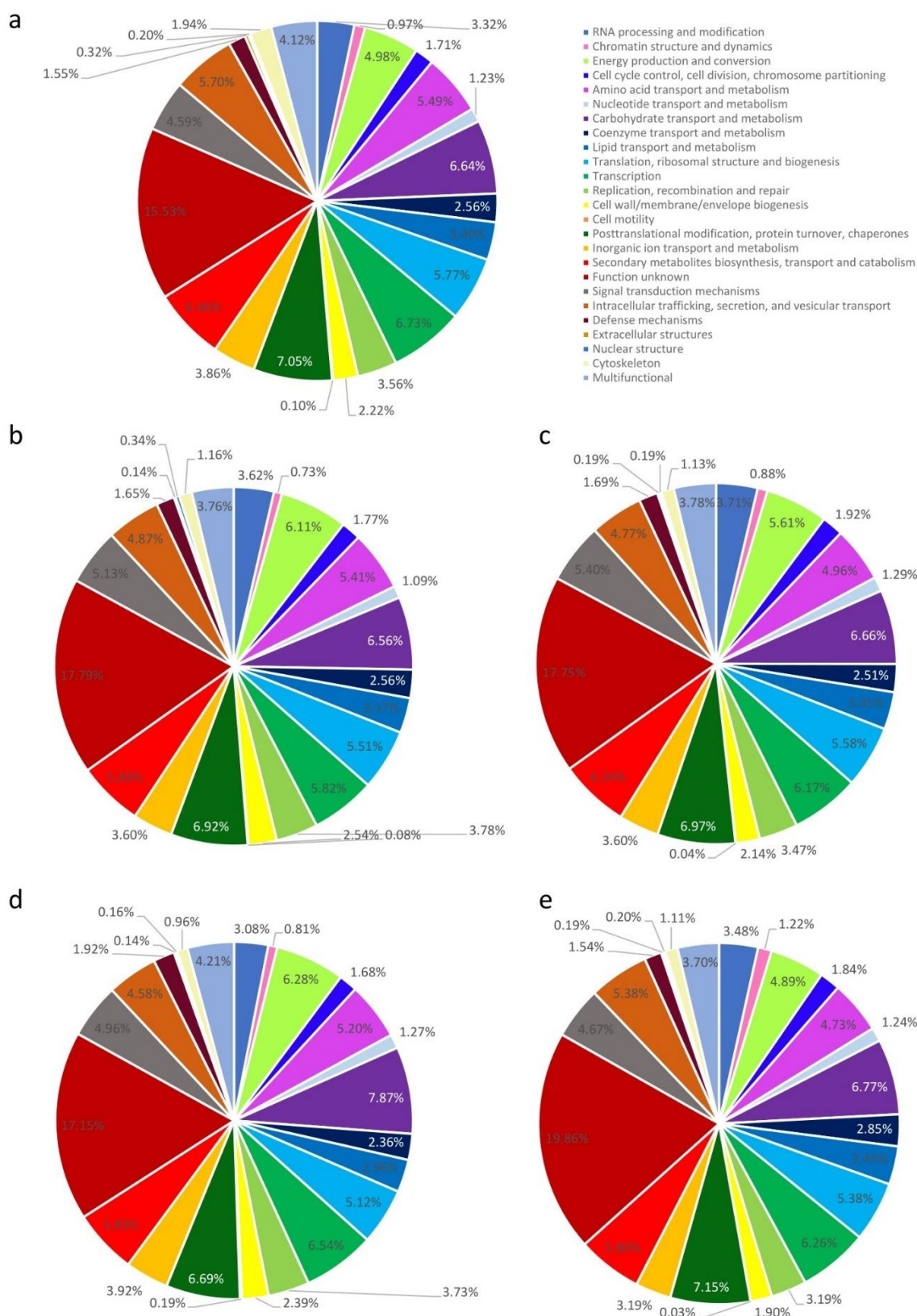
Analizy statystyczne danych dotyczących żywotności przeprowadzono w Statistica™ V13.3 (TIBCO Software Inc.). Normalność rozkładu sprawdzono testem Shapiro-Wilka, a homogeniczność wariancji testem Levene'a. W przypadku spełnienia obu założeń zastosowano test t dla prób niezależnych; przy braku homogeniczności – test t Welcha. Dla danych niespełniających normalności użyto testu U Manna-Whitneya. Za istotne statystycznie uznawano wyniki przy $p < 0.05$ (95% CI).

4.4.3 Wyniki i dyskusja

4.4.3.1 Sekwencjonowanie całogenomowe (WGS)

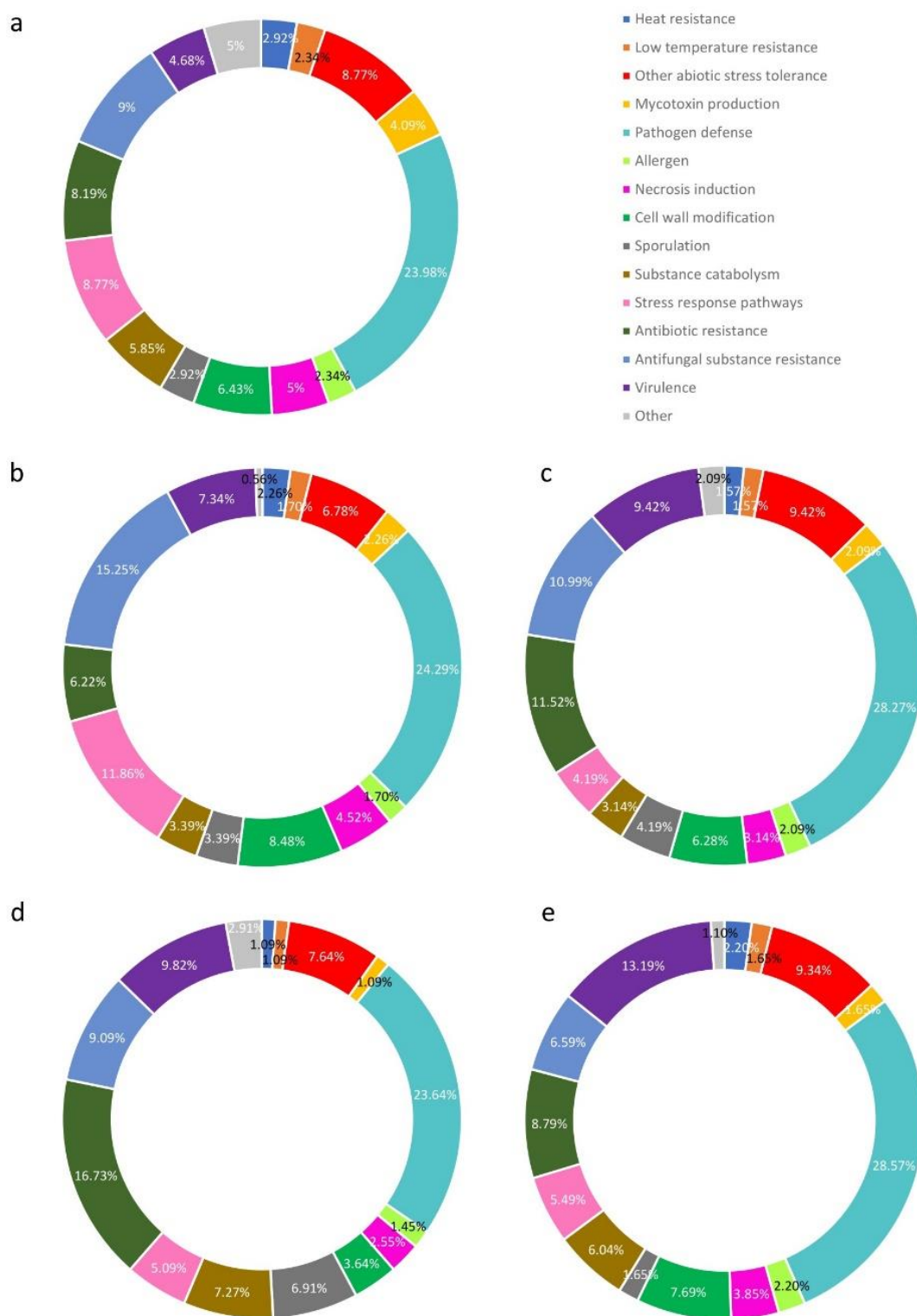
Sekwencjonowanie całogenomowe (WGS) pięciu izolatów *Neosartorya* spp. ujawniło wyraźne podobieństwa w ogólnej strukturze funkcjonalnej genomów, pomimo zróżnicowania fenotypowego izolatów (Rys. 1). We wszystkich genomach dominowały geny o nieznannej funkcji (15,53–19,86%), natomiast najmniej liczne były te związane z ruchem komórkowym (0,03–0,19%). Najczęściej reprezentowane grupy funkcjonalne obejmowały geny związane z metabolizmem węglowodanów, obróbką potranslacyjną białek, replikacją i naprawą DNA oraz biosyntezą metabolitów wtórnych. Podobna kompozycja funkcjonalna sugeruje konserwatywność podstawowych mechanizmów metabolicznych u tych organizmów. Niektóre różnice w rozkładzie genów między izolatami można jednak powiązać z wcześniej ustalonym profilem odporności. Przykładowo, izolat G135/14 (Rys. 1d), określony wcześniej jako najbardziej odporny na testowane naturalne ekstrakty roślinne i środki konserwujące, wykazywał największą ogólną liczbę genów (14 339) oraz najwyższy udział genów związanych z replikacją i naprawą DNA, a także z metabolizmem energetycznym i węglowodanów (7,87%). Z kolei izolat G127/14 (Rys. 1a), oceniany jako najmniej odporny, posiadał najwięcej genów związanych z transportem wewnątrzkomórkowym, biosyntezą

metabolitów wtórnych oraz strukturą cytoszkieletu. Może to wskazywać na inwestowanie zasobów w inne mechanizmy przetrwania niż odporność na zastosowane naturalne środki grzybobójcze.



Rys. 1. Analiza składu funkcjonalnego całego genomu każdego z 5 genomów *Neosartorya* spp. objętych badaniem: G127/14 (a), G130/14 (b), G132/14 (c), G135/14 (d), G150/14 (e).

Genomy izolatów zostały również przeanalizowane pod kątem funkcji obronnych (Rys. 2). Najwięcej genów związanych z mechanizmami obronnymi posiadał izolat G135/14 (Rys. 2d, 1,92%), natomiast najmniej – G127/14 (Rys. 2a, 1,55%) i G150/14 (Rys. 2e, 1,54%). Geny te odpowiadały głównie za rozpoznawanie patogenów, produkcję substancji przeciwwirusowych i antybiotyków oraz odpowiedź immunologiczną. Wysoka liczba takich genów u izolatu G135/14 mogła przyczynić się do jego wcześniej wykazanej odporności (Maj et al., 2024a, 2024b). Izolat G135/14 zawierał najwięcej genów kodujących antybiotykooporność (16,73%), w tym β -laktamazy i aminoglikozydowe fosfotransferazy, oraz geny zwiększające sporulację i rozwój płciowy w warunkach niedoboru cynku (np. Ecl1/2/3). Równocześnie charakteryzował się najmniejszym udziałem genów związanych z odpornością temperaturową, produkcją mykotoksyn i czynników nekrotycznych. To może świadczyć o jego strategicznej specjalizacji w odporności na związki chemiczne.



Rys. 2. Występowanie genów związanych z mechanizmami obronnymi izolatów *Neosartorya* spp: G127/14 (a), G130/14 (b), G132/14 (c), G135/14 (d), G150/14 (e).

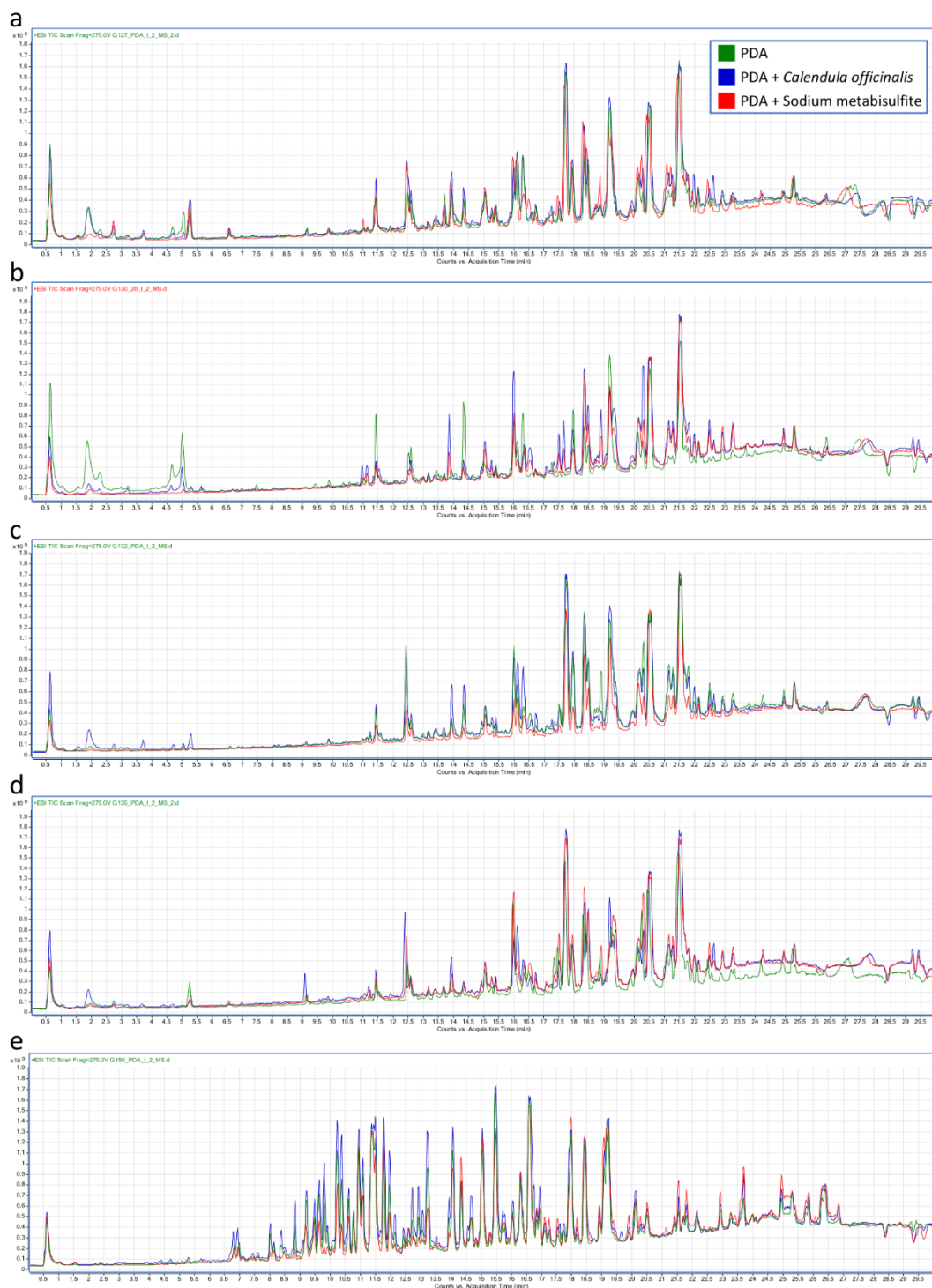
Choć źródła literaturowe donoszą o odporności *Neosartorya* spp. na wysokie temperatury (Salomão et al., 2007), analiza wykazała stosunkowo niewielki udział genów bezpośrednio związanych z tolerancją termiczną u badanych izolatów (1,09–2,92%), głównie kodujących białka szoku cieplnego (HSP). Wśród nich zidentyfikowano domeny funkcjonalne charakterystyczne dla mechanizmów ochrony przed stresem cieplnym, jak STI1_HS-bd, Hikeshi-podobne czy HS1-bd, co potwierdza aktywację złożonych systemów szlaków białek opiekuńczych (Lässle et al., 1997; Muthusamy et al., 2023; Santoro, 2000). Obecność białka STI1 – szaperonu HSP90 – oraz domeny Hikeshi wskazuje na możliwość ochrony białek komórkowych w czasie skoków temperatury, podobnie jak u *Saccharomyces cerevisiae* czy *Aspergillus fumigatus* (Nicolet & Craig, 1989; Tiwari et al., 2015). Co istotne, we wszystkich izolatach obecne były również geny związane z tolerancją niskiej temperatury, takie jak Ltv1, *Low temperature requirement A* i Dlt1, które mogą umożliwiać tym organizmom przetrwanie nawet w warunkach chłodniczych (Merwin et al., 2014; Zheng & Kathariou, 1995). Pojawienie się tych genów u *Neosartorya* spp. może wskazywać na szeroki zakres tolerancji temperaturowej, co utrudnia eliminację tych mikrobiologicznych zanieczyszczeń żywności zarówno w warunkach podwyższonej, jak i obniżonej temperatury.

Oprócz odporności temperaturowej, w analizowanych genomach zidentyfikowano także geny kodujące mechanizmy adaptacyjne do innych stresów abiotycznych. Przykładem może być obecność czujników zasadowego pH (palH/rim21), genów odpowiedzi na hipoksję (HIG1) oraz białek reagujących na stresy środowiskowe, takich jak niskie stężenie glukozy lub promieniowanie UV. Niektóre izolaty posiadały również geny sugerujące zdolność do reagowania na stresy roślinne, np. domenę izomerazy triozowo-fosforanowej reagującą na alkaloidy roślinne, takie jak nikotyna (Cornilescu et al., 2004).

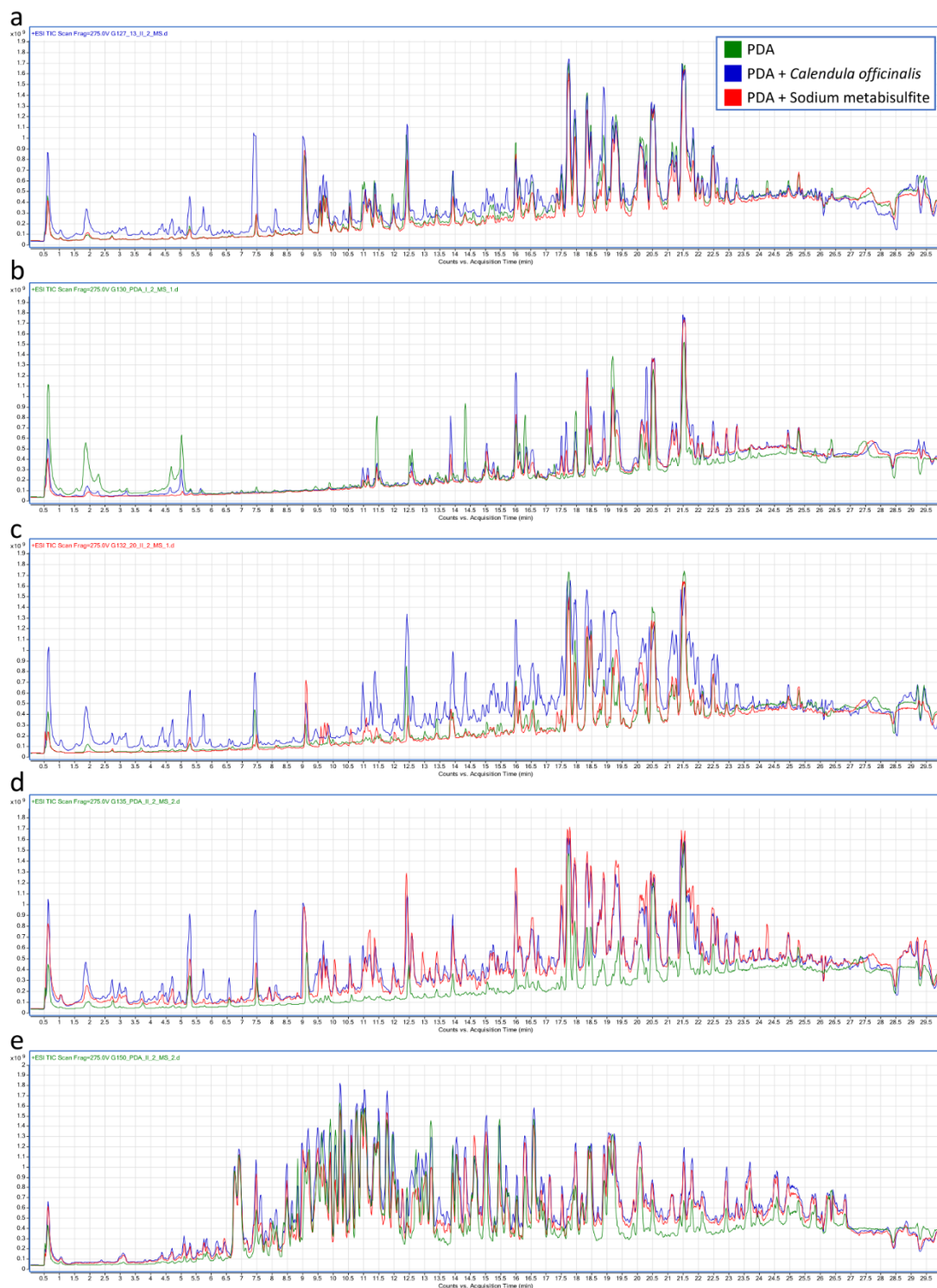
4.4.3.2 Chromatografia cieczowa sprzężona ze spektrometrią mas z analizatorem kwadрупolowym czasu przelotu (LC-QTOF MS)

Stosując niecelowane podejście metabolomiczne, przeanalizowano profile metaboliczne pięciu izolatów *Neosartorya* spp. hodowanych na trzech wariantach podłoży mikrobiologicznych: PDA, PDA z ekstraktem z *Calendula officinalis* oraz PDA z pirosiarczynem sodu. Wyniki wykazały silny wpływ warunków hodowli na metabolom z jednoczesną obserwacją różnic między izolatami o zróżnicowanej wrażliwości na ekstrakty roślinne i konserwanty.

Chromatogramy (Rys. 3 i 4) ujawniły wyraźne zróżnicowanie profili metabolomicznych. Szczególnie odmienny okazał się izolat G150/14 (Rys. 3e, 4e), dla którego obserwowano liczne metabolity eluujące między 6,5 a 15,5 min – niewystępujące u pozostałych izolatów w podobnym nasileniu. Zmiany intensywności pików metabolitów były widoczne we wszystkich warunkach, co potwierdza znaczenie składu podłoża dla metabolizmu grzybów z rodzaju *Neosartorya*.



Rys. 3. Badanie LC-QTOF MS wpływu ekstraktu z nagietka lekarskiego (*Calendula officinalis*) i pirosiarczynu sodu (Sodium metabisulfite) na profile metaboliczne *Neosartorya* spp. po 10-dniowej inkubacji. Chromatogramy przedstawiają izolaty: G127/14 (a), G130/14 (b), G132/14 (c), G135/14 (d), G150/14 (e).



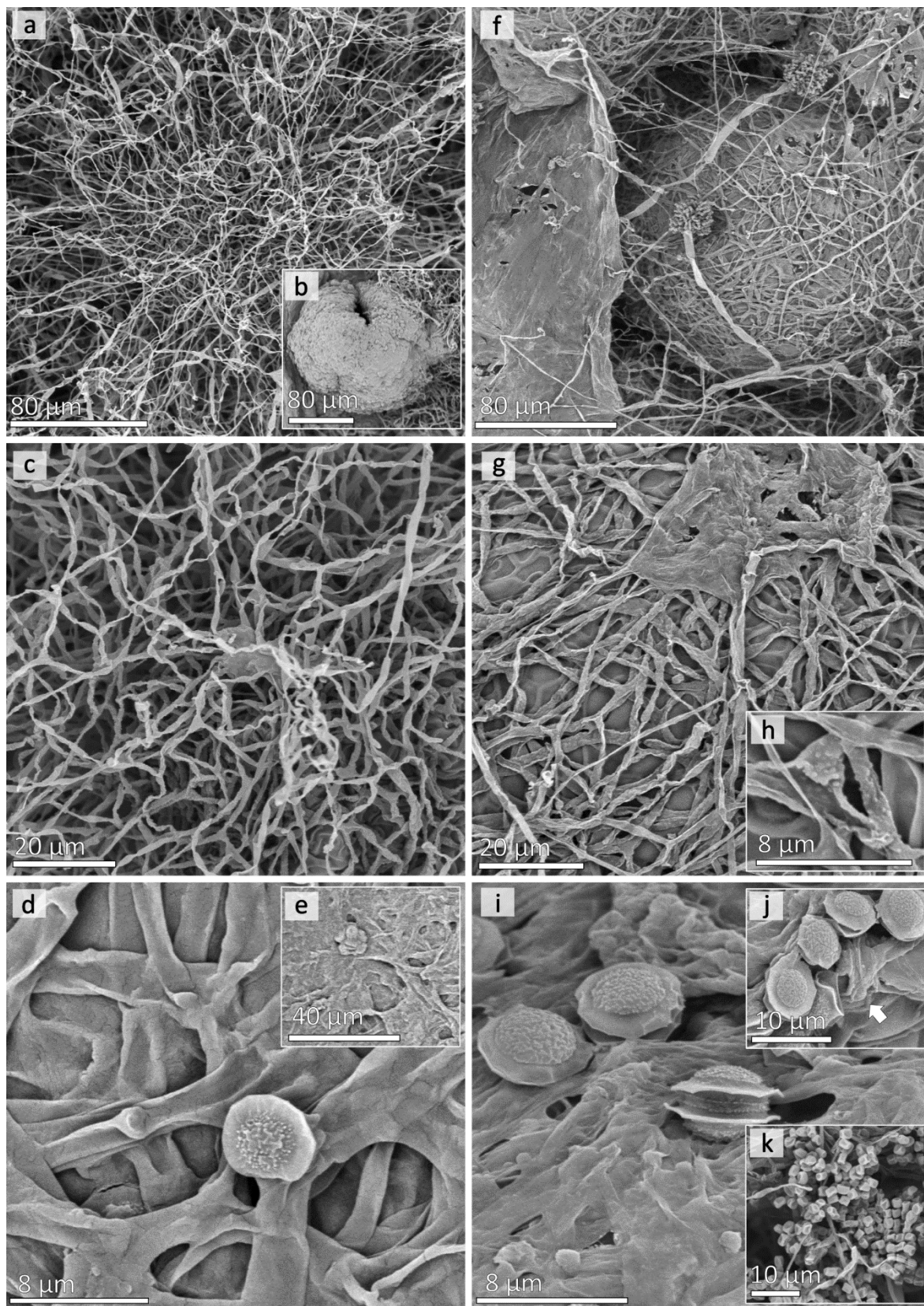
Rys. 4. Badanie LC-QTOF MS wpływu ekstraktu z nagietka lekarskiego (*Calendula officinalis*) i pirosiarczynu sodu (Sodium metabisulfite) na profile metaboliczne *Neosartorya* spp. po 30-dniowej inkubacji. Chromatogramy przedstawiają izolaty: G127/14 (a), G130/14 (b), G132/14 (c), G135/14 (d), G150/14 (e).

Analizy PCA potwierdziły zależność profili od czasu inkubacji i podłoża. Wszystkie izolaty grzybów hodowanych przez 10 dni na podłożu kontrolnym (PDA) uległy wyraźnej separacji w przestrzeni wykresu, natomiast po 30 dniach inkubacji izolaty G132/14 i G135/14 utworzyły wspólny klaster, co sugeruje, że wraz z wydłużeniem czasu hodowli ich profile metabolomiczne zaczęły się do siebie upodabniać. Zastosowanie ekstraktu z nagietka lekarskiego (*Calendula officinalis*) istotnie zmodyfikowało profile metabolomiczne. Po 10 dniach hodowli na tym podłożu największe podobieństwo wykazywały izolaty G130/14 i G132/14, natomiast po 30 dniach każda grupa izolatów tworzyła odrębny klaster, co może sugerować aktywację specyficznych szlaków metabolicznych dopiero po dłuższym okresie adaptacji do obecności ekstraktu roślinnego. Izolaty G130/14 i G132/14 wykazywały także podobieństwo metabolomu po 10 dniach hodowli na podłożu zawierającym pirosiarczyn sodu, lecz po 30 dniach profil metaboliczny G132/14 był bardziej zbliżony do G127/14. Wyniki te potwierdzają dynamiczny charakter zmian metabolomicznych oraz możliwość przebudowy szlaków metabolicznych w czasie. Dodatkowo, gdy analizowano izolaty indywidualnie, wyniki PCA jednoznacznie wskazywały na silny wpływ rodzaju podłoża – izolaty hodowane na PDA, PDA z ekstraktem z nagietka i PDA z pirosiarczynem sodu tworzyły odrębne skupiska niezależnie od czasu inkubacji. Oznacza to, że skład podłoża stanowił dominujący czynnik wpływający na metabolizm *Neosartorya* spp., co może mieć kluczowe znaczenie w kontekście projektowania strategii kontroli tych grzybów w środowiskach żywnościowych.

4.4.3.3 Analizy mikroskopowe

Po 10 dniach hodowli na podłożu PDA zaobserwowano silnie rozgałęzione strzępki powietrzne (Rys. 5a) oraz pojedyncze pęknięte kleistotecjum ($\varnothing 170,5 \mu\text{m}$; Rys. 5b). Strzępki miały gładką powierzchnię (Rys. 5c) i średnicę $0,7\text{--}2,7 \mu\text{m}$. Obecne były dojrzałe, soczewkowate askospory z dwoma wyraźnymi grzbietami ($\varnothing 5,0\text{--}6,0 \mu\text{m}$; Rys. 5d) oraz kuliste worki ($\varnothing 10,6 \mu\text{m}$; Rys. 5e). Po 30 dniach grzybnia pokryta była fragmentami perydium (Rys.

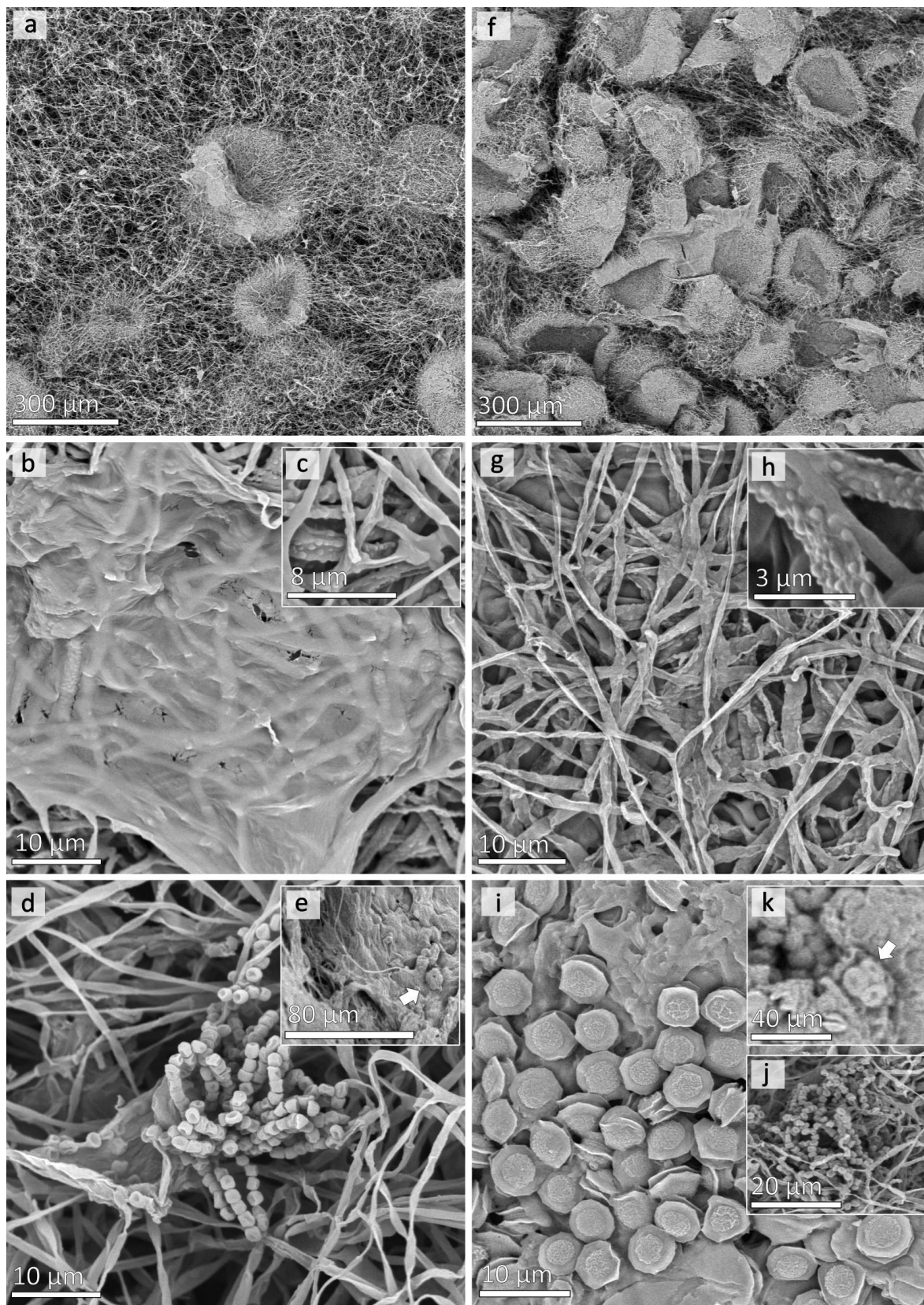
5f), a widoczne strzępki (0,5–1,9 μm) były gładkie (Rys. 5g) lub siateczkowate z brodawkami ($\varnothing$ 0,4 μm ; Rys. 5h). Obecne były dojrzałe askospory ($\varnothing$ 4,7–6,4 μm ; Rys. 5i), worki ($\varnothing$ 6,0 μm ; Rys. 5j) oraz konidiofory z pęcherzykami ($\varnothing$ 5,4 μm), tworzące sześciennie konidiospory z zagłębieniami ($1,7 \times 1,6 \mu\text{m}$; Rys. 5k).



Rys. 5. Morfologia *Neosartorya* sp. G127/14 na podłożu PDA obserwowana za pomocą SEM po 10 (a, b, c, d, e) lub 30 (f, g, h, i, j, k) dniach inkubacji. Ogólna struktura grzybni (a, f),

kleistotecjum (b), powierzchnia strzępek (c, g), wypukłości strzępek (h), askospory (d, i), worki (e, j - zaznaczone strzałką), konidiospory (k).

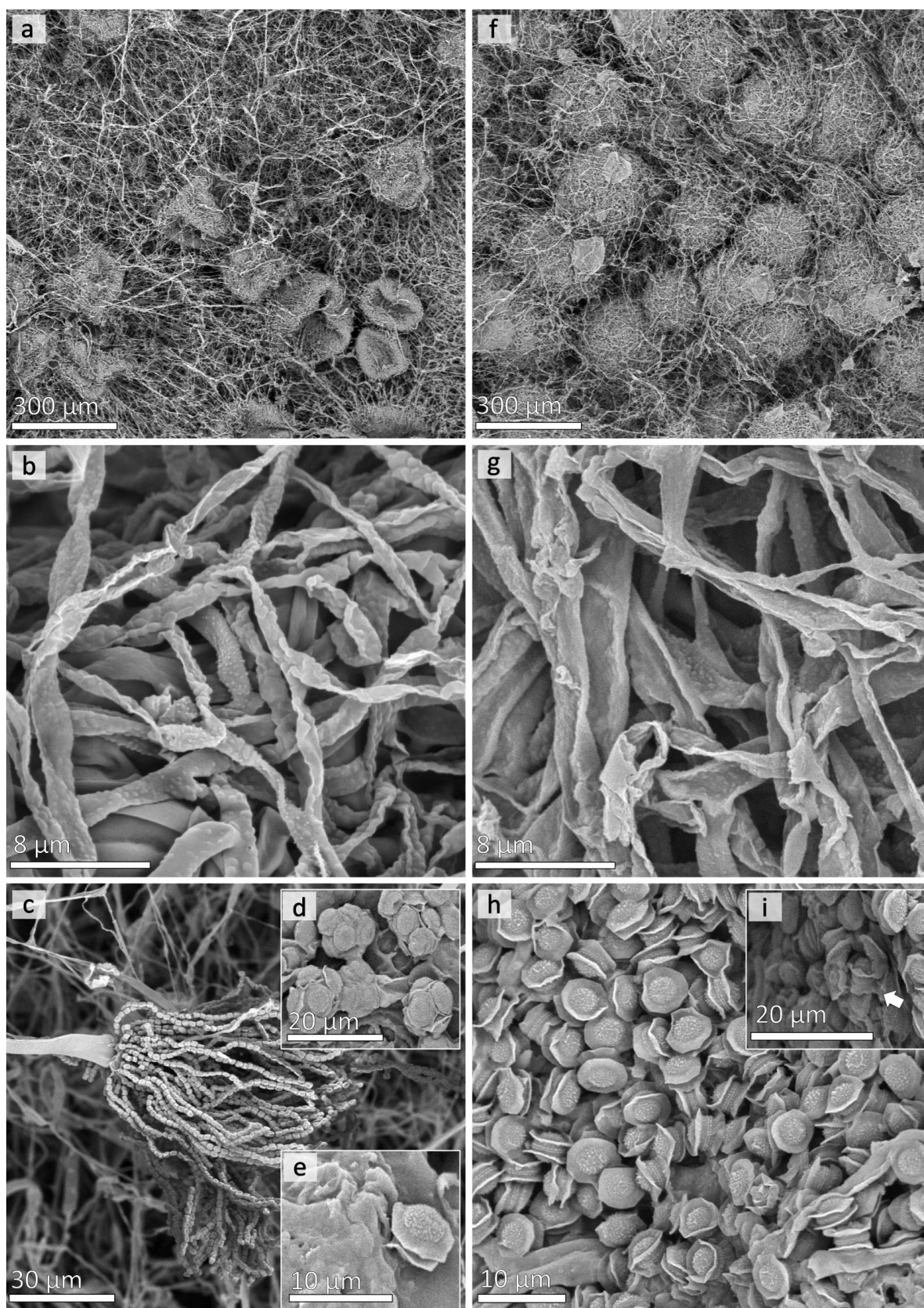
Wzrost grzybów na podłożu PDA z ekstraktem z nagietka prowadził do intensywniejszego tworzenia struktur przetrwalnikowych. Po 10 dniach widoczne były liczne, zapadnięte kleistotecja ($\varnothing 348,9 \mu\text{m}$; Rys. 6a), strzępki często pokryte perydium (Rys. 6b), o powierzchni gładkiej lub z wypustkami ($\varnothing 0,6 \mu\text{m}$; Rys. 6c). Stwierdzono obecność konidioforów ($\varnothing 13,2 \mu\text{m}$) z łańcuchami konidiów ($1,4 \times 1,5 \mu\text{m}$; Rys. 6d) oraz worków ($\varnothing 10,1 \mu\text{m}$; Rys. 6e), ale brak było dojrzałych askospor, co może świadczyć o hamowaniu lub opóźnieniu ich rozwoju przez ekstrakt z nagietka lekarskiego. Po 30 dniach grzybnia tworzyła struktury wielowarstwowe (Rys. 6f), obecne były kleistotecja ($\varnothing 217,5 \mu\text{m}$) w różnych stadiach rozwoju, strzępki o powierzchni głównie brodawkowatej ($0,8\text{--}1,6 \mu\text{m}$; Rys. 6g), z wyraźnymi guzkami ($\varnothing 0,4 \mu\text{m}$; Rys. 6h). Stwierdzono askospory ($\varnothing 5,4\text{--}6,6 \mu\text{m}$) z dwoma grzbietami (Rys. 6i), worki ($\varnothing 11,4 \mu\text{m}$; Rys. 6j) oraz konidiofory ($\varnothing 6,7 \mu\text{m}$) wytwarzające nieregularne konidiospory ($1,7 \times 1,8 \mu\text{m}$; Rys. 6k).



Rys. 6. Morfologia *Neosartorya* sp. G127/14 na podłożu PDA z dodatkiem *Calendula officinalis* obserwowana za pomocą SEM po 10 (a, b, c, d, e) lub 30 (f, g, h, i, j, k) dniach

inkubacji. Ogólna struktura grzybni (a, f), powierzchnia strzępek (b, g), wypukłości strzępek (c, h), konidiospory (d, k), worki (e, j - zaznaczone strzałką), askospory (i).

Obecność pirosiarczynu sodu wpływała na morfologię grzybni. Po 10 dniach obecne były nieregularne, często zapadnięte kleistotecja ($\varnothing 226,5 \mu\text{m}$; Rys. 7a), faliste strzępki ($0,9\text{--}2,0 \mu\text{m}$) z brodawkami ($\varnothing 0,4 \mu\text{m}$; Rys. 7b), konidiofory ($\varnothing 10,5 \mu\text{m}$) z konidiosporami ($1,9 \times 1,5 \mu\text{m}$; Rys. 7c), a także kuliste worki ($\varnothing 9,7 \mu\text{m}$; Rys. 7d) i askospory ($\varnothing 4,8\text{--}7,3 \mu\text{m}$; Rys. 7e). Po 30 dniach zaobserwowano silniej brodawkowate strzępki ($0,5\text{--}2,7 \mu\text{m}$; Rys. 7g), liczne kleistotecja ($\varnothing 244,3 \mu\text{m}$; Rys. 7f), dojrzałe askospory ($\varnothing 5,4\text{--}6,1 \mu\text{m}$; Rys. 7h) i worki ($\varnothing 4,7 \mu\text{m}$; Rys. 7i). Konidiospory nie występowały.



Rys. 7. Morfologia *Neosartorya* sp. G127/14 na podłożu PDA z pirosiarczynem sodu obserwowana za pomocą SEM po 10 (a, b, c, d, e) lub 30 (f, g, h, i) dniach inkubacji. Ogólna

struktura grzybni (a, f), powierzchnia strzępek (b, g.), konidiospory (c), worki (d, i - zaznaczone strzałką), askospory (e, h).

Dla izolatu G130/14, po 10 dniach inkubacji na podłożu PDA zaobserwowano luźną sieć strzępek (1,2–2,2 μm). Obecne było jedno pęknięte, elipsoidalne kleistotecjum ($\varnothing 11,4 \mu\text{m}$), a także dobrze wykształcone, kuliste askospory ($\varnothing 3,9\text{--}6,3 \mu\text{m}$) o wypukłej, szorstkiej powierzchni i dwóch wyraźnie oddzielonych horyzontalnych grzebieniach. Zidentyfikowano luźne, owalne worki ($\varnothing 9,2 \mu\text{m}$) oraz konidiofory z maczugowatymi pęcherzykami ($\varnothing 7,4 \mu\text{m}$) i butelkowatymi fialidami wytwarzającymi walcowate zarodniki konidialne ($1,7 \times 1,5 \mu\text{m}$). Po 30 dniach morfologia grzybni była podobna (1,3–2,3 μm), lecz nie obserwowano kleistotecjów ani struktur konidialnych, jedynie luźne dojrzałe askospory ($\varnothing 5,9\text{--}10,6 \mu\text{m}$). Dodatek ekstraktu *Calendula officinalis* skutkował zagęszczeniem grzybni (0,6–2,1 μm) oraz obecnością kleistotecjów ($\varnothing 62,2 \mu\text{m}$) już po 10 dniach inkubacji. Nie zidentyfikowano dojrzałych worków ani askospor, jednak na brzegach perydium widoczne były zawiązki struktur przypominających załączki worków. Obserwowano konidiofory i gładkie zarodniki konidialne ($2,4 \times 2,0 \mu\text{m}$). Po 30 dniach grzybnia wykształcała wypukłe zgrubienia ($\varnothing 0,6 \mu\text{m}$). Liczba kleistotecjów wzrosła ($\varnothing 247,4 \mu\text{m}$), a ich kształt i stadium rozwoju były zróżnicowane. Obecne były okrągłe worki ($\varnothing 9,0 \mu\text{m}$) i dobrze wykształcone askospory ($\varnothing 4,3\text{--}6,3 \mu\text{m}$). Pęcherzyki konidioforów miały $\varnothing 11,2 \mu\text{m}$, a zarodniki $1,7 \times 1,6 \mu\text{m}$. W warunkach dodatku pirosiarczyny sodu, już po 10 dniach występowały liczne kleistotecja ($\varnothing 216,8 \mu\text{m}$) oraz silnie rozgałęziona grzybnia o brodawkowatej powierzchni (0,9–3,4 μm ; $\varnothing 0,5 \mu\text{m}$). Widoczne były dojrzałe askospory ($\varnothing 4,7\text{--}6,4 \mu\text{m}$), sferyczne worki ($\varnothing 10,1 \mu\text{m}$) oraz konidiofory z pęcherzykami ($\varnothing 6,0 \mu\text{m}$) i zarodnikami konidialnymi ($1,8 \times 1,9 \mu\text{m}$). Po 30 dniach struktura była zdominowana przez dojrzałe kleistotecja ($\varnothing 214,9 \mu\text{m}$) otoczone skórzastym perydium. Strzępki (1,1–2,8 μm) zachowały brodawkowatą strukturę ($\varnothing 0,7 \mu\text{m}$), a askospory miały $\varnothing 5,5\text{--}6,8 \mu\text{m}$. Worki były

głównie kuliste ($\varnothing 7,9 \mu\text{m}$), a konidiofory liczne, z izodiametrycznymi zarodnikami ($2,1 \times 2,0 \mu\text{m}$).

Izolat G132/14 po 10 dniach inkubacji na podłożu PDA wytworzył warstwową strukturę grzybni zbudowaną z gładkich strzępek o szerokości $0,7\text{--}1,8 \mu\text{m}$. Nie zaobserwowano klejstotecjów, jednak w dolnych warstwach próbki obecne były luźne worki ($\varnothing 9,0 \mu\text{m}$) o nieregularnym kształcie oraz dojrzałe, kuliste askospory ($\varnothing 4,5\text{--}6,1 \mu\text{m}$) z dwiema brzeżnymi listewkami i chropowatą powierzchnią. Stwierdzono obfitą konidiację – zarodniki konidialne ($2,0 \times 1,7 \mu\text{m}$) były owalne, połączone w łańcuszki i lekko zapadnięte. Po 30 dniach struktura grzybni pozostała zbliżona ($0,8\text{--}1,9 \mu\text{m}$), bez obecności kleistotecjów i konidioforów, ale z luźnymi, kulistymi zarodnikami konidialnymi ($2,5 \times 1,8 \mu\text{m}$), dojrzałymi askosporami ($\varnothing 5,5\text{--}6,9 \mu\text{m}$) oraz kulistymi workami ($\varnothing 10,5 \mu\text{m}$). Dodatek ekstraktu z nagietka lekarskiego po 10 dniach spowodował agregację grzybni ($0,9\text{--}2,7 \mu\text{m}$), częściej z widocznymi przegrodami i drobnymi, kulistymi brodawkami ($\varnothing 0,4 \mu\text{m}$). Klejstotecja nie występowały, lecz na obrzeżach próbki widoczne były pojedyncze dojrzałe askospory ($\varnothing 4,1\text{--}5,7 \mu\text{m}$) i jeden kulisty worek ($\varnothing 7,2 \mu\text{m}$). Obecne były liczne konidiofory z owalnymi zarodnikami ($2,5 \times 1,8 \mu\text{m}$) o zapadniętych ścianach. Po 30 dniach grzybnia była bardziej zbita i miejscami przykryta luźnym, pofałdowanym perydium. Strzępki ($0,8\text{--}2,7 \mu\text{m}$) były rozgałęzione, o gładkiej lub brodawkowatej powierzchni ($\varnothing 0,4 \mu\text{m}$). Liczne kleistotecja ($\varnothing 175,5 \mu\text{m}$) miały zróżnicowany kształt – od kulistych po zapadnięte – i były na różnych etapach rozwoju. Obecne były dojrzałe askospory ($\varnothing 5,6\text{--}6,6 \mu\text{m}$) oraz kuliste worki ($\varnothing 5,7 \mu\text{m}$). Nie obserwowano form bezpłciowych. Dodatek pirosiarczyny sodu już po 10 dniach powodował zmiany morfologiczne – grzybnia przybrała włóknisty charakter ($1,1\text{--}2,4 \mu\text{m}$) i miała chropowatą powierzchnię z brodawkami ($\varnothing 0,5 \mu\text{m}$). Górna warstwa zawierała liczne strzępy perydium, szczególnie wokół kulistych klejstotecjów ($\varnothing 220,9 \mu\text{m}$). Obecne były kuliste worki ($\varnothing 12,7 \mu\text{m}$), dojrzałe askospory ($\varnothing 5,6\text{--}7,0 \mu\text{m}$) oraz konidiofory z pęcherzykami ($\varnothing 5,8 \mu\text{m}$) i łańcuszkami sześciennych zarodników

($2,0 \times 1,7 \mu\text{m}$). Po 30 dniach struktura była podobna, choć perydium występowało rzadziej. Kleistotecja ($\varnothing 178,7 \mu\text{m}$) były kuliste, a strzępki ($0,9\text{--}2,1 \mu\text{m}$) pokryte owalnymi brodawkami ($\varnothing 0,4 \mu\text{m}$). Obecne były kuliste askospory ($\varnothing 5,5\text{--}6,9 \mu\text{m}$) i elipsoidalne worki ($\varnothing 6,2 \mu\text{m}$). Izolat wytwarzał wiele konidiów – konidiofory miały łańcuszki sześciennych lub kulistych zarodników ($1,9 \times 1,8 \mu\text{m}$).

Izolat G135/14 po 10 dniach inkubacji na podłożu PDA wytworzył zwartą grzybnię zbudowaną z gładkich strzępek o szerokości $0,6\text{--}1,3 \mu\text{m}$. Nie obserwowano kleistotecjów, jednak w pobliżu podłoża znajdowały się liczne dojrzałe askospory ($\varnothing 4,5\text{--}7,3 \mu\text{m}$) oraz owalne worki ($\varnothing 7,4 \mu\text{m}$), przytwierdzone do podłoża. Askospory miały chropowatą, brodawkowatą powierzchnię oraz wyraźne dwa listewkowate grzebienie. Obecne były liczne konidiofory, z których część tworzyła łańcuszki owalnych zarodników ($2,1 \times 1,6 \mu\text{m}$) z lekko zapadniętymi ścianami. Po 30 dniach grzybnia była gęstsza, z licznymi, falistymi strzępkami o szerokości $0,5\text{--}1,4 \mu\text{m}$ o gładkiej powierzchni. Struktura była jednorodna, bez owocników; jedynie przy powierzchni podłoża, w sąsiedztwie perydium, widoczne były kuliste, brodawkowate askospory ($\varnothing 5,3\text{--}6,6 \mu\text{m}$). Dodatek ekstraktu z nagietka zmienił morfologię grzybni – po 10 dniach przybrała ona formę zbitych agregatów strzępkowych ($0,7\text{--}1,4 \mu\text{m}$), pomiędzy którymi obserwowano liczne kleistotecja ($\varnothing 96,4 \mu\text{m}$), przeważnie we wczesnym stadium rozwoju, otoczone dimorficznymi strzępkami o gładkiej lub silnie brodawkowatej powierzchni z owalnymi wypustkami ($\varnothing 0,5 \mu\text{m}$). Obecne były sferyczne worki ($\varnothing 8,8 \mu\text{m}$) oraz dojrzałe, kuliste askospory ($\varnothing 5,1\text{--}7,1 \mu\text{m}$). Widoczne były kolumnowe konidiofory z krótkimi łańcuszkami kulistych zarodników ($2,2 \times 1,7 \mu\text{m}$) o zapadniętych ścianach. Po 30 dniach struktura grzybni zdominowana była przez liczne dojrzałe kleistotecja ($\varnothing 143,3 \mu\text{m}$), z których wiele było pękniętych. Strzępki ($0,3\text{--}0,9 \mu\text{m}$) łączyły poszczególne kleistotecja, często splecione napiętym, skórzastym perydium. Strzępki były cienkie i gładkie lub szersze, pokryte brodawkowatymi wypustkami ($\varnothing 0,4 \mu\text{m}$). Askospory ($\varnothing 5,5\text{--}6,4 \mu\text{m}$) miały zróżnicowaną

budowę: kuliste i ząbkowane bez grzbietów; kuliste, brodawkowate z wyraźnymi listewkami; soczewkowate, z falistymi fałdami. Nie stwierdzono obecności struktur rozmnażania bezpłciowego. Dodatek pirosiarczynu sodu wywołał intensywne zagęszczenie grzybni i masowe tworzenie klejstotecjów już po 10 dniach inkubacji. Askokarpy ($\varnothing 169,1 \mu\text{m}$) były kuliste, otoczone wielowarstwowym perydium. Strzępki ($0,8\text{--}2,3 \mu\text{m}$) były gładkie lub z wypustkami ($\varnothing 0,4 \mu\text{m}$). Obecne były kuliste worki ($\varnothing 10,4 \mu\text{m}$) i chropowate askospory ($\varnothing 5,2\text{--}6,4 \mu\text{m}$). Obserwowano obfitą konidiację – gładkie zarodniki ($2,2 \times 1,7 \mu\text{m}$) tworzyły liczne łańcuszki i miały kulisty lub sześcienny kształt z lekko zapadniętymi ścianami. Po 30 dniach obserwowano większą obecność struktur perydialnych w górnych warstwach próbki. Strzępki ($0,5\text{--}2,8 \mu\text{m}$) prezentowały dwojaką ornamentację powierzchni, a wypustki były bardziej wyraźne ($\varnothing 0,4 \mu\text{m}$). Obecne były kuliste klejstotecja ($\varnothing 171,4 \mu\text{m}$), niemal dojrzałe, kuliste worki ($\varnothing 6,4 \mu\text{m}$) oraz wielopostaciowe askospory ($\varnothing 5,0\text{--}6,5 \mu\text{m}$) – najczęściej kuliste z wyraźnymi grzbietami, ale też soczewkowate z falistymi fałdami. Konidiofory były na wpół wyprostowane, z kulistymi zarodnikami ($2,7 \times 2,2 \mu\text{m}$).

W przypadku izolatu G150/14, po 10 dniach inkubacji na pożywce PDA grzybnia była zbudowana z nitkowatych, gładkich strzępek ($0,8\text{--}2,4 \mu\text{m}$). Obserwowano wczesne stadia rozwoju klejstotecjów ($\varnothing 186,1 \mu\text{m}$), a na obrzeżach próbki, przy podłożu, obecne były dojrzałe, kuliste askospory ($\varnothing 5,2\text{--}6,6 \mu\text{m}$) o szorstkiej powierzchni i wyraźnych podwójnych horyzontalnych grzebieniach, a także nieliczne sferyczne worki ($\varnothing 10,1 \mu\text{m}$). Po 30 dniach grzybnia uległa umiarkowanemu rozluźnieniu ($0,6\text{--}3,1 \mu\text{m}$), bez wykształcenia owocników. Utrzymała się obecność askospor ($\varnothing 4,9\text{--}6,4 \mu\text{m}$) i nieznacznej liczby worków ($\varnothing 5,4 \mu\text{m}$) o bardziej kulistym kształcie niż wcześniej. Konidiospor nie zaobserwowano. Dodatek ekstraktu z *Calendula officinalis* już po 10 dniach inkubacji pobudzał tworzenie struktur ochronnych — luźno zorganizowanego, powierzchniowego perydium i licznych klejstotecjów ($\varnothing 180,3 \mu\text{m}$). Strzępki ($1,1\text{--}2,3 \mu\text{m}$) były gładkie, natomiast askospory ($\varnothing 3,8\text{--}5,9 \mu\text{m}$) kuliste,

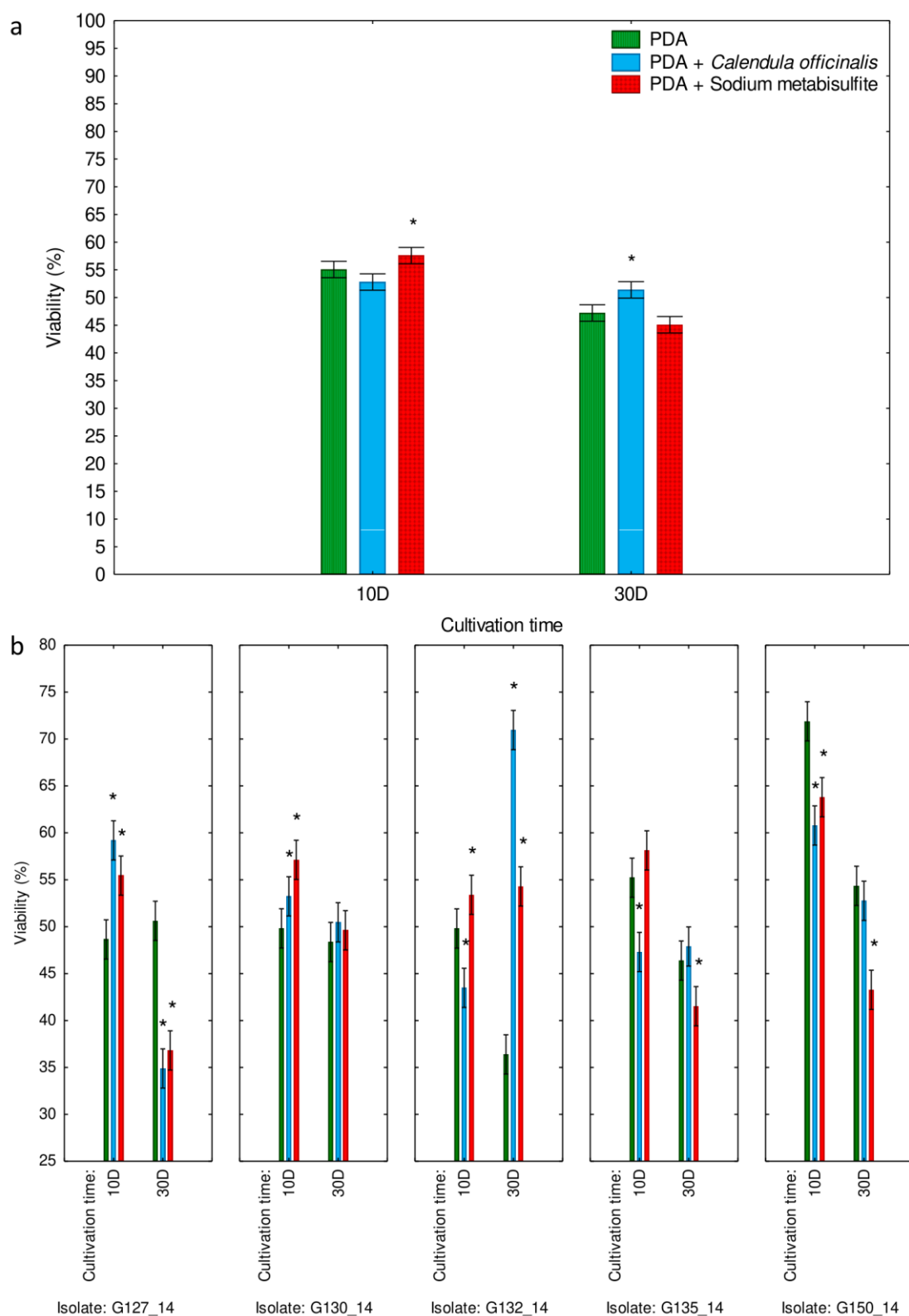
brodawkowate, z dwoma grzebieniami. Po 30 dniach średnica klejstotecjów wzrosła ($\varnothing 253,4 \mu\text{m}$), perydium było luźne i miejscami oderwane. Strzępki ($0,6\text{--}2,8 \mu\text{m}$) cechowały się dwojakością tekstury (gładkie lub brodawkowate, $\varnothing 0,3 \mu\text{m}$). Obecne były worki ($\varnothing 5,5 \mu\text{m}$) i askospory ($\varnothing 5,7\text{--}6,2 \mu\text{m}$) o morfologii zgodnej z wcześniejszym etapem. Nie zaobserwowano struktur bezpłciowych. Obecność pirosiarczynu sodu wpłynęła na wykształcenie warstwowej, włóknistej grzybni i licznych klejstotecjów ($\varnothing 198,2 \mu\text{m}$), silnie splecionych ze strzępkami ($1,1\text{--}2,8 \mu\text{m}$) o falistej morfologii. Widoczne były kuliste worki ($\varnothing 10,1 \mu\text{m}$) i dojrzałe askospory ($\varnothing 4,8\text{--}6,1 \mu\text{m}$) o szorstkiej powierzchni i podwójnych grzebieniach. Występowały konidiofory z długimi łańcuchami konidiów ($1,6 \times 1,5 \mu\text{m}$), o nieco zapadniętych ścianach. Po 30 dniach grzybnia uległa przerzedzeniu, lecz liczba klejstotecjów wzrosła ($\varnothing 220,2 \mu\text{m}$). Część z nich była pokryta segmentowanym perydium. Strzępki ($0,7\text{--}1,3 \mu\text{m}$) były gładkie lub brodawkowate ($\varnothing 0,3 \mu\text{m}$). Obserwowano pojedyncze worki ($\varnothing 6,3 \mu\text{m}$) i liczne askospory ($\varnothing 5,5\text{--}6,4 \mu\text{m}$), lecz brakowało konidiospor.

Obrazowanie SEM umożliwiło ocenę morfologii badanych izolatów. Pomimo różnic genomowych i metabolicznych, strukturalnie izolaty wykazywały duże podobieństwo, a ornamentacja konidioforów i askospor przypominała *Neosartorya fischeri* var. *glabra* (Hong et al., 2006; Samson et al., 1990). W badaniu zwrócono uwagę na pojawiające się na powierzchni strzępek drobne wypukłości, które na ogół nie występowały w warunkach kontrolnych, a ich obecność obserwowano po ekspozycji na ekstrakt roślinny lub konserwant. Po ekspozycji na wymienione środki ograniczające wzrost grzybów, powierzchnia strzępek często stawała się brodawkowata, co może świadczyć o reakcji stresowej. Choć kształtem wypukłości te przypominały początkowe formy komórek Hülle, wielojądrowych struktur magazynujących, charakterystycznych dla rodzaju *Aspergillus*, były one znacznie mniejsze (Bayram & Braus, 2012; Troppens et al., 2020). Nie zaobserwowano również cech charakterystycznych dla appresoriów, a morfologia wypukłości bardziej odpowiada znanym z literatury ciałkom

Woronina lub egzosomom (Gandhi & Joseph, 2022; Jedd, 2011; Money, 2016). W celu potwierdzenia tożsamości struktur wymagane byłyby dalsze analizy, np. z użyciem TEM lub mikroskopii konfokalnej z zastosowaniem przeciwciał (Jedd, 2011).

4.4.3.4. Ocena żywotności metodą mikroskopii konfokalnej fluorescencyjnej

Analiza żywotności grzybów z wykorzystaniem konfokalnej mikroskopii fluorescencyjnej ujawniła zależne od czasu i rodzaju pożywki różnice w przeżywalności izolatów G127/14, G130/14, G132/14, G135/14 i G150/14 (Rys. 8). Po 10 dniach inkubacji wszystkie izolaty hodowane na PDA z dodatkiem pirosiarczynu sodu wykazywały wyraźnie wyższą żywotność niż na standardowym PDA, natomiast dodatek *Calendula officinalis* nie miał istotnego wpływu na wzrost grzybni (Rys. 8a). Po 30 dniach nastąpiło odwrócenie tego trendu: ekstrakt z *Calendula officinalis* podtrzymywał wyższą żywotność grzybów, a efekt pirosiarczynu sodu nie powodował zmian względem kontroli.



Rys. 8. Wpływ ekstraktu z nagietka lekarskiego i pirosiarczyny sodu na żywotność *Neosartorya* spp. przy użyciu konfokalnej mikroskopii fluorescencyjnej. Wpływ składu podłoża na wszystkie izolaty łącznie po 10 lub 30 dniach hodowli (a). Wpływ składu podłoża na każdy

izolat osobno po 10 lub 30 dniach hodowli (b). Gwiazdka wskazuje statystycznie istotne różnice między żywotnością grupy, na którą miał wpływ jeden z testowanych czynników przeciwmikrobiologicznych, a odpowiadającą jej kontrolą w danym dniu eksperymentu.

Efekty substancji przeciwgrzybiczych były również zależne od konkretnego izolatu (Rys. 8b). G127/14 był stymulowany przez oba dodatki po 10 dniach, lecz po 30 dniach ich obecność obniżała przeżywalność grzyba. G130/14 wykazywał stymulację wzrostu przez obie substancje po 10 dniach, lecz ich efekt zanikał po 30 dniach inkubacji. Z kolei G132/14 wykazywał niższą żywotność po ekspozycji na ekstrakt z *Calendula officinalis*, a wyższą po pre-inkubacji w obecności pirosiarczynu sodu po 10 dniach inkubacji, jednak po 30 dniach oba dodatki wspomagały żywotność badanego izolatu. G135/14 wykazywał zmniejszoną żywotność po 10 dniach inkubacji z ekstraktem z *Calendula officinalis* oraz po 30 dniach inkubacji z pirosiarczynem sodu. W przypadku G150/14 oba dodatki początkowo negatywnie wpływały na wzrost grzyba, ale po 30 dniach inkubacji efekt ten utrzymywał się jedynie dla wariantu z pirosiarczynem sodu.

Uzyskane wyniki wskazują, że pirosiarczyn sodu może początkowo poprawiać przeżywalność grzybów poprzez aktywację mechanizmów tolerancji stresu oksydacyjnego, jak np. dysmutaza ponadtlenkowa, jednak jego długotrwałe działanie może prowadzić do kumulacji toksycznych produktów przemiany materii, przekraczających możliwości obronne komórki (Elewski, 1993; Ivanov et al., 2022). Z kolei, ekstrakt z nagietka lekarskiego (*Calendula officinalis*) wykazał efekt grzybobójczy głównie w początkowym okresie ekspozycji, co może wynikać z obecności związków czynnych, takich jak saponiny, flawonoidy czy kwasy fenolowe, które zaburzają integralność błon komórkowych lub hamują enzymy metaboliczne (Doligalska et al., 2021; Al-Huqail et al., 2023; Tavallali et al., 2024). Po dłuższym czasie skuteczność ekstraktu jednak słabła, prawdopodobnie z powodu degradacji substancji czynnych lub adaptacji grzybów, jak ma to miejsce np. w przypadku oporności

Candida spp. na flukonazol (Bossche et al., 1998). Wskazuje to na potrzebę łączenia ekstraktu roślinnego *Calendula officinalis* z innymi środkami w celu osiągnięcia trwałego efektu grzybobójczego.

4.4.4 Podsumowanie

Przeprowadzone badania pozwoliły na dokonanie kompleksowej oceny mechanizmów odporności *Neosartorya* spp., obejmując analizę genomów, oraz odpowiedzi metabolicznych i morfologicznych na działanie naturalnych ekstraktów roślinnych i konserwantów żywności. Wykorzystanie WGS umożliwiło rozpoznanie genomów pięciu izolatów i scharakteryzowanie ponad 10 000 genów dla każdego z badanych grzybów, ze szczególnym uwzględnieniem mechanizmów obronnych. Izolaty grzybowe poddano także działaniu pirosiarczynu sodu i ekstraktu roślinnego z nagietka lekarskiego (*Calendula officinalis*), a ich reakcje na ekspozycje środków grzybobójczych oceniono na podstawie analizy metabolomicznej (LC-QTOF MS) oraz zmian morfologicznych wykonanych za pomocą obrazowania SEM oraz mikroskopii fluorescencyjnej. Wykazano, że ekstrakty roślinne i konserwanty wpływają na szlaki metaboliczne i strukturę grzybni – m.in. poprzez zwiększoną sporulację i pojawienie się wypukłych struktur na strzępkach grzybni. Analiza żywotności komórek wykazała, że pirosiarczyn sodu może początkowo zwiększać przeżywalność, lecz przy dłuższej ekspozycji prowadzi do stresu metabolicznego. Z kolei ekstrakt z *Calendula officinalis* działała krótkotrwale – początkowo obniżając żywotność grzybów, ale efekt ten zanikał w czasie.

Uzyskane wyniki potwierdzają złożone mechanizmy adaptacyjne *Neosartorya* spp., łączące zmiany genetyczne, metaboliczne i strukturalne. Obecność genów związanych z wirulencją w genomach testowanych grzybów sugeruje także ich potencjalną rolę jako patogenów. Wyniki te stanowią podstawę do dalszych badań nad odpornością grzybów i rozwojem skuteczniejszych, zrównoważonych strategii przeciwdziałania psuciu się żywności.

4.5 Badania uzupełniające – P.5: Antifungal Agents Alter Strawberry Microbial and Metabolic Profiles During *Neosartorya* spp. (*Aspergillus* spp.) Contamination

4.5.1 Wstęp

Owoce truskawki (*Fragaria x ananassa*) charakteryzują się wysoką podatnością na psucie, głównie z powodu dużej zawartości wody i składników odżywczych, co sprzyja rozwojowi grzybów w okresie przechowywania (Cybulska et al., 2022; Ladika et al., 2024; Moraga et al., 2006). Szczególne zagrożenie stanowią *Neosartorya* spp. (*Aspergillus* spp.), które dzięki termoopornym askosporom wykazują odporność na konwencjonalne metody utrwalania żywności, w tym obróbkę cieplną i chłodniczą (Frisón et al., 2012; Amaeze, 2013; Chen et al., 2022). Obecność tych grzybów wpływa negatywnie nie tylko na jakość owoców, ale może też wiązać się z produkcją szkodliwych metabolitów wtórnych (Doğan & Zorba, 2024).

W celu ograniczenia zakażeń grzybowych w przemyśle spożywczym stosuje się zarówno konserwanty chemiczne, np. pirosiarczyn sodu czy kwasy organiczne, jak i naturalne środki pochodzenia roślinnego (Ahmadi et al., 2021; Braïek & Smaoui, 2021). Ze względu na rosnące obawy konsumentów dotyczące bezpieczeństwa dodatków syntetycznych, coraz większym zainteresowaniem cieszą się substancje bioaktywne obecne w ekstraktach roślinnych (Falleh et al., 2020; Novais et al., 2022). Przykładem jest nagietek lekarski (*Calendula officinalis*), którego ekstrakty wykazały skuteczność przeciwgrzybiczą wobec wielu patogenów (Dey et al., 2024). Mimo znanego działania ograniczającego rozwój grzybów, wpływ tych substancji na mikrobiologiczny i metaboliczny profil owoców, zwłaszcza w warunkach rzeczywistej kontaminacji, pozostaje słabo poznany (Droby & Wisniewski, 2018; Kusstatscher et al., 2020).

W niniejszych badaniach określono wpływ wybranych środków przeciwgrzybiczych na mikrobiotę i aktywność metaboliczną owoców truskawki sztucznie zakażonych izolatami

Neosartorya spp. o różnej wrażliwości na ekstrakty roślinne i konserwanty. Analizy przeprowadzono z wykorzystaniem sekwencjonowania następnej generacji (v4 16S rRNA, ITS2) oraz testów Biolog™ EcoPlates (Pylak et al., 2023; Tenea & Reyes, 2024; Q. Zhao et al., 2024). Wyniki dostarczają nowych danych na temat interakcji pomiędzy zanieczyszczeniami grzybowymi, substancjami konserwującymi i zbiorowiskami mikroorganizmów występujących w owocach truskawki, co może wspomóc rozwój zrównoważonych metod konserwacji świeżej żywności.

4.5.2 Materiały i metody

4.5.2.1 Izolaty grzybowe i zakażenie truskawek

Do doświadczenia wybrano trzy izolaty *Neosartorya* spp. z kolekcji Laboratorium Mikrobiologii Molekularnej i Środowiskowej (IA PAN, Lublin), różniące się wrażliwością na konserwanty i ekstrakty roślinne: G127/14 (wysoka wrażliwość), G132/13 (średnia wrażliwość) i G130/14 (najmniejsza wrażliwość) (Maj et al., 2024a, 2024b). Analizie poddano wpływ pirosiarczynu sodu (Sigma-Aldrich, USA) i ekstraktu z *Calendula officinalis* (Zrób Sobie Krem, Polska) na mikrobiom owoców truskawki sztucznie zakażonych tymi izolatami. Oba środki przygotowano w wodzie jałowej ($1000 \mu\text{g mL}^{-1}$) i przefiltrowano przez filtry o średnicy porów $0,22 \mu\text{m}$. Zawartość SO_2 w roztworze pirosiarczynu, oznaczona metodą destylacyjną zgodnie z normą PN-A-75101-23:1990/Az2:2002, wynosiła poniżej 10 mg kg^{-1} , co mieści się znacznie poniżej dopuszczalnych wartości dla żywności (Sooriya et al., 2024).

Przed doświadczeniem izolaty grzybów były hodowane na podłożu PDA (BioMaxima, Polska) przez 10 dni w temperaturze 30°C . Następnie grzybnię wraz z zarodnikami zawieszono w sterylnej wodzie, uzyskując zawiesinę o transmisji 75%, odpowiadającą około $4,44 \times 10^4$ askospor mL^{-1} .

Świeże, nieuszkodzone owoce truskawki (*Fragaria × ananassa*) sztucznie zakażano, rozpylając ok. 2,5 mL zawiesiny *Neosartorya* spp. przy użyciu spryskiwacza zapewniającego równomierne pokrycie powierzchni owocu. Następnie owoce truskawki poddawano jednemu z trzech zabiegów, traktując je: wodą (kontrola), ekstraktem z *Calendula officinalis* (1 mg mL⁻¹) lub pirosiarczynem sodu (1 mg mL⁻¹). Owoce umieszczano na jałowych tackach i inkubowano przez 7 dni w temperaturze 4°C.

4.5.2.2 Izolacja DNA i sekwencjonowanie metataksonomiczne (Illumina® MiSeq)

Każdego dnia inkubacji pobierano próbki do izolacji DNA (łącznie 36 owoców dziennie – po 9 dla każdego izolatu lub kontroli). W ramach każdej grupy trzy owoce truskawki traktowano ekstraktem z *Calendula officinalis*, trzy pirosiarczynem sodu, a trzy wodą. Owoce ważono, łączono po trzy w jałowych woreczkach i homogenizowano (2 minuty, Smasher®, AES Laboratoire). Z każdego homogenatu pobierano 0,25 g do izolacji DNA metodą Fast DNA Spin Kit for Feces (MP Biomedicals, USA), z dodatkowym etapem homogenizacji (FastPrep-24 (MP Biomedical, USA), 40 s, 6.0 m s⁻¹) w celu poprawy wydajności izolacji grzybni. Stężenie i jakość DNA oceniano przy użyciu spektrofotometru NanoDrop (Thermo Fisher Scientific, West Palm Beach, FL, USA) i fluorometru Quantus (Promega, Madison, WI, USA).

Do analizy mikrobiomu amplifikowano region V4 genu 16S rRNA (bakterie, startery 515F–806R, Apprill et al., 2015) oraz ITS2 (grzyby, startery ITS3/ITS4, Op De Beeck et al., 2014). Biblioteki przygotowano zgodnie z procedurą opisaną przez Siegiedę et al. (2023), stosując KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Cape Town, South Africa) (Siegieda et al., 2023). Produkty PCR oczyszczano (CleanNGS, CleanNA, Gouda, Netherlands), znakowano unikalnymi adapterami Illumina®, a następnie oceniano jakość (TapeStation 4150, Agilent Technologies, Santa Clara, USA). Próbkę normalizowano do 8 pM. Sekwencjonowanie przeprowadzano w systemie Illumina® MiSeq (Illumina®, San Diego, CA, USA) z dodatkiem 5% PhiX (Illumina®, San Diego, CA, USA) jako kontroli jakościowej.

4.5.2.3 Test aktywności metabolicznej (Biolog™ EcoPlates)

Aktywność metaboliczną zbiorowisk mikroorganizmów występujących w owocach zakażonych *Neosartorya* spp. oceniano z wykorzystaniem systemu Biolog™ EcoPlates (Biolog™, USA) (Mącik et al., 2023; Pylak et al., 2025). Z homogenatu owoców truskawki pobierano 1 g materiału, który umieszczano w sterylnym słoiczku z 99 mL jałowej wody peptonowej, zgodnie z zaleceniami producenta. Zawiesinę poddawano mieszaniu przez 20 minut w temperaturze pokojowej, a następnie inkubowano przez 30 minut w temperaturze 4°C. Po inkubacji, do każdego dołka płytki EcoPlate wprowadzano po 120 µL przygotowanego inokulum. Płytki, zawierające 31 różnych źródeł węgla, umożliwiały ocenę profilu metabolicznego badanych zbiorowisk mikroorganizmów. Inkubację prowadzono w temperaturze 30°C przez 216 godzin, dokonując pomiarów absorbancji przy długości fali 590 nm co 24 godziny za pomocą czytnika MicroStation (Biolog™, Hayward, CA, USA).

4.5.2.4 Analiza statystyczna i bioinformatyczna

Dane sekwencyjne pierwotnie analizowano narzędziami bioinformatycznymi. Liczbę odczytów znormalizowano metodą raryfikacji do 1 000 (grzyby) lub 2 000 (bakterie) odczytów na próbkę. Obliczono wskaźniki α -różnorodności (Shannon, Simpson, Fisher) i β -różnorodności z analizą Bray-Curtis i wizualizacją metodą PCoA. Następnie analizy i wizualizacji metataksonomicznej dokonano z wykorzystaniem RStudio V.4.4.0.

Różnice analizowano testami statystycznymi ($p < 0,05$). Przed ANOVA sprawdzano normalność (test Shapiro–Wilka) i jednorodność wariancji (test Levene’a). Przy niespełnieniu warunku równości wariancji zastosowano test ANOVA Welcha, a przy ich spełnieniu test ANOVA. Skład taksonomiczny analizowano na różnych poziomach taksonomicznych, a dane wizualizowano wykresami pudełkowymi, wskazując na 10 najliczniejszych taksonów w każdej próbce. Diagramy Venna przedstawiały wspólne i unikalne taksony dla izolatu i warunków hodowli w czasie 7 dni i osobno dla każdego dnia inkubacji kontaminowanych owoców

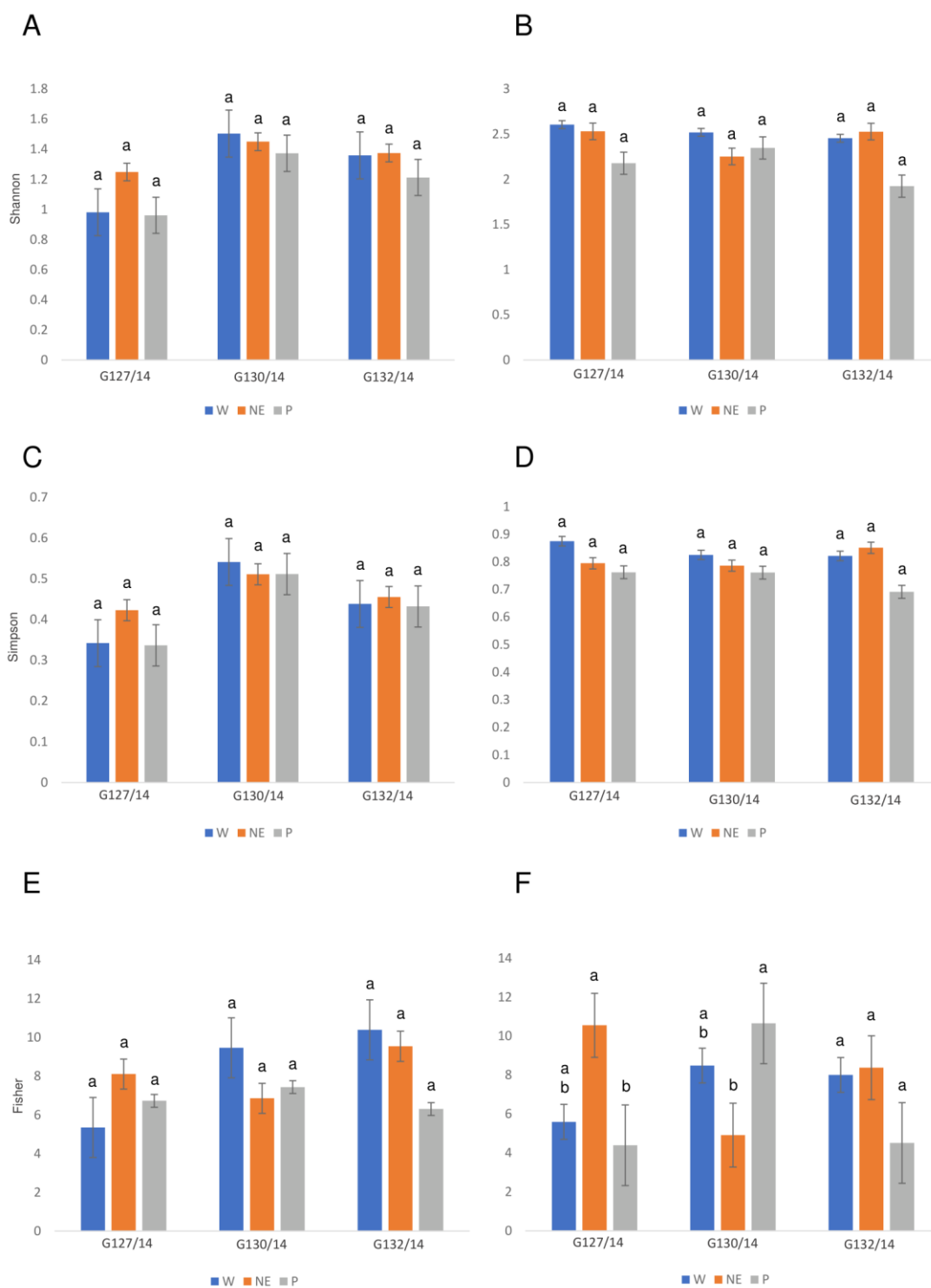
truskawki. Wyniki testu Biolog™ przedstawiono jako mapy cieplne, a także wykresy pokazujące wartości wskaźnika bogactwa substratowego dla poszczególnych wariantów eksperymentalnych. Różnice oceniano testem Wilcozona i zaznaczano istotność statystyczną na wykresach słupkowych dla każdego izolatu i dnia inkubacji owoców.

4.5.3 Wyniki i dyskusja

4.5.3.1 Różnorodność drobnoustrojów (wskaźniki α - i β -różnorodności)

W celu oceny wpływu izolatów *Neosartorya* spp. oraz zastosowanych środków przeciwgrzybiczych na strukturę zbiorowisk mikroorganizmów w owocach truskawki, obliczono wskaźniki α -różnorodności (Shannon, Simpson, Fisher). W przypadku zbiorowisk bakterii nie zaobserwowano istotnych różnic pomiędzy żadnym z zastosowanych wariantów – woda, ekstrakt naturalny oraz konserwant – niezależnie od analizowanego wskaźnika i izolatu grzyba (Rys. 9A, 9C, 9E), co wskazuje na wysoką stabilność zbiorowisk bakteryjnych w obliczu zaburzeń środowiskowych. Obserwowana stabilność potwierdza tezę, że funkcjonalność zbiorowisk mikroorganizmów wykracza poza możliwości ich pojedynczych członków (Zaramela et al., 2021). Zbiorowiska grzybowe nie wykazywały zmian w indeksach Shannona (Rys. 9B) i Simpsona (Rys. 9D), ale ich różnorodność była bardziej podatna na działanie ekstraktu roślinnego i konserwantu, co potwierdziły wyniki wartości wskaźnika Fishera (Rys. 9F). Dla próbek zakażanych izolatami G127/14 i G130/14 odnotowano istotne różnice między wariantami z ekstraktem i konserwantem, przy czym kierunek zmian był odwrotny – ekstrakt roślinny dawał wyższe wyniki różnorodności grzybów niż konserwant w przypadku zakażenia owoców izolatem G127/14, a niższe dla owoców truskawki zakażanych izolatem G130/14. W przypadku owoców zakażonych izolatem G132/14 nie stwierdzono różnic między testowanymi wariantami. Zmienność ta wskazuje, że zbiorowiska grzybów są nieznacznie bardziej wrażliwe na działanie ekstraktu z *C. officinalis* oraz pirosiarczynu sodu niż zbiorowiska bakteryjne, co znajduje odzwierciedlenie również w zróżnicowaniu głównych

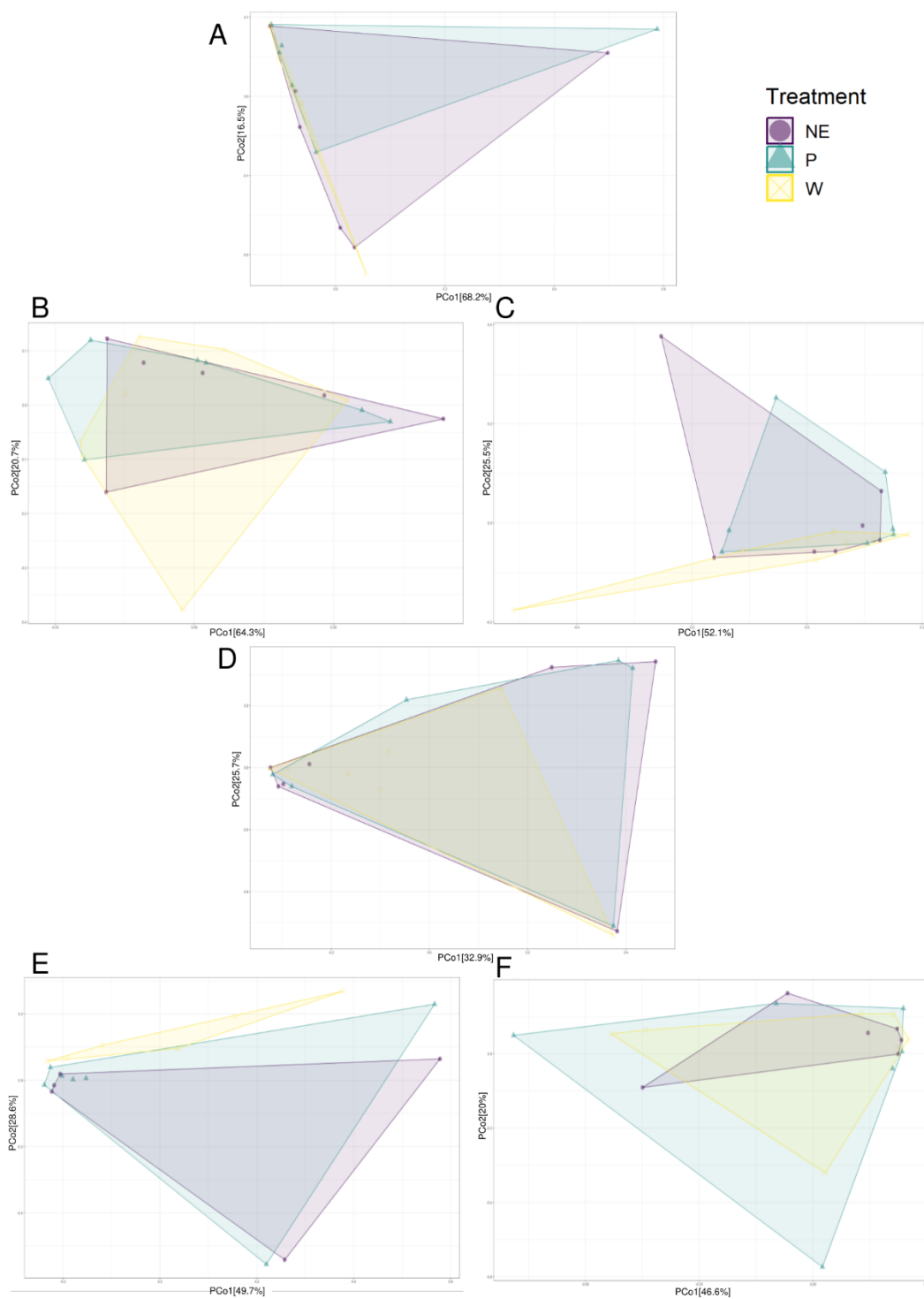
taksonów na diagramach Venna. Jest to spójne z wynikami badań innych autorów, w których stwierdzono zróżnicowaną odpowiedź zbiorowisk bakterii i grzybów na stres suszy czy degradację gleby (Chen et al., 2020; de Vries et al., 2018).



Rys. 9. Wskaźniki α -różnorodności dla zbiorowisk bakterii (A, C, E) i grzybów (B, D, F) w owocach truskawki zanieczyszczanych izolatami grzybów z rodzaju *Neosartorya* (G127/14, G130/14 i G132/14) oraz pod wpływem wody (W), ekstraktu naturalnego (NE) i konserwantu (P). Zastosowano wskaźniki Shannona (A, B), Simpsona (C, D), Fishera (E, F). Litery oznaczają różnice istotne statystycznie.

Analiza β -różnorodności umożliwiła ocenę zmian w składzie zbiorowisk mikroorganizmów w próbkach owoców truskawki zanieczyszczanych izolatami G127/14, G130/14 i G132/14, poddanych działaniu wody, roślinnego ekstraktu naturalnego i konserwantu żywności. W przypadku bakterii, skład zbiorowisk w próbkach z izolatami G127/14 nie różnił się istotnie pomiędzy wariantami – grupy tworzyły zbliżone klastry, co wskazuje na dużą spójność pomimo stosowania różnych zabiegów (Rys. 10A). Dla G130/14 zaobserwowano oddzielenie klastra próbki kontroli wodnej od pozostałych, natomiast próbki traktowane ekstraktem i konserwantem były do siebie zbliżone (Rys. 10B). Podobny trend wystąpił przy zanieczyszczaniu owoców izolatami G132/14, gdzie wariant wodny był bardziej odseparowany, a warianty z ekstraktem i konserwantem grupowały się bliżej siebie (Rys. 10C). W przypadku grzybów, dla owoców zanieczyszczanych izolatami G127/14 nie odnotowano wyraźnych różnic pomiędzy zabiegami – wszystkie tworzyły zbliżone skupiska (Rys. 10D). Dla G130/14 wariant wodny był oddzielony od pozostałych, natomiast próbki owoców traktowanych ekstraktem i konserwantem tworzyły nachodzące na siebie klastry (Rys. 10E). Najbardziej złożony układ wykazały truskawki kontaminowane izolatami G132/14 – trzy warianty oprysku ułożyły się w koncentryczne klastry, co sugeruje hierarchiczne zróżnicowanie składu zbiorowisk grzybów (Rys. 10F). Pomimo wcześniej zaobserwowanych subtelnych różnic w α -różnorodności, ogólny skład zbiorowisk bakteryjnych i grzybowych pozostawał względnie stabilny między próbkami testowanych owoców, co potwierdzają wskaźniki β -różnorodności. Wskazuje to, że niezależnie od zastosowanego zabiegu obecne były głównie te same taksony, choć w różnych

proporcjach, co jest zgodne z wynikami badań Walters & Martiny (2020). Podobne zjawisko odnotowano w badaniach innego zespołu naukowców, gdzie herbicyd oparty na glifosacie wpływał na poszczególne taksony mikrobioty owoców truskawki, lecz nie zmieniał ogólnej struktury zbiorowiska (Mathew et al., 2023).



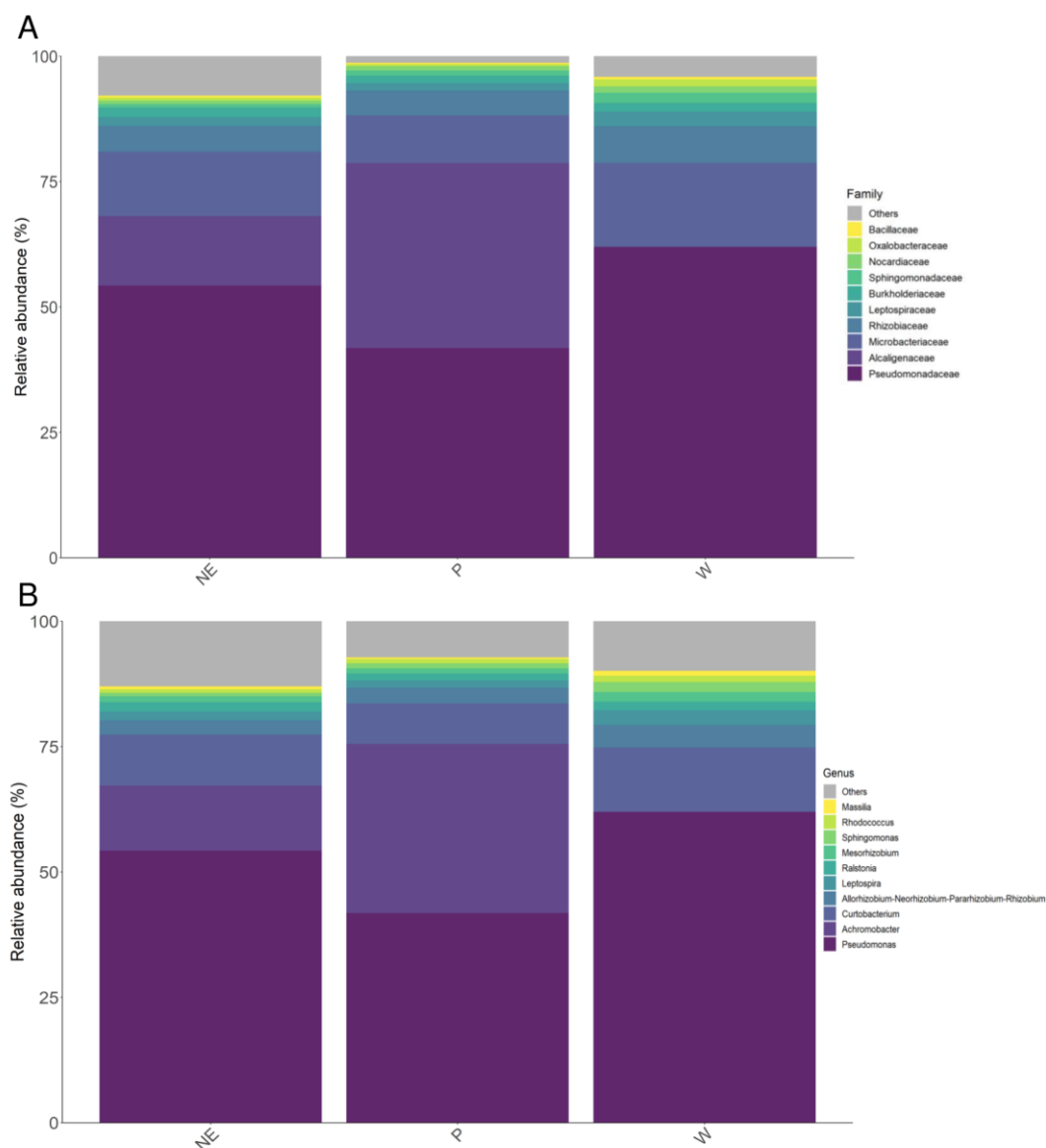
Rys. 10. Wskaźniki β -różnorodność zbiorowisk bakterii (A, B, C) i grzybów (D, E, F) w owocach truskawki zanieczyszczanych izolatami G127/14 (A, D), G130/14 (B, E) i G132/14 (C, F) oraz traktowanych wodą (W), naturalnym ekstraktem roślinnym (NE) i konserwantem żywności (P).

4.5.3.2 Struktura zbiorowisk bakterii i grzybów w owocach truskawki

Największe różnice w składzie mikrobioty bakteryjnej pomiędzy wariantami traktowania zaobserwowano w czwartym dniu eksperymentu, dlatego szczegółowej analizie poddano próbki po 4 dniach przechowywania owoców truskawki w warunkach chłodniczych (Rys. 11). Na poziomie typu dominowały przedstawiciele Proteobacteria, których udział zwiększył się z 77,03% w kontroli wodnej do 78,06% w owocach traktowanych ekstraktem roślinnym i do 87,88% w owocach poddanych działaniu konserwantu. Udział Actinobacteriota, będących na drugim miejscu względem liczności, znacząco obniżył się po zastosowaniu substancji przeciwdrobnoustrojowych, szczególnie po traktowaniu owoców konserwantem (10,21%), w porównaniu z kontrolą wodną (18,27%). Podobny efekt zaobserwowano dla przedstawicieli Spirochaetota. Natomiast obfitość Firmicutes i Bacteroidota zwiększyła się po zastosowaniu ekstraktu roślinnego i były mniej liczne po aplikacji konserwantu. Co ciekawe, tylko w próbkach owoców traktowanych konserwantem wykryto Cyanobacteria, a Verrucomicrobiota pojawiły się wyłącznie w wariantcie, w którym owoce miały kontakt z ekstraktem roślinnym. Na poziomie klas dominowały Gammaproteobacteria, których udział wzrastał po ekspozycji na ekstrakt roślinny i konserwant żywności, co obrazuje obfitość tej klasy na poziomie 68,00% w kontroli, 72,26% w wariantcie z ekstraktem roślinnym i 81,35% w owocach traktowanych konserwantem żywności. Z kolei udział Actinobacteria obniżał się po ekspozycji na ekstrakt roślinny i konserwant – największą obfitość przedstawicieli Actinobacteria odnotowano w owocach kontrolnych (18,26%), mniejszą po ekspozycji owoców na działanie ekstraktu roślinnego (16,34%) i najmniej w wariantcie z konserwantem

(10,20%). Mniejszą obfitość przedstawicieli Alphaproteobacteria, Leptospirae, Bacilli i Bacteroidia odnotowano w owocach traktowanych konserwantem żywności. Zauważalna była także zmiana na poziomie rzędów: choć Pseudomonadales pozostały dominujące, ich udział obniżał się w kolejnych wariantach (z 61,45% w owocach kontrolnych do 53,77% w traktowanych ekstraktem roślinnym i 41,95% w owocach poddanych działaniu konserwantu), ustępując miejsca Burkholderiales, które zyskały na znaczeniu (od najniższej obfitości 4,17% w owocach kontrolnych, przez 16,35% w owocach mających kontakt z ekstraktem roślinnym, do 38,80% w owocach traktowanych konserwantem). Oznacza to wyraźne przesunięcie udziału dominujących taksonów w kierunku bakterii potencjalnie lepiej tolerujących warunki stresowe. Na poziomie rodzin (Rys. 11A), Pseudomonadaceae pozostały najliczniejsze, ale ich udział stopniowo obniżał się w zależności od wariantu eksperymentalnego: 61,43% (owoce kontrolne), 53,77% (owoce poddane działaniu ekstraktu roślinnego), 41,94% (owoce traktowane konserwantem). Przeciwny trend dotyczył Alcaligenaceae (*Achromobacter* spp.), których udział zwiększał się z zaledwie 0,02% w kontroli aż do 13,41% w owocach traktowanych ekstraktem roślinnym i 36,80% w owocach poddanych działaniu konserwantu. Rodziny Microbacteriaceae, Rhizobiaceae, Leptospiraceae i Oxalobacteraceae wykazały obniżenie względnej obfitości po aplikacji substancji przeciwdrobnoustrojowych. Na poziomie rodzaju (Rys. 11B), *Pseudomonas* dominował we wszystkich warunkach eksperymentalnych, choć jego względna liczebność w owocach truskawki obniżała się po aplikacji ekstraktu roślinnego i konserwantu, odpowiednio: 61,43% (kontrola), 53,77% (ekstrakt roślinny) i 41,94% (konserwant). Podobną tendencję spadkową odnotowano dla rodzaju *Curtobacterium* (Microbacteriaceae). *Achromobacter* był niemal nieobecny w owocach kontrolnych, ale jego udział zwiększył się do 12,72% po ekspozycji owoców na działanie ekstraktu roślinnego i aż do 33,61% po działaniu konserwantu. Inne rodzaje, takie jak *Rhizobium*, *Leptospira*, *Ralstonia* czy *Sphingomonas*, były mniej liczne i ich udział dodatkowo obniżał się po zastosowaniu

środków przeciwdrobnoustrojowych. Dla ogółu dni eksperymentu, skład mikrobiomu owoców był bardziej zbliżony między wariantami, a bakterie *Pseudomonas* spp. dominowały w całym przebiegu doświadczenia. W składzie mikrobiomu owoców truskawki pojawiały się drobne różnice, jak wzrost udziału Actinobacteriota i Alphaproteobacteria po ekspozycji owoców na działanie ekstraktu roślinnego, czy większy udział Gammaproteobacteria i Burkholderiales w owocach traktowanych konserwantem. Opisane zmiany odzwierciedlają wyniki uzyskane w badaniach innych autorów. Przykładowo, Proteobacteria są często dominującym typem w mikrobiomie owoców truskawki, tak samo jak Pseudomonadaceae, Alcaligenaceae i Microbacteriaceae (Bai et al., 2023; Wang et al., 2024). W niniejszej pracy konserwant promował dominację Proteobacteria i *Pseudomonas* spp., kosztem taksonów uznawanych za korzystne, jak *Curtobacterium* (Borah et al., 2018). Co istotne, zarówno *Pseudomonas*, jak i *Actinobacteria* wykazują właściwości ochronne wobec chorób truskawek, dlatego przesunięcie w kierunku Burkholderiales i *Achromobacter* spp. może mieć znaczenie ekologiczne (Haggag et al., 2012; Sylla et al., 2013; Marian et al., 2020). Warto dodać, że taksony z rzędu Burkholderiales reagują na czynniki stresowe, takie jak fosforany czy środki chwastobójcze, co może tłumaczyć zwiększenie względnej obfitości tych taksonów w owocach w odpowiedzi na działanie konserwantu (Mathew et al., 2023).



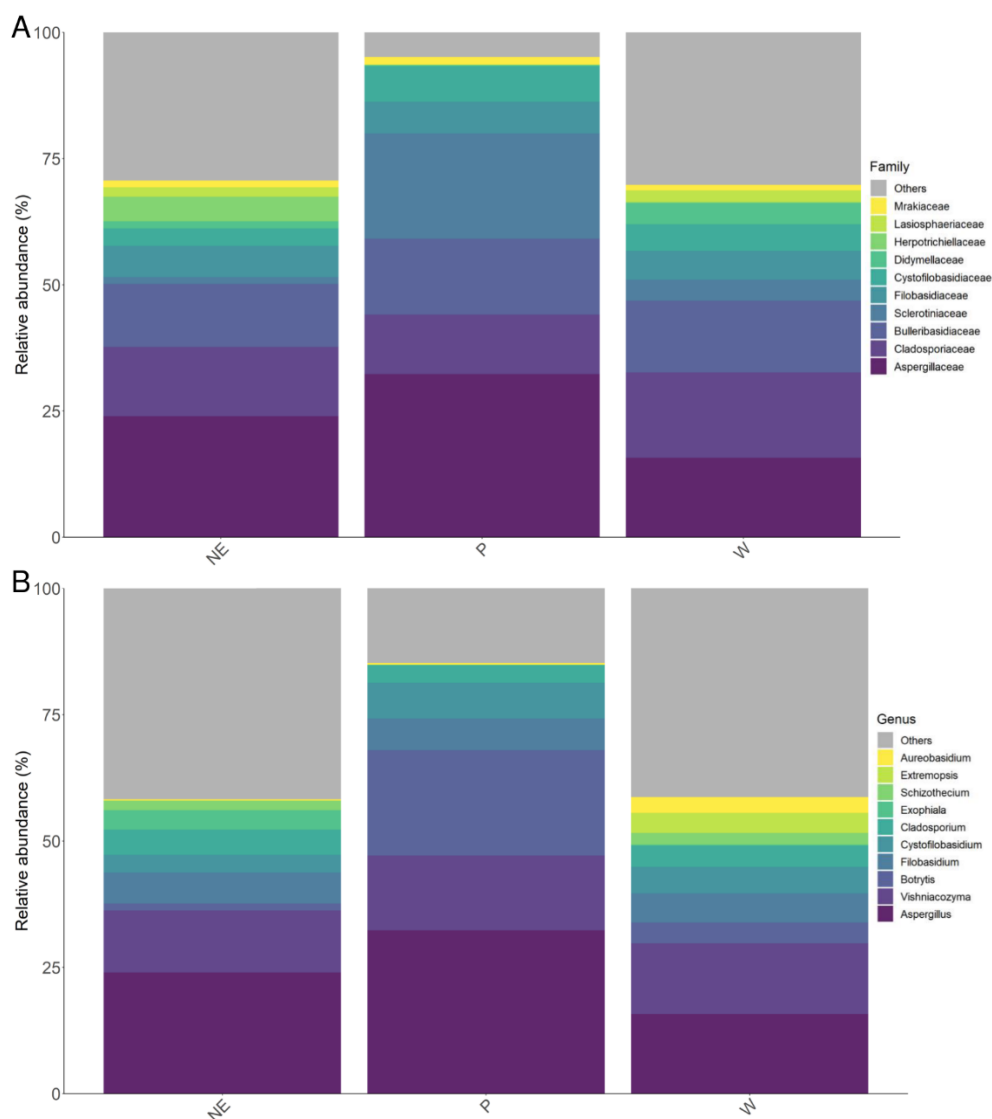
Rys. 11. Względna liczebność 10 głównych rodzin (A) i rodzajów (B) bakterii w owocach truskawki w 4 dniu eksperymentu po ekspozycji owoców na naturalny ekstrakt roślinny (NE), konserwant (P), oraz traktowanych wodą (wariant kontrolny) (W).

W kontekście społeczności grzybowych, czwartego dnia wszystkie próbki owoców truskawki były zdominowane przez Ascomycota, których udział zwiększał się stopniowo pod wpływem oprysków z substancjami o właściwościach antymikrobiologicznych (woda: 61,94%, ekstrakt roślinny: 67,23%, konserwant: 68,38%). Zmiany w obrębie Basidiomycota były bardziej zróżnicowane – ich udział obniżał się w próbach owoców poddanych działaniu

ekstraktu roślinnego (27,59%), natomiast zwiększał się w obecności konserwantu (31,52%) względem owoców kontrolnych traktowanych wodą (29,44%). Na poziomie klas dominowały Tremellomycetes, Eurotiomycetes i Dothideomycetes. Szczególnie widoczny był wzrost udziału Eurotiomycetes w owocach po zastosowaniu konserwantu (32,45% vs. 15,92% w kontroli), co sugeruje wysoką tolerancję tej grupy grzybów na substancje grzybobójcze. Z kolei udział Dothideomycetes obniżał się pod wpływem obu zabiegów, a Leotiomycetes ulegał zwiększeniu w owocach poddanych działaniu konserwantu (20,99% vs. 4,85% w wodzie). Na poziomie rzędów dominowali przedstawiciele Eurotiales, których udział wyraźnie zwiększał się (32,32%) w obecności konserwantu. Na poziomie rodzin (Rys. 12A) do najliczniej reprezentowanych taksonów należały Aspergillaceae, których względna obfitość zwiększała się istotnie w owocach poddanych działaniu konserwantu (do 32,32%). Podobna tendencja zaznaczyła się dla przedstawicieli Sclerotiniaceae (20,85%) i Cystofilobasidiaceae (7,08%). Z kolei Cladosporiaceae i Lasiosphaeriaceae systematycznie traciły udział, a ta ostatnia zanikła całkowicie w obecności konserwantu. Na poziomie rodzajów (Rys. 12B) dominowali przedstawiciele *Aspergillus* spp., których względna obfitość zwiększała się w owocach poddanych każdemu z testowanych zabiegów, a także *Filobasidium* spp. i *Botrytis* spp., których udział w mikrobiomie owoców zwiększał się szczególnie pod wpływem zastosowanego konserwantu żywności. Przeprowadzone badania wykazały, że rodzaje *Cladosporium* i *Exophiala* ulegały niewielkiemu zwiększeniu w owocach poddanych działaniu ekstraktu roślinnego, lecz ich udział obniżał się w owocach traktowanych konserwantem.

W ujęciu ogólnym wszystkich dni eksperymentu, zbiorowiska grzybów występujące w zanieczyszczanych owocach truskawki różniły się odpowiedzią na oba rodzaje zabiegów w zależności od ich pozycji taksonomicznej. W próbach kontrolnych (woda) dominowały Ascomycota (ok. 64%), reprezentowane głównie przez rodzaje *Aspergillus* i *Botrytis*, oraz Basidiomycota (ok. 35%), z istotnym udziałem drożdżaków z rodzaju *Vishniacozyma*, co jest

spójne z danymi literaturowymi na temat mikrobiomu owoców truskawki (Cybulska et al., 2022; Siegieda et al., 2023; Tenea et al., 2024). Po zastosowaniu ekstraktu roślinnego, w owocach truskawki zwiększył się udział drożdży należących do Basidiomycota, w tym *Vishniacozyma* i *Filobasidium*, natomiast udział pleśni z rodzajów *Aspergillus* i *Cladosporium* ulegał obniżeniu. Z kolei konserwant podtrzymywał dominację grzybów strzępkowych w owocach, w szczególności przedstawicieli Ascomycota, takich jak *Aspergillus* i *Botrytis*, oraz ograniczał udział niektórych drożdży i mniej odpornych klas jak Dothideomycetes. Wskazuje to, że ekstrakt roślinny może selektywnie wspierać niepatogeniczne drożdże i ograniczać rozwój grzybów odpowiedzialnych za psucie się owoców, natomiast konserwant sprzyja utrzymaniu się grzybów strzępkowych w owocach.

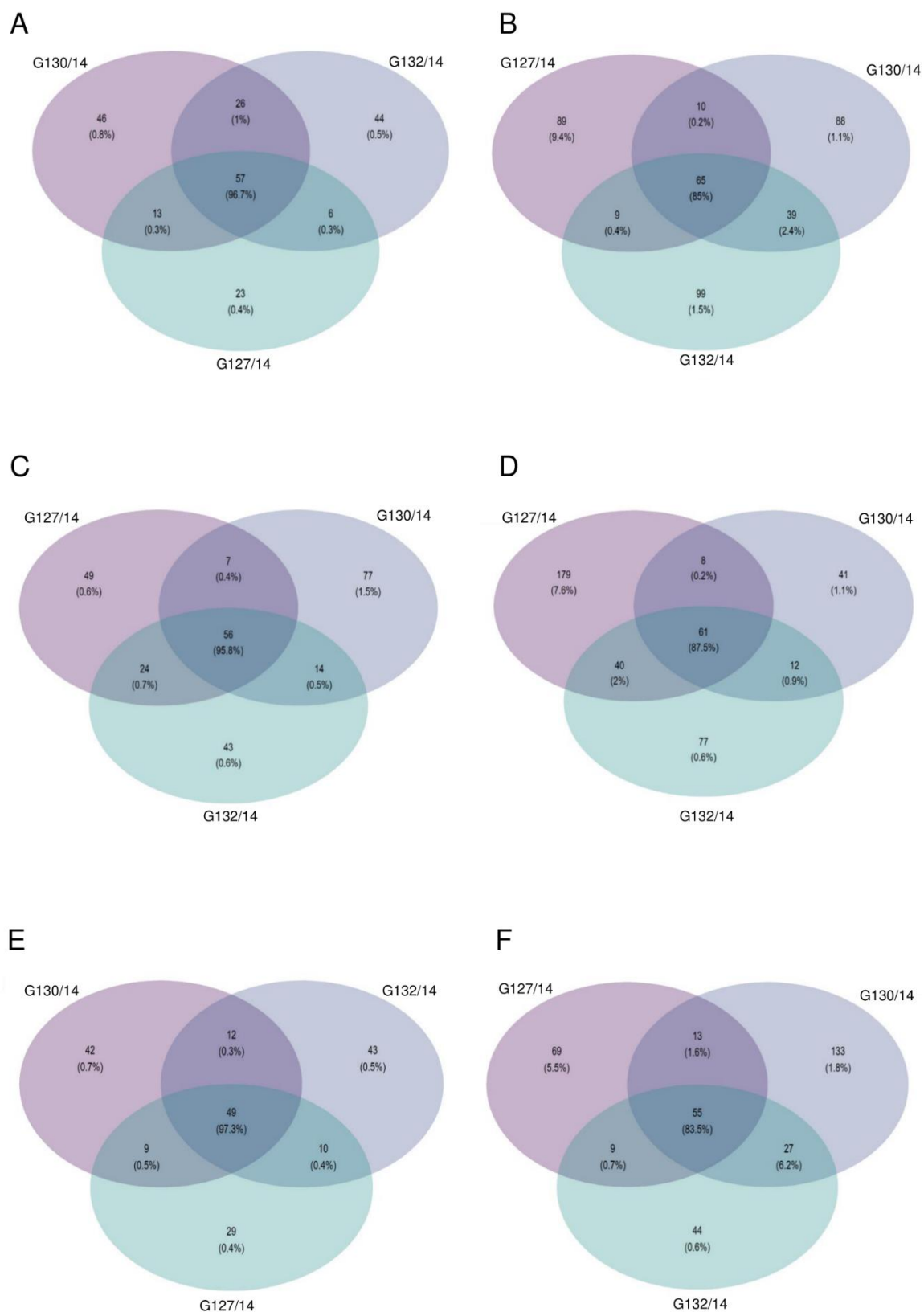


Rys. 12. Względna liczebność 10 głównych rodzin (A) i rodzajów (B) grzybów w owocach truskawki w 4 dniu eksperymentu po ekspozycji owoców na naturalny ekstrakt roślinny (NE), konserwant (P), oraz traktowanych wodą (warian kontrolny) (W).

4.5.3.3 Wspólne i unikalne taksony występujące w owocach truskawki

W owocach kontrolnych, które były traktowane wodą (Rys. 13A), zbiorowiska bakteryjne cechowały się dużym udziałem wspólnych wariantów sekwencji ampliconu (ASVs – amplicon sequence variants) (57 ASVs; 96,7% sekwencji), natomiast unikalne ASVs stanowiły niewielki odsetek u każdego z izolatów (G127/14: 23, G130/14: 46, G132/14: 44). Dla grzybów udział wspólnych ASVs był niższy (65 ASVs; 85%), a najwięcej unikalnych ASV

wykryto w przypadku izolatu G132/14 (99) (Rys. 13B). Po zastosowaniu roślinnego ekstraktu naturalnego liczba wspólnych ASVs pozostawała wysoka u bakterii (56 ASV; 95,8%, Rys. 13C) i umiarkowana u grzybów (61 ASV; 87,5%, Rys. 13D), przy czym wśród grzybów najwięcej unikalnych ASV zaobserwowano w przypadku izolatu G127/14 (179). Z kolei konserwanty powodowały największe ujednolicenie zbiorowisk bakteryjnych (49 ASV; 97,3%, Rys. 13E), a najmniejsze w przypadku zbiorowisk grzybów (55 ASV; 83,5%, Rys. 13F), z najwyższym udziałem unikalnych ASVs dla izolatu G130/14 (133). Wyniki potwierdzają odporność bakterii na działanie zarówno naturalnych, jak i syntetycznych środków przeciwgrzybiczych, pomimo że niektóre dane literaturowe donoszą o silnym działaniu przeciwdrobnoustrojowym ekstraktu z *Calendula officinalis* (Karnwal, 2022; del Mar Ruíz-Posadas et al., 2023) i pirosiarczynu sodu (Shahbazi et al., 2021), jednak w odniesieniu do pojedynczych patogenów, a nie złożonych zbiorowisk mikroorganizmów.



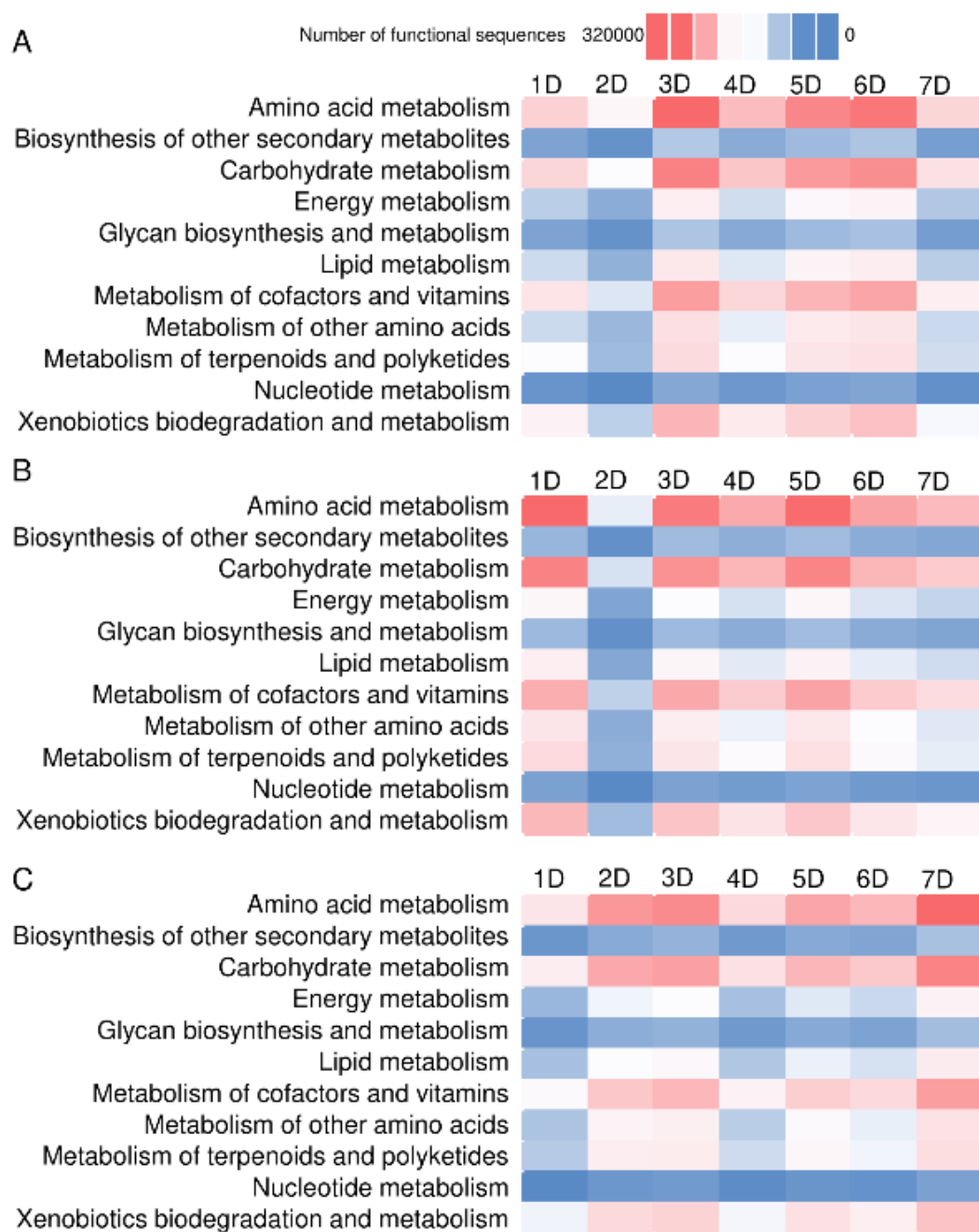
Rys. 13. Diagramy Venna przedstawiające liczbę głównych taksonów dla zbiorowisk bakterii (A, C, E) i grzybów (B, D, F), dla próbek zakażonych izolatami G127/14, G130/14 i G132/14,

zaprezentowane indywidualnie dla owoców traktowanych wodą (A, B), naturalnym ekstraktem roślinnym (C, D), środkiem konserwującym (E, F). Liczba całkowita to liczba ASVs, podczas gdy dane procentowe odnoszą się do całkowitej liczby sekwencji.

4.5.3.4 Przewidywane cechy funkcjonalne mikrobiomu bakteryjnego

Analiza PICRUSt wykazała dominację sekwencji powiązanych z funkcjami metabolicznymi we wszystkich testowanych próbkach owoców (~75–76%). Szczegółowa analiza ujawniła jednak różnice między dniami aplikacji i wariantami zastosowanego oprysku. W próbkach owoców kontrolnych traktowanych wodą (Rys. 14A) najwyższe wartości dotyczyły metabolizmu witamin i kofaktorów (222181.06, dzień 6) oraz metabolizmu aminokwasów (318421.43, dzień 3), natomiast najniższe wartości dotyczyły metabolizmu nukleotydów (26769.2) i metabolitów wtórnych (24 069.42). W przypadku owoców traktowanych naturalnym ekstraktem roślinnym (Rys. 14B), metabolizm aminokwasów był dominujący już w pierwszym dniu ekspozycji owoców na działanie ekstraktu (270779.78), ale jego poziom ulegał szybkiemu obniżeniu, podobnie jak biosynteza metabolitów wtórnych (34615.96, dzień 1). Metabolizm energii uległ obniżeniu z 88551.11 do 54183.72 sekwencji w ciągu tygodnia, a metabolizm kofaktorów i witamin osiągnął maksimum w piątym dniu inkubacji (195854.88), po czym obniżył się w dniu siódmym (122028.20). Z kolei zastosowanie konserwantu (Rys. 14C) spowodowało wyraźny wzrost aktywności metabolicznej zbiorowisk mikroorganizmów w owocach truskawki w czasie. Liczebność sekwencji kodujących metabolizm aminokwasów zwiększyła się z 116877.50 do 298188.36 w 7 dniu inkubacji, a obfitość sekwencji kodujących metabolizm węglowodanów kształtowała się na poziomie 260833.02, przekraczając wartości w kontroli. Podobne trendy dotyczyły metabolizmu energii (do 97970.62) oraz biosyntezy metabolitów wtórnych (45277.90). Najwyższą wartość w całym zbiorze danych osiągnął metabolizm kofaktorów i witamin (221464.01), a także metabolizm terpenoidów i poliketydów (126715.33). Pomimo zmian taksonomicznych sugerujących

przebudowę mikrobiomu w odpowiedzi na testowane zabiegi, prognozy funkcjonalne PICRUSt wskazują na ogólnie stabilny skład szlaków metabolicznych. Zabiegi z użyciem ekstraktu naturalnego i konserwantu prowadziły jednak do istotnych zmian funkcjonalnych, szczególnie w metabolizmie aminokwasów i metabolitów wtórnych. Ekstrakt wywoływał początkowe zwiększenie aktywności metabolicznej, po którym następowało szybsze jej obniżenie w porównaniu z kontrolą wodną, co sugeruje przejściową odpowiedź metaboliczną zbiorowisk mikroorganizmów występujących w owocach traktowanych naturalnym ekstraktem roślinnym (Adi Wicaksono et al., 2022). Z kolei konserwant początkowo obniżał aktywność, a następnie obserwowano jej stopniowe zwiększanie, wskazując na początkowy efekt hamujący i późniejszą adaptację mikrobiomu owoców do zastosowanego konserwantu żywności (Gilbert & Das, 2017).

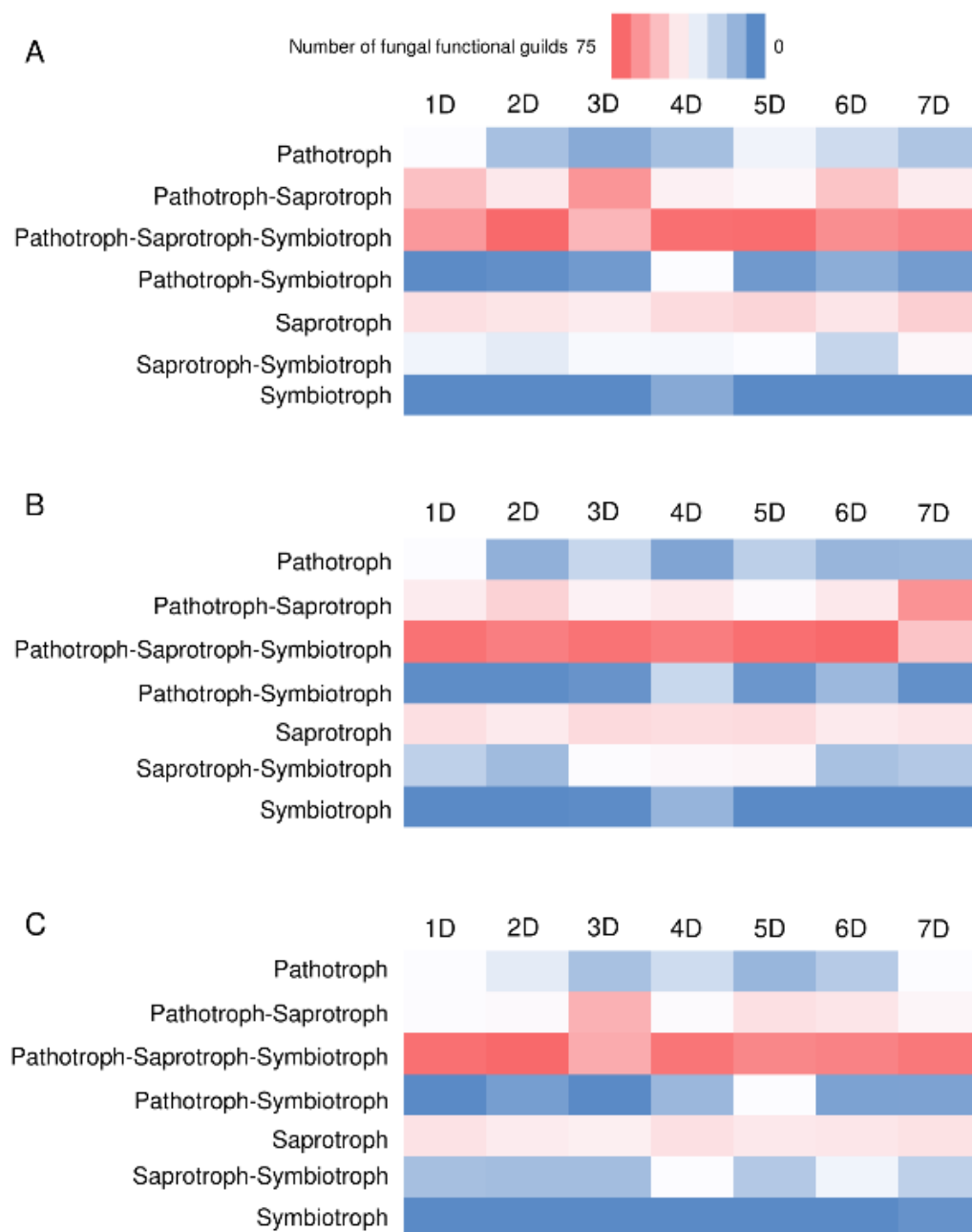


Rys. 14. Predykcja PICRUSt profilu funkcjonalnego bakterii z liczbą sekwencji funkcjonalnych związanych z metabolizmem różnych związków. Dane wizualizowane dla każdego dnia eksperymentu dla każdego wariantu hodowli oddzielnie: woda (A), naturalny ekstrakt (B), konserwant (C).

4.5.3.5 Tryby troficzne zbiorowisk grzybowych

Wykorzystując bazę danych FUNGuild, zbiorowiska grzybów obecne w owocach sztucznie zakażanych izolatami *Neosartorya* spp. zostały zaklasyfikowane do sześciu głównych trybów troficznych. Dominującą grupą we wszystkich testowanych próbkach owoców był złożony tryb patotrof–saprotrof–symbiotrof, którego udział sięgał od ~42% (po inokulacji owoców izolatem G127/14 w kontroli wodnej) do ~78% (po inokulacji owoców izolatem G130/14 w wariancie poddanym działaniu konserwantu). W owocach truskawki zakażonych izolatem G127/14 w kontroli wodnej odnotowano wyższy udział czystych saprotrofów (~30%) niż przy pozostałych wariantach oprysku (~12–14%). Izolaty G130/14 i G132/14 konsekwentnie charakteryzowały się dominacją potrójnego trybu troficznego. Czyste patotrofy utrzymywały się poniżej 2% we wszystkich próbkach owoców. Biorąc pod uwagę każdy dzień eksperymentu (Rys. 15), zaobserwowano dominację trybu patotrof–saprotrof–symbiotrof we wszystkich testowanych wariantach. W kontroli wodnej (Rys. 15A), udział wskazanego mieszanego trybu troficznego wahał się od 35,8% do 73,7%, a tryb patotrof–saprotrof był zmienny (4,5–52,3%). Saprotrofy pozostawały stabilne (10–24%), a pozostałe grupy troficzne grzybów były marginalne (<2%). Po zastosowaniu ekstraktu naturalnego (Rys. 15B) udział potrójnego trybu troficznego początkowo wynosił ~71–76%, ale obniżył się do 30% w 7 dniu inkubacji, równoległe ze zwiększeniem względnej obfitości trybu patotrof–saprotrof (do 55%). Po zastosowaniu konserwantu (Rys. 15c) udział potrójnego trybu troficznego był najwyższy (46–79%), natomiast udział grupy reprezentującej tryb patotrof–saprotrof obniżył się do 2–5% (w porównaniu do nawet 52% w kontroli), a saprotrofy i przedstawiciele trybu saprotrof–symbiotrof występowały rzadziej niż w próbach kontrolnych. Analiza trybów troficznych grzybów wykazała, że w przeciwieństwie do zastosowanego naturalnego ekstraktu roślinnego, który powodował późne zwiększenie udziału grupy patotrof–saprotrof, traktowanie owoców konserwantem powodowało utrzymywanie stale

wysokiego poziomu troficznego trypletu patotrof-saprotrof-symbiotrof i jednocześnie tłumilo udział trybu troficznego saprotrof-symbiotrof. Różnice te podkreślają, że rodzaj zabiegu silnie wpływa na równowagę między grzybami patogenicznymi i saprofitycznymi występującymi w owocach truskawki, a także powoduje zmiany struktury zbiorowisk mikroorganizmów towarzyszących.



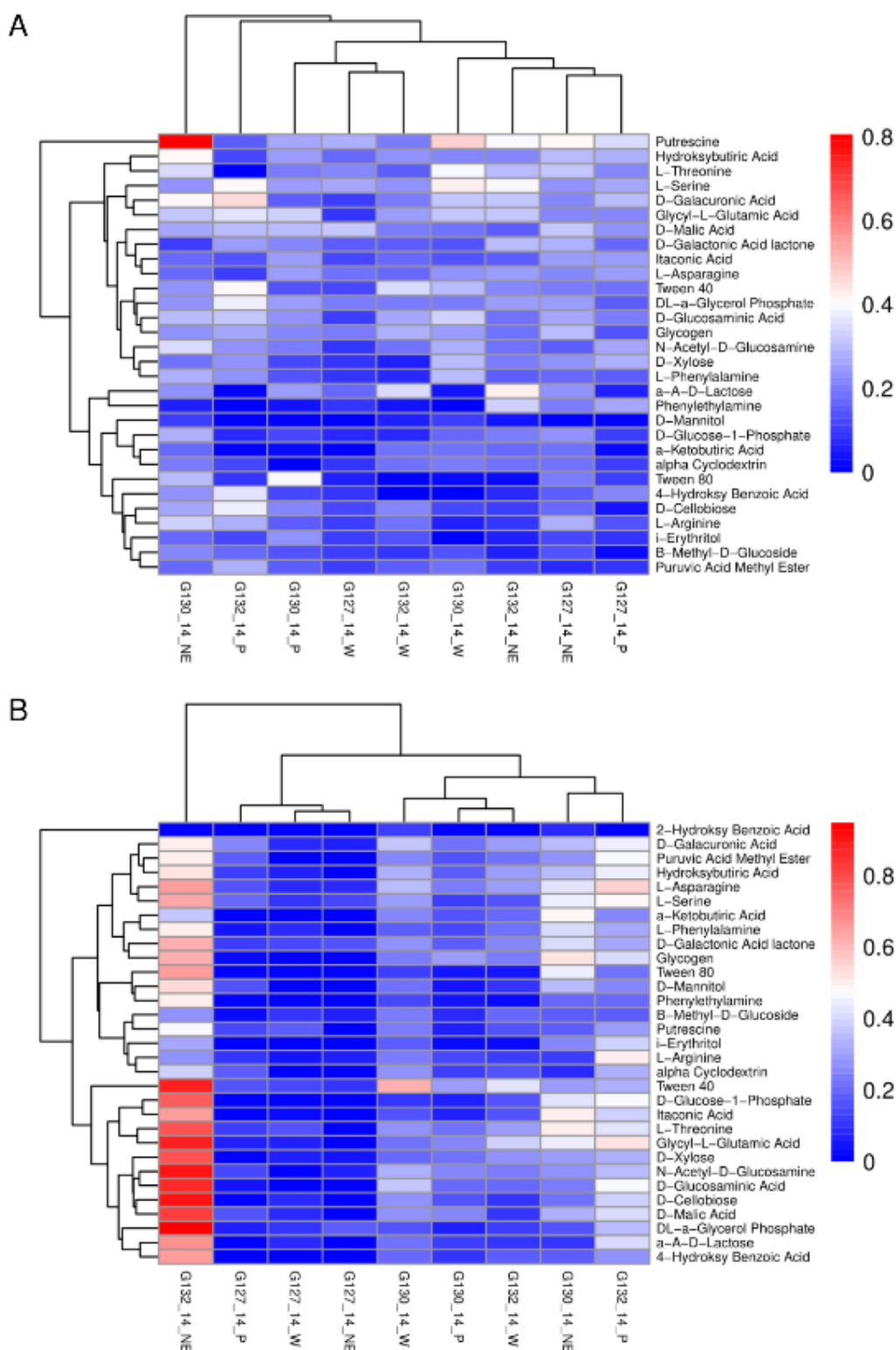
Rys. 15. Grupy troficzne grzybów określone z wykorzystaniem narzędzia FUNGuild z liczbą cech funkcjonalnych grzybów. Dane zostały przedstawione dla każdego dnia eksperymentu,

dla każdego wariantu hodowli oddzielnie: woda (A), naturalny ekstrakt roślinny (B), konserwant (C).

4.5.3.6 Aktywność metaboliczna zbiorowisk mikroorganizmów w owocach truskawki zakażanych grzybami z rodzaju *Neosartorya*

Badania obejmowały analizę profilu metabolicznego owoców truskawki, z wykorzystaniem płytek Biolog™ EcoPlate, po 120 godzinach inkubacji, co pozwoliło na wygenerowanie map cieplnych ilustrujących zróżnicowaną produkcję biomasy w zależności od czasu przechowywania w warunkach chłodniczych i rodzaju zastosowanego oprysku. Po pierwszym dniu przechowywania owoców kontaminowanych grzybami i traktowanych wodą, naturalnym ekstraktem roślinnym lub konserwantem (Rys. 16A), profile metaboliczne były podobne – najwyższą aktywność metaboliczną zbiorowisk mikroorganizmów w owocach zaobserwowano dla putrescyny, kwasu galakturonowego i L-seryny, natomiast nie odnotowano wykorzystania m.in. mannitolu i glukozy-6-fosforanu. Po 7 dniach przechowywania w warunkach chłodniczych (Rys. 16B) odnotowano wyraźne różnice profilu metabolicznego owoców zarówno w obrębie testowanych izolatów grzybów, jak również zastosowanego zabiegu. Efekt ten był silniejszy niż różnice odnotowane między poszczególnymi, testowanymi źródłami węgla. Zbiorowiska mikroorganizmów zasiedlające owoce truskawki zanieczyszczone izolatem G127/14 wykazywały niższą intensywność wzrostu w porównaniu do tych występujących w owocach zakażonych grzybami G130/14 i G132/14, cechujących się bardziej stabilnym wzrostem, z subtelnymi różnicami między dniami inkubacji i zastosowanymi zabiegami. Najwyższą intensywność wzrostu mikroorganizmów zaobserwowano w 7 dniu hodowli dla owoców zanieczyszczonych izolatem G132/14, traktowanych ekstraktem roślinnym (Rys. 16B). Na podstawie uzyskanych wyników obliczono wskaźnik różnorodności substratowej (R). W pierwszym dniu inkubacji zbiorowiska mikroorganizmów występujących w owocach zakażonych izolatem G127/14 i traktowanych

wodą wykazywały niższą różnorodność substratową niż w owocach traktowanych konserwantem lub ekstraktem roślinnym. Owoce kontaminowane izolatami G130/14 nie wykazywały różnic pomiędzy testowanymi zabiegami, natomiast owoce zakażone grzybem G132/14 cechowały się wzrostem wskaźnika R w wyniku traktowania naturalnym ekstraktem roślinnym oraz jego obniżeniem pod wpływem działania konserwantu. Podobne tendencje utrzymywały się w kolejnych dniach trwania eksperymentu, z wyjątkiem dnia 4, gdy różnice dla owoców zanieczyszczonych izolatami G127/14 i G132/14 zanikły, a traktowanie owoców konserwantem zwiększało wskaźnik R w przypadku zakażenia grzybem G130/14. W dniu 5 zastosowanie ekstraktu roślinnego i konserwantu spowodowało zwiększenie różnorodności substratowej zbiorowisk mikroorganizmów w owocach zakażonych izolatami G127/14, natomiast tylko ekstrakt roślinny zwiększał wskaźnik R dla owoców zakażonych izolatami G130/14. Z kolei konserwant powodował obniżenie wskaźnika R dla owoców zakażonych izolatami G132/14. W dniu 6 i 7 owoce kontrolne traktowane wodą charakteryzowały się wyższymi wartościami wskaźnika R w obiektach kontaminowanych izolatami G127/14 i G132/14, podczas gdy w owocach zakażonych izolatami G130/14 większe wartości tego wskaźnika ujawniły się w wariantach poddanych działaniu naturalnego ekstraktu roślinnego i konserwantu. Na podstawie uzyskanych wyników z wykorzystaniem płytek Biolog™ EcoPlate potwierdzono wcześniejsze obserwacje, wskazujące, że produkcja biomasy i różnorodność funkcjonalna zbiorowisk mikroorganizmów obecnych w owocach zależały od rodzaju izolatu i zastosowanego zabiegu. Szczególnie wyraźnie zaznaczył się trend wskazujący na niższą aktywność metaboliczną zbiorowisk mikroorganizmów w owocach zakażonych izolatami G127/14, który był najbardziej wrażliwy na działanie naturalnych ekstraktów roślinnych i konserwantów, w porównaniu do owoców zakażonych grzybami charakteryzującymi się średnią (G132/14) i niską (G130/14) wrażliwością na zastosowane środki.



Rys. 16. Produkcja biomasy (OD 750) na różnych źródłach węgla obecnych w płytkach Biolog™ ECO, przez zbiorowiska mikroorganizmów występujących w owocach truskawki. Przedstawione dane obejmują wyniki po 120 godzinach inkubacji mikropłytek, inokulowanych

zawiesiną mikroorganizmów z owoców przechowywanych w warunkach chłodniczych przez 1 (A) lub 7 (B) dni. Na początku eksperymentu owoce truskawki były zakażane jednym z izolatów grzybów z rodzaju *Neosartorya* (G127/14, G130/14, G132/14) i traktowane jednym wariantem oprysku: wodą (W), naturalnym ekstraktem roślinnym (NE) lub konserwantem żywności (P).

4.5.4 Podsumowanie

Uzyskane wyniki wskazują, że badany naturalny ekstrakt roślinny (ekstrakt z nagietka) oraz konserwant żywności (pirosiarczyn sodu) wpływały różnicująco na zbiorowiska bakterii i grzybów w owocach truskawki, przy czym zbiorowiska grzybów wykazywały większą wrażliwość na testowane środki. Przeprowadzone badania wykazały, że efekt działania zależał nie tylko od zastosowanego środka (ekstrakt roślinny, konserwant), ale także cech czynnika zanieczyszczenia mikrobiologicznego (izolaty grzybów z rodzaju o różnej wrażliwości na ekstrakty roślinne i konserwanty). Ekstrakt roślinny wywoływał krótkotrwałą odpowiedź metaboliczną i sprzyjał rozwojowi potencjalnie korzystnych mikroorganizmów. Z kolei konserwant wzmacniał udział grzybów strzępkowych i powodował opóźniony, lecz intensywny wzrost ich aktywności metabolicznej. Obserwacje te są istotne dla projektowania zrównoważonych strategii konserwowania żywności, które ograniczają rozwój mikroorganizmów powodujących psucie się owoców, a jednocześnie wspierają korzystny mikrobiom owoców.

5. Podsumowanie i wnioski

Współczesne wyzwania związane z zapewnieniem jakości i bezpieczeństwa świeżych owoców miękkich wymagają ciągłego poszukiwania nowych, skutecznych metod przeciwdziałania zakażeniom opornymi mikroorganizmami. Grzyby z rodzaju *Neosartorya* potrafią przetrwać działanie wysokich temperatur dzięki zdolności tworzenia askospor, co

umożliwia im adaptację do niekorzystnych warunków środowiskowych. Ich obecność została udokumentowana w owocach nawet po zastosowaniu standardowych procedur utrwalających. Z tego względu stanowią istotne zagrożenie dla przemysłu rolno-spożywczego oraz sektora ogrodniczego.

W ramach przeprowadzonych badań dokonano charakterystyki grzybów z rodzaju *Neosartorya*, koncentrując się w szczególności na rozpoznaniu ich potencjalnych mechanizmów oporności i zdolności przetrwania w warunkach zbliżonych do stosowanych w praktyce przechowalniczej. Badania pozwoliły na kompleksowe określenie morfologii, zdolności metabolicznych, a także scharakteryzowanie genomu i wrażliwości na ekstrakty roślinne, konserwanty żywności i związki chemiczne izolatów grzybów z rodzaju *Neosartorya* wyizolowanych uprzednio z gleby i owoców truskawki. Uwzględniono zarówno właściwości fenotypowe, jak i różnorodne odpowiedzi izolatów na działanie związków konserwujących oraz wybranych ekstraktów roślinnych. W toku eksperymentów zaobserwowano istotne zróżnicowanie izolatów blisko spokrewnionych, które pozwoliło na wyróżnienie grup charakteryzujących się wzrastającą tolerancją na czynniki stresowe. Uzyskane wyniki wskazują, że nawet blisko spokrewnione izolaty mogą wykazywać istotne różnice w zakresie fizjologii, metabolizmu i odpowiedzi na działanie wybranych związków chemicznych o właściwościach konserwujących. Różnorodność ta przejawia się zarówno w zróżnicowanej gęstości mycelium, jak i w odmiennym wykorzystaniu źródeł węgla oraz oporności na ekstrakty roślinne i konserwanty. Obserwowane zmiany w strukturze mikrobiomu oraz ogólnej aktywności metabolicznej zbiorowisk mikroorganizmów w kontaminowanym grzybami *Neosartorya* spp. owocach truskawki sugerują, że zastosowane zabiegi – zarówno chemiczne (konserwant żywności – pirosiarczyny sodu), jak i ekstrakty naturalne (ekstrakt z nagietka) – mogą mieć nie tylko bezpośredni wpływ na grzyby wprowadzone do owoców, ale również

mogą prowadzić do reorganizacji struktury i aktywności zbiorowisk mikroorganizmów towarzyszących.

Wielopoziomowa analiza obejmująca testy inhibicji, profile metaboliczne, obserwacje mikroskopowe oraz analizy genetyczne pozwoliła na kompleksowe ujęcie fenotypowej i genotypowej zmienności badanych izolatów. Połączenie danych molekularnych z obserwacjami biologicznymi i ekologicznymi umożliwiało lepsze zrozumienie potencjału adaptacyjnego *Neosartorya* spp., a także ocenę skuteczności wybranych strategii przeciwdziałania rozwojowi tych grzybów w owocach truskawki. Uzyskane wyniki potwierdzają, że właściwości metaboliczne, morfologiczne oraz genetyczne izolatów grzybów z rodzaju *Neosartorya* mają istotny wpływ na kształtowanie ich wrażliwości na ekstrakty roślinne i konserwanty, a także na zdolność adaptacyjną do działania tych czynników, wynikającą z różnic w profilu metabolicznym i cechach morfologicznych. Zróżnicowanie tych cech warunkuje zarówno efektywność środków antymikrobiologicznych, jak i mechanizmy obronne poszczególnych izolatów, co potwierdza postawioną główną hipotezę badawczą.

Całokształt przeprowadzonych badań wskazuje na potrzebę indywidualnego podejścia do oceny ryzyka związanego z obecnością grzybów z rodzaju *Neosartorya* w surowcach rolniczych i ogrodniczych oraz żywności, z uwagi na znaczną zmienność fenotypową i genotypową tych mikroorganizmów. Wysoki potencjał przetrwalnikowy i adaptacyjny wybranych izolatów może stanowić istotne wyzwanie dla przemysłu przetwórczego i spożywczego, szczególnie w kontekście długoterminowego przechowywania produktów. Wyniki niniejszych badań podkreślają także potencjał naturalnych substancji roślinnych jako uzupełnienia tradycyjnych metod konserwacji oraz wskazują kierunki dalszych badań nad zrównoważonymi metodami ochrony żywności.

Na podstawie przeprowadzonych badań sformułowano następujące wnioski:

- Badane grzyby z rodzaju *Neosartorya* wykazywały istotnie zróżnicowaną wrażliwość na działanie konserwantów, naturalnych ekstraktów roślinnych i związków chemicznych pomimo wysokiego pokrewieństwa. Wśród pierwotnie badanych 20 izolatów możliwe było wyszczególnienie 5 grup o wzrastającej oporności, aby następnie wybrać 10 izolatów grzybów najbardziej reprezentatywnych, stanowiących 3 główne grupy wrażliwości na badane ekstrakty roślinne i konserwanty, w tym charakteryzujące się wysoką, średnią i niską wrażliwością wobec testowanych środków.
- Zarówno naturalne ekstrakty roślinne (suchy ekstrakt z kwiatu lawendy (*Lavandula angustifolia*), suchy ekstrakt z nagietka lekarskiego (*Calendula officinalis*), olejek lawendowy, olejek rozmarynowy, olejek z drzewa herbacianego, olejek goździkowy), jak i syntetyczne konserwanty (pirosiarczyn sodu, sorbinian potasu, wodorosiarczyn sodu i kwas sorbowy) cechowały się właściwościami mykobójczymi.
- Pomimo wysokiej odporności niektórych izolatów *Neosartorya* spp. na działanie ekstraktów roślinnych i konserwantów żywności, możliwe jest ograniczenie ich występowania poprzez zastosowanie określonych stężeń naturalnych ekstraktów roślinnych lub związków konserwujących żywność. Zastosowanie niskich stężeń ekstraktów naturalnych (np. 1 mg mL⁻¹) powodowało podobne lub wyższe zahamowanie wzrostu, w porównaniu do wyższego stężenia (6.25 mg mL⁻¹) zastosowanych syntetycznych konserwantów.
- Kombinacje zawartości cukru (°Bx) i pH miały ograniczony wpływ na wzrost *Neosartorya* spp., wykazując działanie inhibicyjne dla mniej niż połowy testowanych izolatów w badaniach dyfuzyjno-krażkowych. Jedynie kombinacje 39°Bx o pH 3.2 i 43°Bx o pH 3.2 wykazywały słabe właściwości antymikrobiologiczne w mikropłytkach MT2. Uzyskane wyniki wskazują, że, modyfikacja zawartości cukru i pH przetworów

owocowych jest niewystarczająca by efektywnie zapobiegać rozwojowi grzybów z rodzaju *Neosartorya* w surowcach zakażonych tymi mikroorganizmami.

- Grzyby z rodzaju *Neosartorya* cechowały się zróżnicowanym profilem metabolicznym, wykazując największą aktywność oddechową i wzrost na źródłach węgla z grupy węglowodanów oraz kwasów karboksylowych. Hodowla *Neosartorya* spp. w obecności pirosiarczynu sodu lub ekstraktu z nagietka znacznie obniżyła zdolność izolatów do wykorzystania dostępnych źródeł węgla, ograniczając zarówno oddychanie, jak i produkcję biomasy przez testowane izolaty grzybów. Wrażliwość grzybów na badane środki była zróżnicowana – niektóre izolaty (np. G150/14) wykazały silne zahamowanie uzdolnień metabolicznych, inne (np. G135/14, G129/14) pozostały względnie odporne. I-erytrytol był jedynym związkiem skutecznie metabolizowanym przez testowane izolaty, nawet po działaniu ekstraktu roślinnego lub konserwantu, które ograniczały wzrost grzybów, co wskazuje na jego uniwersalne znaczenie dla grzybów z rodzaju *Neosartorya*.
- Badanie aktywności metabolicznej techniką fluorescencyjną wykazało zależne od czasu działanie pirosiarczynu sodu i ekstraktu z nagietka na żywotność grzybów z rodzaju *Neosartorya*. Pirosiarczyn sodu wykazał efekt hamujący jedynie po 30 dniach inkubacji, prowadząc do zmniejszenia intensywności fluorescencji, co sugeruje jego działanie po długotrwałej ekspozycji. Wpływ ekstraktu z nagietka widoczny był już po 10 dniach inkubacji, powodując zmniejszenie intensywności fluorescencji w obydwu terminach doświadczenia, co sugeruje jego szybsze działanie. Indywidualne wyniki dla poszczególnych izolatów grzybów wykazywały zróżnicowanie, co potwierdza tezę o istnieniu grup zróżnicowanej wrażliwości grzybów na testowane ekstrakty roślinne i konserwanty.

- Choć większość badanych związków chemicznych powodowała zahamowanie wzrostu testowanych grzybów, izolaty *Neosartorya* spp. wykazywały zróżnicowaną reakcję na ich działanie. Na ogół, związki takie jak neomycyna i chlorochina stymulowały wzrost *Neosartorya* spp., podczas gdy związki takie jak propikonazol czy 5-fluorocytozyna wykazywały działanie hamujące.
- Wyniki analizy względnych porównań profili metabolomicznych wykazały istotne różnice w zależności od zastosowanego środka konserwującego, testowanego izolatu oraz czasu inkubacji. Podejście niecelowane jest zatem wartościową techniką poznawania ogólnych tendencji i profili metabolomicznych grzybów *Neosartorya* spp. poddanych warunkom stresowym.
- Obserwacje mikroskopowe ujawniły zmiany morfologiczne izolatów wywołane ich ekspozycją na działanie środków antymikrobiologicznych, w tym zmiany zagęszczenia grzybni, zwiększenie zarodnikowania, wykształcanie perydium oraz powstanie zgrubień na strzępkach. Żywotność testowanych grzybów była specyficzna dla izolatu oraz zależna od czasu ekspozycji na działanie środka antymikrobiologicznego, co wskazuje na aktywację szlaków adaptacyjnych w odpowiedzi na długotrwałą ekspozycję.
- Analiza całogenomowa izolatów grzybów potwierdziła obecność genów kodujących odporność na warunki stresowe oraz wskazała na różnice w mechanizmach oporności między izolatami o różnej wrażliwości na ekstrakty roślinne i konserwanty. Na podstawie funkcji kilkunastu tysięcy genów oceniono całkowity odsetek genów związanych z mechanizmami obronnymi, który różnił się pomiędzy badanymi izolatami *Neosartorya* spp., przy czym te wcześniej sklasyfikowane jako bardziej wrażliwe posiadały mniej genów związanych z reakcjami obronnymi, podczas gdy bardziej odporne izolaty zawierały więcej genów przypisanych do tej grupy.

- Traktowanie owoców truskawki konserwantem żywności (pirosiarczynem sodu) oraz naturalnym ekstraktem roślinnym (ekstraktem z nagietka) miało modyfikujący wpływ na mikrobiom owoców, powodując zmiany zarówno w składzie mikrobioty, jak i w profilu metabolicznym zbiorowisk mikroorganizmów zasiedlających owoce. Obserwowano istotne różnice w odpowiedzi mikrobiologicznej i metabolicznej w zależności od rodzaju zastosowanego środka, co wskazuje na możliwość selektywnego kształtowania mikrobiomu owoców przez konserwanty żywności i naturalne ekstrakty roślinne w zależności od rodzaju zastosowanego środka oraz specyficznych cech izolatu, którym infekowane były owoce.

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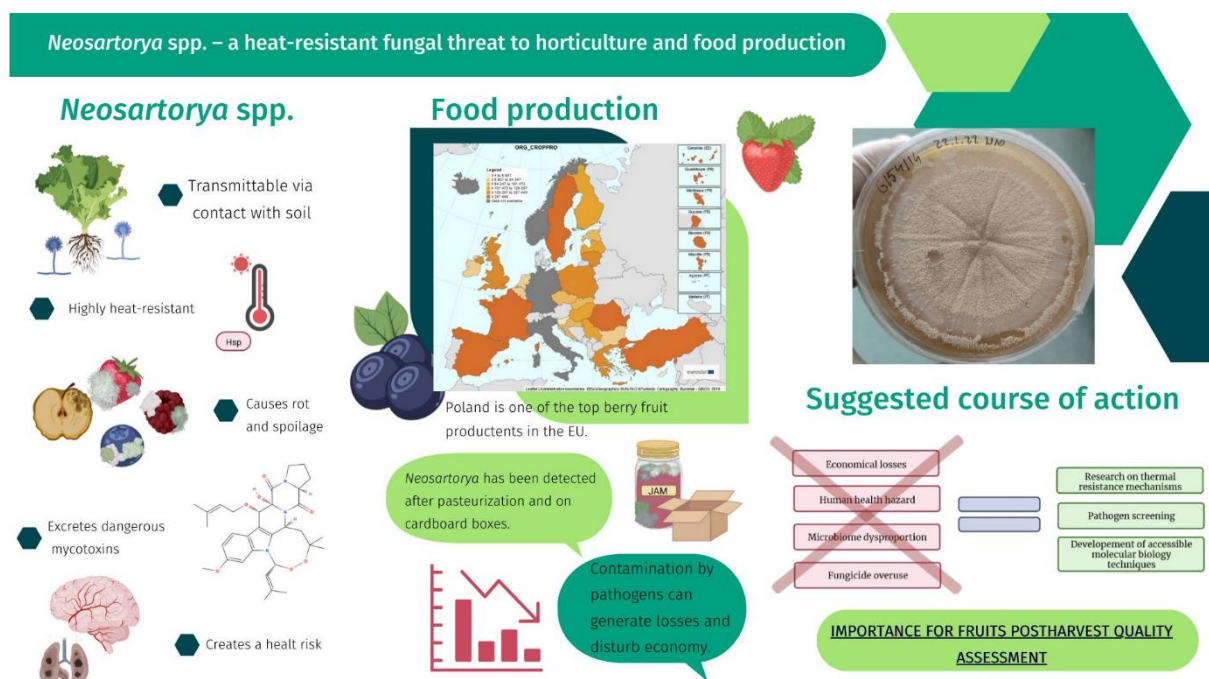
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7. Teksty publikacji

7.1 Publikacja P.1

7.1.1 Abstrakt graficzny



7.1.2 Tekst publikacji



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Review

Soil-Borne *Neosartorya* spp.: A Heat-Resistant Fungal Threat to Horticulture and Food Production—An Important Component of the Root-Associated Microbial Community

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Abstract: Soil-borne *Neosartorya* spp. are the highly resilient sexual reproductive stage (teleomorph) of *Aspergillus* spp. Fungi of this genus are relevant components of root-associated microbial community, but they can also excrete mycotoxins and exhibit great resistance to high temperatures. Their ascospores easily transfer between soil and crops; thus, *Neosartorya* poses a danger to horticulture and food production, especially to the postharvest quality of fruits and vegetables. The spores are known to cause spoilage, mainly in raw fruit produce, juices, and pulps, despite undergoing pasteurization. However, these fungi can also participate in carbon transformation and sequestration, as well as plant protection in drought conditions. Many species have been identified and included in the genus, and yet some of them create taxonomical controversy due to their high similarity. This also contributes to *Neosartorya* spp. being easily mistaken for its anamorph, resulting in uncertain data within many studies. The review discusses also the factors shaping *Neosartorya* spp.'s resistance to temperature, preservatives, chemicals, and natural plant extracts, as well as presenting novel solutions to problems created by its resilient nature.

Keywords: metabolic profile; mycobiome; mycotoxins; thermo-resistance



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1. Introduction

Conventional farming, the use of synthetic fertilizers, harsh pesticides, and commercial preservatives have proven to have a detrimental influence on the natural environment. This introduced an era of promoting sustainability and ecologically sound manufacturing methods. The European Union urges its member states to develop and practice solutions incorporating natural substances, dependences, and structures, which have an advantageous impact on the environment and human health. Numerous economical branches can “go green”; however, the paramount ones are horticulture and food production [1]. Therefore, it is crucial to identify common problems in these areas and search for optimal solutions. Predominantly reported issues include contamination and spoilage during the production and storage of food, mostly attributed to microbes. Technological advances have resolved some concerns; nonetheless, efficient methods of combating durable species are still to be established. In this review, one of the most important groups of fungi, heat-resistant fungi, is presented to explain the role of these microorganisms not only as harmful microbes for postharvest plant and fruit quality, but also as potentially beneficial organisms. *Neosartorya* spp., one of this fungal group, inhabits the rhizosphere; therefore, it has a role in shaping plant quality and sustainable horticulture.

Many countries in the European Union are large producers of strawberries. In addition, those with a share exceeding 50% of EU production are the leading producers of frozen fruits, including strawberries and concentrated juices from soft fruit [2]. These data place many European Union countries in the position of a market leader in berry production worldwide; this requires the producers and processors of fruits to monitor the quality of

their raw materials, intermediates and products at every stage of postharvest production, processing and distribution, to ensure food security and enhance their market position [3]. Therefore, the issues connected with maintaining a high quality of raw materials and fruit products for both national and international markets are an important field of study, not only for the consumers and producers of food, but also for scientists. Moreover, in accordance with the latest policy initiatives and laws, such as The European Green Deal and EU Biodiversity Strategy for 2030, very important targets include reducing the use of fertilizers by 20% and making 25% of EU agriculture organic by the year 2030 [4]. Therefore, one of the aims in modern horticulture is the application of natural methods to protect crops and food, and therefore it is necessary to deepen the knowledge about the metabolic, morphological and genetic properties of *Neosartorya* fungal strains that shape their resistance to natural plant extracts, preservatives and compounds. This is important for healthy food production, given this is one of the main members of the fungal community in the plant rhizosphere, and so could be useful to developing solutions to sustainable horticulture and improving the postharvest quality of crops.

The complex morphology of ascospores and their high thermal resistance enables these fungi to survive high temperatures, including industrial pasteurization processes. *Neosartorya* spp. colonizes soil, rhizosphere and crop residues, and has the additional ability to degrade various chemical compounds, even toxic ones [5]. They overcome these barriers and are able to infect fruit; they may also pose a potential threat to thermally processed fruit products. Organisms belonging to the genus *Neosartorya*, which are present in fresh fruit, despite the lack of visible mould growth, can produce heat-resistant ascospores and, in favourable conditions, may cause the spoilage of processed fruit through rapid mycelium growth and metabolism.

Fungi have developed various adaptations that allow for them to survive when exposed to fungicides and climate change. Initially, these were mainly adaptations for protection against the harmful effects of various natural environmental stressors. However, they can also allow for them to survive in postharvest crops as dormant forms. With regards to evolution, fungi have developed additional mechanisms of response to temperature, light, humidity, oxygen, or to the presence of chemical compounds [6], which allowed for them to effectively adapt to changing environmental conditions, including resistance to high temperatures. Due to the chemical sensitivity of *Neosartorya* spp., only partial data are available. There are articles concerning the thermal death rates of ascospores of *N. fischeri* under the influence of organic acids [7] and preservatives [8]. It has been established that citric and tartaric acids destroy ascospores in fruit juices. Preservatives such as potassium sorbate and sodium benzoate are also used to control this fungus in fruit juices [9]. Delgado et al. [10] reported that hydrogen peroxide must be considered to reduce the probability of package contamination by *N. fischeri*. In recent years, the occurrence of fungal infections has been increasing everywhere; this may be explained by the changing climatic conditions and the resistance of fungi to fungicides due to their extensive use in agriculture and horticulture [11]. Bromley et al. [11] reported that the use of the most dominant class of antifungal agents, azoles, may lead to resistance in environmental fungi, which is of clinical importance. They also isolated azole-resistant examples of the *N. fischeri* species. Due to these cases, it is reasonable to carry out a study that may lead to the control of *Neosartorya* spp. by finding substances that can be used as alternative to the active compounds of plant protection agents. Therefore, this may also be achieved by testing the influence of plant extracts and food preservatives on fungal growth, and metabolic, morphological and genetic changes in these fungi. Although heat-resistant fungi have been an object of intense research, the specific nature of their metabolic profile and morphology, as well as their genome and transcriptome under the impact of chemicals, plant extracts and preservatives, is almost unknown. This review summarizes the existing knowledge concerning an important fungal group, *Neosartorya* spp., including negative and positive aspects for the environment. This review is in line with the implementation of certain research directions decided on by the European Commission, and complies with

the FAO policy related to improving postharvest crops and food quality, to understand signalling mechanisms via root exudates and interactions between plant–rhizospheric microbial communities.

2. Characteristic of *Neosartorya* spp.

Neosartorya spp. are known as *Ascomycetes* and belong to the *Aspergillaceae* family. They exhibit unique heat-resistance abilities, allowing for them to withstand high temperatures. *Neosartorya* spp. fungi are considered to be a teleomorph (sexual state) of *Aspergillus* spp. and, therefore, produce ascospores. The spores are formed in groups of eight inside asci, which, in turn, are covered by an ascocarp, a large fruiting body. The ornamentation of ascospores is one of the key features enabling differentiation between *Neosartorya* species [12]. Usually, asci are differentiated into cleistothecium or gymnothecium. For example, the asci of *N. fischeri* are covered with cleistothecium, which helps them survive in a hot environment [13]. A cleistothecium is a smooth-walled, completely closed fruiting body with no designated opening. As spores are not automatically released into the environment, fungi rely on outside forces to disseminate their spores. Gymnothecium is similar to cleistothecium, with no openings and also containing asci. However, its peridial wall is a loose clump of hyphae, often entwined with coils or spines [14].

The *Neosartorya* spp. life-cycle contains various phases. In general, filamentous fungi reproduce sexually and asexually. Asexual reproduction involves mitotic processes, creating conidia, whereas sexual reproduction involves meiotic processes, creating spores [15]. When a species can access both the asexual and sexual life cycles, the stages of reproduction are usually dependent on distinct environmental and nutritional circumstances. Despite the numerous benefits of sexual reproduction, over one-fifth of all fungi are only known to reproduce asexually, with no ‘teleomorph’ identified [16]. The life-cycle of *Neosartorya* spp. fungi is presented in Figure 1.

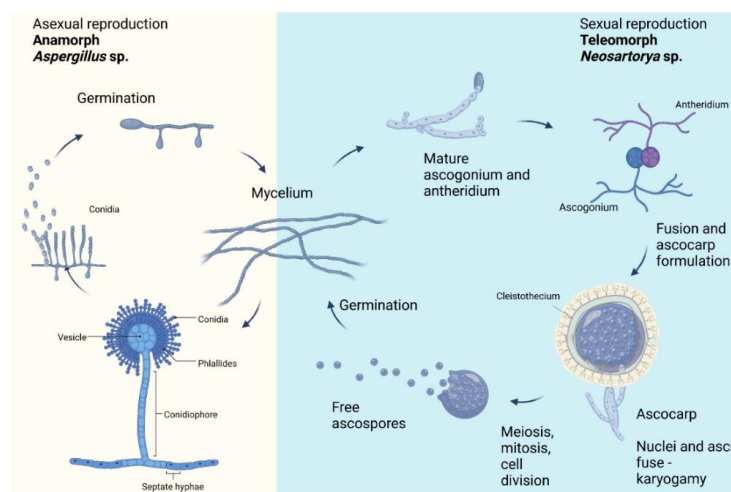


Figure 1. Life cycle of *Neosartorya* spp. with the stages of sexual and asexual reproduction (own elaboration by W. Maj and M. Frac using BioRender, Toronto, ON, Canada).

Although this approach is heavily discussed, in some cases it enables emphasis to be placed on certain aspects of reproduction, e.g., the creation of ascospores. In the case of *Neosartorya*, it is the ascospores that pose the biggest obstacle, as their high thermal resistance makes them more resilient than mycelium and able to survive the high-temperature treatments used in food preservation and the postharvest storage of fruits and vegetables.

23 *Aspergillus* species enter the sexual stage and produce the *Neosartorya* teleomorph. They can all complete their sexual cycle in from approximately 2 to 3 weeks at 25 °C on a traditional mycological medium such as Malt Extract Agar (MEA) or Potato Dextrose Agar (PDA). Depending on species, strains complete the sexual cycle and generate cleistothecia with ascospores in from four weeks to six months. The cleistothecia generated by *A. fumigatus* include ascospores that are morphologically indistinguishable from those seen in other *Neosartorya* species unless studied under scanning electron microscopy (SEM). The patterns on the ascospore surface are modest yet distinct to each species [17].

Fungal isolates identified by the β -tubulin gene sequence (Sanger sequencing, NCBI Blast) as *Neosartorya glabra* can create ascospores and cleistothecia that can be seen by the naked eye after approximately one month of culturing (Figure 2). However, they differ in the early stages of mycelium growth (A,E), producing either broad and woolly-like, floccose growth (A) or dense, velutinous colonies (E). Colonies produce larger globular ascospores that are loosely binded to mycelium (C) or finer, more powdery-like ascospores, which are better attached to mycelium (G). After a month, most ascospores can be easily detached from mycelium.

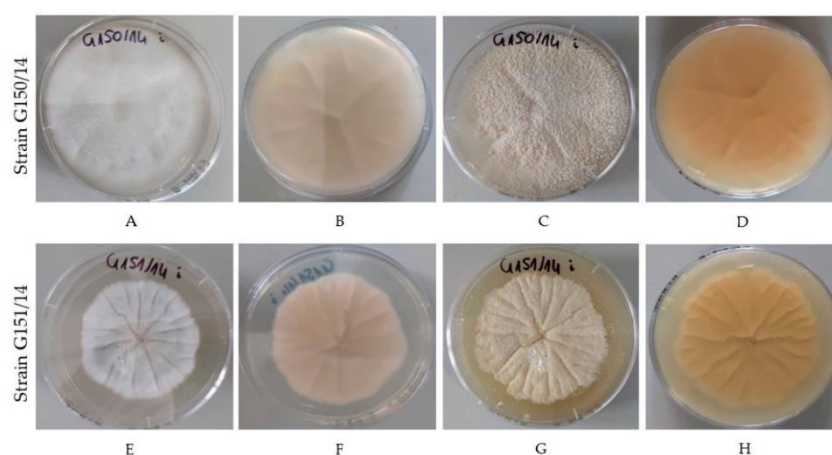


Figure 2. *Neosartorya* spp. growth on Potato Dextrose Agar with antibiotics (streptomycin, chlortetracycline). Strain G150/14 (A–D) after 4 days of culturing at 30 °C (A,B) and a month after (C,D). Strain G151/14 (E–H) after 4 days of culturing at 30 °C (E,F) and a month after (G,H). The number of strains: G150/14; G150/14; G151/14; G151/14 are visible on the plates presented on the photographs.

3. Biodiversity of *Neosartorya* spp.

To better systematize fungi, section Fumigati was created [18]. This consists of species with “uniserial aspergilli, columnar conidial heads in shades of green and flask shaped vesicles”. The section includes 23 *Neosartorya* species. However, there are more *Neosartorya* species that are classified as doubtful and require further research, e.g., *N. australensis*, *N. ferenczii*, *N. papuaensis*, and *N. warcupii*. Usually, they differ from other taxa; in this instance based, on either their β -tubulin, calmodulin or actin gene sequences [18].

The most well-known species of the genus are *Neosartorya fischeri* and *Neosartorya pseudofischeri*, belonging to section Fumigati. They are morphologically very similar to *A. fumigatus*. The genetic diversity of *A. fumigatus* is remarkably low, especially compared to *N. fischeri* and *N. spinosa*. Moreover, *A. fumigatus* shows no geographic pattern for genetic differentiation [19]. There have been reports of *Neosartorya* spp. being mistaken for *Aspergillus* spp., proving that the differentiation between them is not obvious [20]. Despite their many close similarities, more species have been isolated and classified. For example, *Neosartorya nishimurae* and *Neosartorya otanii*, isolated from African forest soil,

were characterized by their morphological differences. Both exhibited rapid growth on Czapek and Malt Extract Agars, had broad equatorial crests and lenticular ascospores. The differences between structures of cleistothecia surfaces and walls of conidia were visible. Due to their morphological affinity, some researchers question the distinctiveness of certain species. Some examples of species regarded as synonymous are presented in the table below (Table 1).

Table 1. Synonymous *Neosartorya* spp. species.

Species Name	Synonymous Species	High Similarity or Identical Features	Status	Ref.
<i>Neosartorya otanii</i>	<i>N. fennelliae</i>	Identical β -tubulin gene sequences, no differences in ascospores	Mating experiments needed for proof	[21]
<i>N. spinosa</i>	<i>N. botucatensis</i> , <i>N. paulistensis</i> , <i>N. takaki</i>	Circular arrangements on the convex walls of ascospores	Accepted	[22]
<i>Neosartorya primulina</i>	<i>N. quadricincta</i>	Nearly identical gene sequences for β -tubulin, calmodulin and actin, morphology, ascospore ornamentation, restricted growth on Czapek agar	Accepted	[22]
<i>Neosartorya delicata</i>	<i>N. tatenoi</i>	Identical ascospore morphology, nearly identical gene sequences for β -tubulin, calmodulin and actin	Accepted	[22]

N. spinosa, *N. glabra*, *N. assulata*, *N. quadricincta*, *N. hiratsukae* and *N. laciniosa* are commonly isolated from fruit and soil surfaces (Table 2). They have been previously isolated from Polish soil and strawberry samples [3]. Analyses of β -tubulin gene and EcoRI RFLP patterns were most helpful in their identification. These species, in particular, are responsible for the spoilage of food processed by heating [23].

N. fisheri was isolated from sunflower rhizosphere, especially after exposing plants to adverse environmental conditions, and was able to produce inulinase, which is important for the food industry as an alternative for the production of fructose syrups [24]. *N. hiratsukae* has also been reported indoors, in the air, on drywall in an Italian hospital. The small white colonies were hardly visible on white walls, so the spores could easily spread. Their presence in the environment caused a health risk, as they could lead to aspergillosis and other infectious diseases [25].

Table 2. Common *Neosartorya* spp. species and their properties.

Name	Telomorph	Relation to Other Species	Key Characteristics	Type of Growth	Reference
<i>N. spinosa</i>	<i>Aspergillus fisheri</i> var. <i>spinosus</i>	Has identical partial beta-tubulin and calmodulin gene sequences to <i>N. botucatensis</i> and <i>N. paulistensis</i>	Rough ascospores	On MEA: broad growth in pale yellow or yellowish white colour; thin layer of mycelium and abundant, granular cleistothecia	[12,26,27]
<i>N. laciniosa</i>	<i>Aspergillus lacinosus</i>	Closely related to <i>N. coreana</i>	Microtuberculate ascospores with two bent crests and two distinct equatorial rings of small projections	On MAA: beige with light yellow ascospores; on CYA: light yellow and white growth	[12,22,26]
<i>N. glabra</i>	<i>Aspergillus fisheri</i> var. <i>glaber</i>	Extrolites typical for <i>N. fennelliae</i> , but is more closely related to <i>N. denticulate</i> , despite having divergent ornamentations of ascospores	Confirmed to be a disease agent; homothallic species	Yellow–white to pale yellow cleistothecia, smooth ascospores	[12,22,28]
<i>N. assulata</i>	<i>Aspergillus assulatus</i>	Closely related to <i>A. waksmanii</i> with only 2% bp difference in the act1 locus	Common extrolites produced by its colonies are indole alkaloids and apolar metabolites	Snow-white growth on MEA medium	[22,29]

4. Two Sides of the Same Coin

The *Neosartorya* genus consists of extraordinarily heat-resistant fungi, which are immune to high temperatures and, consecutively, food preservation techniques, utilizing them. Acidic crops and produce that cannot undergo thermal conditions higher than 60–65 °C are especially vulnerable. In these circumstances, *Neosartorya* spp. can sporulate with great efficiency. Certain species require high temperatures to sporulate, meaning that thermal processing may result in the sudden appearance of new fungal growth [30]. This leads to the secretion of mycotoxins, e.g., aflatoxins, fumitremorgins and gliotoxin, which can pose a threat to both plant and human livelihood [31]. Fornal et al. [32] developed a method that enables the fast and easy quantification of mycotoxins typical for *Neosartorya* spp. isolates, including fumitremorgin C and verruculogen in strawberries, strawberry juice, potato dextrose broth and soil. As *Neosartorya* spp. is present in the soil, the transference and subsequent contamination of plants that come into contact with the ground is effortless. Contaminated plants pass the pollutant to crops, which, in turn, are harvested, processed, and eaten by humans. Without effectively breaking the life-cycle of *Neosartorya*, its extrolites can be transmitted to the food chain, posing a threat to peoples' well-being. As a precaution, new laws have been established regarding the quality of produce. In accordance with these principles, food that is not of satisfactory purity is not utilized [33,34].

It is important to discriminate between the species of *Neosartorya* and *A. fumigatus* in the food industry. Even subtle differences in genotype may lead to different reactions to chemical agents and treatment methods. Moreover, *Aspergillus fumigatus* has never been reported as a spoilage agent in heat-processed food products, meaning that its detection may not foreshadow future concerns [23]. Furthermore, although *N. fischeri* and *A. fumigatus* are phylogenetically close, they have different patterns of carbon sources' metabolism [35].

Neosartorya spp. is also an infectious agent. Recent studies show that, due to the misidentification of fungi, *Neosartorya* genus may be as infectious as *Aspergillus* spp. Aspergillosis, an illness caused by *Aspergillus*, is a major cause of human morbidity and mortality, with over 200,000 life-threatening infections each year worldwide [36]. There had been reports of *Neosartorya hiratsukae* causing the same disease. It is often wrongly identified as *A. fumigatus* due to its close morphological similarity. It also cannot be differentiated by the popular matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) that is used in many medical analyses [37].

A comparable situation arose with *Neosartorya udagawae*, which is also often mistaken for *A. fumigatus*. In 2014, it was reported to cause acute respiratory distress syndrome (ARDS) in a 43-year-old woman [38]. It was distinguished by sequencing ITS, calmodulin and β -tubulin genes. Due to this fact, further research about identification tactics is needed for therapeutics to be more successful, because of differences in the susceptibility to antifungal drugs. *Neosartorya* has also been proven to cause other diseases, such as endocarditis (*N. fischeri*) [39] or dermatitis (*N. hiratsukae*) [40].

Neosartorya spp. is notoriously known for producing durable, toxic metabolites. *N. fischeri* can synthesize acid protease and glycoside hydrolase (GH) 27, which, if left uncontrolled, may be potentially dangerous to wood, fiber and plants [41,42], but also very useful for industry. The *Neosartorya* spp. strain BL4 is known to biodegrade petroleum hydrocarbons. This might be useful when developing bioremediation techniques; however, it also proves how unsusceptible these fungi really are [43]. *A. flavus* and *A. parasiticus* produce aflatoxins that are toxic to the liver and are carcinogenic: the consumption of contaminated groundnuts has been linked with hepatic carcinoma in the populations of Africa and Asia [44]. Furthermore, *Neosartorya pseudofischeri* produces dangerously cytotoxic metabolites, which are proven to cause harm to Sf9 cells from *S. frugiperda* [45]. This, in combination with enzymes, mycotoxins and other extrolites, suggests that *Neosartorya* spp. can be seen as a health hazard, and a possible threat to food production and the economy.

However, in a controlled environment, *Neosartorya* spp. may prove useful in agriculture, horticulture and medicine (Figure 3). It exhibits antibacterial and antifungal properties [46,47] and can be used to produce nanoparticles to control brown spot in

rice [48]. New research suggests that *Neosartorya* spp. could be used to develop novel cancer treatments [49,50]. It can also help in the production of medicine for diabetes [51].

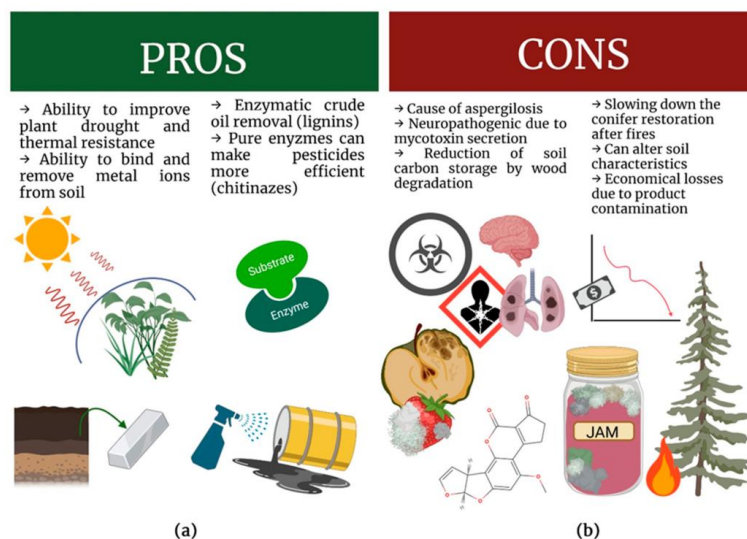


Figure 3. Positive and negative aspects of *Neosartorya* spp. (a) Positive aspects, (b) negative aspects; Own elaboration by W. Maj and M. Frac using BioRender, Toronto, Canada. Aflatoxin B1 structure molview.org (accessed on 12 October 2022).

Neosartorya spp., as with most filamentous and heat-resistant moulds, can be seen as either a threat or a tool (Figure 3). On the one hand, molecular biology can utilize *Neosartorya*-derived proteins or use it as a binding factor in environment protection. On the other hand, its potential as a general health and economic hazard makes it an unwanted contaminant.

5. Heat Response in *Neosartorya* spp. Fungi

Heat resistance, or thermotolerance, is facilitated by a range of factors and processes. These include the presence of heat-shock proteins, molecular chaperones, chaperonins, protective substances, the innate properties of proteins that contribute to making them thermostable, cell-wall stoichiometry and architecture, the formation of multicellular structures, and the development of spores [52]. In general, there are two primal types of thermotolerance: basal and acquired. The first describes an organisms' ability to survive high temperatures without prior acclimation. The latter refers to thermotolerance acquired during prior exposure to mild temperatures, which are not harmful to the organism [53]. To more precisely describe the types of heat responses, organisms can be further divided into different categories. We can observe a variety of lifeforms, such as: (a) cryophiles (psychrophiles), which are capable of life functions at $-20\text{ }^{\circ}\text{C}$ [54]; (b) sychrotolerants (psychrotrophiles), which are capable of growth at low temperatures but possess optimal and maximal growth temperatures at the $15\text{--}20\text{ }^{\circ}\text{C}$ range [55]; (c) mesophiles, which are capable of growth at moderate temperatures between $20\text{ }^{\circ}\text{C}$ and $45\text{ }^{\circ}\text{C}$, with an optimum growth temperature in the range of $30\text{--}39\text{ }^{\circ}\text{C}$ [56]; (d) thermoduric organisms, which are capable of growth in the mesophilic temperature range ($15\text{--}37\text{ }^{\circ}\text{C}$), yet retain the ability to grow at refrigeration temperatures [57]; (e) thermophile, which possess the ability to resist elevated temperatures, enabling them to colonize new environmental hyperthermic niches. The development of thermophilia was probably based on pre-existing molecular blocks,

as it shares many mechanisms with the heat shock (HS) response [58]. Both thermotolerant and thermophilic microorganisms can withstand pasteurization, especially as spores [59].

In dimorphic fungi (e.g., *H. capsulatum*), morphology and temperature are linked with each other. This connection enables a conversion from filamentous to yeast at an elevated temperature and vice versa [60]. Moreover, heat resistance can differ between the strains of a species [61].

The vast majority of yeasts and moulds are resistant to heat in the same way as mesophilic vegetative bacteria. The heat resistance of sexual spores and asexual conidia is not greater than that of vegetative cells. However, ascospores of some moulds, such as *Byssoschlamys*, *Neosartorya*, and *Talaromyces* species, have a relatively high heat resistance, with a 7–22-min D value at 88 °C, and may survive 30 min of heat treatment at 90 °C, causing microbial spoilage in processed fruit drinks and canned fruits [61].

5.1. Impact of the Environment

Alvarenga et al. [62] extracted data from publications between 1969 and 2017 about thermal resistance parameters and their effects on heat-resistant fungi belonging to the *Neosartorya* genus. Data included a comparison of the effects of decimal reduction time (D), inactivation method, temperature of inactivation, pH, °Brix, maturity of spores, and kind of medium (model, juice, concentrates). Each of these parameters can impact fungal heat resistance [62]. These results also indicated that, for *Neosartorya* spp., the estimate for pooled D* values (D at 90 °C, pH 3.5 and 12° Brix) was: 5.35 min; 95% CI: 4.10–7.08 min. Moreover, increasing the content of soluble solids in concentrates tends to cause a smaller decrease in the heat resistance of *Neosartorya* and ascospores appear to be more thermal-sensitive to a decrease in medium pH [62].

Brix can be defined as a measurement of the dissolved sugar-to-water mass ratio. Thus, it can be connected with the dilution of medium. Naturally, freshly squeezed vegetable juice has a lower Brix value, between 5 to 12. Concentrates, caused by the thermal evaporation of water, have higher Brix values, between 25 and 60 [63]. Results obtained by Lane Paixão dos Santos et al. [64] confirmed that increasing °Brix lowers the livelihood chances of *Neosartorya*. Within tested species, *N. udagawae* was the most resilient and possessed the ability to grow at the highest evaluated °Brix (59°/aw = 0.86) [65]. Similarly to sugars, the concentration of other substances can have an effect on heat resistance. For example, NaCl, used to decrease the water activity (aw), causes an increase in the heat resistance of some microorganisms. NaCl, used in up to 10%, increases the thermotolerance of *Salmonella* and acts as a heat-protectant for *L. monocytogenes* [66]. To summarize, a reduction in water activity considerably boosts heat resistance. This is a common issue with foods abundant in sugar, proteins, or fat. However, acidic pH substantially lowers heat resistance. The pH of 4.5 marks a crossing point, as goods with a pH of less than 4.5 can be pasteurized at 100 °C or lower, but foods with pH greater than 4.5 must be sterilized at temperatures higher than 100 °C. The primary reason for this is a microbe, *C. botulinum*, which cannot grow or create toxins at pH 4.5, and any of its spores that survive heat treatment cannot germinate properly. The interplay between heat and other variables can be advantageously used in food production [61].

5.2. Heat Shock Proteins—HSPs

Heat shock proteins (HSPs) are part of the protective mechanism of cells in case of stress. They have a biological function and are involved in transcription, translation, protein folding and posttranslational modifications. HSPs maintain the quality of proteins. In fungi, HSPs are triggered by either specific (temperature shock) or general (pH, starvation, other stress factors) mechanisms [66].

The most common HSPs in fungi are: Hsp90, Hsp70, and Hsp20–40. They play a crucial role in changes in the morphology, adaptation procurement and shaping of antifungal resistance [60]. Hsp90 has been studied in *A. fumigatus* by Lamothe et al. [67]. Hsp90 plays a key role in morphogenesis, helps with transcriptional regulation and controls

conidiation [67,68]. According to the UniProt Database, the repression of its gene showed decreased spore viability, decreased hyphal growth and defects in germination and conidiation. Moreover, Hsp90 is distributed throughout cytosol and moves to specific organs during stress. Hsp90 and Hsp70 were found in *N. fumigata*. Alone or combined, they play a major role in morphogenesis and dimorphism. *N. fumigata* also produces Hsp104, Hsp70, and Hsp40, which play a role in replication [60]. HSPs 70 exhibit ATPase activity and disaggregate denatured proteins, which, in turn, helps with proper chain folding de novo [69].

5.3. Trehalose and Mannitol

The processes utilized by thermophiles to create resistance to increased temperatures are similar to those used by mesophilic fungi in the heat shock response. For example, under heat shock conditions, mesophilic fungi increase the amount of trehalose up to 6–8% of dry weight, which is similar to the values in the thermophilic fungus *Myceliophthora thermophila* (up to 3.5%) under optimum temperature conditions. Mesophilic fungi can acquire thermotolerance by HSP synthesis, trehalose accumulation, changes in the state of water in cell compartments, and membrane composition [58].

Trehalose is the most widespread naturally occurring disaccharide. In some fungi, only acid (unregulated) trehalase has been found (e.g., in *Aspergillus oryzae*) [69]. Wyatt et al. [70] identified and characterized a series of trehalose-containing oligosaccharides responsible for the unique preservation properties of *Neosartorya fischeri* ascospores. In vivo, they acted as a shield for cytosolic biomolecules [71].

The activity of trehalose gene NTH1 is multiplied by ten during the heat shock reaction. During the conidia germination of *A. nidulans*, neutral trehalase is responsible for trehalose mobilization and glycerol build-up. According to tests on *Aspergillus oryzae*, mannitol substantially inhibits acid trehalase from conidia cell walls. Trehalose accumulates during the idiophase, when growth activities are inhibited. Having reached its peak in resting form, it is known as the dormancy sugar. In the early stages of *A. niger* conidia germination, trehalose levels are found to be significantly lower. *A. niger* has proven to be capable of modifying its trehalose and glycerol levels in conidia, indicating the existence of adaptation mechanisms comparable to those seen in vegetative cells. The antioxidant defense process under heat shock comprises not only of the activation of desaturase activity but also the stimulation of trehalose production [69].

Increasing the heat resistance capacity has as much to do with trehalose as it has to do with mannitol. These substances and their relationship are involved in securing the livelihood of cells during oxidative stress. The mannitol and trehalose metabolism cycles are closely connected. Lowering the concentration of mannitol causes an increase in the amount of trehalose and trehalose-based oligosaccharides present [70].

5.4. Other Metabolites

Neosartorya spp. produce many resilient metabolites, which can upkeep the metabolism even under high temperatures. For example, a purified exo-polygalacturonase (EplNg) of *Neosartorya glabra* was effectively identified. The enzyme was active from 30 to 90 °C, with the highest activity at 65 °C and pH 5.0 [27]. Another highly active thermophilic enzyme has been discovered in *N. fischeri* P1. It has been dubbed the soybean isoflavone glycoside-degrading-glucosidase of GH3. The enzyme exhibited a greater optimal temperature and specific activity than any other known fungal homologue, was stable across a wider pH and temperature range, and was resistant to the majority of tested compounds. It had broad substrate specificity, including glucosidase, cellobiase, xylanase, and glucanase activity [72]. *Neosartorya fischeri* M-1 developed a thermophilic glucoamylase that was most active at temperatures ranging from 55 to 60 °C, and had the maximum activity at pH levels ranging from 4.0 to 4.4. Producing enzymes that are stable in hot conditions are definitely beneficial for the heat-resistance shaping of fungi. These enzymes can act as tools, enabling survival and growth in a heated environment [73].

6. Interactions between *Neosartorya* and Plants

Representatives of *Neosartorya* genus are a widely detected fungal group, mostly inhabiting soil. Therefore, it is easily transmittable to plants, impacting their postharvest quality. There are numerous studies in which either *Aspergillus* or *Neosartorya* phases were detected on plants and fruit, e.g., strawberry [30,32,35], coffee plants [74], apples [75], grapes [76]. The spectrum of fungus–plant interaction is broad, beginning with the roots and ending with the very top aboveground plant organs. The presence of *Neosartorya* on plant roots can either be characterized as opportunistic for the fungus or mutually beneficial for both parties. In the first instance, the fungus may be attracted to damaged or diseased roots due to its saprophytic nature. It then accelerates rot and spoils the plant further. Such a situation has been described by [77], where pineapple plants suffering from the red leaf disease had reduced root systems and tested positively for *Neosartorya fischeri*, which does not cause said disease. In this case, the mycelium did not spread to aboveground organs. Often, *Neosartorya* can create a symbiosis with its host, acting as a natural antimicrobial agent or promoting plant growth by enzyme secretion. *Neosartorya fischeri* has been reported to inhabit a traditional medicinal herb *Macleaya cordata*, mainly distributed in China [78]. Interestingly, it acted as an endophytic organism, providing its antibacterial properties to both the plant and, later, to people consuming the plant for its medicinal value. The antimicrobial activity of *N. fischeri* in this study has been proven against eight bacteria: *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Salmonella typhimurium* and *Xanthomonas vesicatoria*. Hamayun et al. [79] reported gibberellins' production and the growth-promoting capacity of another endophytic *Neosartorya* strain (CC-8), which was isolated from the roots of Chinese cabbage (*Brassica rapa*). The fungus cultures significantly promoted plant length and biomass gain.

Neosartorya spp. can migrate to plant organs other than the roots (Figure 4). It has been reported on leaves of kale, where, in consortium with *Talaromyces*, it was showed to control leaf spot in kale, caused by *Alternaria brassicicola* [80]. *N. spinosa* yielded the best results against this pathogen amongst all tested strains (others were *N. hiratsukae*, *N. pseudofischeri*, *N. aureola*, *N. spinosa*, *N. fennelliae*, *Neosartorya* sp., *T. trachyspermus*, *T. muroii*). Genus *Neosartorya* is also one of the five main groups of fungi present on fresh common reed (*P. australis*) leaves, where they exhibit co-occurrences with other members of *Ascomycota* and *Basidiomycota* [81]. They also act as saprotrophs, transforming dead plant matter into compost.

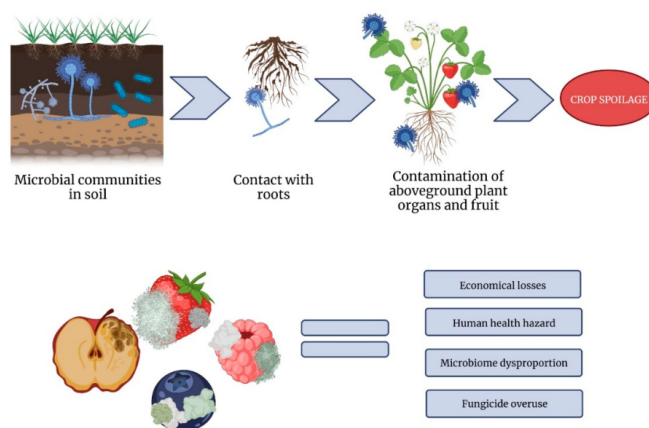


Figure 4. Ways of causing postharvest food spoilage by *Neosartorya* spp. (own elaboration by W. Maj and M. Frąc using BioRender, Toronto, ON, Canada).

7. Fruit and Vegetable Production in the European Union

7.1. Organic Crop Production

Organic farming can be seen as a viable alternative to high-input horticultural systems relying on synthetic fertilizers, fungicides, and insecticides. It is built on the premise that the soil is a living system, closely intertwined with fauna and flora. It considers the microbiome and its interactions with the soil–plant system. Laws define the word “organic” mostly in terms of ‘natural’ vs. ‘synthetic’ inputs [82]. The most common practices used in sustainable horticulture are crop rotation, utilizing animal manure, and biological pest management [83]. There are many ways of delivering additional nutrients to the soil, e.g., mineral fertilization (increases the ground’s mineral content) and organic manuring (upkeeping the soil’s biological fertility) [84].

Organic crops possess more value than regular fruits and vegetables. They are often richer in nutrients [85] and contain fewer heavy metals [86]. Due to the current holistic view of ecological behaviours, the volume of organic crops is steadily growing. Organic horticulture promotes not only the production of food, but also the production of fibre and timber [83]. The branding of produce as “organic” is heavily controlled. Organic farming methods are sustainable, have a minimal environmental effect and may be viewed as a means of cleaning up and rehabilitating deteriorated agricultural land [82].

Horticultural goods are an important aspect of the European Unions’ regional and cultural character. According to Eurostat data from 2019, Poland was one of the European producers with the highest yield of organic crops. The most important producers in the EU were France, Spain and Italy. According to Eurostat in these countries the total amount of harvested crops in the EU in 2019 included grain (299.3 mln t), vegetables (61.5 mln t) and fruit, berries, nuts (25.2 mln t), respectively. Furthermore, in this year, organic crop farming accounted for 8.5% of the EU’s total utilised agricultural area, with 13.8 million hectares available for growing organic crops.

7.2. Poland as a Leader in Fruit Production in the European Union

The food industry in Poland was among those sectors that saw significant upheavals and rebounded quickly following the country’s political revolution in the 1980s. As a result, the industry became an important part of the economy, influencing economic growth. Poland has evolved into a sophisticated and innovative food manufacturer in Europe as a result of technical and organizational advancements. This is proven by the increase in food exports. Another significant aspect that aided the growth of this business was Poland’s entrance to the European Union and the resulting prospects for the greater exploration of other markets. Polish food makers were eligible for various forms of grants and subsidies as a result of their EU membership. The standards for food production in Poland are mostly established by European Union legislation. Compliance with EU rules and regulations is especially critical for food exporters, since about 80% of Polish food exports are destined for EU markets. In addition to strict norms and novel pro-ecological legislation, consumers’ interest in food produced by industrial methods has rapidly declined. Chemical-plant-manufactured goods or genetic alterations being utilized in production make customers hesitant to purchase. Thus, it is important to implement new, ecological technologies in food production and preservation [87]. Ecologically sound alternatives should not omit the threat posed by thermoresistant organisms. As reported by Eurostat, statistically, in 2016, Poland harvested over 1 of every 4 apples produced in the EU. Poland was also the main EU-producer of cherries and the second most important producer of strawberries, right after Spain. In general, according to the National Centre of Agricultural Support (KOWR) Poland is a major producer of strawberries, gooseberries, and chokeberries. The fruit harvest from orchards was estimated to be 4.5 million tonnes in 2018. Based on data reported by FAO, Figures 5 and 6 present fruit production in 2020 in Poland and Europe, respectively.

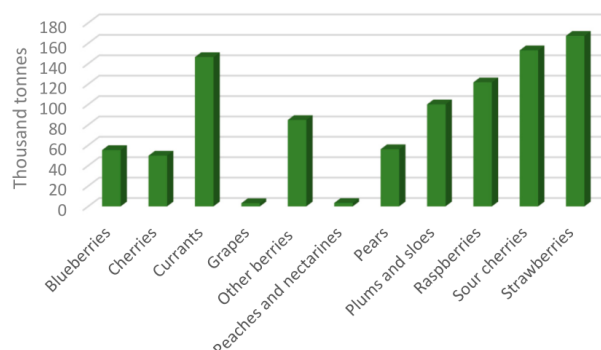


Figure 5. Polish fruit production in 2020 (data from [fao.org](https://www.fao.org)) (accessed on 12 October 2022).

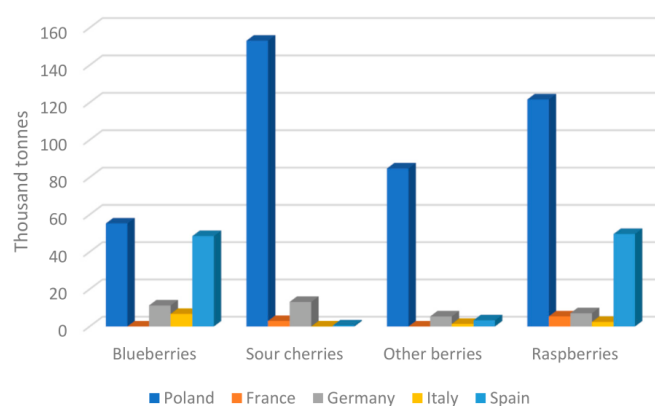


Figure 6. European fruit production in 2020 (data from [fao.org](https://www.fao.org)) (accessed on 12 October 2022).

The production of cherries and apples accounted for a sizable portion of this total. In 2017, Spain represented the most noteworthy extent (40.1%) of the region inside the EU in terms of organic food production, due to high yield of nuts and citrus products. Italy represented the following most noteworthy country (17.5%), followed by Poland (9.6%). According to KOWR, the current estimate is that 350,000 tonnes of apple juice concentrate are produced, representing a 29% growth over the years 2014–2017. While these numbers seem optimistic at first, it is important to remember that a high yield does not always equal rapid income. After harvest, fruit is still susceptible to rot and pests, which can generate economic losses.

7.3. European Union Policy Framework

The European Union is currently focused on supporting ecological solutions in many sectors, including horticulture. Legal documents backing the EU's support for sustainable farming include, e.g., regulation no. 1308/2013, focused on the common organisation of the markets in horticultural products, directive 2009/128/E, touching on the subject of pesticide usage, and Water Framework Directive 2000/60/EC (WFD), directly impacting which substances may be used in plant protection in relation to water quality and purity. The “umbrella” directive that summarizes the EU's goals is the common agricultural policy (CAP). The CAP aims to combat climate change, conserve natural resources, and promote variety in the EU. It supports sustainable agriculture and horticulture by recommending

reduced pesticide and fertilizer usage and supporting organic farming. The CAP greatly contributes to the decrease in the overall impacts of food manufacturing. It is important to uphold EU standards regarding horticultural development, and especially important to follow the trends of sustainability and creating positive environmental impact. Inventing novel, greener alternatives to commonly used preservation methods of obtained crops can further help to implement these policies in real life [88–90].

7.4. Common Problems in Fruit Production

Fruit production faces many difficult challenges. The issues can be divided into the following: (a) biological, including vulnerability to pest, diseases, microbes, and postharvest losses due to these factors, and (b) economical, including poor pricing and low fertilizer use, the unavailability of horticultural credit, land tenure insecurity, the slow development of horticultural research, infrastructure, the productivity of labour, and consumer expectations [91–93].

Looking at the presented data, it is crucial to develop new technologies for food protection, harvest and processing. This can be achieved with further research on pathogens' heat resistance, methods of detection and natural food preservatives. As explained before, fruit production plays a major role in European and Poland's economy, and any losses in this sector could be grossly disadvantageous.

7.5. Methods of Postharvest Food Preservation

The destruction caused by postharvest microbiological food contaminants, including heat-resistant fungi and diseases, amounts to a 20–25% yield reduction, depending on the country [94]. Therefore, the methods, strategies and ways of postharvest food protection are very important. The current postharvest strategies of microbiological contaminants' mitigation heavily rely on chemicals, which pose a threat to the environment and human health. Several techniques can be used to protect against deteriorating factors, e.g., freezing or chilling, pasteurization, canning, or dehydrating [30,61]. Pasteurization and canning usually have little effect on ascospores, which later sprout into fungi and create mycotoxins that contaminate the produce. Mould ruins the product by developing colonies on the surface, floating mycelia, or clarifying the material. As a result of their microaerophile nature, they destroy fruit juices even when stored under low-oxygen conditions. *N. fischeri* is frequently responsible for the deterioration in apple juice, strawberry pulp, and passion fruit juice. As previously mentioned, harvest losses in these commodities can cause serious economic problems in Poland, which is a key producer of apples, cherries, and strawberries in the EU.

In accordance with European Union laws and suggestions touching on the subject of sustainable horticulture and food production, the use of heat or natural preservatives to protect the produce is highly recommended. Plant extracts may present a valid means of protection against fungi. It has been confirmed that *Calendula arvensis* hydrosol extracts possess antifungal properties, mainly against *Penicillium expansum* and *Aspergillus niger*. However, it is unclear whether essential oils or hydrosols have the best antifungal properties. This pertains to the concentration of substances, but also the type of plant material. Extracts diluted in water are generally seen as milder and safer to use in food production than highly concentrated essential oils, which mainly dominate the field of cosmetics [95]. Despite marigold being more active as a hydrosol, plants such as mint [96], thyme and lavender [97] express wider antifungal properties in the form of essential oils [98].

Tipping the heat-resistance temperature point may be another green alternative to chemical protection. For example, raising the pasteurization temperature to 95 °C for at least 45 s successfully lowers the risk of purees being spoiled by *A. fischeri*. Furthermore, it increases the microbiological stability of such purees [30].

Subsequently, simply editing the environment of fungal growth may be enough to stop its growth altogether. Previously mentioned variables, such as °Brix/acidity ratio [99], pH [100], °Brix [101], alternating temperatures, and the concentration of soluble dry matter

can all be altered to elicit a reaction from fungi [102]. There are also studies that show the prospect of using the UV-C light as a form of non-thermal food processing. This is considered to cause few quality alterations while reducing microbial burden [103]. During transport, it has been proven that using a coating with lipopeptides and nisin on cardboard boxes can diminish the duration of *Neosartorya hiratsukae* [104], which might prove advantageous during the shipment and delivery phase of production. Coating composites are used to improve the postharvest quality of fruits [105–107], and can be effective in the control of fungi, including the genus of *Neosartorya* [108].

8. Conclusions and Future Directions

Neosartorya spp. fungi are riveting and extraordinary organisms, which require more focus and research. Being easily mistaken for *Aspergillus* has masked their actual contribution to food spoilage and effects on human health. They simultaneously pose dangers to crops and offer novel perspectives in medicine. In the future, more studies focused on *Neosartorya* are needed to estimate its inactivation parameters in food industry, and deepen the knowledge of its infectious properties and possible medicinal uses.

Moreover, future research directions should focus on heat-resistant fungal threats to horticulture and postharvest food security to develop new approaches to ensuring a robust global food supply chain. An important issue of future research is to consider and obtain insight into the plant mycobiome as important player in enhancing resistance to microbiological food contaminants and pathogens. Another challenge is to determine how to protect crops from postharvest fungal damage, especially from the *Neosartorya* genus. The best strategy at present may be to focus on studying natural fungicides and substances, such as microbial-based solutions, plant extracts, and essential oils, which have little to no effect on the environment and do not lead to the decay of postharvest horticultural products and food, but do affect thermoresistant moulds such as *Neosartorya*.

Finally, effort should be taken to find connections between heat-resistant fungi and climate change, including responses to the question of how new and existing fungi can be identified, whose geographic range is expanding due to climate change, and how they adapt to these changes and increase temperatures, considering global trade's ability to exacerbate the spread of postharvest fungal contaminants of crops. Another important future direction is to fill the knowledge gap regarding the role of heat-resistant fungi (mainly belonging to the *Neosartorya* genus) in the enhancement of carbon sequestration, given the prospects and challenges of prolonging the postharvest shelf life of fruits, vegetables, crops, and food.

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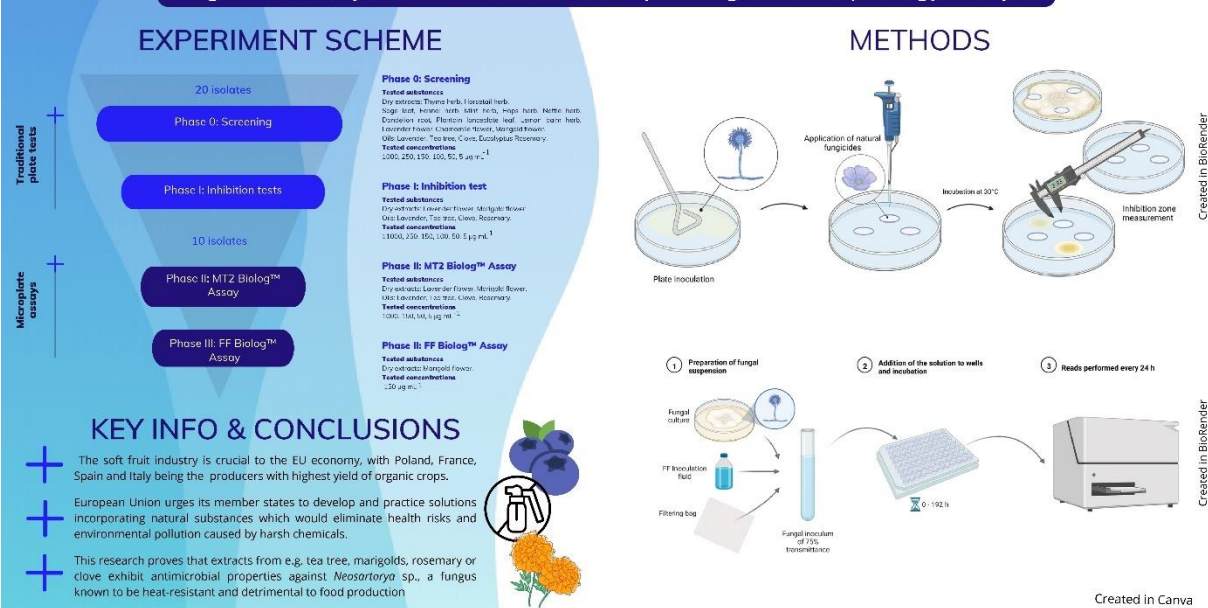
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7.2 Tekst publikacji P.2

7.2.1 Abstrakt graficzny

Natural plant extracts as antifungal agents against *Neosartorya* spp. (teleomorph of *Aspergillus* spp.):
fungal sensitivity assessment, metabolic profiling, and morphology analysis



scientific reports



OPEN Comprehensive antifungal investigation of natural plant extracts against *Neosartorya* spp. (*Aspergillus* spp.) of agriculturally significant microbiological contaminants and shaping their metabolic profile

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Fungi belonging to the genus *Neosartorya* (teleomorph of *Aspergillus* spp.) are of great concern in the production and storage of berries and fruit-based products, mainly due to the production of thermoresistant ascospores that cause food spoilage and possible secretion of mycotoxins. We initially tested the antifungal effect of six natural extracts against 20 isolates of *Neosartorya* spp. using a traditional inhibition test on Petri dishes. Tested isolates did not respond uniformly, creating 5 groups of descending sensitivity. Ten isolates best representing of the established sensitivity clusters were chosen for further investigation using a Biolog™ MT2 microplate assay with the same 6 natural extracts. Additionally, to test for metabolic profile changes, we used a Biolog™ FF microplate assay after pre-incubation with marigold extract. All natural extracts had an inhibitory effect on *Neosartorya* spp. growth and impacted its metabolism. Lavender and tea tree oil extracts at a concentration of 1000 µg mL⁻¹ presented the strongest antifungal effect during the inhibition test, however all extracts exhibited inhibitory properties at even the lowest dose (5 µg mL⁻¹). The fungal stress response in the presence of marigold extract was characterized by a decrease of amino acids and carbohydrates consumption and an uptake of carboxylic acids on the FF microplates, where the 10 studied isolates also presented differences in their innate resilience, creating 3 distinctive sensitivity groups of high, average and low sensitivity. The results confirm that natural plant extracts and essential oils inhibit and alter the growth and metabolism of *Neosartorya* spp. suggesting a possible future use in sustainable agriculture as an alternative to chemical fungicides used in traditional crop protection.

In recent years, due to climate change, a continuous increase in temperature and a reduction in rainy periods can be observed, which has led to the frequent occurrence of poor-quality agricultural products. This combined with contamination by pathogenic and toxigenic fungi, is placing significant economic strain on agriculture, particularly for fruit and post-harvest products¹. Approximately 25% of the global food supply is lost due to microbiological deterioration that occurred in the post-harvest phase. Furthermore, 92% of European and American juice producers reported having experienced mould or yeast contamination in the finished product and 89% of the producers reported having observed ingredient spoilage in general production². Among fungi belonging to the group of microbiological food contaminants, the most important in the field of agriculture and food production are Heat Resistant Fungi (HRF). This group is heavily studied because of their ability to produce spores that allow them to resist temperatures above 75 °C, greatly diminishing the effectiveness of various pasteurization

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and sterilization techniques of fruit-derived food. HRF cause problems in the food chain connected to fruit (for example fruit juice and pulp)³. The most notorious fungi from the HRF group are *Byssoschlamys*, *Eupenicillium*, *Hamigera*, *Neosartorya*, *Talaromyces*, *Thermoascus*, *Rasamsonia*^{4–6}. *Neosartorya* spp. (teleomorph of *Aspergillus* spp.) belong to section Fumigati and are one of the most recurring fungal threats in food production⁷. They are present worldwide and have been discovered in different environments: in soil⁸, sea water⁹, on common weeds¹⁰, grass¹¹, wood¹² and crops¹³. Additionally, *Neosartorya* spp. have been isolated from canned products¹⁴ and juices¹⁵ highlighting the importance of conducting studies on these fungi in order to find a natural method of controlling their spread in the agricultural field. *Neosartorya* spp. are unique fungi, with additional defence mechanisms such as resilient cell walls containing substances such as trehalose, isobemisiolose, neosartose and fischerose which enable them to survive drought and heat. In some cases, this genus is more resilient to stress than other genera in the HRF group, e.g. with *Neosartorya fischeri* outlasting *Talaromyces macrosporus* in drought conditions¹⁶. Many studies are being conducted on methods to block the propagation of spores, since this group of fungi are resistant to the high temperatures reached during pasteurization or sterilization and further increasing the temperature would lead to adverse effects on the quality of food through e.g. caramelization, evaporation and densification¹⁷. The importance of stopping the contamination not only concerns food spoilage, but also consumers' health, as these potentially toxigenic fungi can produce mycotoxins such as fumitremorgin C, verruculogen and fischerin^{18–20}. However, due to chemical fungicide overuse the European Union has launched the "Farm to Fork" strategy²¹. The need for alternative fungicide solutions stems from the need of protection of soil microbial diversity, soil quality and fertility, and groundwater quality. In accordance with this policy, alternative antimicrobial substances should be studied. One possible choice could be natural plant extracts, such as dry and oil extracts. Active ingredients of plant extracts can be removed from the whole plant or from parts of the plant (such as roots, stem, leaves, and flowers) due to use of different solvents, such as water, acetone, acetic acid, ethanol, ethyl acetate, methanol, and hexane²². The principal antifungal activity effect depends on the specific bioactive components such as alkaloids, phenols, flavonoids, terpenes, ketones, amines, and sulphides²³.

In the field of microbiology, extensive research has been conducted on the antifungal properties of essential oils and plant extracts. Abdolahi et al.²⁴ analysed the antifungal effect of different essential oils from ajowan, fennel, caraway against *Penicillium digitatum* and *Alternaria alternata*. Other studies identified antifungal effects of eugenol, thymol, and summer savory against the pathogenic fungus *Botrytis cinerea*^{25–28}. Tripathi et al.²⁹ reported a 100% inhibitory effect of various essential oils to *Botrytis cinerea*. Zabka et al.³⁰ reported the effect of 14 plant extracts on different pathogenic fungi, including *Fusarium oxysporum*, *F. verticillioides*, *Penicillium expansum*, *P. brevicompactum*, *Aspergillus flavus*, and *A. fumigatus*. However, little research has been conducted on the antifungal effects of dry plant extracts and essential oils on heat resistant fungi. This could be particularly valuable considering the known antifungal properties of essential oils and plant extracts, and because these natural substances are non-toxic, biodegradable, environmentally non-persistent and declared as "Generally Regarded as Safe" (GRAS) and can be used in food production^{22,23}.

Considering the economic losses and possible health hazard caused by highly resilient *Neosartorya* sp., it is necessary to search for additional methods of fungal control while simultaneously adhering to the new novel ecological directives. The main objective of this research was to compare sensitivity among *Neosartorya* spp. isolates to plant extracts, taking into account possible differences between individual isolates. Furthermore, the antifungal properties of natural extracts were assessed. Additionally, metabolic profiling of selected isolates was performed to observe possible changes in metabolic capability of isolates varying in sensitivity. Morphological features of *Neosartorya* spp. were also visualized to confirm whether isolates of *Neosartorya* sp. presented pre-existing morphological features that could potentially influence their response towards antifungal plant extracts.

Materials and methods

Natural extracts

The antifungal effects of dry extracts (marigold and lavender) and essential oils (lavender, rosemary, clove, and tea tree) against *Neosartorya* spp. were analysed. Dry *Lavandula angustifolia* flower extract was produced by Greenvit Sp. z o.o. (Zambrów, Poland) using a maltodextrin carrier. *Calendula officinalis* dry extract was produced by Zrób Sobie Krem Kosmetyki Naturalne (Prochowice, Poland). All dry extracts were determined to be free from *Salmonella* spp. and *Escherichia coli*. Oil extracts were produced by Dr Beta³, FSZ Pollena-Aroma Sp. z o.o., (Nowy Dwór Mazowiecki, Poland) and registered in the Cosmetic Products Notification Portal (CPNP). Studied oils were as follows: tea tree oil (*Melaleuca alternifolia* leaf oil, limonene), eucalyptus oil (*Eucalyptus globulus* leaf oil, limonene), rosemary oil (*Rosmarinus officinalis* leaf oil, limonene, linalool, geraniol, eugenol), clove oil (*Eugenia caryophyllus* bud oil, eugenol, isoeugenol), lavender oil (*Lavandula angustifolia* oil, linalool, geraniol, limonene, citronellol).

The chemical nature of natural extracts makes them highly antimicrobial, thus rigorous material sterilization processes are not needed. To prepare stock solutions, each of the powdered plant extracts was resuspended and dissolved in sterile water to achieve a concentration of 1000 µg mL⁻¹ and filtered via 0.22 µm filters (Millex³, Ireland). Natural oils were weighed and added to sterile water to establish the percent weight by volume (% w/v) of 1000 µg mL⁻¹, thoroughly mixed by vigorous shaken until a cloudy emulsion without visible phase separation was formed and then filtered via 0.22 µm filters (Millex³, Ireland). All stock solutions were diluted to the following concentrations: 250, 150, 100, 50 and 5 µg mL⁻¹. This method of oils preparation was used to not disturb naturally occurring reactions of volatile organic compounds (VOCs) found in essential oils during the incubation phase as e.g. ethanol and methanol are linked with fast VOCs dispersion³¹. Naturally, VOCs can disperse through water matrices while maintaining lasting effects on the environment³². Incubating fungi on moist media in contact with natural oils within sealed petri plates could potentially replicate the controlled atmosphere of transport or storage conditions of food as with time volatile compounds diffuse into air³³. A water-based oil dispersion method

was also chosen on the basis that common oil solvents such as ethanol or dimethyl sulfoxide (DMSO) may not be adequate in fruit production since side-effects such as altered ripening time³⁴ or nutrient (e.g. carotenoids) synthesis disruption can occur due to their involvement³⁵.

Fungal isolates identification

Neosartorya spp. isolates were obtained from the Laboratory of Molecular and Environmental Microbiology, Institute of Agrophysics of Polish Academy of Sciences (Lublin, Poland). A total of 20 distinct isolates were selected by the sequencing Internal Transcribed Spacer region 1 (ITS1)³⁶, and a fragment of the functional β -tubulin gene specific to this genus⁴. The N2F/2R and Af1F/1R primers have proven efficacy in distinguishing between *Neosartorya* spp. and *Aspergillus fumigatus*⁴. Accurate discrimination between *A. fumigatus* and other *Neosartorya* species is of utmost importance in the food industry and in this study because although they are morphologically similar, *A. fumigatus* is not well documented as a spoilage agent in heat-processed food products, contrary to *Neosartorya* genera well-known for food spoilage capabilities. Only isolates presenting PCR product bands for N2F/2R and not for Af1F/1R primers were utilized in this study to ensure that the samples were all within the *Neosartorya* genus. This was further proven by high percent identity scores via BLAST³⁷. To facilitate comparison between the origin of the isolates, 8 isolates originating from soil and 12 from strawberry fruit were chosen. Prior to genetic identification, the isolates were cultivated on Potato Dextrose Agar medium (PDA, BioMaxima S. A., Lublin, Poland) at 30 °C. After 5 days of incubation, the mycelium was transferred into 2 mL tubes containing glass beads (250 mg 1.45 mm ϕ and 500 mg 3.15 mm ϕ) and Lyse F buffer (EURx, Poland) and later homogenized using FastPrep-24 homogenizer (MP Bio, United States) for 20 s at 4 m s⁻¹. The DNA was extracted using the EURx GeneMATRIX Plant and Fungi DNA Purification Kit (EURx, Poland)³⁸. Three different PCRs were prepared for following genes: ITS1 (primers ITS1/ITS2³⁹), β -tubulin gene (primers specific for *Neosartorya* spp. N2F/N2R and *Aspergillus fumigatus* Af1F/R⁴). PCRs were conducted using REDTaq⁺ ReadyMix™ (Sigma-Aldrich Co. LLC, USA), 10 μ M of each primer and 2 μ l of diluted fungal DNA. The amplification conditions were reported in Supplementary Table S1.

Each PCR product was analysed on a 2% agarose gel. PCR products were sequenced using Sanger sequencing method. The amplicons were purified using Exo-BAP mix (EURx, Gdańsk, Poland) and re-amplified with Big Dye⁺ Terminator v1.1 Reaction Mix (Thermo Fisher Scientific, Waltham, MA, USA). All samples were cleaned through the Performa⁺ DTR Cartridges (Edge BioSystems, Gainthersburg, MD, USA). Purified products were incubated at 95 °C for 180 s followed by an incubation at 4 °C for 180 s, and loaded into the Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Phylogenetic trees were obtained using MEGA 11 software⁴⁰. In this analysis, some sequences of other HRF genera were added for comparison, all obtained from the National Centre for Biotechnology Information database (NCBI; <http://www.ncbi.nlm.nih.gov>). Additional internal transcribed spacer region sequences included: *Eupenicillium meridianum* (AM992114.1), *Hamigera insecticola* (NR137684.1), *Paecilomyces variotii* (NR130679.1), *Byssoschlamys lagunculariae* (FJ389944.1), *Aspergillus nidulans* (NR133684.1), *Aspergillus niger* (NR111348.1), *Aspergillus flavus* (NR111041.1), *Neosartorya fischeri* (KF640700.1), *Neosartorya spinosa* (JN943589.1), *Neosartorya glabra* (JN943577.1), *Talaromyces udagawae* (MH860584.1), *Aspergillus* sp. (ON920551.1), *Neosartorya* sp. (MH472615.1), *Neosartorya* sp. (2) (MF681541.1), *Neosartorya laciniosa* (JN943581.1), *Neosartorya assulata* (HF545007.1). Additional beta tubulin sequences included: *Neosartorya glabra* (AF057323.1), *Neosartorya spinosa* (AF057329.1), *Neosartorya fischeri* (AF057322.1), *Talaromyces udagawae* (OK338783.1), *Aspergillus fumigatus* (OP646312.1), *Aspergillus* sp. (OL792696.1), *Neosartorya* sp. (KT253238.1), *Neosartorya* sp. (2) (EU220283.1), *Neosartorya laciniosa* (JX845620.1), *Neosartorya* sp. (3) (DQ114123.1), *Penicillium meridianum* (GU981660.1), *Hamigera insecticola* (LC076663.1), *Paecilomyces variotii* (MN153297.1), *Byssoschlamys lagunculariae* (AY753353.1), *Aspergillus nidulans* (MK749993.1), *Aspergillus niger* (LC774552.1) and *Aspergillus flavus* (AF036803.1). Maximum likelihood tree illustrating the ITS1 phylogenesis was created using Tamura 3-parameter model with discrete gamma distribution, whereas the β -tubulin phylogenesis was created using Kimura 2-parameter model with discrete gamma distribution.

Disc diffusion assay for testing fungal isolates sensitivity to plant extracts

The disc-diffusion inhibition test was used to determine an inhibitory effect of a specific plant extract on the growth of 20 *Neosartorya* spp. isolates. For this analysis several extracts were used, such as: lavender flower dry extract, pot marigold flower dry extract, lavender oil, tea tree oil, clove oil and rosemary oil. All natural substances were tested at concentrations of 1000 μ g mL⁻¹, 250 μ g mL⁻¹, 150 μ g mL⁻¹, 100 μ g mL⁻¹, 50 μ g mL⁻¹ and 5 μ g mL⁻¹. To test the effect of the inhibitory properties of these extracts on growth, Petri plates with PDA medium (BioMaxima S.A., Lublin, Poland) were inoculated with 300 μ L of a *Neosartorya* spp. water suspension, as described in Pertile et al.⁴¹. To ensure a uniform number of cells inside the inoculum, fungal material included ascospores with mycelium collected from the previous 10 days old culture was placed in FF inoculating fluid (Biolog⁺, Hayward, CA, USA) to achieve 75% transmittance equating to approximately 4.44×10^4 ascospores mL⁻¹ (Supplementary Table S3). Moreover, to confirm the inoculum uniformity, the ascospores were counted in a cell counting chamber acc. to Thoma (Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany). Taking into account that *Neosartorya* spp. fungi are heat resistant due to their ability to form resilient sexual spores, fungal growth with present ascospores was obtained. This was confirmed by SEM (Phenom ProX, Thermo Fisher Scientific, Waltham, MA, USA) (Fig. S5). Sterile paper discs (Whatman No. 1, 5 mm ϕ) were placed on top of the medium and 30 μ L of plant extract at each of the above concentrations was applied to each disc. Each combination of isolate, extract type and concentration was replicated three times. Three discs with the same concentration were placed in the same Petri dish. Petri plates were incubated at 30 °C for 10 days and the areas of growth inhibition were measured daily using a digital caliper.

Sensitivity of *Neosartorya* spp. isolates to different concentrations of natural plant extracts using MT2 microplates

To assess the resource utilization, sensitivity, or resistance of *Neosartorya* spp. to the presence of analysed natural plant extracts, MT2 microplate assays were conducted (Biolog™, Hayward, CA, USA) following the methods described in Fraç et al.⁴². In accordance with the results from the inhibition test, multiple compounds and dilutions with the best antifungal properties were chosen. Selected natural extracts were marigold dry extract, lavender flower dry extract, lavender oil, rosemary oil, tea tree oil, and clove oil. Based on the previous results, each natural extract was tested at the following concentrations: 1000 µg mL⁻¹, 150 µg mL⁻¹, 50 µg mL⁻¹ and 5 µg mL⁻¹. Furthermore, on the basis of inhibition tests, the 20 analysed isolates were divided into 5 different groups according to their sensitivity to tested substances, from which 2 isolates per group were chosen to be studied via the MT2 analysis (Biolog™, Hayward, CA, USA).

Pure cultures of 10 fungal isolates were grown on PDA medium (BioMaxima S.A., Lublin, Poland) at 30 °C for 10 days. After the incubation period, ascospores with mycelium were collected and placed in a sterile filter bag with FF inoculating fluid (FF IF, Biolog™, Hayward, CA, USA) to perform homogenization. Subsequently transmittance of 75% equating to approximately 4.44×10^4 ascospores mL⁻¹ was achieved (Supplementary Table S3). Each Biolog™ microplate well was filled with 100 µL of natural extract and 50 µL of fungal inoculum. To allow a comparison of fungal development with and without the presence of natural extracts, a control was prepared which composed of fungal ascospores with mycelium suspended in 50 µL FF IF and mixed with 100 µL of water. Plates were incubated at 30 °C and analysed every 24 h for 10 days at the optical density (OD) of 490 nm (utilisation of substances) and 750 nm (fungal biomass production) using the Biolog™ MicroStation (Biolog™, Hayward, CA, USA). For each wavelength separately, the obtained results were corrected by subtracting values of background obtained for each the used dilution with each plant extracts, as they could interfere spectrophotometrical measurements and results. The blank samples were formed by extracts dilutions (100 µL) mixed with inoculating fluid solution (FF IF 50 µL) instead of inoculum in order to avoid background interference in this study. Then subtracted values were used to calculate the Average Fungal Respiration Intensity (AFRI; for the optical density at 490 nm) and Average Fungal Growth Intensity (AFGI; for the optical density at 750 nm). Results of samples treated with natural extracts were then compared to the results attained by the fungi cultivated in control conditions without the contact with any natural extracts.

Neosartorya spp. metabolic profile analysis—following pre-incubation with marigold extract

To observe changes in the metabolism of *Neosartorya* spp. after pre-incubation in the presence of marigold extract, the FF microplate (Biolog™, Hayward, CA, USA) assay was carried out^{43–45}. For this experiment, only marigold extract was selected as its low concentration permitted mycelial growth and accession of satisfactory quantity of fungal material. For the control group, pure cultures of fungi were grown on PDA medium (BioMaxima S. A., Lublin, Poland) at 30 °C for 10 days; whereas the test group was grown under the same conditions with an extra addition of marigold extract at a concentration of 150 µg mL⁻¹ on PDA media. Each treatment was prepared in triplicate. Ascospores with mycelium were collected from the surface of each plate and placed in a sterile filtering bag with FF inoculating fluid (Biolog™, Hayward, CA, USA) with the subsequent adjustment of transmittance to 75%. FF plates were inoculated with 100 µL of fungal suspensions and incubated at 30 °C for 10 days. Optical density was measured every 24 h at 490 nm and 750 nm. Carbon sources in the FF plate can be divided into 6 groups (Supplementary Fig. S1): amines and amides, carbohydrates, polymers, amino acids, carboxylic acids and miscellaneous^{46,47}. The number of different substrates used by individual isolates was used to determine functional diversity, which was expressed as substrate richness (R). The diversity amongst the isolates was determined by the utilization of substrates and expressed as the Average Fungal Respiration Intensity (AFRI; for the optical density at 490 nm) and Average Fungal Growth Intensity (AFGI; for the optical density at 750 nm). Within both the test and control groups at each measured wavelength, the optical densities of each individual well were subsequently adjusted by subtracting the optical density of the water control (inoculum resuspended in water) at the corresponding measurement hour.

Scanning electron and fluorometric microscopy analyses

Five isolates were selected based on difference in their sensitivity levels to natural extracts were cultivated for 10 days on PDA medium (BioMaxima S. A., Lublin, Poland) and observed using an electron microscope, Phenom ProX (Thermo Fisher Scientific, Waltham, MA, USA). Samples were mounted on aluminium supports using conductive double-sided carbon adhesive tape, dried under silica gel and coated with a 5 nm layer of gold (sputter coater, CCU-010 LV, Safematic GmbH, Zizers, Switzerland) prior to SEM analysis. The pictures were captured using a BS detector at an accelerating voltage of 10 kV, at 200× magnification and 5000× magnification.

Three of these five isolates were selected for further examination under a ZEISS LSM 510 Meta Confocal Microscope. After 5 days of cultivation on PDA medium approximately half of the ascospores with mycelium was harvested and visualized without fluorescence (light microscopy). The remaining fungal material was added to a wash buffer (2% D-glucose and Na-HEPES) and subsequently centrifuged at 10,000×g for 5 min. After the removal of the supernatant, the pellet was re-suspended in 1 mL of wash buffer and 1 µL of FUN 1 cell stain (10 mM solution in DMSO; Thermo Fisher Scientific Corporation, USA) was added. Samples were incubated at 30 °C in the dark for 5 min. Finally, the stained suspension was transferred onto a glass slide and covered with a coverslip. Cells were imaged with filters: excitation 488 nm, emission 530 nm. In parallel, photographs of the tested fungal isolates were taken under unfiltered light of to visualize possible differences in the production of spores.

Statistical analysis

For the results from the inhibition tests, heatmaps with cluster analysis were calculated through Euclidian distance and Wards' method respectively and prepared with R software v 4.3.1 (packages: *ComplexHeatmap*, *circulize*). Statistical tests were used to determine the differences between the isolates, effects of natural extracts and their concentrations on creating inhibition zones or absorbance values (MT2, FF). The initial phase involved assessing dataset normality using the Shapiro–Wilk test and examining variance homogeneity using the Levene test⁴⁸. Since the datasets did not meet the criteria for parametric tests, the results were analysed by performing Kruskal–Wallis test and post hoc Wilcoxon Mann Whitney test using TIBCO Statistica' software (Version 13.3). To visualize microplate tests results, we created heatmaps and bar graphs to demonstrate the similarities and differences in carbon utilization patterns among the different isolates. Data is presented at 95% confidence intervals with statistical significance at $p < 0.05$. Additional analyses based on OD readings included Substrate Richness (R index), Average Fungal Respiration Intensity (AFRI) and Average Fungal Growth Intensity (AFGI) to assess the functional diversity, impact of incubation time, isolate and carbon source type as in Oszust et al.⁴³, Frac et al.⁴⁹. To further explore the carbon utilization a cluster analysis was performed, using a dendrogram created through the Ward method and Sneath's dissimilarity criterion at 66% and 33% (TIBCO Statistica' software (Version 13.3)).

Results

Fungal isolates identification

The identity of isolates was initially determined using the specific PCR's and then confirmed by sequencing. Obtained products for the ITS1 gene were approximately 240 bps (Supplementary Fig. S2A) and enabled an approximate placement of isolates at *Neosartorya*/*Aspergillus* genus level. The isolates presented a PCR-product for the N2F/N2R primers (Supplementary Fig. S2B) while they did not present any PCR product for *A. fumigatus* primers (primer AflF/AflR; Supplementary Fig. S2C) which effectively excluded them from relation to *A. fumigatus* species. The isolates were identified to genus level as *Neosartorya* spp. based on the Internal Transcribed Spacer (ITS) and β -tubulin sequences during Sanger sequencing. All obtained sequences were deposited in the National Centre for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>; see Supplementary Table S2).

After analysing the sequences obtained through specific phylogenetic trees (Figs. 1, 2) it was observed that the ITS1 gene sequences of the isolates were grouped closely with *Aspergillus* spp. and *Neosartorya* spp. creating a separate cluster from other HRF (*Eupenicillium meridianum*, *Hamigera insecticola*, *Paecilomyces variotii*, *Byssoscllamys lagunculariae*, *Talaromyces udagawe*; Fig. 1). Sequences obtained for the functional β -tubulin gene confirmed that the isolates belonged to the genus *Neosartorya* creating a cluster completely separated from *Aspergillus* spp. and other fungi (Fig. 2).

Sensitivity analysis of *Neosartorya* spp. isolates to plant extracts

The plant extracts (dry lavender flower, dry marigold, as well as lavender, tea tree, clove and rosemary oils) were effective at inhibiting *Neosartorya* spp. during the inhibition tests (Figs. S3 and S4). Essential oils had a stronger inhibitory effect than plant extracts, with rosemary and lavender oils the most potent (Fig. 3A). Clove and tea tree oils inhibited the growth of *Neosartorya* spp. to a lesser degree as the two other oils. As illuminated by results focused on the concentration, the medium dose ($250 \mu\text{g mL}^{-1}$) of natural extracts tended to yield the strongest antifungal effect against all the tested isolates of *Neosartorya* spp. (Fig. 3B).

Based on cluster analysis, the 20 tested isolates were divided into 6 groups according to their susceptibility to the plant extracts. As observed in Fig. 3A, the isolates could be divided as follows: the most sensitive isolate G150/14 and the sensitive group with isolates G154/14, G159/14 and G151/14; a slightly higher than average sensitivity for G127/14 and G125/14; an average sensitivity for the group with G259/14, G126/14, G155/14, G161/14, G226/14, G131/14 and G130/14; and the lowest sensitivity group containing G132/14, G160/14, G253/14, G239/14, G135/14, and G129/14. Figure 3A,B showed that isolate G150/14 presented a different degree of sensitivity when compared to the other isolates of the *Neosartorya* genus. This preliminary analysis, aside from guiding the decision on which natural extracts and concentrations exhibited the best antifungal effect, also underscored a non-uniform response among the 20 tested fungal isolates, indicating a potential intraspecific difference.

The results were also analysed for of both the extract type and concentration together (Fig. 4A). The concentration of $1000 \mu\text{g mL}^{-1}$ seemed to be the most effective for all extracts in inhibiting *Neosartorya* spp., with clove oil at $1000 \mu\text{g mL}^{-1}$ presenting the highest antifungal effect on tested isolates among all analysed natural extracts. Interestingly, lavender flower dry extract presented a more or less uniform inhibitory ability regardless of dosage (ranging from a maximum of $1000 \mu\text{g mL}^{-1}$ and minimum of $100 \mu\text{g mL}^{-1}$). Marigold dry extract, lavender oil, clove oil, tea tree oil and rosemary oil presented an increasing trend of inhibition with increasing dosage. However, the antifungal capabilities did not increase in a linear manner for clove oil, lavender dry extract and rosemary oil. The results indicated that tea tree oil exhibited the highest inhibition towards *Neosartorya* spp. in all of the analysed concentrations (with the exception of 1000 and $5 \mu\text{g mL}^{-1}$).

The inhibition zones for each isolate were later compared with their respective controls (Fig. 4B). A large difference between the control and the group of isolate G150/14 was immediately evident, thus confirming the results in Fig. 3A,B and deeming the isolate the most sensitive towards contact with the plant extracts. The other isolates were categorized into groups based on the recorded inhibition zones: those with an high inhibition area (including G127/14, G151/14, G154/14, and G159/14), and those with an average inhibition zone (comprising of G153/14, G125/14, G126/14, G131/14, G155/14, G161/14 and G259/14) and low inhibition zone (G130/14, G132/14, G160/14, G226/14, G239/14, G253/14). Two isolates (G129/14 and G135/14) showed no growth difference and can therefore be described as a distinct group displaying the highest resistance to incubation with the plant extracts.

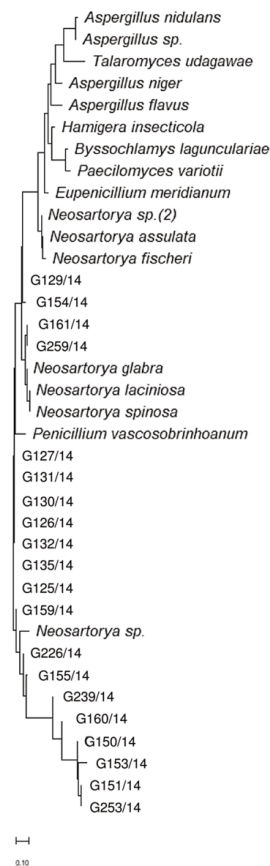


Figure 1. Phylogenetic tree based on ITS1 sequences of isolates used in this study and other published fungal sequences.

Ten strains were selected on the basis of the results from the inhibition test and further investigated on MT2 and FF microplates. Taking into account all results gathered thus far, 5 groups could be distinguished in terms of sensitivity (from the most to the least sensitive): G150/14 and G127/14 (I group); G226/14 and G132/14 (II group); G154/14 and G151/14 (III group); G130/14 and G129/14 (IV group); G135/14 and G160/14 (V group).

Fungal sensitivity assay during the exposure to different concentrations of essential oils and natural extracts in MT2 microplates

The combined data from all 20 isolates was analysed. All analysed substances showed an inhibition towards the AFGI in the MT2 microplates, with the exception of lavender and rosemary oils at 1000 $\mu\text{g mL}^{-1}$ and clove oil at 50 $\mu\text{g mL}^{-1}$, which did not present an effective inhibition on the biomass production (Fig. 5A). The rest of the studied substance decreased the growth ability of *Neosartorya* spp., with the most effective substances being lavender flower dry extract (5 $\mu\text{g mL}^{-1}$), and lavender and rosemary oils (150 $\mu\text{g mL}^{-1}$). Rosemary and lavender oils showed an inhibitory effect at 5, 50, and 150 $\mu\text{g mL}^{-1}$ and clove oil presented an inhibitory effect at: 5, 150, and 1000 $\mu\text{g mL}^{-1}$. Tea tree oil was the most effective at a concentration of 5 $\mu\text{g mL}^{-1}$, the same concentration at which marigold dry extract and clove oil exhibited the highest antifungal properties.

A low ratio between the AFRI and AFGI indicates a more efficient metabolism, due to a relatively large fungal biomass production despite low uptake of available substrates in plate wells. In contrast, a higher ratio indicates a smaller biomass (as indicated by low OD values at 750 nm) but with high respiration rates (as indicated by high OD values at 490 nm). This indicates a state of heightened metabolic activity or stress, where the fungal organism is experiencing a challenging metabolic situation^{47,50}. The ratio between AFRI/AFGI differed for all

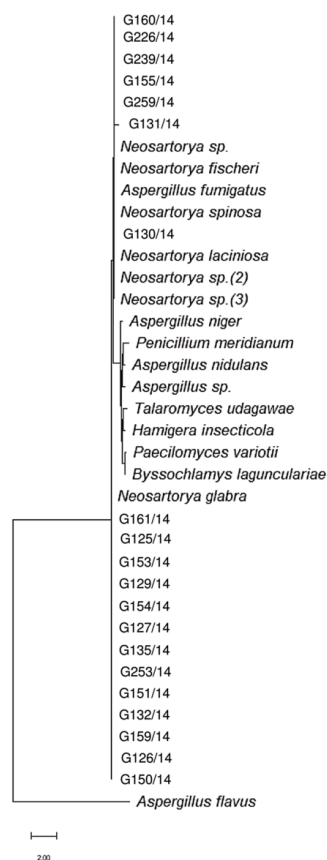


Figure 2. Phylogenetic tree based on β -tubulin gene sequences of isolates used in this study and other published fungal sequences.

tested concentrations of extracts and was different from the control (Fig. 5B). Higher ratio values mark fungal substrate stress which was especially prominent when dry marigold flower extract was used ($1000 \mu\text{g mL}^{-1}$, $150 \mu\text{g mL}^{-1}$, $50 \mu\text{g mL}^{-1}$). Substrate related stress was also detectable when other substances were used: lavender oil ($1000 \mu\text{g mL}^{-1}$ and $50 \mu\text{g mL}^{-1}$), rosemary oil ($50 \mu\text{g mL}^{-1}$ and $5 \mu\text{g mL}^{-1}$), lavender flower dry extract ($1000 \mu\text{g mL}^{-1}$ and $150 \mu\text{g mL}^{-1}$), and clove oil ($1000 \mu\text{g mL}^{-1}$). Only rosemary oil could induce metabolic stress at a concentration of $5 \mu\text{g mL}^{-1}$ and promote better metabolism than the control when used at the highest concentration ($1000 \mu\text{g mL}^{-1}$). Tea tree oil did not cause metabolic stress in the isolates during the MT2 microplate assay.

The data from the MT2 assay was also analysed for all of the isolates separately, taking into account values obtained from the control and the group influenced by natural extracts (Fig. 6). After examining the AFGI index (Fig. 6A), it becomes evident that each group of isolates exhibited a distinct behaviour when exposed to these natural extracts. One group of isolates (G129/14, G127/14, G135/14, G151/14, G154/14, and G160/14) displayed a reduction in fungal biomass production upon exposure to natural extracts. Conversely, only three isolates (G130/14, G150/14, and G226/14) exhibited an increase in biomass production following exposure to natural substances. Isolate G132/14 showed no difference between the control and natural treatments. From the perspective of the AFRI/AFGI ratio, it was observed that only a few isolates displayed significant differences between the two treatments (Fig. 6B). Specifically, one isolate (G132/14) demonstrated a significantly lower value of AFRI/AFGI ratio after exposure to natural extracts compared to the control, indicating a higher metabolic efficiency and decrease in fungal biomass production, while isolate G135/14 exhibited a higher value of AFRI/AFGI ratio indicating a lower metabolic efficiency and an increase in biomass when exposed to the natural extracts.

The effect of fungal growth in response to the natural extracts was examined using the MT2 microplate. Slight variations in metabolic profiles were observed among the fungal isolates. Four groups of isolates could

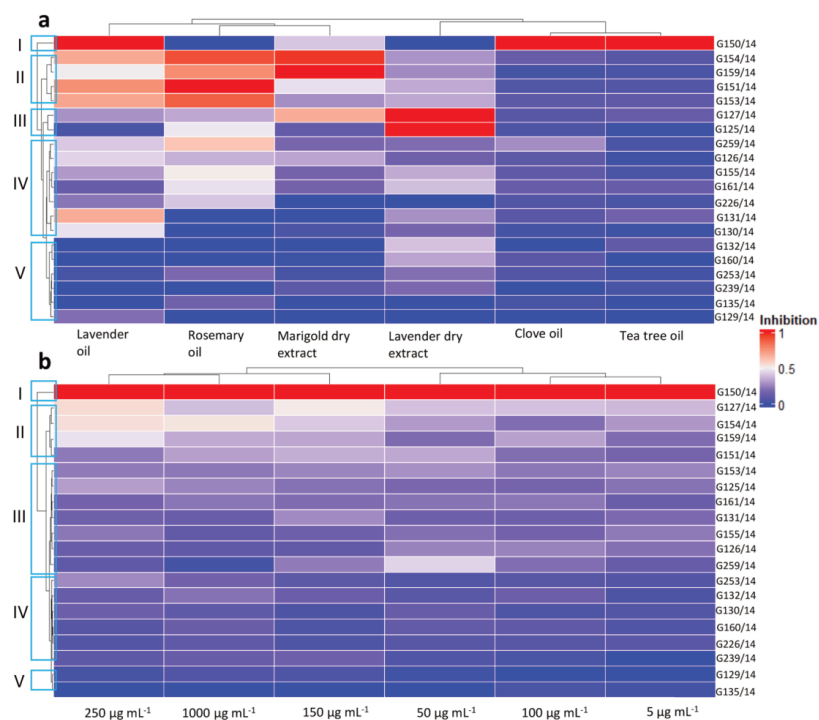


Figure 3. Heatmaps presenting the inhibition profile of different *Neosartorya* spp. isolates. Pooled results are shown for all concentrations of natural substances (a) and concentrations for all natural substances (b). Blue rectangles on the right signify isolate clusters.

be observed by cluster analysis based on Sheath's dissimilarity criterion at 66% and using the Average Fungal Respiration Intensity (AFRI; Fig. 7A) and the Average Fungal Growth Intensity (AFGI; Fig. 7B). As indicated by previous analyses, the response to these treatments by the G150/14 isolate differed from that of the other tested isolates (as evident in Fig. 7A,B). This underscores the significance of intraspecific differences when evaluating the effects of specific treatments on organisms. Furthermore, consistent separation of fungal biomass utilization and growth under exposure to the natural extracts was observed for all other isolates. Two isolates (G160/14 and G135/14), were characterized by a uniform response and confirming their inclusion in the previously mentioned high resistance group (referred to as the V group). The remaining isolates (G154/14, G129/14, G132/14, G151/14, G130/14, G226/14 and G127/14) were distributed into a third cluster based both on AFRI and AFGI.

From this research, three groups of isolates with different sensitivity to plant extracts could be distinguished, including those characterized by high (G150/14), medium (G126/14, G127/14, G129/14, G130/14, G132/14, G151/14, G154/14) and low (G135/14, G160/14) sensitivity to the tested dry extracts and plant oils.

Metabolic profile analysis—changes after pre-incubation with marigold extract

Pre-incubation of isolates with marigold extract ($150 \mu\text{g mL}^{-1}$) led to different behaviours amongst the analysed isolates. Over half of the isolates (seven out of ten) exhibited a significant loss of substrate richness of metabolized substances compared to the control (Fig. 8A), while the remaining three isolates (G129/14, G135/14, and G154/14) exhibited a significant loss of substrate richness (R index) of metabolized substances compared to the control. Cluster analysis (Fig. 8B) showed a clear division between isolates that had been pre-incubated on marigold extract and control isolates that were cultured on PDA medium in their biomass production abilities. Only 3 isolates (G129/14, G135/14, and G154/14) exposed to marigold extract were found to cluster with isolates not exposed to this plant extract. The distinct response of these three isolates after pre-culturing in marigold extract is also depicted in Fig. 8A. In general, the isolates clustered as 3 separate groups according to the Sneath criterion (33%), with one group representing the control, the second made up of the treatment group and a third containing a few isolates from both the treatment and control groups. The utilization percentages within the six groups of analysed substances using the FF microplate showed variations in utilization patterns based on

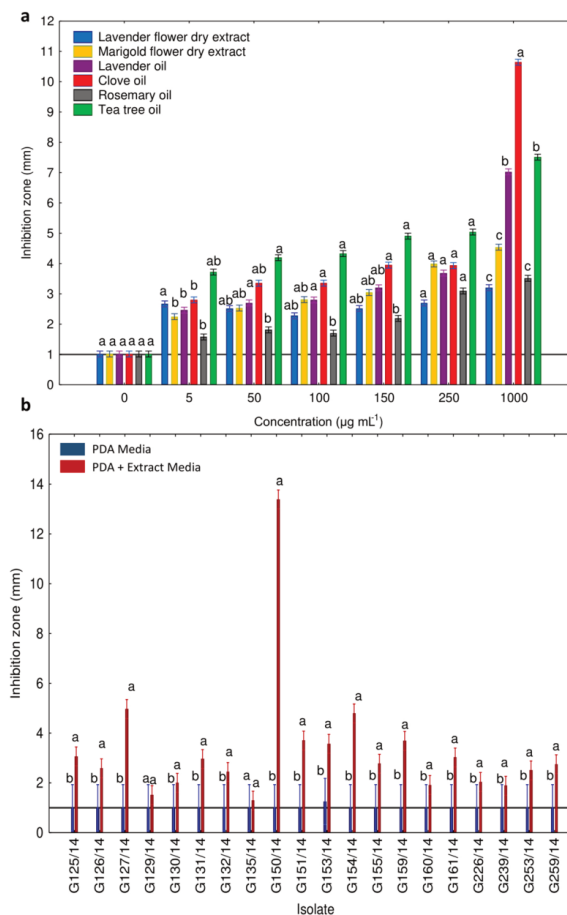


Figure 4. *Neosartorya* spp. isolates' sensitivity to natural extracts during disc diffusion inhibition test. Insights from analysed concentrations (a) and individual isolate responses to natural extracts (b). Significant differences indicated by letters (Wilcoxon test, $p < 0.05$).

isolates' pre-incubation conditions (Fig. 8C). A notable increase in average utilization of the group of miscellaneous substrates (referred to miscellaneous group from now on) was observed for nearly all isolates that had been pre-incubated with marigold extract. In the case of G135/14 (characterised by the lowest sensitivity to plant extracts), significant changes were observed when compared to the control. These changes included the loss of substrate utilization within the amino acids group, a reduction in utilization within the carbohydrates group, and, nearly double the utilization within the carboxylic acids and polymers groups. In the case of the G150/14 isolate, characterized by the highest sensitivity to the tested plant extracts, pre-incubation with marigold extract caused an increase uptake of miscellaneous group substrates, but it did not cause a change in the use of individual groups of carbon substrates compared to the control fungi, with only a very slight decrease of amino acids and polymer metabolism visible. In the remaining isolates, characterized by medium sensitivity to the tested plant extracts, when treated with marigold extract there was generally a lower use of amines, amides and carbohydrates, as well as an increased use of compounds belonging to the miscellaneous group. G150/14 may be more sensitive than G135/14 and the rest of the more resilient isolates (e.g. G129/14) because it maintains a more balanced utilization of carbon substrates even when exposed to marigold extract, whereas G135/14 exhibits significant changes in substrate utilization when compared to the control. Thus isolate G150/14 possibly exhibits an inability to adapt and change its metabolism in stressful conditions. The obtained results indicate that the metabolic properties of fungi of the genus *Neosartorya* have an impact on shaping their resistance to plant extracts. Especially the high

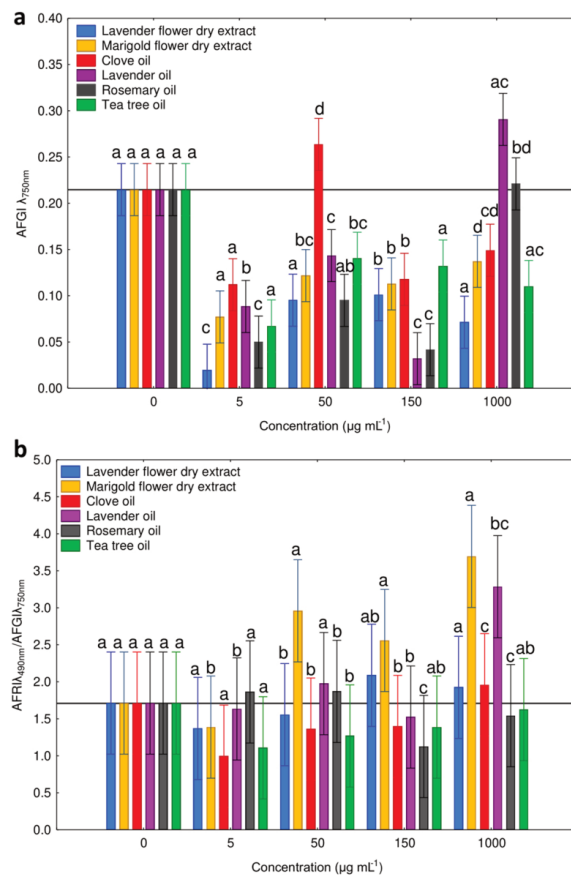


Figure 5. *Neosartorya* spp. isolates' sensitivity to natural extracts during MT2 microplate assay. AFI (Average Fungal Growth Intensity) for different treatments and their concentrations (a); Ratio of AFRI (Average Fungal Respiration Intensity)/AFGI for different treatments and their concentrations (b). Letters indicate significant differences (Wilcoxon test, $p < 0.05$).

use of nitrogen compounds, in particular the proportionally equal ratio of the use of amines, amides and amino acids, suggests a high sensitivity of fungal isolates to plant extracts. However, the predominance of the use of carboxylic acids by fungal isolates may suggest their lower sensitivity to the tested plant extracts.

Disparities among isolates were also evident in the heatmap analysis of the values related to fungal biomass production (corresponding to the optical density at 750 nm) as shown in Fig. 9. *Neosartorya* spp. grown without the marigold extract (control group) showed the highest growth in the presence of L-rhamnose, D-xylose, D-sorbitol, N-acetyl-D-glucosamine, adonitol, maltose and D-trehalose. Despite it not inducing the highest growth in the control group, D-erythritol emerged as the only carbon source universally promoting growth of *Neosartorya* spp. incubated on media with marigold extract. D-Mannitol was responsible for a moderate production of fungal biomass (ranging from 0.7 to 0.9) in isolates obtained from strawberry fruits, such as G129/14, G130/14, G135/14, and G226/14. Meanwhile, D-trehalose yielded positive results across almost all fungal isolates pre-incubated without the addition of marigold extract, resulting in optical density values between 0.7 and 1.1. Three isolates possessed a somewhat similar growth capacity to the control (G154/14, G129/14, G135/14), although it was slightly diminished. Such similarity could be a sign of high resistance of three isolates to incubation with marigold extract. The three isolates differed from the control group most notably in their inability to metabolise N-acetyl-D-glucosamine and adonitol which were both well metabolised by the isolates cultivated on PDA media without marigold extract.

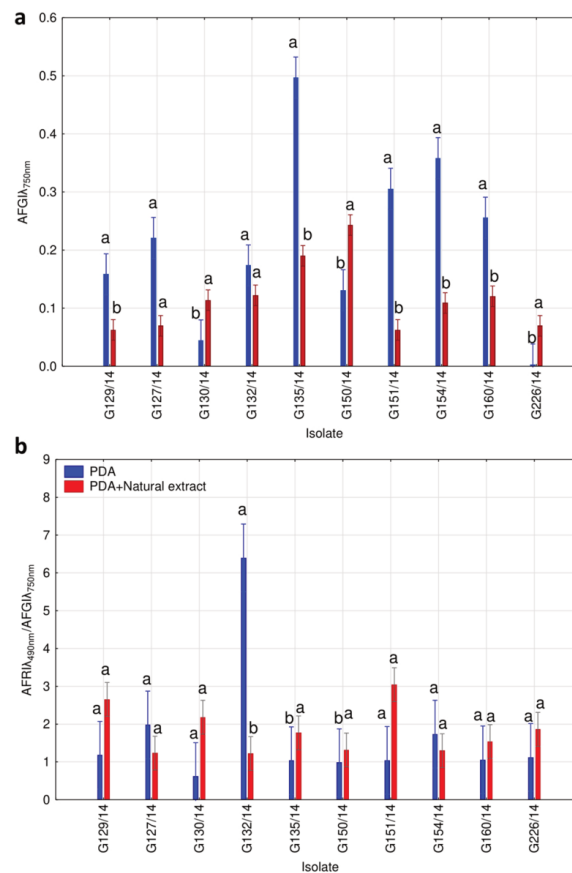


Figure 6. Evaluation of the *Neosartorya* spp. isolates' sensitivity to natural extracts using an MT2 microplate assay. AFGI (Average Fungal Growth Intensity) of analysed isolates—control and test group (a); AFRI (Average Fungal Respiration Intensity)/AFGI of analysed isolates—control and test group (b). Different letters indicate significant differences (Wilcoxon test, $p < 0.05$).

Scanning electron and fluorometric microscopy analyses

When observed under a scanning electron microscope (SEM), mycelium of *Neosartorya* isolates appeared as a complex network of branching, thread-like structures. The mycelium displayed a high interconnectivity, with numerous fine hyphae extending in multiple directions (Fig. 10). Hyphae were generally characterized by a smooth surface and varied from 1.5 to 5 μm in width. The mycelium of isolates G135/14 (the least sensitive) and G130/14 (an isolate at the lower end of average sensitivity) appeared more dense and tightly woven, (Fig. 10A,B) than the mycelium of isolates G132/14 (average sensitivity) and G127/14 (high sensitivity) (Fig. 10C–E). Additionally, after the 10 day long incubation period, isolates produced well-developed ascospores (Fig. S5).

Optical imaging after 5 days of incubation showed that isolate G127/14 had the most prominent conidia and conidiospore formation (Fig. 11A), G130/14 possessed a moderate amount of conidia (Fig. 11B) and G132/14 had the least visible conidia from all 3 isolates and exhibited a slower rate of growth compared to the other isolates (Fig. 11C). During fluorescence imaging with FUN-1 dye, no significant differences in filament viability were observed among the isolates (Fig. 11D–F).

Discussion

Neosartorya spp. is a pathogenic fungus capable of affecting fruit prior to harvesting, potentially leading to contamination of finished products (such as juice and preserved fruit) through spores and mycotoxins⁷. So far, studies have focused on traditional food protection techniques used during manufacture, such as modifying sugar

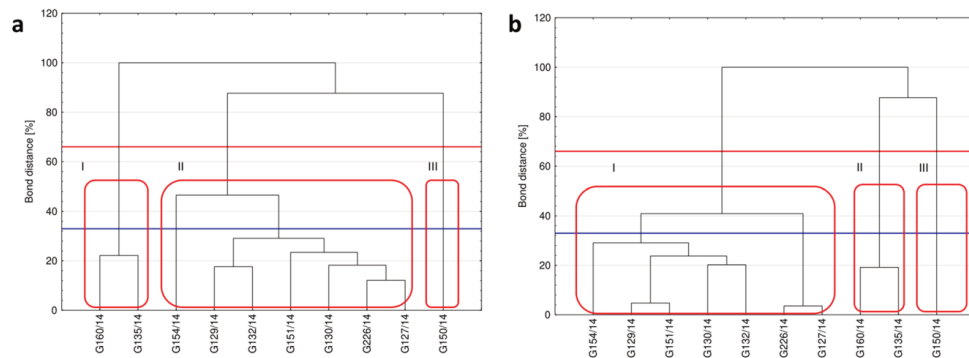


Figure 7. Cluster analysis of *Neosartorya* spp. isolates. Clusters according to the Average Fungal Respiration Intensity (a) and Average Fungal Growth Intensity (b) with the lines representing the Sneath criterion at 33% (blue line) and at 66% (red line) and numeration I, II, III for observable clusters.

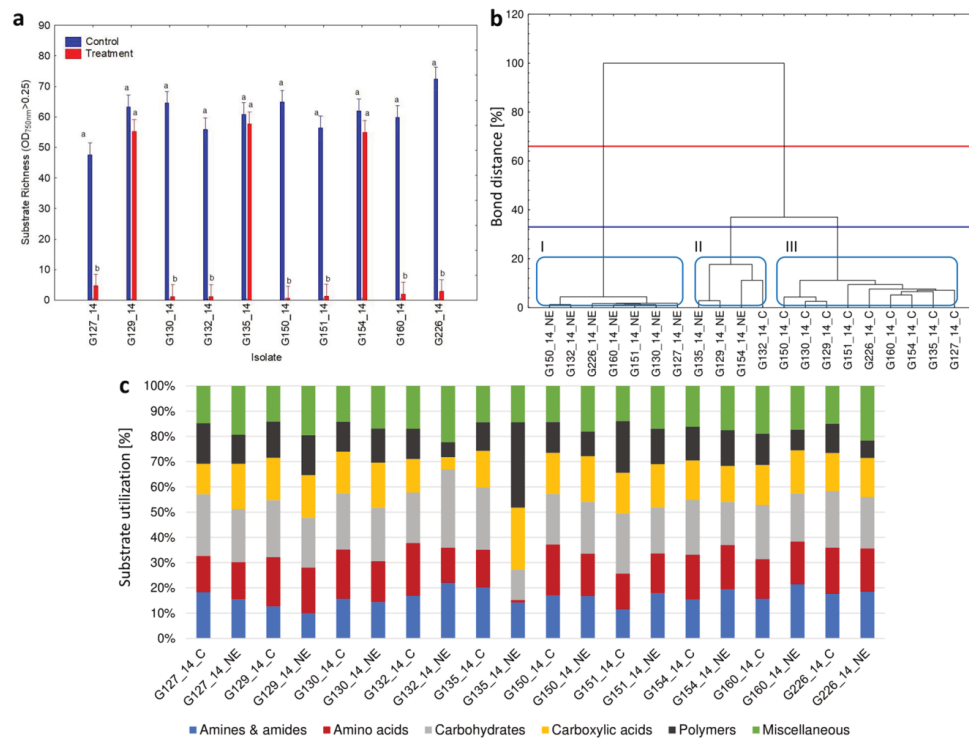


Figure 8. The effect of marigold extract on *Neosartorya* spp. metabolic profiles. Substrate richness per isolate (a). Cluster analysis of fungal biomass production similarity between different isolates of *Neosartorya* spp. pre-cultured in the presence of marigold extract dilution (NE) and without it (C) after 120 h of incubation, with the lines representing the Sneath criterion at 33% (blue line) and at 66% (red line) and numbers I, II, III for observable clusters (b). Utilization of carbon source groups by isolates treated (NE) and untreated (C) with marigold extract after 120 h of incubation (c).

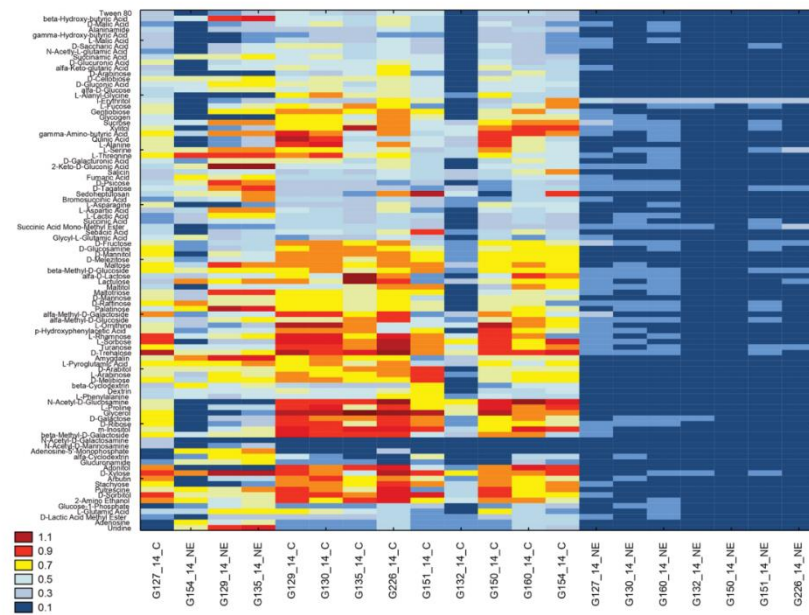


Figure 9. *Neosartorya* spp. biomass production on the FF microplate. Isolates cultured in the presence of marigold extract (NE) and without it (C) after 120 h of incubation.

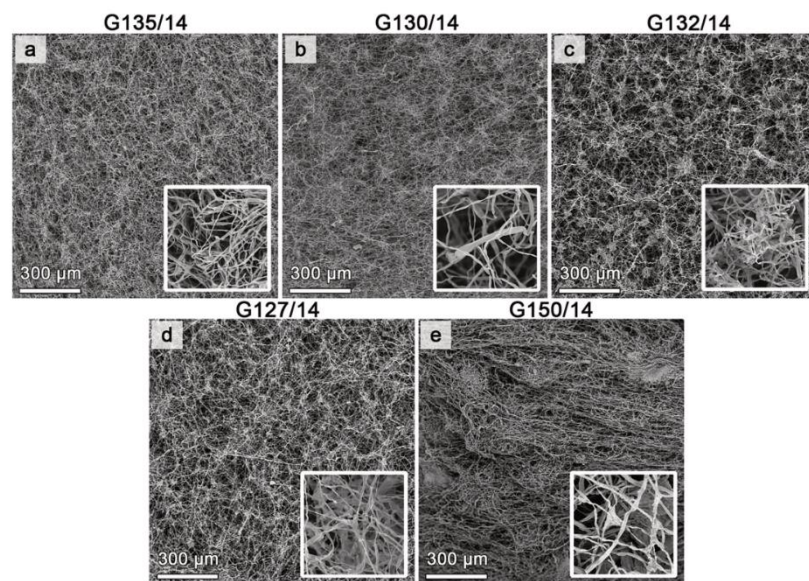


Figure 10. *Neosartorya* spp. observation using SEM – isolates G135/14 (a), G130/14 (b), G132/14 (c), G127/14 (d), G150/14 (e) cultivated on PDA media, 200X magnification and 5000X magnification zoom.

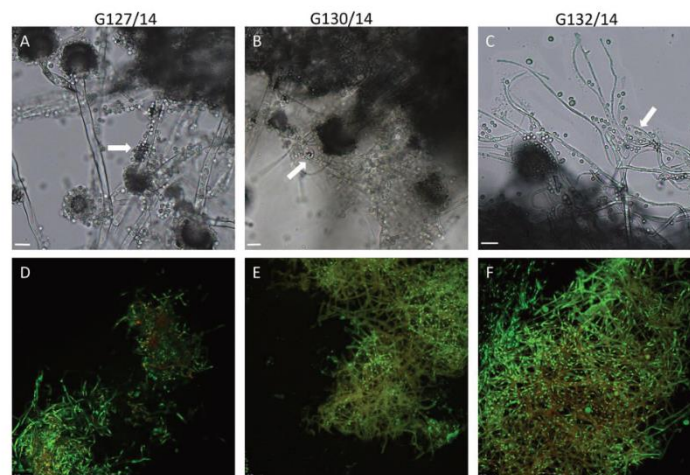


Figure 11. Observation of *Neosartorya* spp. isolates. G127/14 (A, D), G130/14 (B, E) and G132/14 (C, F) through optical microscope (A, B, C; 40X magnification) and fluorescence imaging (D, E, F). Visible spores are indicated by white arrows.

content, temperature or pressure of processing^{51,52}. However the use of such methods poses a risk of altering the physical properties of food, leading to a decrease in value and consumer dissatisfaction¹⁷. Therefore it is essential to search for alternative, health-positive or neutral methods of protection that are easy to apply in high-scale farming. Examples of effective fungicides that could be directly applied to the general market are antagonistic organisms or natural extracts, both easily applied e.g. in form of sprays^{53,54}. In our research, aside from natural dry powder solutions, water emulsions of oils were used as antifungal agents.

Ben Miri et al.⁵⁵ reported sensitivity of *Aspergillus ochraceus* and *A. niger* to menthol and eugenol, common constituents derived from mint or myrtle. Some reports also focus on reduction in aflatoxin synthesis, spore germination and mycelium growth of *Aspergillus flavus* under the influence of natural extracts (e.g. Shirazi thyme and cumin seed)⁵⁵. In our research, natural extracts (lavender and marigold dry extracts; lavender, tea tree, clove and rosemary oils) caused an inhibition of *Neosartorya* sp. isolates' mycelium growth. Overall, all these substances exhibited an inhibitory effect on the growth of fungal mycelium at low concentrations, and as the concentrations increased, a nearly exponential rise in this effect was noted (Fig. 5A). At all concentrations, the size of the inhibition zones were relatively consistent. However, when the concentration of $1000 \mu\text{g mL}^{-1}$ was used, a significant and distinct separation into three groups was evident. Clove oil was particularly effective, followed by lavender and tea tree oil, with a lesser effect observed with lavender flower and marigold extracts, and rosemary oil. While their impact on mycotoxin production and thermal resistance remains unknown, our experiments confirmed the antifungal ability of these extracts, expressed as growth inhibition. In general, essential oils are considered more potent than dry extracts, albeit less stable due to evaporation⁵⁶, potentially due to the use of different extraction methods to generate these substances. Natural extracts are obtained using common solvents, but the choice of solvent and extraction conditions can be critical factors that could affect the yield and composition of the active ingredients extracted from different plant parts⁵⁷. A more detailed examination of Fig. 4A revealed that the division of fungal isolates into five groups based on their sensitivity after incubation with the analysed natural substances closely aligned to their origin. Isolates G150/14, G154/14, G159/14, and G151/14, which were obtained from soil, displayed greater sensitivity to incubation with these natural substances. In contrast, the more resistant strains, G131/14, G130/14, G132/14, G253/14, G239/14, G135/14, and G129/14, were all isolated from strawberry fruit. This differential response could be attributed to the limited resources available in soil, hindering their growth and development across all growth stages. Soil, however, could also have served as a protective environment, shielding the fungus from external changes and events. Conversely, strawberry fruit harbours a wider array of carbon resources for growth, but the sudden change in these resources could have compelled these fungi to exhibit metabolic plasticity. This would have resulted in immediate metabolic adjustments to cope with environmental fluctuations, ensuring the survival of the fungal mycelium.

The inhibitory effect of the analysed natural substances on *Neosartorya* sp. growth was further confirmed by the MT2 microplate analysis. The use of MT2 microplates determined whether various *Neosartorya* isolates utilized these natural resources for their metabolism and if they subsequently exhibited resistance or sensitivity to these substances. All natural extracts caused a substantial decrease in biomass production (Fig. 6A). A strong inhibitory effect was observed in response to the lavender extract, rosemary, and lavender oils. Interestingly, the isolates underwent a significant decrease in mycelium production at concentrations of 5 and $150 \mu\text{g mL}^{-1}$ for all oils and dry extracts, instead of being strongly affected by the highest concentration. It's important to note that

the dose–response relationship and optimal concentrations can vary depending on the specific natural extract. It is plausible that the 5 $\mu\text{g mL}^{-1}$ concentration resulted in a higher solute “liquidity” within the solvent (distilled and sterilized water in our case), facilitating the passage of natural substances into the fungal mycelium, thereby eliciting negative reactions in fungal metabolism, ultimately preventing mycelium proliferation by the analysed substance. In contrast, at concentrations of 1000 $\mu\text{g mL}^{-1}$, the solute may be less “fluid,” reducing penetration of the substances into the fungus, resulting in a less pronounced effect compared to the minimum concentration analysed. *Neosartorya* spp. may also have a susceptibility threshold, beyond which higher doses have limited additional inhibitory effects. Thus the toxicity-growth inhibition relationship may be non-linear, with higher concentrations exhibiting increased toxicity but diminished inhibitory effects.

We observed that at concentrations of 5 and 150 $\mu\text{g mL}^{-1}$ (causing the most significant antifungal effect on growth) the AFRI/AFGI ratio was lower for the majority of treatments than for the control (0 $\mu\text{g mL}^{-1}$). However, these values were higher than the control for rosemary oil (at both concentrations) and for lavender and marigold extract at 150 $\mu\text{g mL}^{-1}$ (Fig. 6B). Considering that the AFRI/AFGI ratio can provide insights into whether the isolates are in a state of stress⁴⁷, we can conclude that the fungus exhibits the most evident stress response when incubated with marigold extract (ranging from 50 to 1000 $\mu\text{g mL}^{-1}$) and lavender oil at 1000 $\mu\text{g mL}^{-1}$. This suggests that exposure of *Neosartorya* sp. to marigold extract negatively affects fungal biomass production (Fig. 6A) and induces a state of stress, manifested through an inefficient metabolism (Fig. 6B). There was, however, high variability between individual *Neosartorya* spp. isolates (Fig. 8) to the treatments. Focusing specifically on the AFGI, only one isolate (G130/14) displayed a lower growth intensity in the control compared to the natural treatments analysed, and isolate G132/14 showed no difference in growth between the control and the treatment. The remaining eight isolates exhibited a negative effect on fungal biomass production during the incubation period with the different natural substances analysed (Fig. 8A), contributing to a lower stress condition (Fig. 8B). Analysis using MT2 microplates highlighted the changes in the metabolic level of *Neosartorya* sp. due to incubation with these natural substances, rendering fungi unable to use the substances effectively, resulting in significantly lower growth compared to the control incubated under favourable conditions. Furthermore, the substantial variation in behaviour among the various isolates (Fig. 8) corresponds with the findings of Panek et al.³⁷. These authors investigated the chemical sensitivity, using Biolog™ Phenotype MicroArray analysis, of two different isolates of *Neosartorya fischeri* from distinct environments: one isolated from canned apples in 1923 and another from thermally processed strawberry products in 2012. Their study revealed that the fungus isolated from the processed strawberry product demonstrated much greater resistance to the chemicals analysed through the PM platform compared to the isolate from canned apples. Despite the similar isolation and storage time of isolates studied in our experiments, many of the fungi derived from strawberry fruit, possessed an increased resistance to stress conditions compared to those from soil. Three isolates (G130/14 and G226/14, both resistant, and G132/14, displaying no effect in this study) originating from strawberry fruit exhibited greater resistance when incubated with natural substances. This supports the results obtained from the inhibition test and confirms that *Neosartorya* spp. isolates from strawberry fruits are more resistant to incubation with these natural substances. When analysing how the metabolism of *Neosartorya* spp. isolates could be influenced (FF microplates) by pre-cultivation in marigold extract (Fig. 9), a clear division into two clusters (Fig. 9B) was noticed, based on whether the isolates were pre-incubated with or without marigold extract. Within this separation, one exception was noted for three isolates pre-incubated in marigold (G135/14, G129/14, and G154/14), which were grouped with isolates pre-incubated without the natural extract. For these three isolates, incubation with marigold extract did not result in negative effects on metabolism, in contrast to the majority of the analysed isolates. Instead, it led to a significant increase in substrate richness (Fig. 8A) compared to the control. Furthermore, as observed in the other analyses we conducted, the isolates that were not affected by incubation with these natural substances were found to be fungi isolated from strawberry fruits, specifically G135/14 and G129/14. Upon examining the optical density values obtained at 750 nm (Fig. 10), it was apparent that the isolates involved in acquiring heat resistance capacity and the maturation of ascospores^{14,16,58} allowed for a high production of fungal mycelium in only a few fungal isolates that were pre-incubated without marigold extract. These findings offer potential regarding the application of marigold extract as a natural fungicide. Five days of pre-incubation with this natural substance led to a distinct alteration of metabolism, limiting the production of fungal biomass across 95 different carbon substrates. Additionally, only three isolates pre-incubated with marigold exhibited increased fungal biomass compared to the control for specific carbon resources, including β -hydroxy-butyric acid, L-serine, 2-keto-D-gluconic acid, fumaric acid, D-tagatose, L-aspartic acid, palatinose, and uridine.

Upon analysing the mycelium structure of five *Neosartorya* isolates grown in a standard culture medium (PDA), it was clear that isolate G150/14 (originating from soil) exhibited a distinctive structure compared to the other isolates (Fig. 11). This structural variation in the mycelium could potentially account for the differential response to the treatments examined in this study.

Considering the cumulative results across the various treatments and dosages, it seems clear that both the dry extracts and natural oils exert an inhibitory influence on the growth and development of *Neosartorya* spp. fungal mycelia. Furthermore, pre-incubation with marigold extract results in a substantial reduction in the growth of fungal mycelium across different carbon substrates, effectively slowing its growth on D-mannitol and D-trehalose, both of which are highly soluble compounds associated with the acquisition of heat resistance by *Neosartorya* spp.^{59,60}. These findings are a cause for optimism regarding the prospective application of these natural substances to mitigate the presence and proliferation of fungi in fruit-derived products. Moreover, this study highlights that fungi isolated from strawberry fruits demonstrate greater resistance compared to those isolated from soil, which warrants further investigation in the future. This discrepancy is further supported by the visualization of fungal mycelium structure using a scanning electron microscope (SEM). The variance in treatment response is likely linked to potential intraspecific differences within the *Neosartorya* genus, prompting an additional need for further exploration.

Conclusions

This is a first report on the effect of natural extracts on *Neosartorya* spp. utilizing both inhibition tests and microplate metabolic assays. Using these methodologies we were able to identify variation in the susceptibility of individual isolates to the effects of plant extracts and establish 3 main groups of increasing sensitivity, underlining the possibility of a non-uniform response to stress factors amongst isolates of the same genus. There seems to be a link between innate fungal substrate utilization abilities (which vary between isolates) and sensitivity to natural extracts, with weaker isolates naturally preferring nitrogen compounds and more resilient ones preferring carboxylic acids. Another observable difference between isolates was the mycelial density, where more resistant isolates were characterized by denser mycelium than sensitive fungi. The results indicate that natural extracts, especially lavender oil, yield an effective outcome in growth inhibition of fungi belonging to *Neosartorya* genus. To conclude, an in-depth examination of *Neosartorya* spp. was conducted, focusing on metabolic capabilities under the influence of a natural extracts possessing antimicrobial capabilities. Information on fungal phenotypes and specific nutrient utilization profiles was also provided. These findings are very important for understanding functional and morphological features in shaping the resistance of *Neosartorya* spp. to plant extracts. Moreover, they could be used to develop better prevention and intervention methods against *Neosartorya* spp., and in the development of new active compounds for the control of microbiological food contaminants and pathogenic species, which is important for the development of a more sustainable agriculture. However, further research is required to determine the underlying cause of such metabolic diversity among the tested *Neosartorya* spp. isolates.

Data availability

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

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Author contributions

WM designed and performed experiments, analysed data, and wrote and corrected the manuscript. GP corrected experiment design, analysed data, corrected the manuscript. SR took part in methodology design and experiments with fluorimetric microscopy. KS took part in methodology design and experiments with scanning electron microscopy. MF obtained the funding, designed the concept of the research, took part in designing all experiments, supervised, reviewed and edited experiments and the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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7.2.3 Materiały dodatkowe

Comprehensive antifungal investigation of natural plant extracts against *Neosartorya* spp. (*Aspergillus* spp.) of agriculturally significant microbiological contaminants and shaping their metabolic profile

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Water	Tween 80	N-Acetyl-D-Galactosamine	N-Acetyl-D-Glucosamine	N-Acetyl-D-Mannosamine	Adonitol	Amygdalin	D-Arabinose	L-Arabinose	D-Arabitol	Arbutin	D-Cellobiose
α -Cyclodextrin	β -Cyclodextrin	Dextrin	L-Erythritol	D-Fructose	L-Fucose	D-Galactose	D-Galacturonic Acid	Gentiobiose	D-Gluconic Acid	D-Glucosamine	α -D-Glucose
Glucose-1-Phosphate	Glucuronamide	D-Glucuronic Acid	Glycerol	Glycogen	m-Inositol	2-Keto-D-Gluconic Acid	α -D-Lactose	Lactulose	Maltitol	Maltose	Maltotriose
D-Mannitol	D-Mannose	D-Melezitose	D-Melibiose	α -Methyl-DGalactoside	β -Methyl-DGalactoside	α -Methyl-DGlucoside	β -Methyl-DGlucoside	Palatinose	D-Psicose	D-Raffinose	L-Rhamnose
D-Ribose	Salicin	Sedoheptulosan	D-Sorbitol	L-Sorbose	Stachyose	Sucrose	D-Tagatose	D-Trehalose	Turanose	Xylitol	D-Xylose
γ -Amino-butyric Acid	Bromosuccinic Acid	Fumaric Acid	β -Hydroxy-butyric Acid	γ -Hydroxy-butyric Acid	p-Hydroxyphenylacetic Acid	α -Keto-glutaric Acid	D-Lactic Acid Methyl Ester	L-Lactic Acid	D-Malic Acid	L-Malic Acid	Quinic Acid
D-Saccharic Acid	Sebacic Acid	Succinamic Acid	Succinic Acid	Succinic Acid Mono-Methyl Ester	N-Acetyl-L-Glutamic Acid	Alaninamide	L-Alanine	L-Alanyl-Glycine	L-Asparagine	L-Aspartic Acid	L-Glutamic Acid
Glycyl-L-Glutamic Acid	L-Ornithine	L-Phenylalanine	L-Proline	L-Pyroglutamic Acid	L-Serine	L-Threonine	2-Amino Ethanol	Putrescine	Adenosine	Uridine	Adenosine-5'-Monophosphate
		AMINES/AMIDES		AMINO ACIDS	CARBOHYDRATES		CARBOXYLIC ACIDS	POLYMERS	MISCELLANEOUS		

Figure S1. List of 96 substances inside the FF microplate and their grouping.

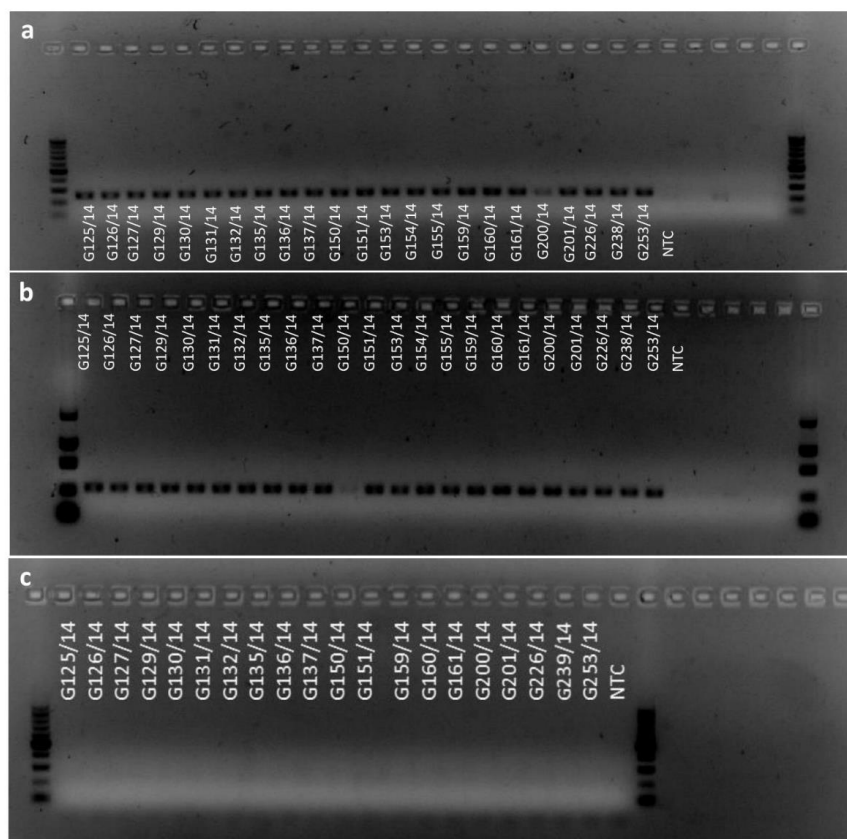


Figure S2. PCR amplifications using primers for: ITS1 (a) , β -tubulin *Neosartorya* spp. specific (b) and *A. fumigatus* specific (c).

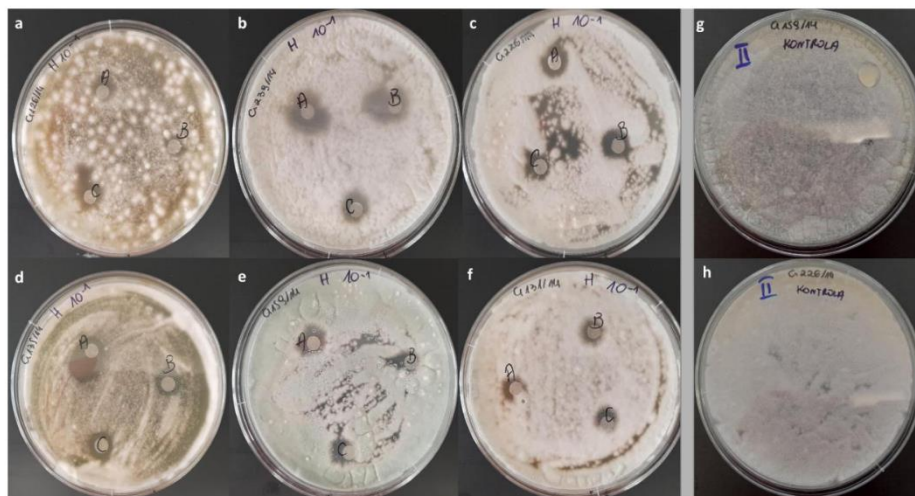


Figure S3. Inhibition zones in *Neosartorya* spp. growth formed under the influence of different dilutions of tea tree oil (a-f) and control plates (g-h).

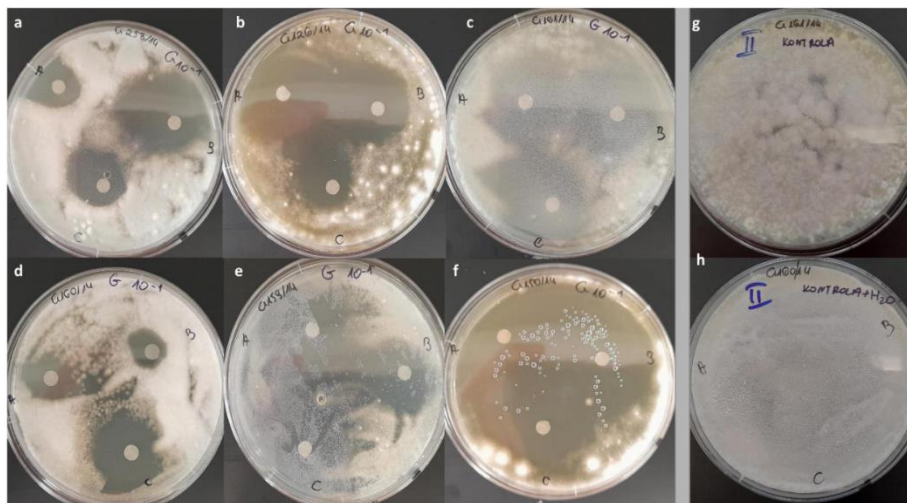


Figure S4. Inhibition zones in *Neosartorya* spp. growth formed under the influence of different dilutions of clove oil (a-f) and control plates (g-h).

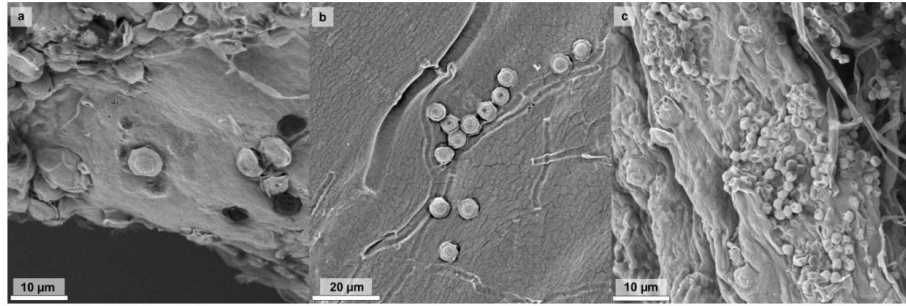


Figure S5. *Neosartorya* spp. ascospores observation. SEM imaging of isolates G130/14 (5000X magnification) (a), G150/14 (3000X magnification) (b), G132/14 (5000X magnification) (c).

Table S1. Amplification conditions for ITS1 and β -tubulin PCRs.

	ITS1	β-TUB <i>Neosartorya</i> spp. and <i>Aspergillus fumigatus</i>	β-TUB <i>Aspergillus fumigatus</i> specific
Initial activation	95°C for 3 min	95 °C for 3 min	94 °C for 3 min
Denaturation	95°C for 15 s	95 °C for 1 min	94 °C for 5 s
Annealing	55°C for 30 s	59 °C for 1 min	59 °C for 5 s
Extension	72°C for 40 s	72 °C for 1 min	72 °C for 10 s
Final extension	72°C for 7 min	72 °C for 10 min	72 °C for 1 min
Number of cycles	35	35	35

Table S2. Description of isolates used in this study and NCBI database accession numbers.

Isolate	Sample origin	Type of Sequence	NCBI Accession No.
G125/14	Strawberry fruit	ITS	OQ300418.1
		B-TUB	OR500229
G126/14	Strawberry fruit	ITS	OQ300422.1
		B-TUB	OR597661
G127/14	Strawberry fruit	ITS	OQ300429.1
		B-TUB	OR597660
G129/14	Strawberry fruit	ITS	OQ300428.1
		B-TUB	OR597662
G130/14	Strawberry fruit	ITS	OQ303998.1
		B-TUB	OR597659
G131/14	Strawberry fruit	ITS	OQ303995.1
		B-TUB	OR597658
G132/14	Strawberry fruit	ITS	OQ300430.1
		B-TUB	OR597657
G135/14	Strawberry fruit	ITS	OQ300432.1
		B-TUB	OR608355
G150/14	Soil	ITS	OQ423135.1
		B-TUB	OR608366
G151/14	Soil	ITS	OQ306552.1
		B-TUB	OR608362
G153/14	Soil	ITS	OQ448879.1
		B-TUB	OR608354
G154/14	Soil	ITS	OQ306551.1
		B-TUB	OR608361
G155/14	Soil	ITS	OQ306553.1
		B-TUB	OR608356
G159/14	Soil	ITS	OQ306554.1
		B-TUB	OR608357
G160/14	Soil	ITS	OQ306555.1
		B-TUB	OR608359
G161/14	Soil	ITS	OQ448880.1
		B-TUB	OR608363
G226/14	Strawberry fruit	ITS	OQ306560.1
		B-TUB	OR608360
G239/14	Strawberry fruit	ITS	OQ306558.1
		B-TUB	OR608358
G253/14	Strawberry fruit	ITS	OQ306559.1
		B-TUB	OR608364
G259/14	Strawberry fruit	ITS	OQ306561.1
		B-TUB	OR608365

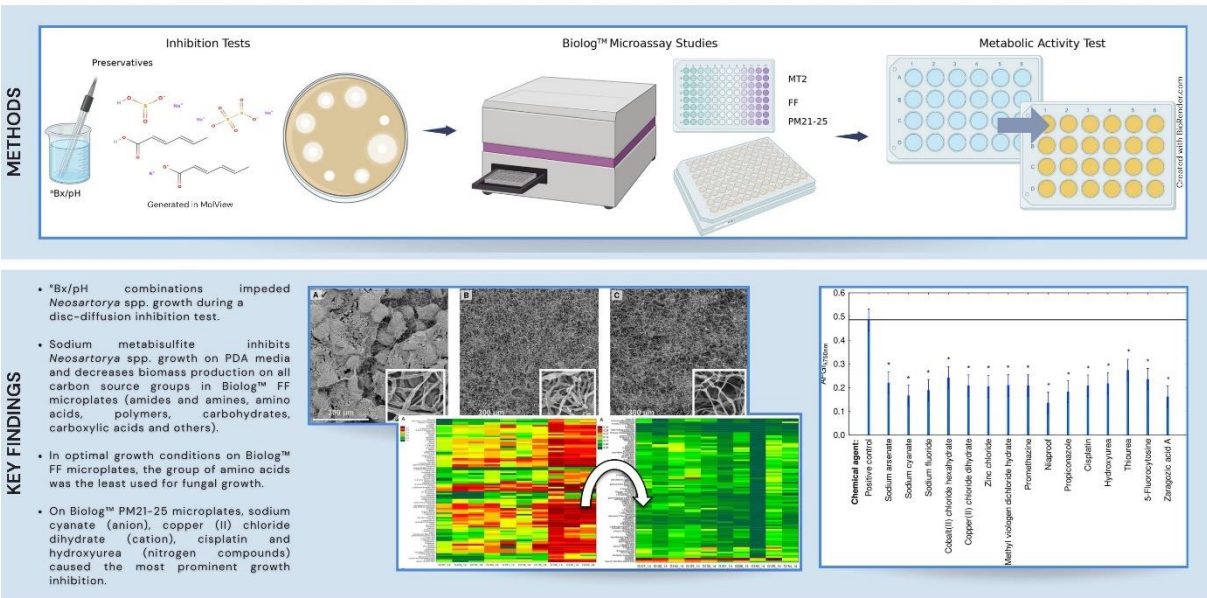
Table S3. Quantitative analysis of *Neosartorya* spp. isolates ascospores counts using Thoma cell counting chamber.

Isolate No.	Square position	Ascospores count in inoculating fluid with 75% transmittance [10^4]							Average ascospores count [10^4]
G150/14	Top	6	3	3	2	5	4	3	3.65
	Bottom	3	4	2	5	4	4	3	
G127/14	Top	5	7	5	6	2	4	4	4.79
	Bottom	5	4	5	3	6	4	7	
G132/14	Top	8	4	5	9	4	3	3	4.50
	Bottom	5	3	4	3	7	3	2	
G130/14	Top	3	5	4	3	2	3	2	3.65
	Bottom	2	5	7	2	4	6	3	
G135/14	Top	7	4	6	5	7	8	4	5.58
	Bottom	5	6	3	8	8	4	3	
Average ascospores counts from all tested <i>Neosartorya</i> spp. isolates									4.44

7.3 Publikacja P.3

7.3.1 Abstrakt graficzny

AN EXTENSIVE RESEARCH ON *NEOSARTORYA* SPP. (*ASPERGILLUS* SPP.): SECRETS OF FUNGAL METABOLIC PROFILE IN SHAPING ITS SENSITIVITY TO FOOD PRESERVATIVES AND CHEMICALS



7.3.2 Tekst publikacji

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The role of food preservatives in shaping metabolic profile and chemical sensitivity of fungi - an extensive study on crucial mycological food contaminants from the genus *Neosartorya* (*Aspergillus* spp.)

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ABSTRACT

Food preservatives are crucial in fruit production, but fungal resistance is a challenge. The main objective was to compare the sensitivity of *Neosartorya* spp. isolates to preservatives used in food security applications and to assess the role of metabolic properties in shaping *Neosartorya* spp. resistance. Sodium metabisulfite, potassium sorbate, sodium bisulfite and sorbic acid showed inhibitory effects, with sodium metabisulfite the most effective. Tested metabolic profiles included fungal growth intensity and utilization of amines and amides, amino acids, polymers, carbohydrates and carboxylic acids. Significant decreases in the utilization of all tested organic compound guilds were observed after fungal exposure to food preservatives compared to the control. Although the current investigation was limited in the number of predominately carbohydrate substrates and the breadth of metabolic responses, extensive sensitivity panels are logical step in establishing a course of action against spoilage agents in food production being important approach for innovative food chemistry.

1. Introduction

Fungal contamination poses a threat in various industries, including agriculture, horticulture and food production. It leads to economic losses and public health concerns due to possible mycotoxin production (Fornal, Parfieniuk, Czekko, Bilinska-Wielgus, & Frac, 2017). *Neosartorya* spp. belong to the *Aspergillaceae* family and are notorious for causing spoilage by contributing to the degradation of a wide range of agricultural produce e.g. purees, jams, fruit juices and canned fruits (Evelyn, Kim, & Silva, 2016; Lane Paixão dos Santos et al., 2020). Furthermore, the *Neosartorya* genus is characterized by high heat-resistance, allowing it to withstand pasteurization and therefore, rendering this food preservation technique ineffective against this fungal group (Berni, Tranquillini, Scaramuzza, Brutti, & Bernini, 2017). The ability of these fungi to colonize a wide range of substrates, such as indoor materials, plant matter and soil, underscores their ecological significance and economic relevance (Bang et al., 2019; Takada, Horie, & Abliz, 2001; Udagawa, Tsubouchi, & Toyazaki, 1996). *Neosartorya* spp. fungi possess a variety of defence mechanisms that enable them to survive under stressful environmental conditions. Such resilience has

been attributed to the production of ascospores which are characterized by various protective features: trehalose-based oligosaccharides and mannitol accumulation, high cytoplasmic viscosity that slows the metabolism, thick cell wall and low water content (Wyatt et al., 2015; Wyatt, Gerwig, Kamerling, Wösten, & Dijksterhuis, 2015; Wyatt, van Leeuwen, Wösten, & Dijksterhuis, 2014). Furthermore, media-dependent resilience, whereby differences in material characteristics (e.g. osmotic pressure) between the carrier and ascospores, might also play a role in providing extra heat protection (Buerman, Worobo, & Padilla-Zakour, 2019; Lane Paixão dos Santos, Samapundo, Van Impe, Sant'Ana, & Devlieghere, 2020; Lane Paixão dos Santos et al., 2020). Therefore, the development of an effective preservative treatment is necessary to prevent microbial spoilage, even in pasteurized products.

Chemical compounds such as preservatives play a crucial role in preventing the spoilage of various foodstuffs. Their use allows for loss reduction, quality increase and shelf life extension of food products, thus meeting the increasingly challenging market demands (Adhikari, 2021; Zhang et al., 2023). One of the simplest preservative techniques is the modification of sugar content and acidity of products so that the goods themselves are altered into a hostile environment for pathogens and

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microbiological food contaminants. The assessment of sugar content, often referred to as Brix, plays a critical role in evaluating the quality of agricultural products and alcoholic beverages (Jaywant, Singh, & Arif, 2022) and can be useful in the inactivation of heat resistant moulds (Alvarenga, Gonzales-Barron, do Prado Silva, Cadavez, & Sant'Ana, 2021). Moreover, sugars are the primary components of fruit products, essential for flavour, and acting as natural food preservatives (Khan, Afridi, Ilyas, Sohail, & Abid, 2012). Soluble sugar concentrations, pH and water activity in products may have an effect on the thermal tolerance in fungi (Buerman et al., 2019; Rajashekhara, Suresh, & Ethiraj, 2000; Slongo & De Aragão, 2006). High amounts of sugar dissolved in an aqueous solution, measured in degrees Brix (°Bx), combined with the low pH of juices is known to decrease the survival of bacteria (Enache & Chen, 2007). As *Neosartorya* spp. typically thrive in low acidity environments and demonstrate resilience to the high °Bx levels commonly found in products like jams, it is crucial to investigate various °Bx and pH combinations to understand their full impact on *Neosartorya* spp. metabolism and survival capabilities (Lane Paixão dos Santos, Samapundo, Van Impe, Sant'Ana, and Devlieghere, 2020; Rana, Yeasmin, Khan, & Riad, 2021). This in turn can help identify products with certain characteristics, such as favourable sugar availability, that thus may be the most susceptible to spoilage by *Neosartorya* spp.

Chemical or synthetic preservatives are often used in addition to natural methods of food protection (Taylor, Ravishankar, Bhargava, & Juneja, 2019). Both natural and synthetic preservatives are divided into categories such as antimicrobial, antioxidant, and anti-enzymatic. Antimicrobials, such as sodium metabisulfite, function by inhibiting or delaying the growth of bacteria, yeast, and moulds (Kumari, Akhila, Rao, & Devi, 2019). The use of any type of preservative requires the knowledge on the maximum dosage permitted by the place of manufacture, such as Regulation (EC) No. 1333/2008 in the European Union territory (European Parliament and the Council of the European Union, 2008). During manufacture, it is also important to note the effect of substances on the organoleptic properties of food, since the addition may have an influence on the texture, aroma and taste of the product (Santoso et al., 2023). The effectiveness of preservatives in controlling fungal growth is attributed to various mechanisms of action, including: membrane composition disruption, inhibition of metabolic reactions, pH-related homeostasis disruption, accumulation of toxic anions, reactions that generate oxygen singletons or hydroxyl radicals and the energy pool exhaustion in cells due to a general stress response (Diakogiannis et al., 2013; Dijksterhuis, Meijer, van Doorn, Houbaken, & Bruinenberg, 2019; Li et al., 2022; Pfukwa et al., 2019; Singh et al., 2023). Due to constant pesticide and fungicide use, many species of microorganisms develop resistance mechanisms that increase their viability even under unfavourable conditions (Davies et al., 2021). Alterations to metabolic activity can be a defence mechanism against synthetic preservatives. The main cell defence mechanism against antimicrobial agents involves drug modification through the excretion of microbial enzymes that alter or destroy molecules, such as β -lactamases that break down antibiotics like penicillin (Tooke et al., 2019). Another common mechanism is by altering treatment targets through gene mutations or modifications to decrease the cell's affinity for antibiotics, as seen in pathogenic fungi (Lima, Oliveira, Amaral, Freitas, & Souza, 2021). Many fungal species are able to withstand antimicrobial peptides and proteins by activating signalling pathways related to a pH stress response or cell wall modification (Thery, Lynch, & Arendt, 2019). Similarly, many species develop resistance to food preservatives, e.g. in *Saccharomyces cerevisiae*, resistance to high levels of acetic acid involves the loss of Fps1 aquaglyceroporin and a transcriptional response mediated by Haa1 (Piper, 2011). Other contributors to preservative resistance include the production of substance degrading enzymes, Tpo family transporters, and changes to the cell wall to minimize preservative entry upon contact (Cowen, Sanglard, Howard, Rogers, & Perlin, 2014). These resistance mechanisms warrant constructing periodical studies that report on the susceptibility of organisms to commonly used

fungicides, with an additional focus on explaining the metabolic behaviour involved.

Understanding the efficacy of certain preservatives in inhibiting *Neosartorya* spp. growth is critical for industries such as agriculture, where fungal contamination poses a significant challenge. The main hypothesis of the study is that closely related *Neosartorya* spp. isolates differ in their sensitivity to preservatives and chemicals and can form tiers of descending sensitivity, as sensitivity differences exist between sexual and asexual forms and closely related isolates (Laura, Emilia, Ana, Manuel, & L., 2008; Vinh et al., 2009). The key idea is that the metabolic properties of *Neosartorya* spp. influence their resistance to these substances and furthermore that the adaptation of *Neosartorya* spp. to various substrates involves alterations in their metabolic profile. Additionally, this research aims to identify preservatives that could be used as alternatives to the main components of chemical agents currently employed in agriculture for controlling the occurrence of microorganisms in plant products. In general, as key players in carbon recycling, fungi are exposed to various environmental stressors, including chemicals, toxins, and preservatives which can alter their susceptibility over the years. Therefore, the presented study documents sensitivity levels of *Neosartorya* spp. isolates to preservatives and chemicals via a full panel of sensitivity assays, concluding in the discovery of varying responses among *Neosartorya* spp. isolates. Metabolic profile of said isolates was established to be an important drive in the developments of sensitiveness or resistance of *Neosartorya* spp. fungi to preservatives and chemicals.

2. Materials and methods

2.1. Fungal isolate selection and experimental design

Twenty *Neosartorya* genus isolates procured from environmental samples, with the accession numbers provided in supplementary materials (Table S1) came from the sample collection of the Laboratory of Molecular and Environmental Microbiology (Department of Soil and Plant System of IA PAS) Lublin, Poland. These isolates were obtained from either soil (8 isolates) or strawberry cv. Senga Sengana (*Fragaria × ananassa* Duch.) fruit (12 isolates) material during the SONATA4 project (Frac M., project ID: 2012/07/D/NZ9/03357: https://projekty.ncn.gov.pl/index.php?projekt_id=202974) supported by National Science Centre. All the isolates were identified through the sequencing of the ITS1 region and β -tubulin genes (Table S1).

Step-wise selection process was performed, whereby the original 20 isolates were reduced to 10 and finally 5 isolates. At each step, isolates were chosen on the basis of represented range of susceptibility to preservatives or other chemicals according to the results of the tests described in the following sections. Based on the inhibition tests using disc-diffusion method and Biolog™ MT2 microassay (Biolog™, Hayward, CA, USA) (Frac, Gryta, Oszust, & Kotowicz, 2016) results, 3 main groups of fungi with different susceptibility to the tested preservative variants (isolates with high, medium and low sensitivity) were established. Isolates were selected from each sensitivity level based on the most consistent response to preservatives across the disc-diffusion and Biolog™ MT2 (Biolog™, Hayward, CA, USA) tests. Ten isolates were selected for phenotyping using various Biolog™ phenotype microarray (PM) microplates on a MicroStation readings system (ELX808BLG, Biolog™, Hayward, CA, USA) as described Panek, Frac, and Bilińska-Wielgus (2016). Finally, five isolates best representing the varying sensitivity levels (G150/14 as highly sensitive, G135/14 as the least and G127/14, G130/14 and G132/14 of intermediate sensitivity) were chosen for metabolic activity assays and scanning electron microscopy (SEM) (Phenom ProX, Thermo Fisher Scientific, Waltham, MA, USA) previously used and described by Maj, Pertile, Różalska, Skic, and Frac (2024).

The five isolates were visualized using SEM according to Pertile et al. (2021) and Maj et al. (2024) with modifications. After a 10-day long

cultivation period on Potato Dextrose Agar (PDA) medium (BioMaxima S. A., Lublin, Poland), the isolates were examined using an electron microscope - Phenom ProX (Thermo Fisher Scientific, Waltham, MA, USA). Samples were fixed to aluminium supports using conductive double-sided carbon adhesive tape, desiccated with silica gel, and coated with a 5 nm gold layer (Sputter Coater, CCU-010 LV, Safematic GmbH, Zizers, Switzerland) prior to SEM analysis. Images were captured using a BS detector at an accelerating voltage of 10 kV and at 200× magnification (Fig. S1).

2.2. Preparation of chemical preservatives and °Bx/pH combinations

Prior to each experiment, fresh sterile water solutions of preservatives and °Bx/pH combinations were prepared. Preservatives (Sigma-Aldrich, St. Louis, MO, USA) were obtained as easily soluble powder thus were simply dissolved in sterile water and filtered via 0.22 µm filters (Millex®, Ireland) to achieve the desired concentrations (Denyer & Hodges, 2004). Sucrose (purity grade of *pro analysis* (p.a.), Chempur, Piekary Śląskie, Poland) solutions were obtained via suspension in sterile water, followed by measurement of the sugar to water ratio values via a refractometer (ALLA, France) to obtain selected °Bx values (Ermiš et al., 2015). A pH-meter (CP-502 Elmetron, Poland) was used to achieve set pH values by correction via addition of 0.1 M NaOH (p.a. grade, Sigma-Aldrich, St. Louis, MO, USA) or 0.1 M HCl (p.a. grade, STANLAB Sp. z o.o., Lublin, Poland) solutions.

2.3. The effect of preservatives on *Neosartorya* spp. isolates

The growth inhibition of chemical preservatives and °Bx/pH combinations against 20 fungal isolates was studied according to a modified method of Pertile and Frac (2023). Firstly, 20 isolates were cultivated under optimal growing conditions (30 °C) for 10 days and their mycelium and ascospores were harvested, suspended in filamentous fungi inoculation fluid (FF-IF) (Biolog™, Hayward, CA, USA) and homogenized. The suspension from each isolate was diluted to reach 75% of transmittance and used to inoculate (300 µL) Petri dishes with PDA medium (BioMaxima, Poland). Subsequently, three sterile paper discs (Whatman No. 1, 5 mmø) were placed on the medium of each agar plate, and 30 µL of a sterile dilution of one of the analysed substances was applied to each disk. Therefore, each experimental variant was repeated three times. The chemical preservatives (p.a. grade) applied to the discs were: sodium metabisulfite, potassium sorbate, sodium bisulfite and sorbic acid (Sigma-Aldrich, St. Louis, MO, USA). Based on preliminary experiments and regulations (EFSA Panel on Food Additives and Flavourings et al., 2019; EFSA Panel on Food Additives and Flavourings et al., 2022), each preservative was tested at concentrations: 25 mg mL⁻¹, 50 mg mL⁻¹, 75 mg mL⁻¹, 100 mg mL⁻¹. The analysed combinations of °Bx/pH applied to the plates were similar to those found in apple juices: 45°Bx, 43°Bx, 39°Bx, 35°Bx, each at 3.2 and 3.5 pH (Park, Ha, & Kang, 2017). Plates were incubated at 30 °C for ten days, during which the areas of growth inhibition were measured every 24 h. The susceptibility of fungi to each substance was determined by measuring the formation of an inhibitory zone around each paper disc with a digital calliper (Kingfisher International Products B.V., The Netherlands). The results from these inhibition tests were used to select 10 isolates for further study, based on varying sensitivities to each substance.

2.4. Fungal sensitivity assay using Biolog™ MT2 MicroPlates

To determine the metabolic response in the presence of chemical preservatives or °Bx/pH combinations, an MT2 microplate (Biolog™, Hayward, CA, USA) assay was carried out as in Frac et al. (2016) on the ten fungal isolates selected in the first stage of the study. These isolates were re-cultured on PDA medium (BioMaxima, Poland) at 30 °C for ten days, after which the mycelium was collected and placed in a sterile filtering bag with FF inoculating fluid (Biolog™, Hayward, CA, USA) to

perform homogenization. The inoculum was then diluted in pure FF inoculating fluid to achieve 75% transmittance. MT2 microplates were filled with 100 µL of each chemical preservative or °Bx/pH combination and 50 µL of isolate inoculum. The positive control was composed of fungal inoculum and FF inoculating fluid (50 µL and 100 µL respectively). The test groups were composed of fungal inoculum mixed with chemical preservatives with the purity grade of *pro analysis* (p.a.) (sodium metabisulfite, potassium sorbate, sodium bisulfite sorbic acid at 25 mg mL⁻¹, 50 mg mL⁻¹, 100 mg mL⁻¹) or water-based sugar solutions of set pH (45°Bx of 3.5 pH, 43°Bx of 3.2 pH, 39°Bx of 3.2 pH). The experiment included two biological replicates per isolate, with technical measurements conducted in triplicate, resulting in a total of six repetitions. Each plate was incubated at 30 °C for 10 days. Readings were performed on Biolog™ MicroStation (ELX808BLG, Biolog™, Hayward, CA, USA) every 24 h. To avoid background interference, water values were obtained by mixing each of the substances' dilutions (100 µL) with FF-IF (50 µL) instead of inoculum and measuring at the same time as the treatment samples. Corrected treatment values were obtained by subtracting readings with their respective water values, and used to calculate the Average Fungal Respiration Intensity (AFRI; for the optical density at 490 nm) and Average Fungal Growth Intensity (AFGI; for the optical density at 750 nm) following by Maj et al. (2024).

2.5. Fungal chemical sensitivity analysis using Biolog™ PM MicroPlates

To determine the chemical sensitivity of the 10 *Neosartorya* spp. isolates, a panel of Biolog™ phenotype microarray (PM) microplates (PM21-PM25) (Biolog™, Hayward, CA, USA) was used, as described in Panek et al. (2016) with modifications. The plates contain an array of substances, such as anions, cations, antibiotics, chelators, cyclic compounds, membrane function compounds and nitrogen compounds. In total, 120 chemical substances were analysed. Ten fungal isolates were cultivated for 10 days on PDA medium (BioMaxima, Poland) at 30 °C, after which the fungal ascospores with mycelium were harvested from the surface and transferred into sterile inoculating fluid (FF-IF, Biolog™, Hayward, CA, USA). The suspension was further diluted to 62% of transmittance. For each fungal isolate, PM inoculating fluid was then prepared according to the manufacturer's protocol, by mixing 60 mL of sterile inoculating fluid with 4.5 mL of D-glucose (32×), 12 mL of yeast nitrogen base (12×), 67.2 mL sterile water and 300 µL of ascospore suspension. 100 µL of each PM inoculation fluid was transferred into the wells of PM plates. Two biological and three technical replicates were used for each isolate, amounting to a total of six replicates. Inoculated PM plates were incubated at 30 °C for 216 h. Readings were performed on a MicroStation (ELX808BLG, Biolog™, Hayward, CA, USA) every 24 h. In order to avoid background interference obtained results were corrected with their respective controls (optical density in water control well) and used to calculate the Average Fungal Respiration Intensity (AFRI; for the optical density at 490 nm) and Average Fungal Growth Intensity (AFGI; for the optical density at 750 nm). Graphs base lines were established using positive control results for fungal isolates development in FF-IF.

2.6. Fungal metabolic profile analysis using Biolog™ FF MicroPlates

The ability of each of the 10 *Neosartorya* spp. to use 95 carbon sources was evaluated using the Biolog filamentous fungi (FF) microplates (Biolog™, Hayward, CA, USA) containing tetrazolium dye. This approach is important for fungal functional diversity and catabolic versatility evaluation (Pinzari et al., 2016). The procedure was based on the manufacturer's protocol and the protocol described in Pawlik et al. (2019). The isolates were cultured for ten days at 30 °C on either regular PDA media (BioMaxima, Poland) or PDA media with an addition of sodium metabisulfite at a dose (150 µg mL⁻¹) which fulfills the European Union's safety regulations in food production (EFSA Panel on Food Additives and Flavourings et al., 2022). After cultivation, the ascospores

with mycelium were harvested for inoculum preparation by suspending the ascospores in inoculating fluid (FF-IF, Biolog™, Hayward, CA, USA) and adjusting the suspension to 75% of transmittance. Fungal ascospores suspensions were then added to each well of the microplate (100 µL per well). The experiment was carried out in two biological replications for each isolate and the measurements were performed in triplicates to obtain a total of six read-outs. Readings were performed on a MicroStation (ELX808BLG, Biolog™, Hayward, CA, USA) every 24 h. In order to avoid background interference obtained results were corrected by subtracting the optical density of the water control (inoculum resuspended in water) at the corresponding measurement hour and utilized to calculate the Average Fungal Respiration Intensity (AFRI; for the optical density at 490 nm) and Average Fungal Growth Intensity (AFGI; for the optical density at 750 nm). Functional diversity was considered to be equal to substrate utilization richness, i.e. the number of different substrates used by each isolate, measured as optical density (OD) ($OD > 0.25$) (Pawlik et al., 2019).

2.7. Fungal metabolic activity analysis using a fluorescence-based method with fluorescein diacetate (FDA)

Five *Neosartorya* spp. isolates of varying sensitivity to preservatives, were cultured separately for ten and thirty days at 30 °C using two different PDA media variants: one with the addition of sodium metabisulfite (150 µg mL⁻¹), and a control without sodium metabisulfite addition. To assess the metabolic activity of the fungal isolates, a modified method based on the use of fluorescence of fluorescein diacetate (FDA) was used (Różalska, Glińska, & Długoński, 2014). FDA has the ability to penetrate cell membranes and is taken up by cells where cellular enzymes (estrases) cleave the acetate groups, yielding fluorescein, a fluorescent compound. Due to its polar nature, fluorescein cannot cross intact cell membranes and accumulates in cells, emitting a yellow-green fluorescence when the cell is viable (Yang et al., 1995). This fluorescence emission provides a direct indicator of cellular metabolic activity. Lack of observable fluorescence can be indicative of cellular enzyme inactivity and overall metabolic inefficiency (Adam & Duncan, 2001). A drop in fluorescence after the use of sodium metabisulfite indicates cell structure impairment due to prolonged contact with the preservative (Soboń, Szweczyk, Różalska, & Długoński, 2018).

Following cultivation, ascospores with mycelium from each fungal isolate were gently removed from the plate, and a water-based solution was created to achieve 10% of transmittance. Subsequently, 1 mL of this suspension was placed into individual wells of a 24-well plate (Biologix®, USA). FDA powder (Sigma-Aldrich, USA) was dissolved in acetone, p.a., (P.P.H Standard, Poland) to obtain a 0.7 M solution. The solution was mixed with phosphate buffer at pH 6.4 (1 mL of FDA solution per 9 mL of phosphate buffer) and 1 mL of this mixture was added to each of the wells of the plate. This was followed by incubation at room temperature in the absence of light. The change in FDA fluorescence was measured using an Infinite® 200 Pro reader (Tecan, Switzerland) after 0, 30 and 60 min of incubation (at excitation wavelength of 485 nm and emission wavelength of 520 nm). Fluorescence measurements were expressed as fluorescence intensity in arbitrary units (AU).

2.8. Statistical analyses

Statistical analyses were performed using Statistica™ software (TIBCO Software Inc. V13.3) and R (version 4.3.1). Normality distribution was tested via Shapiro-Wilk test and homogeneity of variance was checked via Levene's test for all results. Kruskal-Wallis ANOVA and post hoc Wilcoxon tests were performed in datasets with no normal distribution. When homogeneity of variance was not met ($p < 0.05$ in the Levene's test), Welch test and Games-Howell post hoc test were conducted using the package rstatix (Kassambara, 2023) in R (version 4.3.1). Cluster analyses were conducted through Euclidian distance and Wards' method, and visualized with a heatmap using the R packages:

ComplexHeatmap (Gu, 2016; Gu, 2022), *circIzize* (Gu, Gu, Eils, Schlesner, & Brors, 2014). All data were presented as 95% confidence intervals with statistical significance established at $P < 0.05$.

3. Results

3.1. Evaluation of the effect of preservatives on *Neosartorya* spp. isolates using zone of inhibition test method and Biolog™ MT2 MicroPlates

The inhibition effect of chemical preservatives and Bx°/pH combinations was investigated by measuring the plate areas devoid of mycelium growth due to the influence of these substances (Fig. S2). All tested chemical preservatives (sodium metabisulfite, potassium sorbate, sodium bisulfite and sorbic acid) caused growth inhibition of 20 *Neosartorya* spp. isolates (Fig. 1). Results varied depending on the substance used and studied isolate. The least growth was observed sodium bisulfite for most isolates. Sodium metabisulfite yielded moderate success in inhibiting *Neosartorya* spp. growth. Potassium sorbate and sorbic acid clustered together and presented similar, lower levels of inhibitory properties.

In almost all cases, increasing preservative dosage yielded a greater zone of inhibition, with the exception of potassium sorbate where the inhibition zone was much larger at 75 mg mL⁻¹ than at the higher dose of 100 mg mL⁻¹ (Fig. 2A). Sodium metabisulfite and sodium bisulfite presented similar inhibitory properties in both the 75 mg mL⁻¹ and 100 mg mL⁻¹ doses. The strongest inhibitory effect was achieved with the use of sodium metabisulfite at these concentrations, closely followed by sodium bisulfite.

The results were also analysed for each isolate individually (Fig. S3). This allowed the observation of innate reaction differences between closely related isolates. Sodium metabisulfite had the strongest inhibitory effect on a few of the tested fungal samples (e.g. G159/14, G132/14, G131/14, G161/14, G155/14) (Fig. S3A) while for others it was similar to sodium bisulfite in its inhibition (e.g. G125/14, G135/14, G151/14, G153/14, G160/14, G259/14). Sodium bisulfite surpassed other chemical preservatives in its inhibition of isolates G154/14 and G239/14. Isolate G126/14 was affected most by potassium sorbate alone, while isolate G150/14 was most affected by both potassium sorbate and sorbic acid. Most isolates showed little sensitivity to sorbic acid in comparison to the three other substances, as inhibition areas caused by this chemical were significantly smaller than all three other preservatives for isolates G125/14, G126/14, G129/14, G130/14, G132/14, G135/14, G151/14, G153/14, G154/14, G159/14, G160/14, G161/14, G259/14.

According to the inhibition test, all 8 tested combinations of "Bx/pH caused growth inhibition of *Neosartorya* spp. (Fig. 2B) inhibition zone diameters were all statistically similar. Overall, measured diameters of growth inhibition caused by "Bx and pH combinations attained between 3.2 and 3.9 mm on average through the duration of the experiment. However, response variations between isolates were also visible (Fig. S3B), and mycelium growth of less than half (9) of the isolates was statistically different to that of the control. Growth inhibition was clearly evident in isolates G126/14, G127/14, G131/14, G132/14, G150/14, G226/14, G239/14, G259/14 and G155/14, while for 11 isolates no significant differences in growth capabilities under the influence of "Bx/pH combinations occurred.

Taking into account these results, a varying sensitivity pattern emerged among the 20 tested isolates (Fig. 1, S3A, S3B). Isolate G150/14 had an extremely robust reaction and was thus characterized as the most sensitive. Other fungal isolates groups were established as follows: sensitive (G155/14, G154/14, G239/14, G159/14, G126/14, G259/14), moderately sensitive (G129/14, G127/14, G253/14, G132/14, G151/14, G153/14, G226/14, G131/14), least sensitive (G125/14, G135/14, G160/14, G161/14, G130/14).

The inhibition effect of preservatives was also analysed using Biolog™ MT2 microplates (Fig. 2C, D). The same preservatives were tested

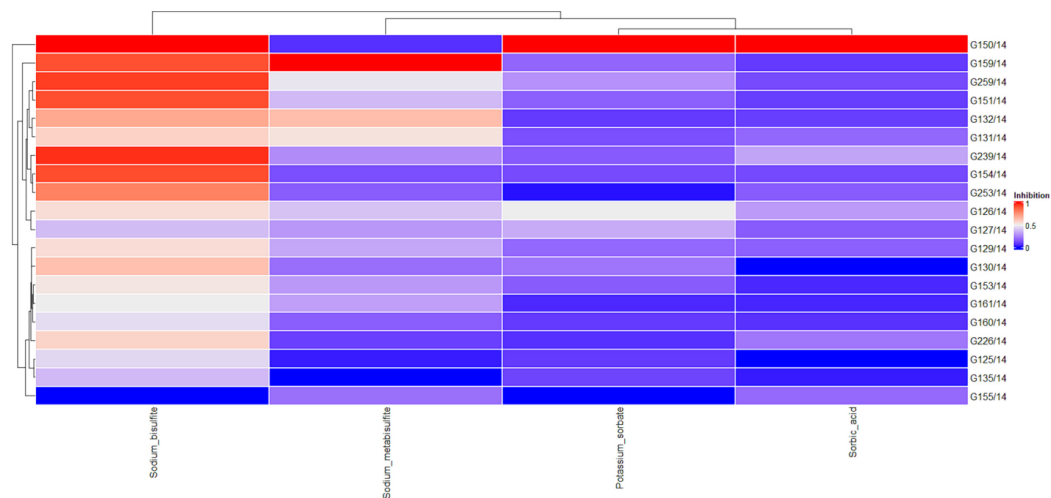


Fig. 1. Heatmap presenting the inhibition profile of different *Neosartorya* spp. isolates under the influence of chemical preservatives and cluster analysis with levels of inhibition of the 20 isolates to different chemical preservatives.

as in the inhibition tests, yet their effect on general fungal biomass production did not directly mirror those results (Fig. 2C). All preservatives inhibited fungal growth to some degree, but the level at which this occurred varied and was less consistent than in the inhibition test (Fig. 2C). Average Fungal Growth Intensity (AFGI) differed significantly from the control at all levels of sodium metabisulfite. Sodium bisulfite and potassium sorbate both had a stronger antifungal effect than sodium metabisulfite, but this occurred only at 25 and 50 mg mL⁻¹ respectively. For sorbic acid AFGI was lower than the control at 25 and 75 mg mL⁻¹, but showed a marked increase at 50 mg mL⁻¹. Sorbic acid at 50 mg mL⁻¹ yielded abundant fungal biomass production reflected as higher values than the controls' optical densities. Overall, potassium sorbate at 50 mg mL⁻¹ and sodium bisulfite at 50 mg mL⁻¹ and 25 mg mL⁻¹ seemed to turnout the best antifungal properties in this experiment. Individual isolates also showed varying in their sensitivity to the preservatives. For three isolates (G127/14, G154/14 and G160/14) no significant differences were observed compared to the control for all preservatives (Fig. S3C), while the remaining isolates did experience inhibition but this varied per added substance. Notably, sorbic acid induced the largest increase in AFGI among these isolates, as evidenced by isolates G129/14, G130/14, and G151/14. Conversely, sodium metabisulfite and sodium bisulfite typically resulted in a decrease in AFGI values, exemplified by isolates such as G130/14, G132/14, and G135/14. Fungal isolate G150/14 was the most sensitive for all tested food preservatives.

Three variants of °Bx/pH combinations were tested for their properties to effectively reduce AFGI on MT2 microplates (Fig. 2D). The solution of 45°Bx at pH 3.5 did not provide statistically different results to those of control samples while those of 39°Bx at pH 3.2 and 43°Bx at pH 3.2 had had significantly reduced *Neosartorya* spp. biomass production. However, analysis of the results for the effect of the preservatives on each isolate separately revealed a generally low level of inhibition whereby only two isolates (G135/14, G151/14) experienced a clear decrease in AFGI for all treatments (Fig. S3D). Therefore, the °Bx and pH combinations were not studied further.

On the basis of these two inhibition tests, 39°Bx of 3.2 pH can be established as the most inhibitory combination. Among tested preservatives during inhibition test and MT2 analysis, doses of 100, 50 and 75 mg mL⁻¹ presented to be the concentrations of the most potent antifungal properties (Fig. 2A, C). Overall, the most uniform inhibitory

features are displayed by sodium metabisulfite and sodium bisulfite. Despite that sorbic acid at 50 mg mL⁻¹ causes robust zones of inhibition, it allowed for abundant AFGI rise within the MT2 microplate experiment. Sodium bisulfite and sodium metabisulfite were identified as the most effective chemical preservatives across both study methods, with sodium metabisulfite showing the strongest inhibition for most isolates in the inhibition test and being particularly potent at higher concentrations. In contrast, potassium sorbate and sorbic acid exhibited lower inhibitory properties, with sorbic acid at 100 mg mL⁻¹ being the most effective in the MT2 plate assay. The efficacy of °Bx/pH combinations in inhibiting fungal growth was less consistent, with some combinations allowing for biomass generation that exceeded control values.

A relatively consistent pattern of sensitivity was observed from the inhibition tests, allowing a selection of 10 isolates for further testing. On the basis of MT2 microplate and inhibition experiments, sodium metabisulfite at a low dose of 150 µg mL⁻¹ was chosen as a preferred media additive to induce possible intra-metabolic changes within fungi while still permitting mycelium growth due to low concentration.

3.2. Fungal metabolic profile analysis using Biolog™ FF MicroPlates after the pre-incubation with sodium metabisulfite

The effect of sodium metabisulfite on 10 *Neosartorya* spp. isolates' metabolic abilities to utilize and grow on 95 carbon substrates was measured via Biolog™ FF MicroPlates (Figs. 3, 4). The results were analysed separately for each of the studied wavelengths (490 and 750 nm). Under control conditions most isolates exhibited high AFGI values when grown in the presence of carbohydrates, carboxylic acids and the group of other substances, whereas amino acids yielded the least mycelium production (Fig. 3A). Under optimal conditions, isolates G132/14, G150/14 and G130/14 were characterized by high mycelium growth on carbon sources available in the microplate, while isolates G154/14 and G160/14 were characterized by low mycelium production. Among isolates treated with sodium metabisulfite, the two substrate groups carbohydrates and a group of other substances emerged as the most optimal for biomass production (Fig. 3B). This is similar to that of the control group although the optical density values with sodium metabisulfite were much lower for all isolates and ranged from 0.01 to 0.09 compared to the control group range of 0.05 to 0.75. Therefore, the

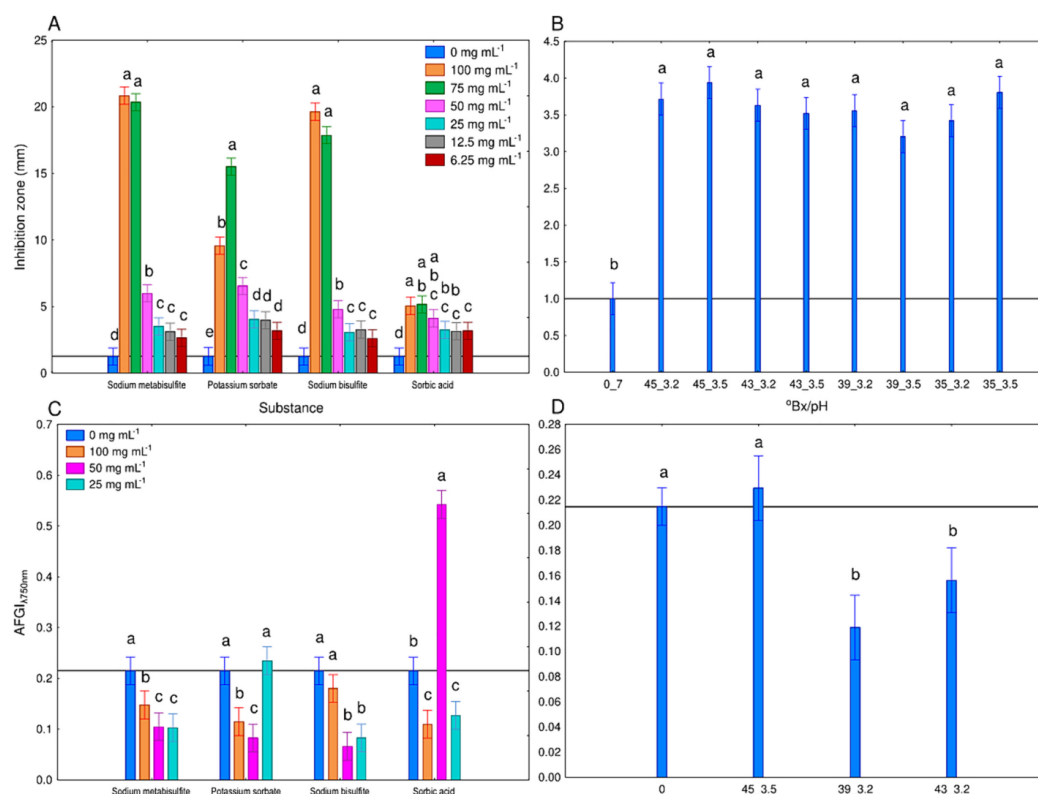


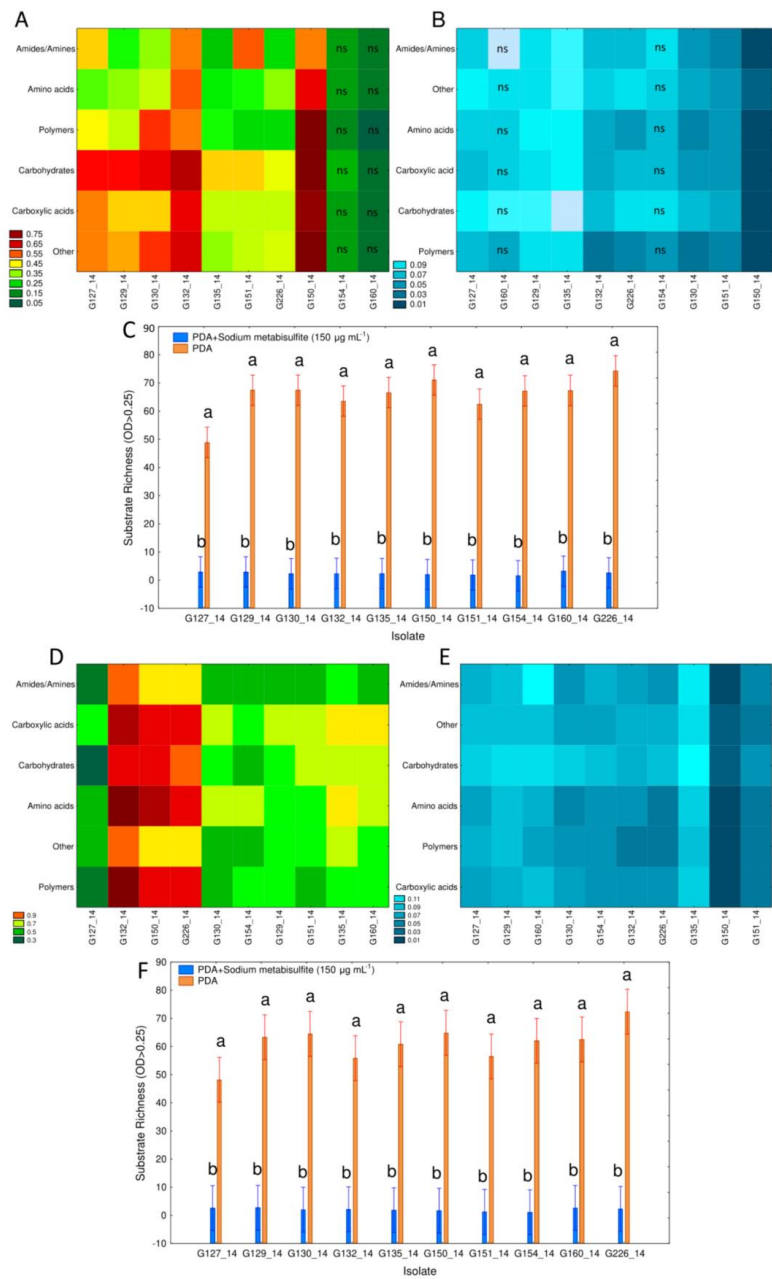
Fig. 2. Influence of tested substances on *Neosartorya* spp. isolates. (A) Response to preservatives across all tested isolates, with letters indicating significant differences between doses per substrate. (B) Average inhibition zones in response to °Bx/pH combinations across all tested isolates, with letters indicating significant differences between the control and each combination. (C) Average Fungal Growth Intensity on MT2 plates in response to preservatives across all tested isolates, with letters indicating significant differences between doses per food preservative. (D) Average Fungal Growth Intensity on MT2 plates in response to °Bx/pH across all tested isolates, with letters indicating significant differences between the control and each combination. Black lines represent the control value measured within FF-inoculation fluid without the analysed substances.

growth ability of all isolates was reduced by preincubation with the preservative. However, individual isolates did not respond uniformly and presented a varied ability for biomass production (Fig. 3B). The lowest density could be observed for isolates G150/14, G132/14 and G130/14 in the presence of sodium metabisulfite while they showed the highest biomass production under optimal conditions, indicating a high sensitivity to the preservative. In contrast, isolates G135/14 and 129/14 showed great growth capabilities on all substrate groups after preincubation with sodium metabisulfite, deeming them the least sensitive to the effect of the preservative. The substrate richness for fungal biomass production showed that isolates exhibited efficient metabolism of carbon substrates for fungal biomass production without preincubation with sodium metabisulfite (Fig. 3C). However, this decreased for all isolates after preincubation with sodium metabisulfite (Fig. 3C).

Metabolic capabilities of *Neosartorya* spp. isolates were also observed at 490 nm (Fig. 3). In general, respiration values varied from 0.01 to 0.11 for treated isolates while values of fungi in the control group had values between 0.3 and 1.0 (Fig. 3D, E). Respiratory intensity (AFRI) of isolates preincubated on PDA media was increased for carboxylic acids, carbohydrates, and amino acids (Fig. 3D). The group of amines and

amides had the lowest respiration values. Three isolates (G132/14, G150/14, G226/14) demonstrated high levels of carbon source utilization but after preincubation with sodium metabisulfite, their respiratory capabilities dropped from approximately 0.7 to 1.0 range to 0.01 to 0.07 (Fig. 3E). This was especially prominent for G150/14 whose respiration almost stopped. Respiratory intensity seemed to the same general trend as that of AFI values for some isolates (e.g. G135/14, G150/14, G151/14, G226/14) with notable differences on some carbon sources indicative of substrate stress. Polymers caused a heightened respiratory rate while having low abundant mycelium growth in most isolates. This characterizes them as universal stress factor seen in samples with a weakened metabolism. Amides and amines, as well as carboxylic acids permitted some isolates (e.g. G160/14, G154/14) to maintain an efficient metabolism. Substrate richness differed greatly for all isolates depending on the presence of sodium metabisulfite during preincubation (Fig. 3F). The preservative caused a decrease in metabolic capabilities of fungi and weakened their respiration. All isolates presented statistical differences in the amount of substrates available for respiration.

In general, G150/14 was the most sensitive to preservatives and characterized by the highest growth on almost all substrate groups.



(caption on next page)

Fig. 3. *Neosartorya* spp. isolates' analysis at 750 nm (A, B, C) and 490 nm (D, E, F) on FF microplates after 144 h of incubation. (A) Average Fungal Growth Intensity (AFGI) of isolates grown on PDA media. (B) Average Fungal Growth Intensity (AFGI) of isolates grown on PDA media enriched with sodium metabisulfite. (C) Substrate Richness for fungal biomass production among control and treated isolates, with letters indicating significant differences between measurements for each isolate. (D) Average Fungal Respiration Intensity (AFRI) of isolates grown on PDA media. (E) Average Fungal Respiration Intensity (AFRI) of isolates grown on PDA media enriched with sodium metabisulfite. (F) Substrate Richness utilization by fungi among control and treated isolates, with letters indicating significant differences between measurements in the presence of sodium metabisulfite and control for each isolate separately. Colour scale applied to heatmaps indicates optical density on each of the 6 carbon substrate groups, and lack of "ns" (not significant) for particular substrates group indicates significant differences in carbon sources guild utilization/growth intensity between fungal isolates cultivated in the presence of sodium metabisulfite and the control, while "ns" indicates no significant statistical differences.

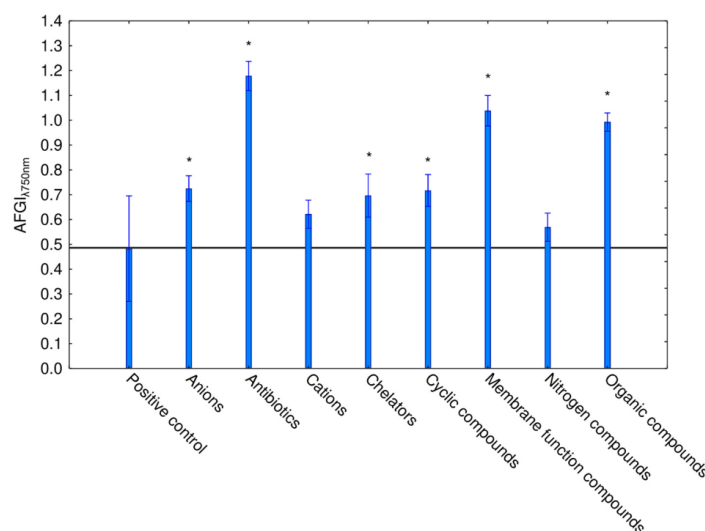


Fig. 4. Average Fungal Growth Intensity of *Neosartorya* spp. isolates on substrate groups in 21–25 PM plates. Data acquired during 144 h of incubation. The black line signifies the optical density value of the positive control and asterisks indicate significant differences between AFGI of the control and a particular substrate group.

G160/14 was characterized by low sensitivity to preservatives and very weak growth on almost all substrate groups. G127/14, G129/14, G130/14 were characterized by average sensitivity to preservatives and intensive growth on half group of the substrates (carbohydrates, carboxylic acids) and weak growth on the rest of the tested substrate groups.

The influence of each of the 95 substrates on the tested isolates showed that the control (Fig. S4) presented a much wider and higher optical density range (0.1–1.1) than the group treated with sodium metabisulfite (0.01–0.31) (Fig. S5), meaning the substrates were much better utilized. For the control group several substances elicited a low respiratory rate, mainly: N-Acetyl-D-Glucosamine, N-Acetyl-D-Mannosamine, α -Cyclodextrin, β -Cyclodextrin, Adenosine-5'-Monophosphate (Fig. S4A). From these, only Adenosine-5'-Monophosphate resulted in extremely low biomass production, hence this substance can be labelled as mostly unmetabolizable by *Neosartorya* spp. with an exception of isolates G132/14 and G150/14 (Fig. S4B). The least abundant mycelial growth was observed on: gamma-hydroxy-butyric acid, succinic acid, sebacic acid and D-saccharic acid (Fig. S4B). Isolates G154/14 and G160/14 were characterized by considerably lower metabolism than the other isolates, due to the high uptake of substrates yielding no significant biomass gain. G132/14, G135/14, G226/14, and G151/14 showed a lower yield in mass compared to their respiration values. Isolates of somewhat similar input/output values were G150/14 and G129/14. The isolates G127/14, G130/14 showed slightly elevated metabolic activity levels because of successful biomass production despite low substrate

utilization. Therefore, the metabolism makeup of the tested isolates differed despite identical preincubation conditions. After preincubation with sodium metabisulfite, a drastic drop in metabolic activity was observed (Fig. S5). Both values of respiratory intensity and biomass production decreased. The only substance that was universally oxidized (Fig. S5A) and that permitted abundant biomass growth (Fig. S5B) was i-erythritol. High respiratory activity and growth on D-sorbitol and some on succinic acid mono-methyl ester, sucrose and L-serine was also observed for several isolates (Fig. S5A, B), none of these substrates were as consistently used in biomass production by all isolates as i-erythritol (Fig. S5B).

3.3. Fungal chemical sensitivity analysis

The BiologTM PM chemical sensitivity panels offer a wide array of ready substrates available for analysis. This assay (PM 21–25) focuses on 120 chemical substances divided into 8 groups. Growth of 10 fungal isolates in the presence of these substrates showed that all caused an increase in fungal biomass production. Universally, isolates were able to overcome any inhibitory properties of chemical substances after 96 h of preincubation and reached a beginning of an AFGI plateau phase by the 144th hour. After 144 h of microplate incubation, six groups of substrates supported greater biomass gain than observed under control conditions (Fig. 4).

Out of eight substrate groups present on the PM 21–25 microplates, the highest *Neosartorya* spp. growth was with antibiotics. Other

substance groups with higher growth than the positive control were anions, chelators, cyclic compounds, membrane function compounds and organic compounds. Cations and nitrogen compounds did not result in a significantly greater biomass than the positive control. However, when we considered each substrate in each of the 8 available chemical groups individually, we found significant fungal growth inhibition after 144 h of incubation (Fig. 5). Among the anions, the most prominent inhibitory properties were observed for sodium arsenate, sodium cyanate and sodium fluoride, which all successfully reduced *Neosartorya* spp. development (Fig. 5A). However many anions either had no discernible influence on the isolates (sodium metaborate tetrahydrate, boric acid, sodium (meta)periodate and sodium cyanide) or generated greater biomass production than under optimal control conditions (sodium dichromate, sodium metavanadate, potassium iodide, sodium arsenite, sodium orthovanadate, sodium selenate, sodium metasilicate and potassium chromate). Similarly in the cations group, several substances caused inhibition (zinc chloride, cobalt (II) chloride hexahydrate and copper (II) chloride dihydrate), stimulation (aluminium sulfate, magnesium chloride and manganese (II) chloride) or had no effect on *Neosartorya* spp. growth (remaining substances, e.g. copper (II) sulfate) (Fig. 5B). Antibiotics either stimulated fungal growth (neomycin, polymyxin B, cephalotin, paromomycin, apramycin sulfate, tobramycin, dequalinium chloride, amphotericin B and kanamycin monosulfate) or had no effect on it (ceftriaxone, hygromycin B, pentamidine isethionate and D-cycloserine) (Fig. 5C). The same situation could be observed for the mode of action of chelators, with stimulating substances being 2,2'-dipyridyl, 1-hydroxypyridine-2-thione and ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), and substances that had no effect being ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA), 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) and sodium pyrophosphate decahydrate (Fig. 5D). Cyclic compounds could also be split into three effect group: abundant growth (compound 48/83, cycloheximide, doxycycline hyclate and berberine chloride), inhibition (promethazine and methyl viologen dichloride hydrate) or fungal indifference (e.g. caffeine, benzamidine and chlorotetracycline hydrochloride) (Fig. 5E). Membrane function compounds mostly improved growth conditions for studied isolates (guanidine hydrochloride, niastatin, protamine sulfate, cetylpyridinium chloride, domiphen bromide, myclobutanil, benzethonium chloride, poly-L-lisine hydrochloride and miconazole nitrate) (Fig. 5F). However, two of the membrane function related substances caused growth inhibition (propiconazole, niaproof). Amitriptyline hydrochloride had no statistically significant influence on *Neosartorya* spp. AFGI values. Conversely, most nitrogen compounds (diamide, 3-amino-1,2,4-triazole, urea hydrogen peroxide, sodium azide, sodium nitrite, tetrazolium violet, 4-aminopyridine and fluorodeoxyuridine) did not produce any substantial effect on *Neosartorya* spp. growth (Fig. 5G). Nitrogen-based substances that diminished the ability of isolates to develop were thiourea, cisplatin and hydroxyurea, whereas hydroxylamine hydrochloride, isoniazid and mechlorethamine hydrochloride stimulated growth. Only two (zaragozic acid A and 5-fluorocytosine) of the 33 organic compounds elicited a reduction in quantifiable optical density, as illustrated in Fig. 5H. Thirteen of said substances had no effect on *Neosartorya* spp. biomass production (e.g. trifluoperazine, D-serine and tartaric acid). Eighteen of the organic compounds caused high levels of mycelium growth (especially observable for chloroquine, thialysine and chloroalanine hydrochloride). Among all of described substances, the most prominent growth inhibitory properties could be attributed to sodium cyanate (anion), copper (III) chloride dihydrate (cation), niaproof (membrane function compound), methyl viologen dichloride hydrate (cyclic compound) and cisplatin and hydroxyurea (nitrogen compounds) whereas the most abundant growth was achieved with neomycin (antibiotic) and chloroquine (organic compound).

Intraspecific variation in growth on these substrates was also observed for individual *Neosartorya* spp. isolates (Fig. S6). The isolate G150/14 showed no increase in mycelium development in the presence

of widely used substances such as potassium chromate, aluminium sulfate, or neomycin, but displayed a slightly higher resistance to toxic agents such as sodium fluoride, than the other isolates (Fig. S6A). While growth patterns varied, only four isolates effectively used sodium orthovanadate for biomass growth; however, three of these were less effective with sodium selenate (Fig. S6A). Notably, aluminium sulfate was a common substrate, although isolate G150/14 and two others showed different mycelium growth patterns (Fig. S6B). A subgroup including G129/14 and G132/14 showed similar growth responses to magnesium and manganese chlorides. Isolates G127/14, G226/14, G129/14, G132/14, and G151/14 exhibited the broadest metabolic responses to cations. Under antibiotics, most isolates showed reduced growth except G135/14, which was, highly susceptible to specific antibiotics (Fig. S6C). G127/14 displayed the most versatile growth across 14 antibiotics. Responses to chelators varied, except for EDTA, which was uniformly metabolized by most isolates (Fig. S6D). G135/14 showed resilience against sodium pyrophosphate decahydrate but not against 2,2'-dipyridyl. Certain cyclic compounds universally hindered growth, but G150/14 was unaffected by some, yet sensitive to others affecting membrane functions, indicating particular vulnerabilities (Fig. S6E, S6F). Key inhibitors of growth for all isolates included niaproof and propiconazole. Nitrogen compounds were generally incompatible with *Neosartorya* spp., except hydroxyurea, which didn't affect G150/14. G127/13 showed broad metabolic capabilities, using several substances for significant growth (Fig. S6G).

Organic compounds revealed universal antifungal substances such as 5-fluorocytosine and zaragozic acid A, with varying metabolic responses among isolates (Fig. S6H). G150/14 was unable to use advantageous components (e.g. thialysine) and G129/14 was unique in its hindrance with ibuprofen. In conclusion, some disparities between sensitive and insensitive isolates were visible. Resilient isolates (e.g. G135/14, G160/14) did not metabolise e.g. mechlorethamine hydrochloride, sodium orthovanadate and dequalinium chloride as effectively as sensitive isolates (e.g. G127/14). In contrast to sensitive isolates, insensitive isolates attained more abundant biomass growth on e.g. sodium pyrophosphate decahydrate, berberine chloride and sodium metasilicate. In general, growth of resilient isolates (G135/14, G160/14, G154/14) tended to be lower than sensitive isolate growth (G150/14, G127/14), especially on polymers or amines and amines.

3.4. Fungal metabolic activity analysis

The metabolic activity of the isolates was assessed using the FDA staining method (Fig. S7). The results show that the general fluorescence emission was significantly lower after prolonged exposure (30 days) to a low dose of sodium metabisulfite ($150 \mu\text{g mL}^{-1}$) (Fig. 6A). However, the shorter preincubation period of 10 days did not cause statistically significant differences in fluorescence intensity between the control and the group treated with sodium metabisulfite. The results show that the general fluorescence emission was significantly lower after prolonged exposure (30 days) to a low dose of sodium metabisulfite ($150 \mu\text{g mL}^{-1}$).

The influence of sodium metabisulfite on individual isolates after 10 and 30 days was also determined (Fig. 6B, C). Here, the intraspecific differences between single isolates become apparent. After 10 days of preincubation only two isolates (G130/14, G150/14) did not present significant metabolic changes (Fig. 6B). Isolates G127/14, G132/14 presented a diminished fluorescence intensity linked with a lowered metabolic activity. Interestingly, in the presence of sodium metabisulfite, isolate G135/14 developed a very high metabolic response and emitted almost three times as much fluorescence compared to its control. After 30 days of exposure 4 isolates (G127/14, G130/14, G132/14, G135/14) had reduced fluorescence readings (Fig. 6C). The only isolate that remained unaffected was G150/14, which was consistent with the highest overall metabolic activity and biomass production on different substrates (Fig. 3A, D).

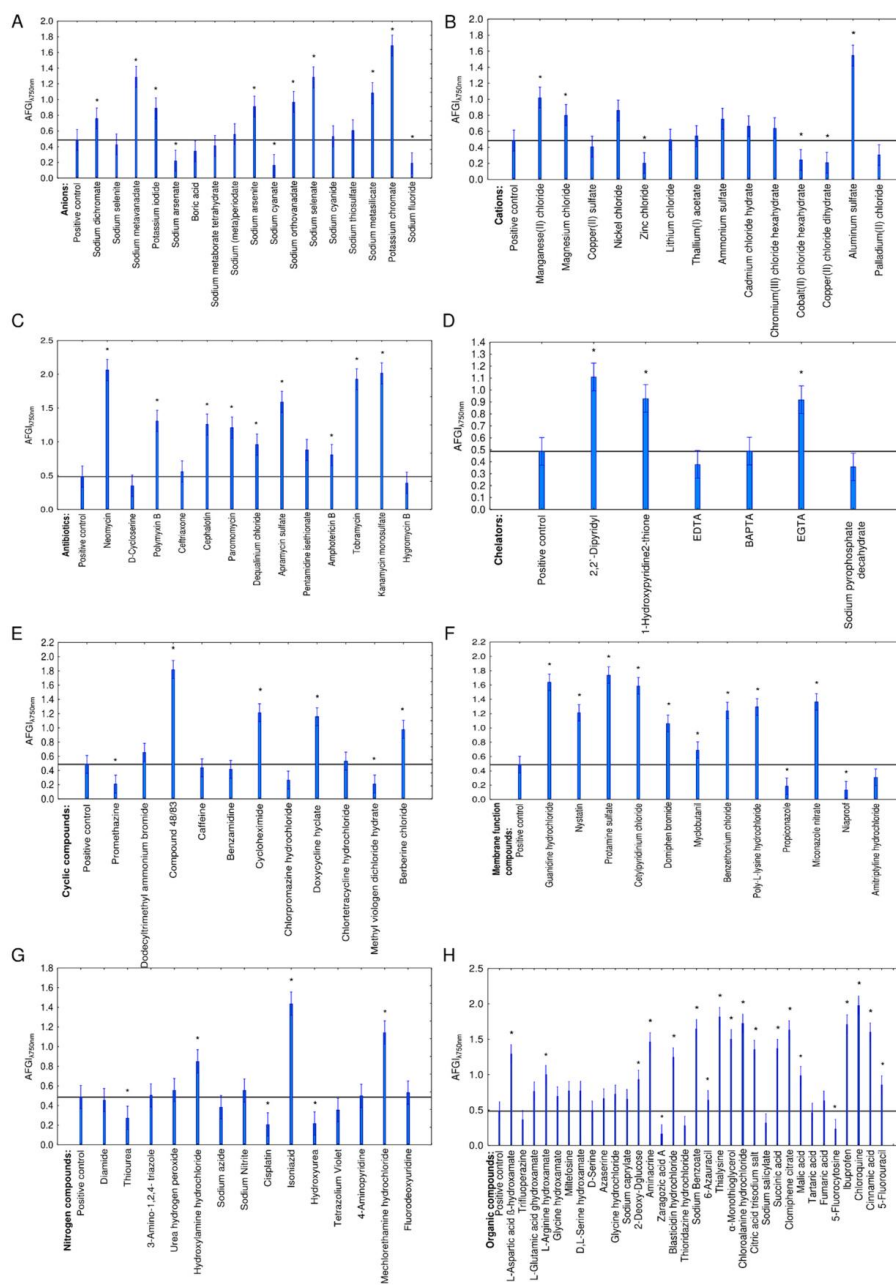


Fig. 5. *Neosartorya* spp. biomass production within each substrate group inside the 21–25 PM microplates after 144 h incubation period. Graphs are divided according to substrate groups and are as follows: (A) anions, (B) cations, (C) antibiotics, (D) chelators, (E) cyclic compounds, (F) membrane function compounds, (G) nitrogen compounds, (H) organic compounds. Black lines signify the control value and asterisks indicate significant differences between AFGI of the control and a particular substrate.

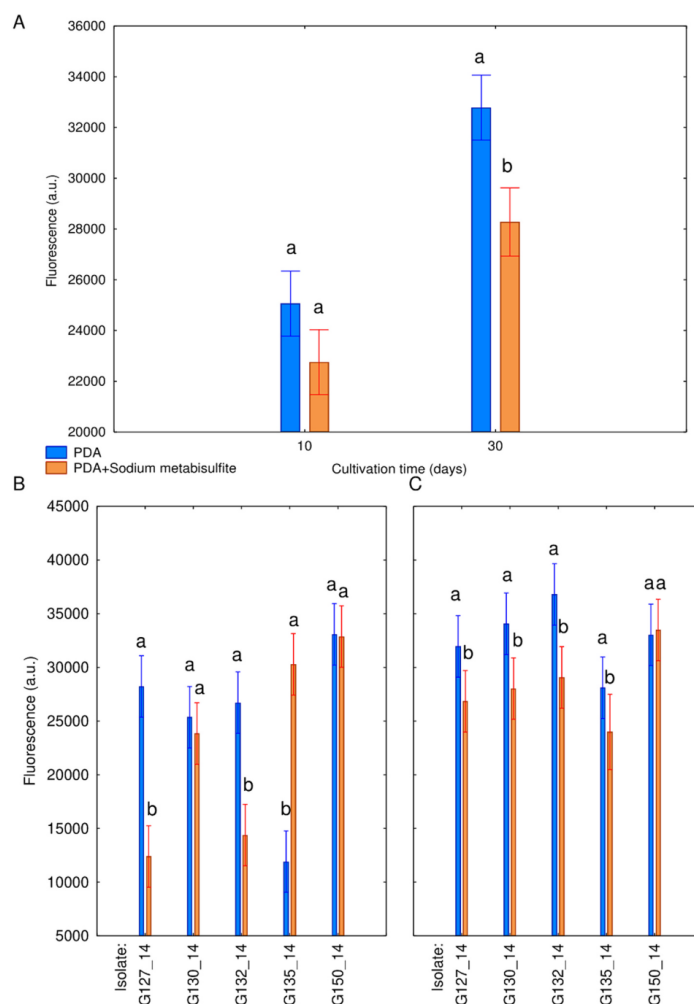


Fig. 6. Fluorescence intensity of *Neosartorya* spp. samples measurable after 30 min incubation. (A) Fluorescence intensity for samples cultivated for 10 and 30 days in two variants: on PDA medium and on PDA medium with sodium metabisulfite ($150 \mu\text{g mL}^{-1}$). (B) Fluorescence intensity for individual isolates and their respective controls after 10 days of incubation. (C) Fluorescence intensity for individual isolates and their respective controls after 30 days of incubation.

4. Discussion

Aspergillus section Fumigati contains 25 species, with 8 *Aspergillus* spp. anamorphs and 7 *Neosartorya* spp. teleomorphs (Samson, Hong, & Frisvad, 2006). Due to their close resemblance, these species are sometimes misidentified which could lead to the implementation of improper management tactics, such as pasteurization which is survivable for *Neosartorya* spp. ascospores (Balajee et al., 2005). It is of utmost importance to agriculture and food production to characterize the genus *Neosartorya* and find probable antifungal agents effective against different isolates. Divergence in sensitivity between sexual and asexual forms and closely related isolates has been recorded, but mainly in modern medical literature that was focused on clinical trials and drug effectiveness, and not on the agricultural practices that are also

endangered by spoilage by *Neosartorya* spp. (Laura et al., 2008; Vinh et al., 2009).

Some sources report that certain preservatives such as potassium sorbate, can have effective inhibitory properties against *Neosartorya* spp. (Rajashekhara et al., 2000). For example, Nielsen and Beuchat (1989) showed that *Neosartorya* spp. growth can be repressed by potassium sorbate, and at a dose of 75 mg mL^{-1} it completely inhibits the germination of ascospores (Nielsen & Beuchat, 1989). In our inhibition tests, a dose of 25 mg mL^{-1} was enough to warrant a similar growth response (Fig. 2A). López, Lima-Coelho, and Ferreira (2009) stated that sodium metabisulfite is one of the most effective preservatives to inhibit the growth of *Neosartorya* spp., with lasting effects even with low concentrations (López et al., 2009). This explains why the preincubation with sodium metabisulfite led to a similar decrease of the AFGI, at a

concentration of 25 and 50 mg mL⁻¹ (Fig. 2C). However, we found that at the same sodium bisulfite could be an even stronger preservative.

A pH dependent growth ability of fungi has previously been reported in literature, such as the decrease of *N. fischeri* growth with a decrease in pH values (Nielsen, 1991). This was mirrored in our experiment where lower pH values tended to diminish growth capabilities of the isolates (Fig. 2B). During the MT2 microplate analysis, we observed that the higher available sugar content and pH have a positive influence on mycelium growth that did not differ from that of the control. We obtained similar findings to Lane Paixão dos Santos, Samapundo, Van Impe, Sant'Ana, and Devlieghere (2020), indicating that degrees Brix did not exhibit satisfactory antifungal properties against *Neosartorya* spp. The tolerance of microorganisms to high sugar levels present in e.g. jams presents a challenge for the microbial stability of products (Figueroa, 2007). The combined effect of °Bx and pH on fungal growth is likely complex and influenced by interactions between these two factors and needs further study.

The results of the inhibition tests show that all °Bx/pH combinations cause growth inhibition (Fig. 2B), whereas MT2 analysis the 45°Bx at pH 3.5 does not cause a statistically significant AFGI decrease. The observed differences may arise from the specific conditions of each assay. It is conceivable that divergent outcomes arose due to the utilization of distinct analysis methods, which are inherently incomparable owing to their disparate modes of contact between the fungal ascospores with mycelium and the analysed substances. Disc-diffusion methods of inhibition primarily test the physical inhibition of fungal growth. The formation of clear zones around the disc indicates a direct impact on the ability of the fungus to proliferate (Biemer, 1973). The Biolog™ MT2 microplate method assesses metabolic activity through the reduction of tetrazolium dye, providing a colour change as an indirect measure of fungal growth. It focuses on the overall metabolic response of the fungus rather than just growth inhibition (Frac et al., 2016). The notable disparity between the two methodologies is perhaps that in the disc-diffusion inhibition test the substance under analysis directly contacts only those fungal hyphae in direct contact with the paper disc, whereas in the MT2 plates, all mycelia interface closely with the substance under analysis, as it is introduced into each well. The discrepancy in results between the two methods can be attributed to the fact that while sorbic acid at 50 mg mL⁻¹ may initially inhibit fungal growth, the metabolic responses measured in the MT2 microplate experiment reveal a more nuanced and dynamic interaction between the test substance and the fungi over time. None of the 4 studied preservatives simultaneously demonstrated antifungal activity at a certain dose, meaning that preservative dosage should be established individually for each substance.

Taking into account the results from both variants of the FF experiment, in general the most preferred carbon source for *Neosartorya* spp. seem to be carbohydrates, while the group of polymers was least preferred. Sodium metabisulfite greatly impacts the metabolic capabilities of *Neosartorya* spp. isolates. G150/14 was especially sensitive in this regard - its metabolism was almost completely stopped in the presence of sodium metabisulfite. More resilient isolates (G135/14, G160/14) were able to partially overcome the preservative effects yet they did not achieve the same AFGI or AFRI levels as their respective controls. Therefore, even low doses of this preservative can be effective in inhibiting *Neosartorya* spp. It is important to note, that groups of sensitivity did display unique preferences towards certain substances. After preincubation with sodium metabisulfite, resilient isolates seemed well able to grow on L-arabinose, whereas sensitive isolates did not. On the contrary, after treatment with the preservative, sensitive isolates produced only a little more biomass on α-methyl-D-galactoside than resilient isolates. When isolates were cultivated without the addition of sodium metabisulfite, most sensitive isolates grew more robustly on uridine than resilient isolates, whereas an opposite situation was observable for D-ribose. During the FF microplate analysis, isolates displayed evidence of inefficient metabolism with low AFGI in relation to AFRI values, especially during growth on carbohydrates (Fig. 3). In

Aspergillus spp., the enzymes α-amylase (Higuchi, 2021) and aldolase (Torres, 1994) are mostly responsible for carbohydrate metabolism. Further study is required to determine whether the isolates in our study were limited in the enzymatic machinery necessary for the efficient breakdown of carbohydrates. The isolates may face challenges in recognizing or efficiently taking up carbohydrates due to faulty membrane transport systems that can be related to issues such as the *GpMST1* (José et al., 2018) gene dysfunction or mitogen-activated protein kinases' unavailability (Schüßler, Martin, Cohen, Fitz, & Wipf, 2006). In the control group, isolate G226/14 could not effectively metabolise available substances for biomass production (Fig. 3). This could be indicative of a metabolic specialization where this isolate preferentially uses certain substrates for energy production as observed in mammalian, fungal and bacterial cells from other studies (McGuire et al., 2010; Singh, Halestrap, & Paraskeva, 1997; Szenk, Dill, & De Graff, 2017). Alternatively, research suggests that as substrate concentrations and growth rate increases, a shift from high-efficiency to low-efficiency central metabolic pathways could take place (Molenaar, van Berlo, de Ridder, & Teusink, 2009). This means that over time, cellular systems optimized for rapid growth could undergo regulatory adjustments. After reaching a certain biomass level, metabolic rates may change, as was perhaps the case with this isolate.

Several substances are linked with the well-known heat resistance of *Neosartorya* spp., such as D-mannitol and D-trehalose (Wyatt et al., 2014; Wyatt, Gerwig, et al., 2015; Wyatt, Golovina, et al., 2015). During the FF microplate assessment of control samples, D-mannitol and D-trehalose caused an AFGI of approx. 0.5 optical density. After incubation under stress conditions with sodium metabisulfite, AFGI on these substrates dropped to 0.06–0.11 OD. Hence, it is possible that sodium metabisulfite, even in low doses, impairs the heat resistance of *Neosartorya* spp. as the substances responsible for this feature can no longer be used.

Determining possible weaknesses in the preservation process, such as discovering substances that promote fungal growth, is also key to improve food protection techniques. From this point of view, the high growth rates on i-erythritol on FF plates, even for isolates with impaired metabolism by sodium metabisulfite, is a cause for concern for foods containing this ingredient in a form of e.g. sweeteners (Regnat, Mach, & Mach-Aigner, 2018). As even isolates with impaired metabolism could develop on this particular substrate, food containing it may be subjected to easier germination and growth of *Neosartorya* spp.

During the PM chemical sensitivity assay, groups of antibiotics, anions, membrane function compounds, organic compounds, cyclic compounds and chelators created an environment enabling most abundant biomass production (Fig. 4). Certain antibiotics are naturally exuded by several species of the *Aspergillus* genus, such as tensioles A and B from *Aspergillus niger* (Hasegawa, Fukuda, Hagimori, Tomoda, & Omura, 2007) or fumagillin from *Aspergillus fumigatus* (Eble & Hanson, 1951). Moreover, many antibiotics do not impair *Aspergillus* spp. growth, such as moxifloxacin and tobramycin (Day et al., 2009), or even stimulate it (prophylactic antibiotics) (Bargon et al., 1999). From the antibiotic assay, the substance that resulted in the highest AFGI values was kanamycin monosulfate (Fig. 5C), which has had no reported inhibitory properties towards fungi (Umezawa et al., 1957), and neomycin to which resistance can be acquired by *Aspergillus versicolor* (Dong et al., 2014). Kanamycin and neomycin are both aminoglycoside antibiotics of broad-spectrum antibacterial activity, widely used in husbandry and agriculture (Cui et al., 2009; Tang et al., 2021). Similar strong resistance of *Neosartorya fischeri* to antibiotics was documented by Panek et al. (2016), who reported an inhibitory effect of membrane function compounds on long-stored *N. fischeri*, whereas a fresh isolate remained unaffected, similar to the pooled isolate results in our research (Fig. 5F). From the group of membrane function compounds, the one generating the highest growth levels was protamine sulfate, which can be used in fisheries to supplement the diet of *Clarias lazera* (Metwally & Fouad, 2009). The use of these substances may lead to favourable environment for *Neosartorya* spp. growth. Therefore, proper precautions are advised

to prevent fungal overgrowth and pollution of fisheries. Groups that lead to lower growth were anions, cations, cyclic compounds and nitrogen compounds (Fig. 4), with the least biomass production on sodium cyanate (anion), sodium fluoride (anion), zinc chloride (cation), cobalt (II) chloride hexahydrate (cation), copper (II) chloride dihydrate (cation), cisplatin (nitrogen compound) and hydroxyurea (nitrogen compound) (Fig. 5A, B, G). The effects of these substances on fungal growth are not entirely unexpected, given their known uses and impacts in other biological and environmental contexts. Zinc chloride can be used as a micronutrient fertiliser for cereals and as a fungicide for wheat, maize and beans (Bagreev, Locke, & Bandosz, 2001; Behm, Tylka, Niblack, Wiebold, & Donald, 1995). Copper (II) chloride dehydrate can be used for the production of antimicrobial and antifungal copper nanocoatings (Pham, Duong, Le, Hoang, & Pham, 2019). Cisplatin antifungal effect was expected, as it is widely recognized as a toxic chemical substance of adverse effect on plants (Babula et al., 2007), microorganisms (Joyce et al., 2010) and animals (Aoyama, Shioya, Tsukamoto, Hasegawa, & Sugita, 2021). This enhances the understanding of the antifungal properties of these chemical compounds and highlights their potential to manage fungal growth in settings such as agriculture. It also suggests that the antifungal effects of compounds like zinc chloride and copper (II) chloride dihydrate stem not only from their direct toxicity but also from their ability to disrupt vital biological processes in fungi, as observed via the PM microplate assay. However, several substances that positively influenced AFGI were also observed, such as chloroquine (organic compound) and aluminium sulfate (cation). Chloroquine has proven antifungal effects on *Histoplasma capsulatum* (Weber, Levitz, & Harrison, 2000), a fungus from the same Ascomycota division (Víte-Garín, Estrada-Bárcenas, Cifuentes, & Taylor, 2014) as *Neosartorya* spp. More importantly however, it was shown to be effective against *Aspergillus fumigatus* (Farida et al., 2013), indicating that differences in sensitivity between sexual forms of *Neosartorya* and *Aspergillus* spp. may occur and it is important to differentiate between the two. Aluminium sulfate is often used in water treatment and horticulture (Chow, van Leeuwen, Fabris, & Drikas, 2009; Gebremedhin, 2020; Jain & Janakiram, 2016). Therefore, in these settings it is of utmost importance to avoid fungal contamination, as the growth stimulatory effect may enhance the spread of *Neosartorya* spp. in crops. It is important to note that several of the studied substances that exhibited promising antifungal properties (e.g. sodium fluoride, zinc chloride, copper (II) chloride dehydrate) are also commonly used as fertilizers in agriculture (Bagreev et al., 2001; Behm et al., 1995; Gray, 2018; Pham et al., 2019; Prasad, Kumar, Shivay, & Rana, 2014). Therefore, their dual mode of action needs to be considered during application. More research is needed on the simultaneous use of fungicides and supplements that have an inhibitory effect on the growth of *Neosartorya* spp. as perhaps fungicide doses should be modified due to the aggressive effects of these compounds. This might reduce the unnecessary chemicalization of agriculture with highly toxic agents. It is also worth noting that substances such as copper sulphate (Fig. S6B, 5B), commonly used as a fungicide and pesticide in horticulture for lawn care and protection (Çalik, 2020; USEPA, 2009), is not particularly effective against *Neosartorya* spp. fungi. During this study, divergence in the metabolism of different isolates were observed (Fig. S6). While some isolates grew well on certain substances e.g. aluminium sulfate, there were others that showed diminished growth. Future genetic studies are needed to show where genes involved in these processes differ between the isolates, since they are closely related. Nevertheless, the results highlight the importance of performing routine microbial susceptibility panels, to allow a better design of fungicide preparations and decrease the risk of creating environments accessible to highly resistant fungi.

Additionally, the role of isolation source on the sensitivity and metabolism of isolates needs further study. The adaptive polymorphism theory posits that populations inhabiting broad ecological niches exhibit more diversity compared to those in restricted niches (Wildman, 1995). For example, certain endomycorrhizal fungal species possess higher

resistance to stress conditions than their closely related ectomycorrhizal counterparts (Sharples, Meharg, Chambers, & Cairney, 1999). During inhibition tests with preservatives, isolates derived from soil presented higher levels of inhibition than isolates derived from strawberries. However, during inhibition tests with °Bx/pH combinations isolates derived from strawberries were more inhibited than soil derived isolates. During the metabolic capabilities test on FF microplates and the chemical sensitivity test on PM microplates no differences in optical density values were attributed to the source of fungal material isolation.

The FDA staining method has previously been used for assessing cell viability of several fungi (such as *Metarhizium robertsii* (Różalska et al., 2014), *Candida albicans* (Sushmitha et al., 2023) and *Cunninghamella echinulate* (Soboń et al., 2018)), or bacteria (e.g. *Burkholderia cenocepacia* and *Pseudomonas aeruginosa* (Peeters, Nelis, & Coenye, 2008)). It has also been used on the anamorphic form of studied fungi, *Aspergillus* spp. (*Aspergillus flavus* (Aquino et al., 2005), *Aspergillus awamori* (Freudenberger et al., 1996), *Aspergillus niger* (Yang et al., 1995)). However, to our knowledge, this is the first report adapting this method to assess *Neosartorya* spp. cell viability. The fluorescein diacetate staining dye permitted the observation of the effect of sodium metabisulfite on the metabolic activity of isolates. Only the longer incubation period with sodium metabisulfite affected fluorescence intensity. This can be linked to the time-dependent nature of *Neosartorya* spp. isolates' metabolic response to the preservative. The absence of statistically significant variations in fluorescence intensity after a shorter preincubation period shows that the rapid action of sodium metabisulfite may not be sufficient to appreciably affect metabolic activity. This latency in response indicates that the inhibitory effect of sodium metabisulfite on the metabolic activity of *Neosartorya* spp. isolates may require a considerable duration to manifest fully. The study also suggests the existence of a unique adaptive or resistance mechanism exhibited by isolate G135/14 in response to sodium metabisulfite exposure. It suggests that isolate G135/14 may possess an enhanced capacity to counteract or tolerate the inhibitory effects of sodium metabisulfite on its metabolic activity. During cell viability assessment the samples from different isolation sources only presented any statistical differences after treatment with sodium metabisulfite. Controls, cultivated under optimal growing conditions, yielded similar levels of fluorescence irrespective of their origin. Isolate derived from soil emitted a higher fluorescence reading after preincubation with sodium metabisulfite than isolates derived from strawberry fruit. Therefore, the results suggest that the source of these fungal isolates could impart specific properties in dealing with preservatives, but further studies are needed to confirm the influence of isolation source on fungal metabolic capabilities.

5. Conclusions

This study showed possible vulnerabilities to preservatives in highly heat-resistant *Neosartorya* species. However, the tested food preservatives are not equally effective. Sodium metabisulfite showed the highest levels of inhibition while sugar and pH combinations did not possess long-term inhibitory properties and are hence not advised as a standalone method for food protection. The various *Neosartorya* spp. isolates differed in their sensitivity to the food preservatives and chemicals despite being closely related. Three main groups of isolates exhibiting decreasing sensitivity and resistance were established, each with different substrate preferences and metabolic (respiratory and growth) capabilities. However, the utilization of all tested organic compounds guilds decreased significantly following the exposure of the fungal isolates to food preservatives.

In this study, resilient isolates generally had a lower biomass density under optimal conditions than sensitive isolates. The PM microplate assay demonstrated that isolates varied in their responses to the tested chemicals, with resilient isolates unable to metabolise certain substance as effectively as sensitive isolates. However, it is important to note that sensitivity to one substance does not oppose the possibility of resistance

to others.

Chemicals that are used as fertilizers were shown through the use of sensitivity assays to also have antifungal characteristics against *Neosartorya* spp., and must be carefully considered in agricultural practices. The results from the adapted FDA staining technique suggest intracellular enzyme inactivation occurred after incubation with the preservatives. This underscores the role of estrases and raises questions about differential effectiveness of sodium metabisulfite on these enzymes and the underlying factors influencing its efficacy. Since there was variation in the effect of each chemical, and there were differences between soil and strawberry isolates, additional research should be conducted to determine the impact of isolation source on the sensitivity and metabolism of these fungi. Applied methodology allowed for performing studies that offer insightful information on the biology of *Neosartorya* spp. with useful ramifications for food safety and agriculture.

CRedit authorship contribution statement

Wiktorija Maj: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Giorgia Pertile:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sylvia Różalska:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Kamil Skic:** Writing – review & editing, Visualization, Methodology, Investigation. **Magdalena Frać:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2024.139583>.

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7.3.3 Materiały dodatkowe

Food preservatives as important factor in shaping metabolic profile of fungi and its sensitivity to chemicals - an extensive research on crucial mycological food contaminants from the *Neosartorya* genus (*Aspergillus* spp.)

Table S1. List of *Neosartorya* spp. isolates used in the study. Signs “+” and “-“ indicate whether the isolate was used in a particular experiment.

Isolate	Isolation source	GenBank Accession No. ITS sequence	GenBank Accession No. β -tubulin sequence	Inhibition test	MT2 MicroPlates	FF MicroPlates	PM MicroPlates	Metabolic activity test
G125/14	Strawberry	OQ300418.1	OR500229	+	-	-	-	-
G126/14	Strawberry	OQ300422.1	OR597661	+	-	-	-	-
G127/14	Strawberry	OQ300429.1	OR597660	+	+	+	+	+
G129/14	Strawberry	OQ300428.1	OR597662	+	+	+	+	-
G130/14	Strawberry	OQ303998.1	OR597659	+	+	+	+	+
G131/14	Strawberry	OQ303995.1	OR597658	+	-	-	-	-
G132/14	Strawberry	OQ300430.1	OR597657	+	+	+	+	+
G135/14	Strawberry	OQ300432.1	OR608355	+	+	+	+	+
G150/14	Soil	OQ423135.1	OR608366	+	+	+	+	+
G151/14	Soil	OQ306552.1	OR608362	+	+	+	+	-
G153/14	Soil	OQ448879.1	OR608354	+	-	-	-	-
G154/14	Soil	OQ306551.1	OR608361	+	+	+	+	-
G155/14	Soil	OQ306553.1	OR608356	+	-	-	-	-
G159/14	Soil	OQ306554.1	OR608357	+	-	-	-	-
G160/14	Soil	OQ306555.1	OR608359	+	+	+	+	-
G161/14	Soil	OQ448880.1	OR608363	+	-	-	-	-
G226/14	Strawberry	OQ306560.1	OR608360	+	+	+	+	-
G239/14	Strawberry	OQ306558.1	OR608358	+	-	-	-	-
G253/14	Strawberry	OQ306559.1	OR608364	+	-	-	-	-
G259/14	Strawberry	OQ306561.1	OR608365	+	-	-	-	-

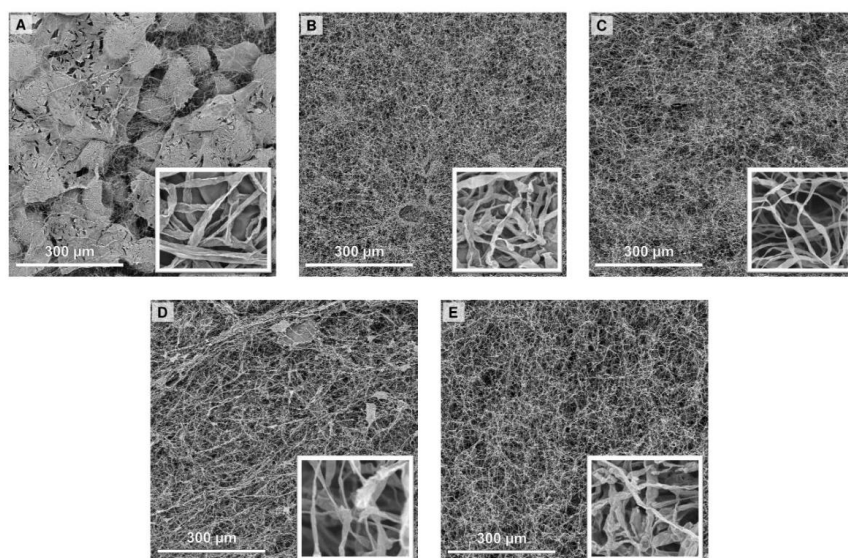


Fig. S1. Isolates of *Neosartorya* sp. observed via SEM. Exemplary isolates (A) G127/14, (B) G135/14, (C) G132/14, (D) G130/14, (E) G150/14 cultivated on PDA media.

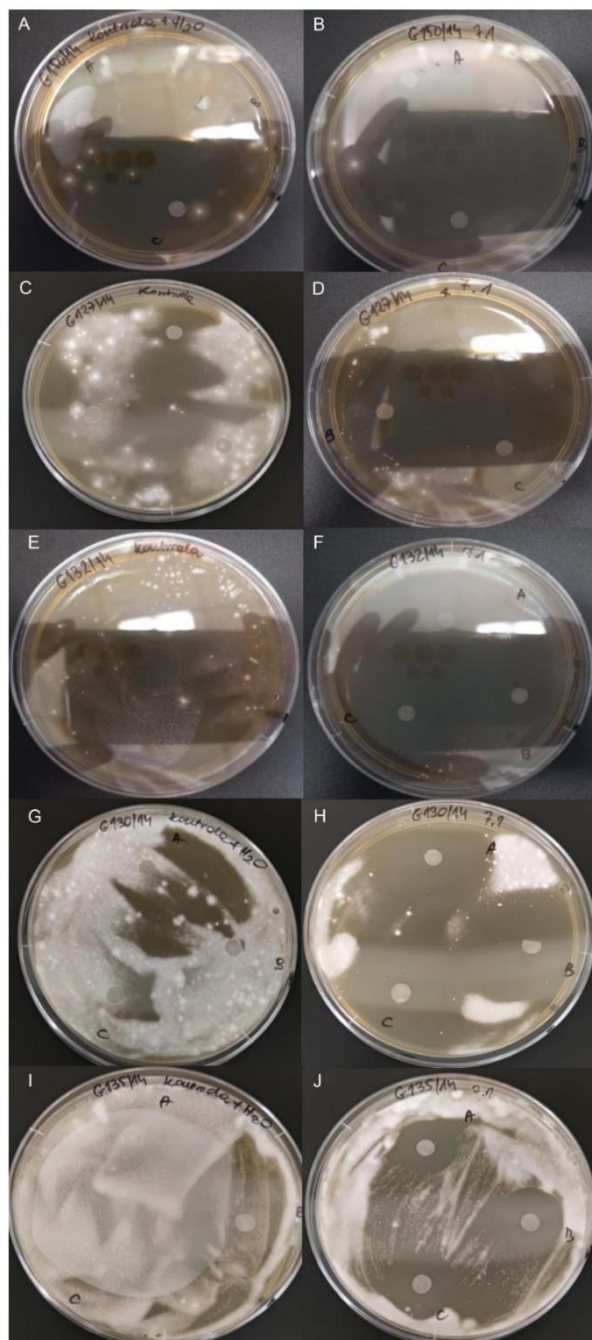


Fig. S2. *Neosartorya* sp. isolates' growth during inhibition tests. A, C, E, G, I – control; B, D, F, H, J – group treated with sodium metabisulfite. Isolates presented in descending order according to their sensitivity levels (high to low): (A, B) G150/14, (C, D) G127/14, (E, F) G132/14, (G, H) G130/14, (I, J) G135/14.

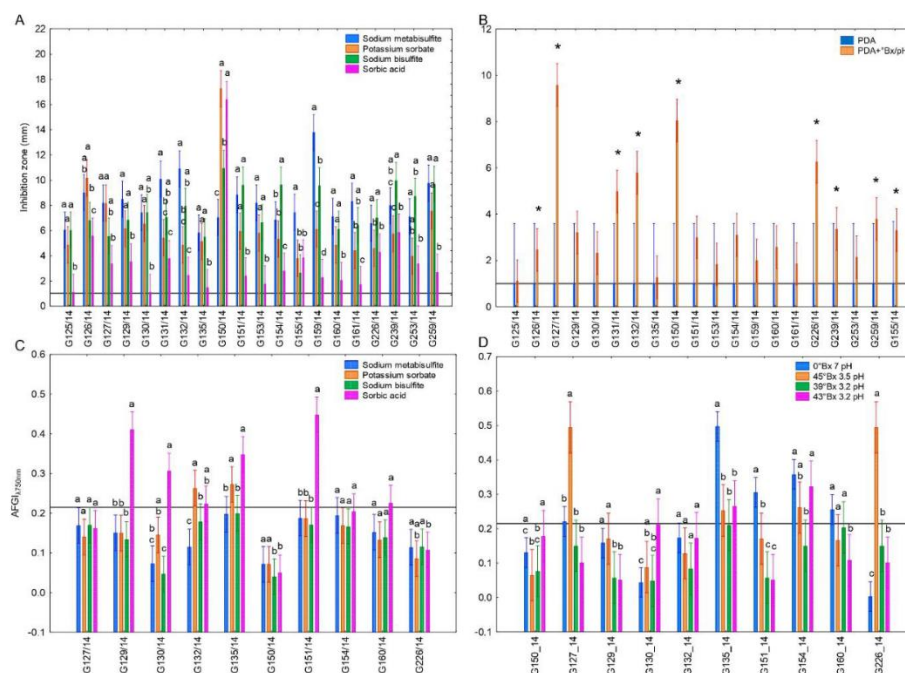


Fig. S3. Individual *Neosartorya* sp. isolates' reaction to tested substances. (A) Comparison of observable growth inhibition caused by preservatives during the inhibition test (black line represents control value of no inhibition). (B) Comparison between control and °Bx/pH combinations during the inhibition test. (C) Average Fungal Growth Intensity (AFGI) values of analysed isolates during MT2 microplate assay dependent on preservative type: sodium metabisulfite, potassium sorbate, sodium bisulfite, sorbic acid. (D) Average Fungal Growth Intensity (AFGI) values of isolates during MT2 microplate assay dependent on solution type: control (0 °Bx 7pH), 39 °Bx 3.2 pH, 43 °Bx 3.2 pH, 45 °Bx 3.5 pH. Letters or asterisks indicate significant differences ($p < 0.05$) between values with analyses being performed for each isolate separately.

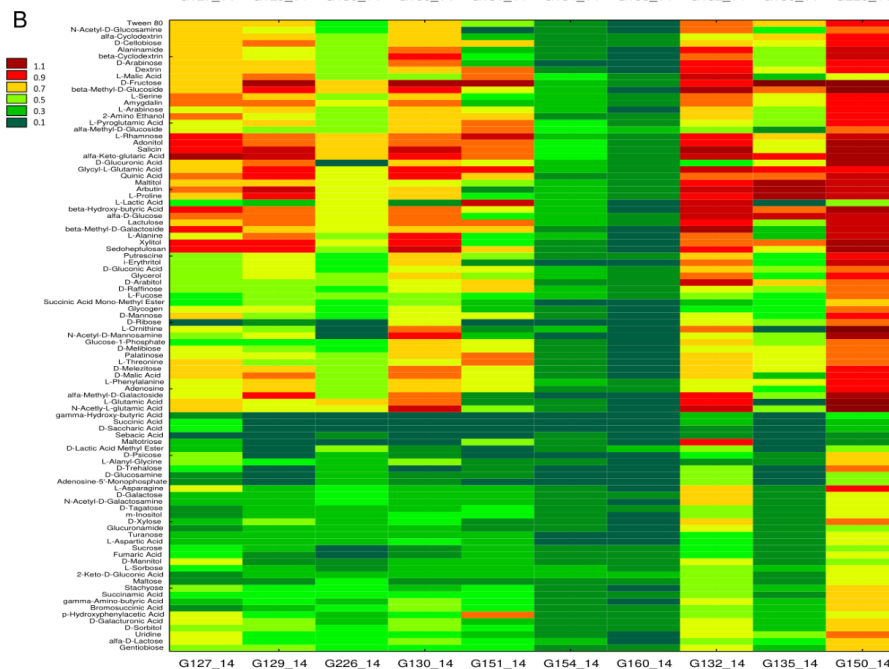
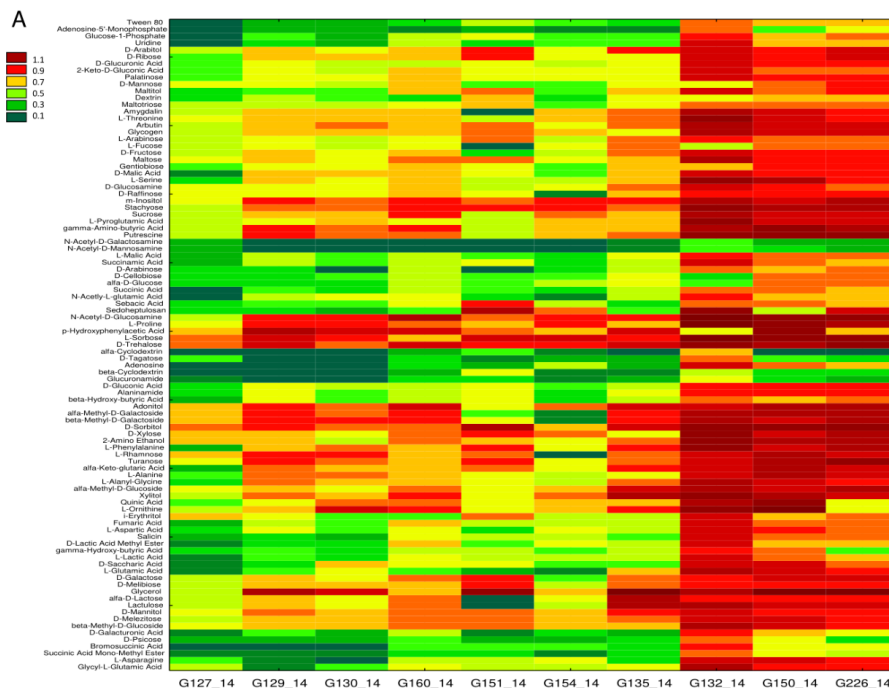


Fig. S4. *Neosartorya* spp. biomass production on FF microplate after 144 hours of incubation. Isolates were preincubated in optimal growth conditions. Colour scale applied to heatmaps indicates respiratory activity (**A**) or fungal growth density (**B**) on each of the substrates.

Fig. S5. *Neosartorya* spp. biomass production on FF microplate after 144 hours of incubation. Isolates were preincubated on sodium metabisulfite enriched media (150 µg/ml). Colour scale applied to heatmaps indicates respiratory activity (**A**) or fungal growth density (**B**) on each of the substrates.

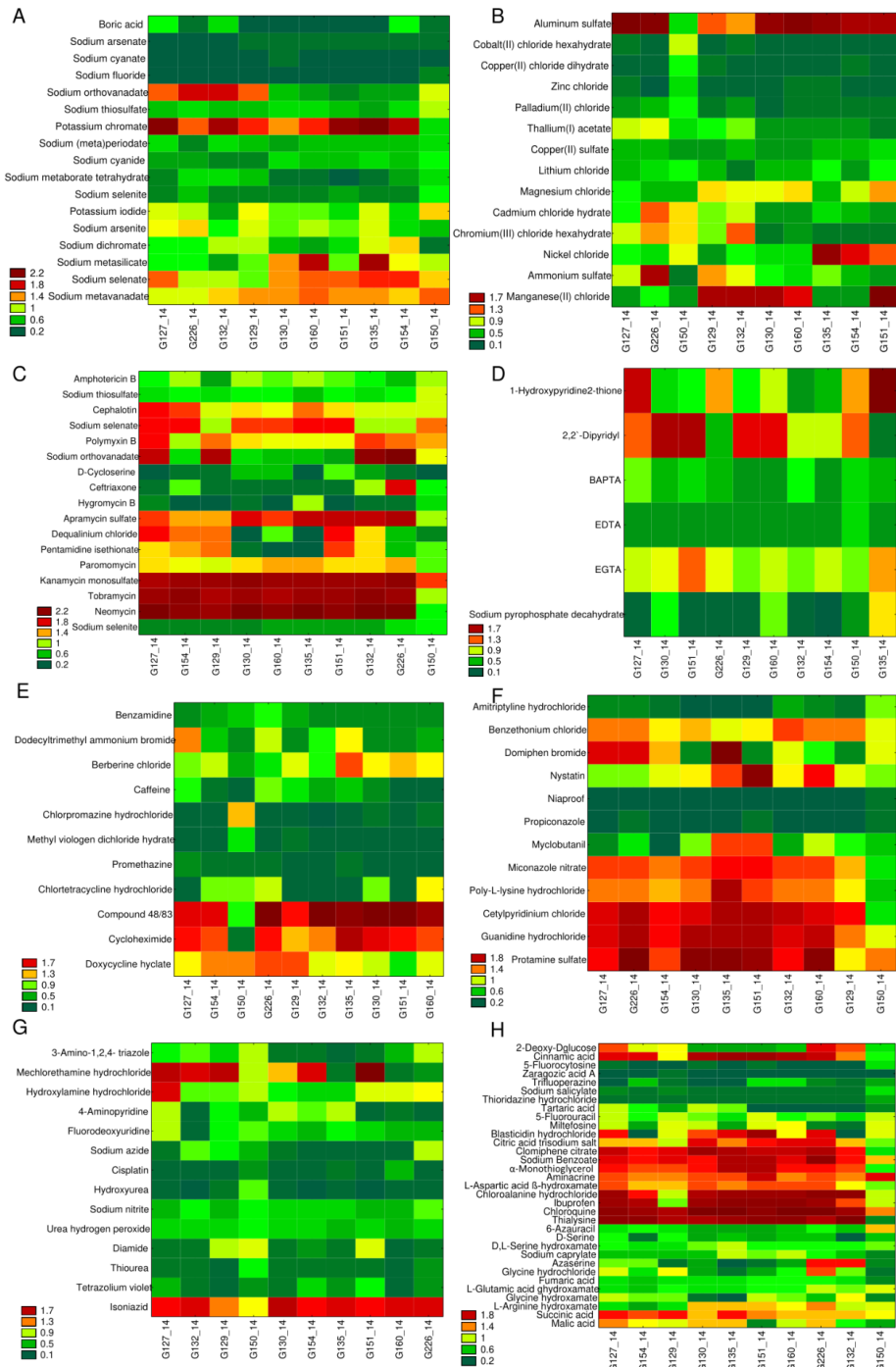


Fig. S6. *Neosartorya* spp. biomass production on PM microplates (21-25) after 144 hours of incubation. Colour scale applied to heatmaps indicates fungal growth density (optical density at 750 nm) on each chemical substance. Heatmaps are divided according to substrate groups and are as follows: **(A)** anions, **(B)** cations, **(C)** antibiotics, **(D)** chelators, **(E)** cyclic compounds, **(F)** membrane function compounds, **(G)** nitrogen compounds, **(H)** organic compounds.

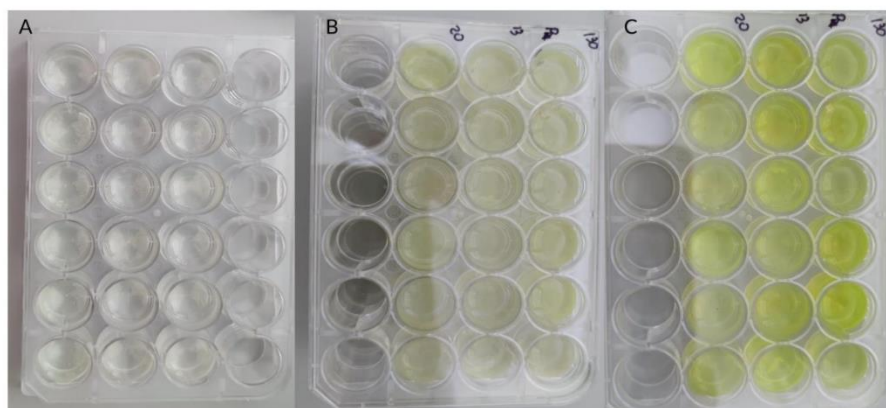


Fig. S7. Fluorescence progression during metabolic activity assessment. Plate with fungal inoculum and FDA solution after 0 minutes (**A**), 30 minutes (**B**) and 60 minutes (**C**) of lightless incubation.

7.4 Manuskrypt publikacji P.4

7.4.1 Abstrakt graficzny

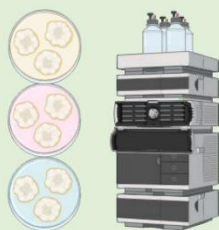
GENOME-WIDE ANALYSIS, METABOLOMIC PROFILING AND MICROSCOPIC EVALUATION OF *NEOSARTORYA* SPP. (*ASPERGILLUS* SPP.) DIVERSITY AND ADAPTATIONS TO NATURAL AND SYNTHETIC ANTIMICROBIALS

WHOLE GENOME SEQUENCING



More resilient *Neosartorya* spp. isolates had a higher proportion of genes linked to defence mechanisms, while sensitive isolates contained fewer.

LC-QTOF MS



PCA revealed medium-dependent metabolomic shifts, overriding innate isolate sensitivity and incubation effects.

SCANNING ELECTRON MICROSCOPE



Exposure to sodium metabisulfite and *Calendula officinalis* altered hyphal surfaces, which is potentially linked to stress response.

CONFOCAL LASER SCANNING MICROSCOPE



Viability assessment showed sodium metabisulfite lost effectiveness over time, while *Calendula officinalis* had transient antifungal effects.

CREATED IN BIOENDER

7.4.2 Tekst manuskryptu publikacji naukowej

1 **Genome-Wide Analysis, Metabolomic Profiling and Microscopic Evaluation of *Neosartorya* spp.**
2 **(*Aspergillus* spp.) Diversity and Adaptations to Natural and Synthetic Antimicrobials**

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10

11 **Abstract**

12 Highly adaptable *Neosartorya* spp. ability to produce heat-resistant ascospores renders them significant
13 food contaminants, posing risks to food safety and quality. This study aims to clarify how genetic, metabolic,
14 and morphologic landscapes contribute to antifungal resistance, utilizing comprehensive profiling techniques to
15 enhance the *Neosartorya* genus understanding within the context of fungal biodiversity and resilience. Whole-
16 genome sequencing was used to characterize the baseline genomes of five distinct *Neosartorya* isolates. Pre-
17 incubation with sodium metabisulfite or *Calendula officinalis* extract enables a comparative analysis of each
18 antifungal's impact on fungal phenotypes. Genes pertaining to defensive functions were detected and
19 characterized for each of the studied isolates. The influence of calendula extract or sodium metabisulfite
20 generated metabolomic changes and a clear split in metabolite spectra based on the type of cultivation media
21 confirmed by liquid chromatography-quadrupole time-of-flight mass spectrometry. Scanning electron
22 microscopy and confocal fluorescence microscopy provide insights into morphological adaptations and cell
23 viability, respectively.

24 **Keywords**

25 Heat-resistant fungi, Whole-genome sequencing, Morphology, Scanning electron microscopy, Confocal
26 fluorescence microscopy, metabolomic profiling

27

1. Introduction

The genus *Neosartorya* (family *Trichocomaceae*) encompasses a unique group of fungi notable for their remarkable resilience and diverse metabolic potential (Poeaim, 2015). Known primarily as the teleomorphic state of the *Aspergillus* spp. section *Fumigati*, *Neosartorya* species possess the capability to produce both sexual (ascospores) and asexual (conidiospores) reproductive forms (de Sá et al., 2022). This dual reproductive capacity contributes to their environmental adaptability, allowing them to thrive in various challenging conditions, soils and seawater alike (Eamvijarn et al., 2013). While the Melbourne Code mandates the use of the name “*Aspergillus*” for both sexual and asexual forms, the designation “*Neosartorya*” persists for species classified before this nomenclatural change took effect in 2013 (de Sá et al., 2022).

Neosartorya spp. exhibits a striking chemical diversity, producing a range of bioactive compounds across various chemical classes, including alkaloids, phenolic compounds, peptides and meroterpenes (Lin et al., 2020; Sabry, 2020). In addition to their metabolic diversity, *Neosartorya* species are recognized as heat-resistant moulds (HRM), a classification that underscores their relevance to the food industry (Frąc et al., 2015; Alam et al., 2024). HRM such as *Neosartorya* spp. can survive standard pasteurization processes and other food preservation methods, posing a serious risk of contamination and spoilage, particularly in high-acid fruit products (Chen et al., 2022; Santos et al., 2020). Ascospores produced by *Neosartorya* spp. are highly resilient, and once activated by pasteurization, they can germinate and proliferate under conditions that inhibit other microbial growth (Racchi et al., 2020). This resilience challenges the effectiveness of conventional preservatives like sorbates, as well as other technological aids, which have been only partially effective or ineffective against HRM (Berni et al., 2017; Racchi et al., 2020).

Addressing *Neosartorya* spp. contamination in food products necessitates a comprehensive understanding of how growth conditions influence their proliferation and the activation of their innate defence mechanisms, which contribute to their remarkable resistance against conventional preservation methods. Despite some efforts being made in the field of genetic analysis of *Aspergillus/Neosartorya* genera, most of the research focuses on biotechnological applications or medical hazards, omitting a routine investigation of general genetic changes that may occur due to changes in climate and environment (Daisuke et al., 2020; Pertile et al., 2020; Hajdu et al., 2019). Whole-genome sequencing (WGS) serves as a crucial methodological foundation for exploring genetic variability, as it enables comprehensive characterization of the entire genome, including conserved regions and potential mutations, providing a baseline to assess genetic stability and intrinsic traits such as resistance

57 mechanisms in *Neosartorya* spp. (Mariana et al., 2017). WGS can be employed to identify resistance mutations
58 in related fungi, such as azole resistance mutations in *Aspergillus* spp. (Daisuke et al., 2020). Additionally, WGS
59 comparisons are proven to be a powerful tool for elucidating genetic diversity among fungal strains, for example
60 by revealing fungicide resistance in plant-derived *Aspergillus fumigatus* (Takahashi et al., 2021). Here, the
61 method was selected for its capacity to capture detailed genetic differences between isolates that had shown
62 varying sensitivity to preservatives and natural extracts in our previous studies (Maj et al., 2024a, 2024b). By
63 examining each isolate's genome, we aimed to identify specific genetic features underlying these distinct
64 resistance profiles.

65 The extensive and prolonged application of fungicides has led to their persistence in the environment, with
66 residues detected in soil (Bhandari et al., 2020), groundwater (Sousa et al., 2021), and even food products (Yigit
67 & Velioglu, 2019), raising concerns about their broader ecological impact, including a potential reduction in soil
68 biodiversity. The evolutionary pressures exerted by the widespread use of pesticides have driven fungi to
69 develop sophisticated resistance mechanisms, showcasing their capacity for rapid adaptation to anthropogenic
70 challenges. Studies show that the control of agricultural crop diseases is endangered by the rapid evolution of
71 fungicide resistance in fungi (Hawkins et al., 2019). It is further suggested that azole resistance in *Aspergillus*
72 *fumigatus* may take its root from agricultural pesticide overuse (Berger et al., 2017). Moreover, the research
73 indicated that *Neosartorya fischeri* is resistant to almost 100 different chemicals including anions, cations,
74 membrane function compounds, chelators, antibiotics, cyclic, organic and nitrogen compounds (Panek et al.,
75 2016). To better understand fungal adaptability, it is essential to inquire how *Neosartorya* spp. respond to both
76 synthetic and natural antifungal agents. For this study, sodium metabisulfite, a widely-used preservative in the
77 food industry, was selected for its effectiveness in microbial control (Ahmadi et al., 2018; do Nascimento &
78 Canteri, 2020; Shahbazi et al., 2021), while *Calendula officinalis* extract represents a promising “farm-to-fork”
79 alternative aligned with the increasing demand for natural preservation strategies (Di Lorenzo et al., 2013;
80 Wesseler, 2022). Despite the popularity of both synthetic and plant-based treatments, no comprehensive studies
81 have compared their effects on *Neosartorya* spp. with respect to both the morphologic and metabolic changes
82 they may induce. One method permitting a comprehensive analysis of these adaptations is scanning electron
83 microscopy (SEM). Although SEM is an important tool for species identification for some organisms, many
84 *Neosartorya* spp. species cannot be differentiated on macro- and micro-morphological characteristics alone due
85 to close resemblance (S. B. Hong et al., 2006). SEM can however, be useful in terms of fungal structures
86 visualization. It is known that negative growth conditions, such as oxidative, heat or osmotic stress may alter

87 fungal morphology (Bai et al., 2003; Duran et al., 2010; Leonardi et al., 2017). These changes can be seen as
88 multiple hyphae tip-formation near the hyphal apex, thickening of cell walls or an increase of visible conidiation
89 (Markham, 1992; Chang et al., 2011; Heilmann et al., 2012). Thus, this study aimed to investigate any
90 morphological changes in *Neosartorya* spp. induced by the application of *Calendula officinalis* extract or sodium
91 metabisulfite. Another approach to gauging fungal responses to stress is confocal fluorescence microscopy,
92 where dyes act as a viability probe, measuring activity of enzymes and membrane integrity via emission of
93 fluorescence dye (Różalska et al., 2018). The technique is an effective for filamentous fungi investigation, as and
94 has been successfully applied to assess the viability of *Fusarium sambucinum* following exposure to biotic and
95 abiotic stressors (Walaszczyk et al., 2024). In this study, observations using confocal fluorescence microscopy
96 were conducted to further examine the structural alterations and metabolic activity of the fungal isolates in
97 response to natural and chemical treatments. The study was expanded by employing liquid chromatography
98 coupled with quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) which enables for metabolic
99 profiling, due to high sensitivity and accurate detection of a wide range of metabolites (Yurchenko et al., 2023).
100 Proteomic approaches are deemed effective in the investigation of the protein constituents of *Ascomycetes*, for
101 example via permitting the observation of proteins/enzymes involved in the germination of *Aspergillus flavus*
102 conidia which pertains to its survivability (Tiwari et al., 2016). Additionally, the high-resolution LC/MS
103 technique is helpful in gene expression analysis, with the capacity to establish protein profiles of spores with and
104 without heat treatment, providing insights into the mechanisms underlying heat resistance and the fungal
105 response to thermal stress (Chen et al., 2022). Here, by analysing the metabolic profiles of *Neosartorya* spp.
106 isolates, we aimed to identify general biochemical changes associated with the use of antifungal agents.

107 This study investigates the morphological, metabolic, and viability changes in *Neosartorya* spp. following
108 exposure to sodium metabisulfite and *Calendula officinalis* extract. Observations are made within the context of
109 unaltered genomes, focusing solely on phenotypic responses to elucidate resilience mechanisms and potential
110 vulnerabilities in these thermotolerant fungi. Insights gained from this exploration may contribute to developing
111 improved control practices for *Neosartorya* spp. in food products, enhancing both preservation strategies and
112 overall food safety through a better understanding of fungal stress responses.

113 2. Materials and methods

114 2.1 Fungal isolates and cultivation conditions

Five isolates from the sample collection of the Laboratory of Molecular and Environmental Microbiology Institute of Agrophysics, Polish Academy of Sciences (Lublin, Poland), were selected for this study. Analysed isolates were first isolated and identified during the SONATA4 project (project ID: 2012/07/D/NZ9/03357) supported by National Science Centre. The isolates were identified as closely related *Neosartorya* spp. specimen through the sequencing of the ITS1 region and β -tubulin genes (Maj et al., 2024a, 2024b). These 5 particular isolates were chosen based on their varying sensitivity to natural plant extracts and food preservatives implemented in previously described inhibition tests (Maj et al., 2024a, 2024b). However, previous research indicated that these isolates did not produce mycotoxins such as verruculogen and fumitremorgin C when incubated on the PDB medium (Fornal et al., 2017). Here, the isolates were cultivated at 30°C for periods of 10 (Fig. SI1) and 30 (Fig. SI2) days (morphology and metabolome assessment) or just 10 days (genome assessment). This incubation temperature and duration were selected to ensure optimal growth conditions for *Neosartorya* spp. fungi while facilitating a detailed examination of any induced morphological, genetic, and metabolic changes.

To investigate morphological and metabolic changes in *Neosartorya* spp. fungi, isolates were cultivated on three variants of Potato Dextrose Agar (PDA) medium (BioMaxima S.A., Lublin, Poland). The variants included: PDA medium without additives, PDA medium supplemented with *Calendula officinalis* dry powder water solution (150 $\mu\text{g mL}^{-1}$), and PDA medium supplemented with sodium metabisulfite solution (150 $\mu\text{g mL}^{-1}$). The *Calendula officinalis* dry extract was obtained from Zrób Sobie Krem Kosmetyki Naturalne (Prochowice, Poland), while sodium metabisulfite was procured from Sigma-Aldrich (St. Louis, MO, USA). Both the preservative and the *C. officinalis* powder were provided as readily soluble powders, which were dissolved in sterile water and filtered using 0.22 μm filters (Millex®, Ireland) to achieve the specified concentration. The concentration of 150 $\mu\text{g mL}^{-1}$ was chosen due to its previously demonstrated ability to stunt fungal growth while still allowing for some mycelial development, thus permitting the observation of mycelial structures under the influence of these substances. To observe innate genetic differences between isolates, before the DNA extraction the 5 *Neosartorya* spp. fungi were cultivated only in optimal conditions on PDA medium without any add-ons.

2.2 Whole Genome Sequencing (WGS)

To study the innate differences between the five *Neosartorya* spp. isolates, the technique of Next-Generation Sequencing was employed. Fungal samples were first homogenized using sterile glass beads as in Malarczyk et al., 2020, and DNA material was isolated using GeneMATRIX Plant & Fungi DNA Purification

144 Kit (EURx, Poland), according to the manufacturer's protocol. The quantity and quality of obtained genetic
 145 material was confirmed using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and
 146 QuantusFluor®DNA dsDNA reagents on a Quantus fluorometer (Promega, Madison, WI, USA). PCR-grade
 147 water was used as isolation control. DNA was diluted to obtain the concentration ranging between 100 and 500 ng
 148 in final volume of 30 µl. Library preparation was done using the Illumina DNA Prep Kit (Illumina, San Diego,
 149 CA) according to the manufacturer's instructions and similarly as in Price et al., 2021 with some modifications.
 150 Therefore, genomic DNA was tagged at 55°C and post tagmentation clean-up was performed, followed by
 151 PCR amplification for 8 cycles according to producer's advice for procured material concentration. Libraries
 152 quality was checked using D1000 ScreenTape and TapeStation 4150 system (Agilent Technologies, Santa Clara,
 153 CA) and QuantusFluor®DNA dsDNA reagents on a Quantus fluorometer (Promega, Madison, WI, USA).
 154 Libraries were normalized to 0.02 nmol L⁻¹ concentrations for the Illumina MiSeq v3 reagent kit and
 155 subsequently pooled and denatured before sequencing. The Illumina MiSeq System was used to produce 300-bp
 156 paired-end reads on Read Length 301-301 mode. Datasets obtained via WGS were processed using bioinformatic
 157 tools. Cutadapt was employed to trim adapter/primer sequences (Martin M., 2011). De novo assembly of genome
 158 sequencing was performed using SPAdes Genome Assembler (A. Prjibelski et al., 2020) with exSPANDER
 159 algorithm (A. D. Prjibelski et al., 2014; Vasilinets et al., 2015). Genome annotation was done with
 160 Funannotate® (Love et al., 2018). Quality assessment of assembly was achieved with QUAST – Quality
 161 Assessment Tool for Genome Assemblies (Gurevich et al., 2013), QUAST-LG (Mikheenko et al., 2018) and
 162 Icarus visualizer (Mikheenko et al., 2016). Average nucleotide identity was tested with FastANI (Jain et al.,
 163 2018). Transfer RNA genes were identified with tRNAscan-SE (Lowe & Eddy, 1997). Orthology data
 164 annotation was conducted using eggNOG 6.0 (Hernández-Plaza et al., 2023). Transmembrane topology and
 165 signal peptide prediction was carried out with Phobius (Käll et al., 2004). Signal peptides prediction was
 166 accomplished with SignalP 6.0 (Teufel et al., 2022). Classification of protein sequences into families using
 167 InterPro database resources was possible with InterProScan 5 (Blum et al., 2021; Jones et al., 2014). Secondary
 168 metabolites and antibiotics classification was performed with antiSMASH 7.0 (Blin et al., 2023). Sequence
 169 comparison and alignment was performed with Basic Local Alignment Search Tool (Altschul et al., 1990;
 170 Camacho et al., 2009) and The National Center for Biotechnology Information resources (Sayers et al., 2022).

171 **2.3 Liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF MS) analysis**

172 Liquid chromatography (LC) quadrupole time-of-flight (QTOF) mass spectrometry (MS) metabolomic
 173 profiling was performed using an Agilent Infinity 1290 ultra-performance liquid chromatograph coupled to an
 174 Agilent 6550 iFunnel QTOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with
 175 JetStream ion source enabling electrospray ionisation (ESI). Mycelium from 10 and 30 days old fungal colonies
 176 on PDA with and without the pre-incubation with sodium metabisulfite or *Calendula officinalis* extract was
 177 harvested and successively weighted in test tubes to obtain 20 mg of each sample. For this experiment, two
 178 biological replicates were prepared, and a third replicate was generated as a technical replicate. Tissue
 179 disintegration was performed using 2.8 mm diameter ceramic beads shaken at 6 m s⁻¹ during 10 seconds cycles
 180 repeated 4 times (Bead Mill MAX, VWR, part of Avantor, Radnor PA, United States). Then, 1 mL of cooled
 181 extraction solution (methanol:chloroform:water of volume proportions 2.5:1:0.5) was added to the reinforced test
 182 tube containing the fungal material. The mixture was mixed for 10 seconds by inversion and incubated on ice for
 183 8 minutes. Samples were centrifuged at 14,000 x g for 4 minutes at 4 °C. The supernatant (containing
 184 metabolites) was separated from the pellet (containing cell debris and proteins). 500 µL of Milli-Q water
 185 (Millipore Direct-Q3-UV purification system, Merck KGaA) was added to each sample. To separate the phases,
 186 resulting in polar and nonpolar metabolites, the samples were mixed for 10 seconds and centrifuged at 14,000 x
 187 g for 2 minutes. The upper (polar) phase was transferred to a new test tube. The homogenized suspensions were
 188 dried under vacuum conditions using a miVac Duo concentrator (Genevac Ltd., Ipswich, UK). Samples were
 189 then solved with 100 µL of 1:1 methanol:ethanol mixture (nonpolar) or 100 µL of 1:1 methanol:water mixture
 190 with 0.1 % formic acid (polar) and successively vortexed for 5 minutes. Chromatographic separation of
 191 metabolites was performed on Agilent ZORBAX Rapid Resolution High Definition (RRHD) Extend-C18
 192 analytical column (2.1 mm × 100 mm, 1.8 µm). The mobile phase was composed of 0.1 % formic acid in water
 193 (A) and 0.1% formic acid in acetonitrile (B). The phase was injected at a flow rate of 0.4 mL min⁻¹. The gradient
 194 program was 0–25 min, 3% B to 95% B; 25–30 min, 95% B. The injection volume was 10 µL and the column
 195 temperature was 45 °C. Analyses were performed at positive electrospray ionization mode (ESI+), and the
 196 operation parameters were as follows: ion source gas (nitrogen) temperature: 225 °C; nitrogen flow rate: 12 L
 197 min⁻¹; nebulizer pressure: 50 psi; sheath gas temperature: 275 °C; sheath gas flow: 12 L min⁻¹ and capillary
 198 voltage: 3500 V. The nozzle voltage was set at 1000 V and the fragmentor voltage at 275 V. The mass
 199 spectrometer was operated with a scan rate of 1.2 spectra per second. Internal mass calibration was permitted by
 200 using two reference masses, at 121.0509 and 922.0098 m z⁻¹. Data was recorded and processed with Agilent
 201 Mass Hunter Data Acquisition software (B.09.00) and Qualitative software (B.10.00), respectively.

2.4 Microscopy imaging

The five *Neosartorya* spp. isolates were examined with an electron microscope, Phenom ProX (Thermo Fisher Scientific, Waltham, MA, USA). To perform the visualization, fungal samples were cut out of Petri plates and placed on aluminium supports with conductive double-sided carbon adhesive tape. Specimens were then dried using silica gel to absorb moisture, and coated with a 5 nm gold layer (sputter coater, CCU-010 LV, Safematic GmbH, Zizers, Switzerland) before SEM analysis (Pertile et al., 2021). Images were taken using a BS detector at an accelerating voltage of 10 kV (Beer-Lech et al., 2022). Measurements of fungal structures were taken using ImageJ 1.54g, National Institutes of Health, USA (Schneider et al., 2012).

Fungal isolates were also studied with a confocal laser scanning microscope Leica TCS SP8 (Wetzlar, Germany). After 10 and 30 days of growth on 3 types of PDA media (PDA, PDA with *Calendula officinalis* extract at 150 µg mL⁻¹, PDA with sodium metabisulfite at 150 µg mL⁻¹), about half of the ascospores with mycelium from each of the 3 culture repetitions were harvested and added to a wash buffer (2% D-glucose and Na-HEPES) and centrifuged at 10,000 x g for 5 minutes. The supernatant was removed, and the pellet was re-suspended in 1 ml of wash buffer with 1 µl of FUN-1 cell stain (10 mM solution in DMSO; Thermo Fisher Scientific Corporation, USA). The samples were incubated at 30°C in the dark for 5 minutes. The stained suspension was placed on a glass slide, covered with a coverslip, and cells were imaged using excitation laser wavelength with excitation at 488 nm and emission at 530 nm (Walaszczyk et al., 2024). Additionally, photographs of the fungal isolates visualize differences in spore production. Viability of the samples was determined by calculating the percentage of metabolically active cells using fluorescence. As cells were stained with FUN-1, viable cells displayed red-fluorescent intravacuolar structures, and non-viable cells exhibited bright, diffuse yellow-green fluorescence (Lee & Bae, 2018). To determine cell viability, the ratio of red to green/yellow fluorescence intensity was calculated within eleven 20 × 20 µm regions across three replicates for each cultivation variant. This ratio was then multiplied by 100 to express viability as a percentage.

2.5 Statistical analyses

For viability data, statistical analyses were performed using Statistica™ software (TIBCO Software Inc. V13.3). The normal distribution was tested via Shapiro-Wilk test and homogeneity of variance was checked via Levene test. For datasets that presented normality and homogeneity of variance, an Independent Samples t-Test was employed to compare the means between the control and individual test groups. When groups did not display homogeneity of variance, Welch's t-Test was used instead. For datasets that did not meet the normality

assumption, the Mann-Whitney U test was used to compare between the control and test groups. All data was presented at 95% confidence intervals with statistical significance established at $p < 0.05$.

Raw LC-QTOF MS data files collected for tested *Neosartorya* spp. isolates on 3 media variants and 2 cultivation times were processed by Mass Hunter Qualitative B.10.00 software and then imported into Mass Profiler Professional 15.1 for data processing. Principal components analysis (PCA) using Pareto-scaled data was performed to reduce the dimensionality of the data and to reveal any clustering of the groups (isolates, incubation conditions, isolates and incubation conditions).

3. Results

3.1 Whole Genome Sequencing (WGS)

Sequences obtained via WGS were deposited in the National Centre for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>; see Table S1). Genomes of each of 5 isolates were further characterized taking into account their size, gene count, the amount of coding sequences and a number of automatically annotated genes.

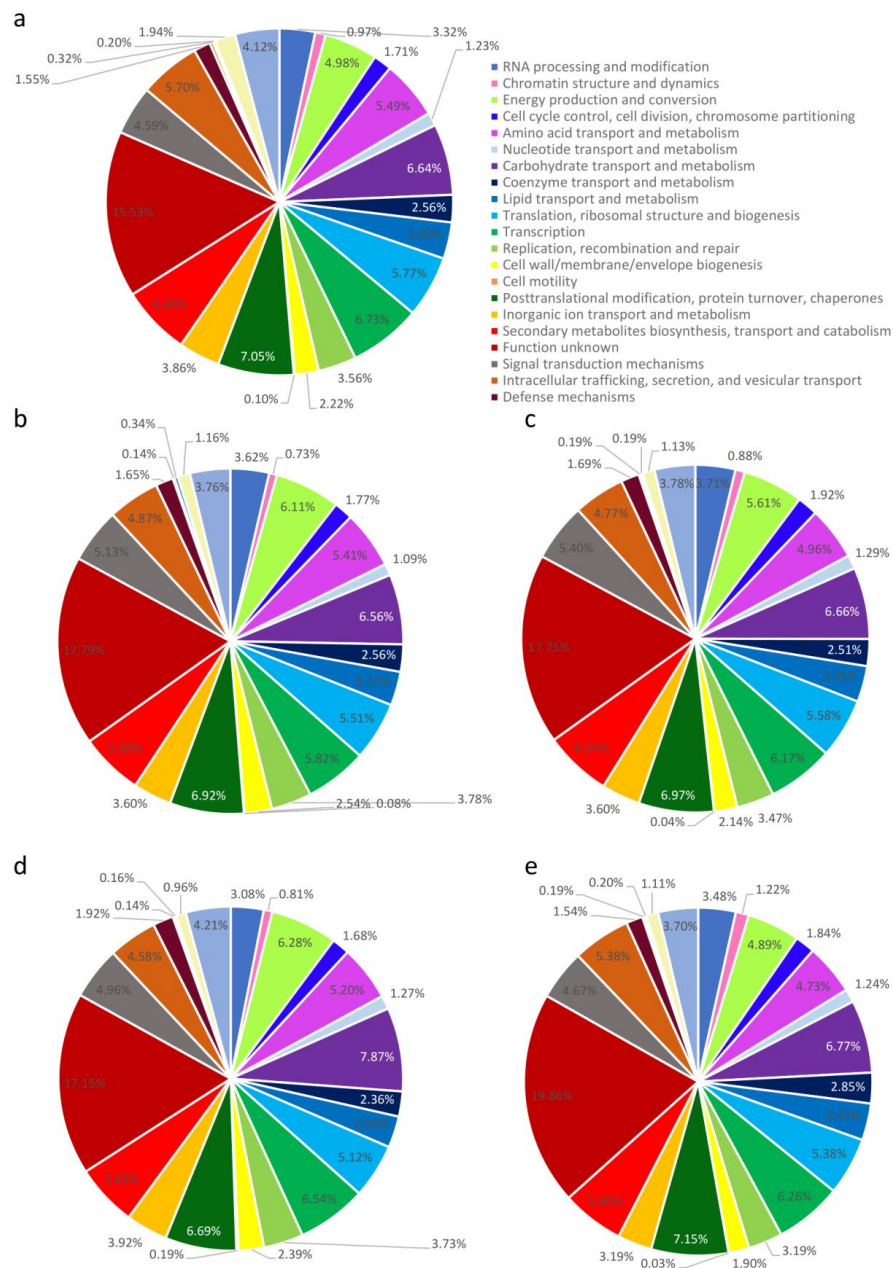
Table 1 Gnome general characteristics of the studied *Neosartorya* spp. isolates

Isolate	GenBank Accession	Genome Size (bp)	Total Gene Count	Coding DNA Sequences	COG
	Number				Automatically Annotated Genes
G127/14	JBKOSS000000000	34432240	11014	20061	9202
G130/14	JBKOST000000000	32824853	10744	19885	8751
G132/14	JBKOSU000000000	34514406	11284	20059	9146
G135/14	JBKOSV000000000	35134313	14339	20817	11889
G150/14	JBKOSW000000000	36838727	11853	20879	9500

All obtained genes were evaluated to fit Clusters of Orthologous Groups (COGs) function classification. If no classification was assigned automatically, manual description was performed. This was done for each isolate separately (Fig. 1). Whole genome sequencing of five *Neosartorya* spp. isolates reveals consistent patterns in gene function composition across all genomes analysed. The majority of genes are annotated as having unknown function, comprising 15.53-19.86% of each genome, whereas genes associated with cell motility are least represented, ranging from 0.03-0.19%. Groups generally counting more genes are

usually carbohydrate transport and metabolism (6.56-7.87%), posttranslational modification and protein turnover (6.69-7.15%), replication, recombination and repair (5.82-6.73%), and secondary metabolites metabolism (5.80-6.46%). Less diverse are groups of genes pertaining to nucleotide transport and metabolism (1.09-1.29%), chromatin structure and dynamics (0.73-1.22%), extracellular structures (0.14-0.32%), nuclear structure (0.16-0.34%). Notably, isolate G135/14, previously characterized as the most antifungal-resistant, also exhibits the highest gene count (14,339). However, despite this genome complexity, the relative composition of functional categories remained consistent across isolates.

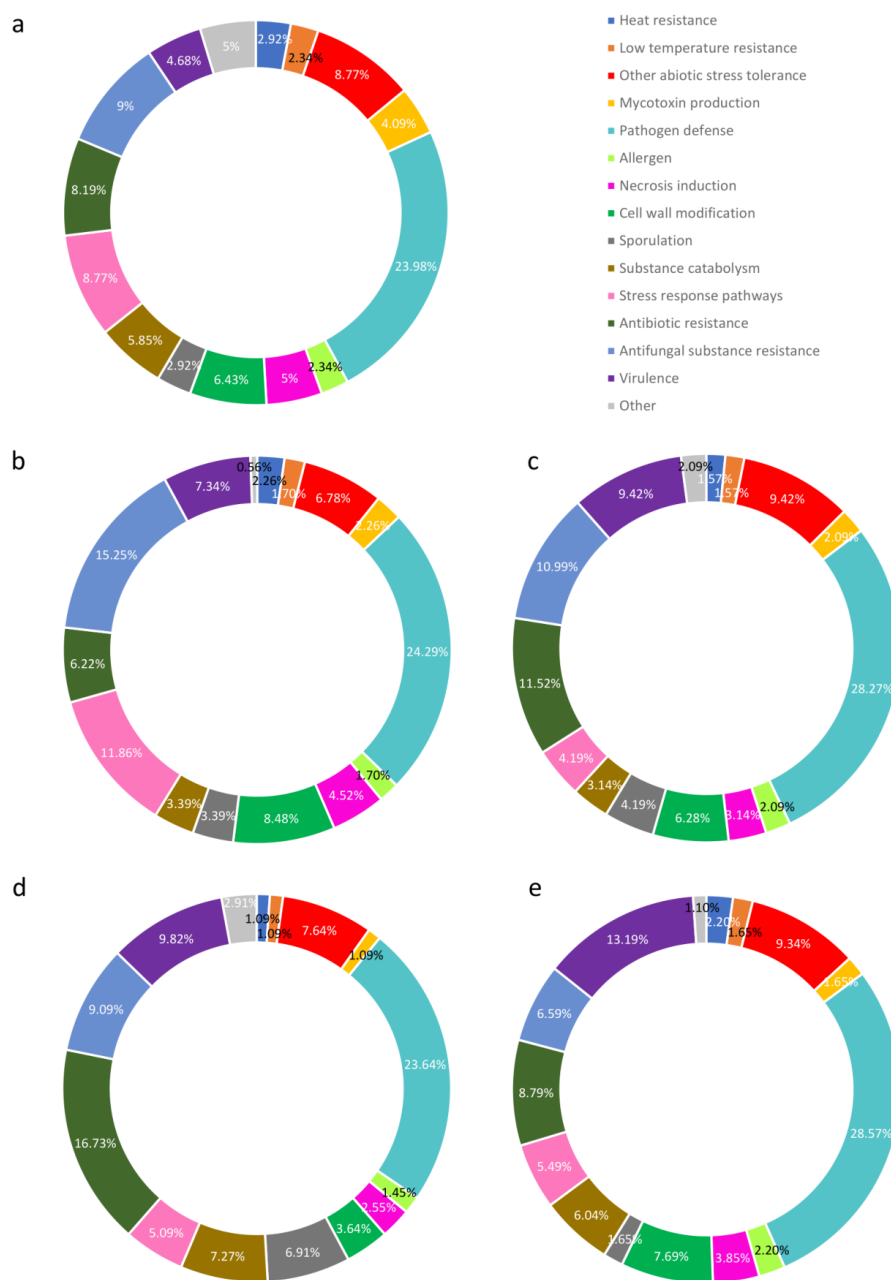
In the genomic analysis of five *Neosartorya* spp. isolates, G127/14 stood out with the largest amount of identified genes and the fewest classified as having an unknown function (Fig. 1a). This isolate also showed the highest proportions of genes involved in intracellular trafficking (5.7%), secondary metabolite production (6.46%), and translation (5.77%). Notably, G127/14 also had the highest proportion of cytoskeleton-related genes (1.94%). The previously established mid-resistance isolates, G130/14 (Fig. 1b) and G132/14 (Fig. 1c), displayed a greater proportion of genes associated with signal transduction (5.13% and 5.40%, respectively) compared to other isolates. G130/14 had the fewest genes related to nucleotide transport and metabolism (1.09%), while all other isolates averaged around 1.2%. G130/14 also had the lowest transcription-related gene proportion (5.82%), though it had elevated levels of RNA modification genes (3.62%) relative to other isolates. Similarly, G132/14 had an elevated RNA modification gene proportion (3.71%), perhaps reflecting its mid-resistance profile. Isolate G135/14 (Fig. 1d), established to be the most resistant in previous studies, harboured the most genes related to DNA repair and replication (3.73%) and extracellular structures (3.92%). It also had the lowest proportion of cytoskeleton-related genes (0.96%) among the isolates. Notably, G135/14 possessed the highest proportions of genes for energy production and conversion (6.28%) and carbohydrate metabolism (7.87%), while most other isolates showed approximately 6.6-6.7% in this category. Both G127/14 and G150/14 (Fig. 1e), previously identified as the least resistant to antifungals, exhibited a higher proportion of transcription-related genes. However, G150/14 had the fewest genes coding for cell wall components (1.9%) and stood out with the highest proportions of genes for posttranslational modification (7.15%) and chromatin structure (1.22%). Across all isolates, approximately 4% of genes were multifunctional, which means they possessed two or more equally significant functions. Each isolate had around 2.4-2.9% of genes dedicated to coenzyme transport, 3-3.4% to lipid metabolism, and 3.19-3.92% to inorganic ion transport.



280

281 Fig. 1 Whole genome functional composition analysis of each of the 5 *Neosartorya* spp. genomes included in the
282 study: G127/14 (a), G130/14 (b), G132/14 (c), G135/14 (d), G150/14 (e)

283 The total proportion of genes related to defence mechanisms varied across the five *Neosartorya* spp.
 284 isolates, with isolates previously classified as more sensitive - G127/14 (1.55%) and G150/14 (1.54%) - having
 285 fewer defence-related genes, while the moderately resistant isolates, G130/14 (1.65%) and G132/14 (1.69%),
 286 contained more (Fig. 2). The most resilient isolate, G135/14, exhibited the highest percentage of defensive
 287 mechanism genes at 1.92%. Defensive genes across all isolates were primarily linked to pathogen defence
 288 mechanisms, which include genes involved in virucidal substance secretion (e.g., cyanovirin, defensin),
 289 antibiotic synthesis, pathogen recognition (e.g., heterokaryon incompatibility), and immune response factors
 290 such as interferon-related genes. Despite being heat-resistant species, these isolates had a limited number of
 291 genes related to heat resistance (1.09–2.92%), which mainly coded for heat-shock proteins. Interestingly, all
 292 isolates possessed nearly as many genes for low-temperature tolerance (1.09–2.34%). Across all isolates,
 293 G127/14 (Fig. 2a) possessed the most genes related to mycotoxin production (4.09%)—notably including
 294 aflatoxin production genes—as well as the highest proportions of allergen-related genes (2.34%) and necrosis-
 295 inducing factors (5%). This isolate also had the highest concentration of genes classified under 'other' stress-
 296 related functions, such as EGF-like domain genes responsible for cell proliferation under stress. Notably, it had
 297 the fewest genes linked to virulence (4.68%). Among all tested fungal isolates, G130/14 (Fig. 2b) had the highest
 298 gene count for cell wall modifications (8.48%), including hydrophobin addition and wax synthesis, and the most
 299 genes for stress response pathways (11.86%), such as stress detection and information relay involving signalling
 300 enzymes (e.g., signalling mucin MSB2). It had the least genes coding for abiotic stress tolerance (6.78%) and the
 301 fewest antibiotic resistance genes (6.22%) but the most genes for antifungal substance resistance (15.25%). This
 302 isolate also had unique genes associated with increased sexual development under zinc depletion (e.g., coding
 303 for the extender of the chronological lifespan protein Ecl1/2/3). G132/14 (Fig. 2c) exhibited the highest gene
 304 proportion for abiotic stress tolerance (9.42%), including UV resistance and hypoxia response proteins. It had the
 305 fewest genes for noxious substance catabolism (3.14%) and the lowest representation of stress response pathway
 306 genes (4.19%) when compared to other *Neosartorya* spp. isolates. G135/14 (Fig. 2d) defensive mechanisms
 307 contained the most genes associated with sporulation (6.91%) and additionally, the isolate possessed sequences
 308 for enhancing sexual development due to zinc depletion (e.g., Ecl1/2/3). It stood out with the highest level of
 309 antibiotic resistance genes (16.73%), including genes coding for Beta-lactamase and aminoglycoside
 310 phosphotransferase, and slight insecticidal activity due to fungal fruit body lectin genes (classified under 'Other').



311

312 Fig. 2 Occurrence of genes pertinent to cell defensive mechanisms of *Neosartorya* spp. isolates: G127/14 (a),

313 G130/14 (b), G132/14 (c), G135/14 (d), G150/14 (e)

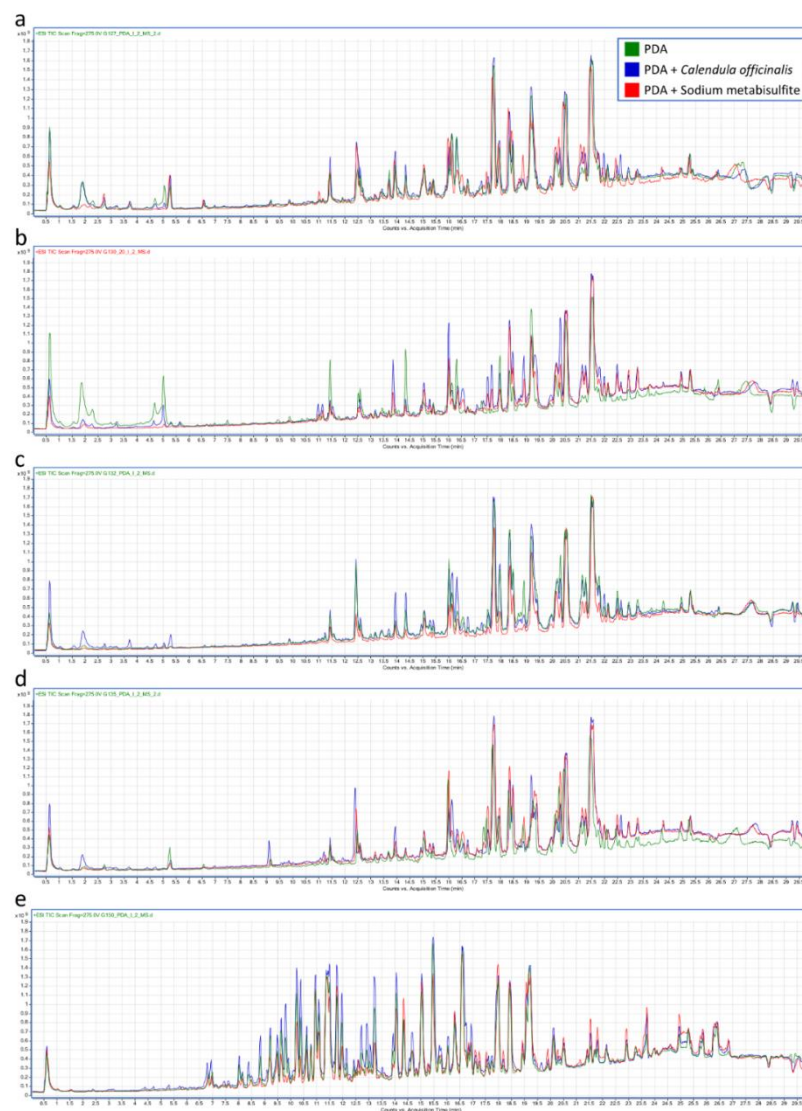
314 On the other hand, this isolate had the lowest proportions of genes coding for temperature resistance (1.09% for
315 both heat and low temperatures), mycotoxin production (1.09%), allergens (1.45%), necrosis-inducing factors
316 (2.55%), and cell wall modification (3.64%). G150/14 (Fig. 2e) displayed the highest percentage of virulence-
317 related genes (13.19%) and had the largest proportion of pathogen defence genes, comprising 28.57% of its
318 genome. It contained the fewest genes for sporulation (1.65%) and antifungal substance resistance (6.59%)
319 among the isolates but also carried genes for potential insecticidal activity (e.g., fungal fruit body lectin in the
320 'Other' category).

321 3.2 LC-QTOF MS analysis

322 The LC-QTOF MS analyses allowed the relative comparisons of metabolic profiles among the different
323 *Neosartorya* spp. isolates cultivated on 3 media variants and two incubation times. They revealed significant
324 variations in the metabolic profiles between the different cultivation conditions, isolates and across the
325 incubation periods. Metabolomic profiles visualized as LC-QTOF total ion current (TIC) chromatograms for 10
326 and 30 day incubation are presented in Figure 3 and 4, respectively. Very distinctive differences in metabolite
327 profiles are apparent for *Neosartorya* spp. isolates. The biggest difference is observed between G150/14 and the
328 four other isolates; the number of metabolites eluting between 6.5 and 15.5 min are present at high quantity only
329 in the former but not in the latter. For all isolates the changes in metabolomics profile occur resulting from the
330 cultivation conditions; the changes in metabolite peak intensities are easily visible.

331 To further examine the metabolite fingerprints of *Neosartorya* spp. differences we performed principal
332 component analyses (PCA). First the differences and similarities between *Neosartorya* spp. isolates were
333 evaluated (Fig. SI3). Clear separation of all isolates was observed for samples cultivated on PDA for 10 days
334 (Fig. SI3a). When the incubation was elongated to 30 days, isolates G132/14 and G135/14 clustered together
335 indicating metabolomic profiles similarity (Fig SI3b). When cultivated for 10 days on PDA with *Calendula*
336 *officinalis*, isolates G130/14 and G132/14 clustered together (Fig SI3c), although after 30 days of incubation
337 clear separation of all isolate groups was visible (Fig SI3d). The two isolates - G130/14 and G132/14 - also
338 exhibited a similar strong metabolic resemblance when cultivated on PDA supplemented with sodium
339 metabisulfite for 10 days (Fig SI3e). However, after 30 days of incubation, the metabolism of G132/14
340 resembled more that of G127/14 (Fig SI3f).

341



342
 343 Fig. 3 LC-QTOF MS study on *Calendula officinalis* and sodium metabisulfite effect on *Neosartorya* spp.
 344 metabolic profiles, 10 day incubation. Chromatograms depict isolates: G127/14 (a), G130/14 (b), G132/14 (c),
 345 G135/14 (d), G150/14 (e)

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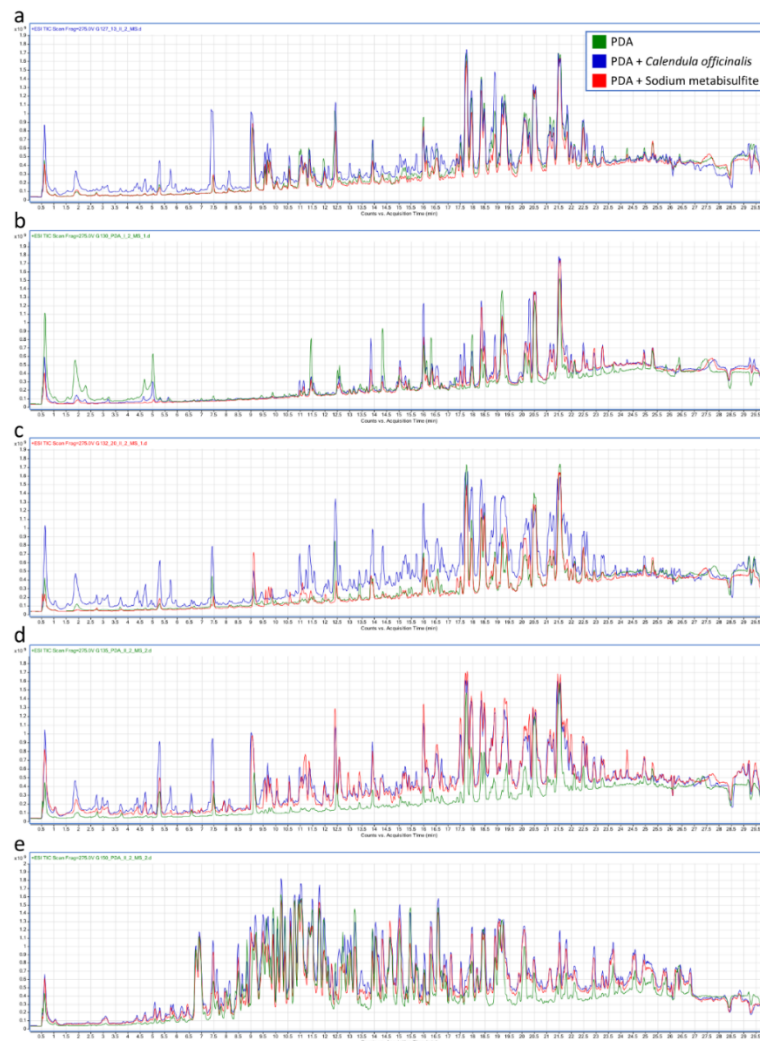


Fig. 4 LC-QTOF MS study on *Calendula officinalis* and sodium metabisulfite effect on *Neosartorya* spp. metabolic profiles, 30 day incubation. Chromatograms depict isolates: G127/14 (a), G130/14 (b), G132/14 (c), G135/14 (d), G150/14 (e)

Then PCA was performed on metabolomic fingerprint data from five isolates individually, taking into account the growth under different cultivation conditions (Fig. SI4). The PCA results revealed distinct, clearly separate clustering patterns between samples grown on PDA, PDA with *Calendula officinalis*, and PDA with

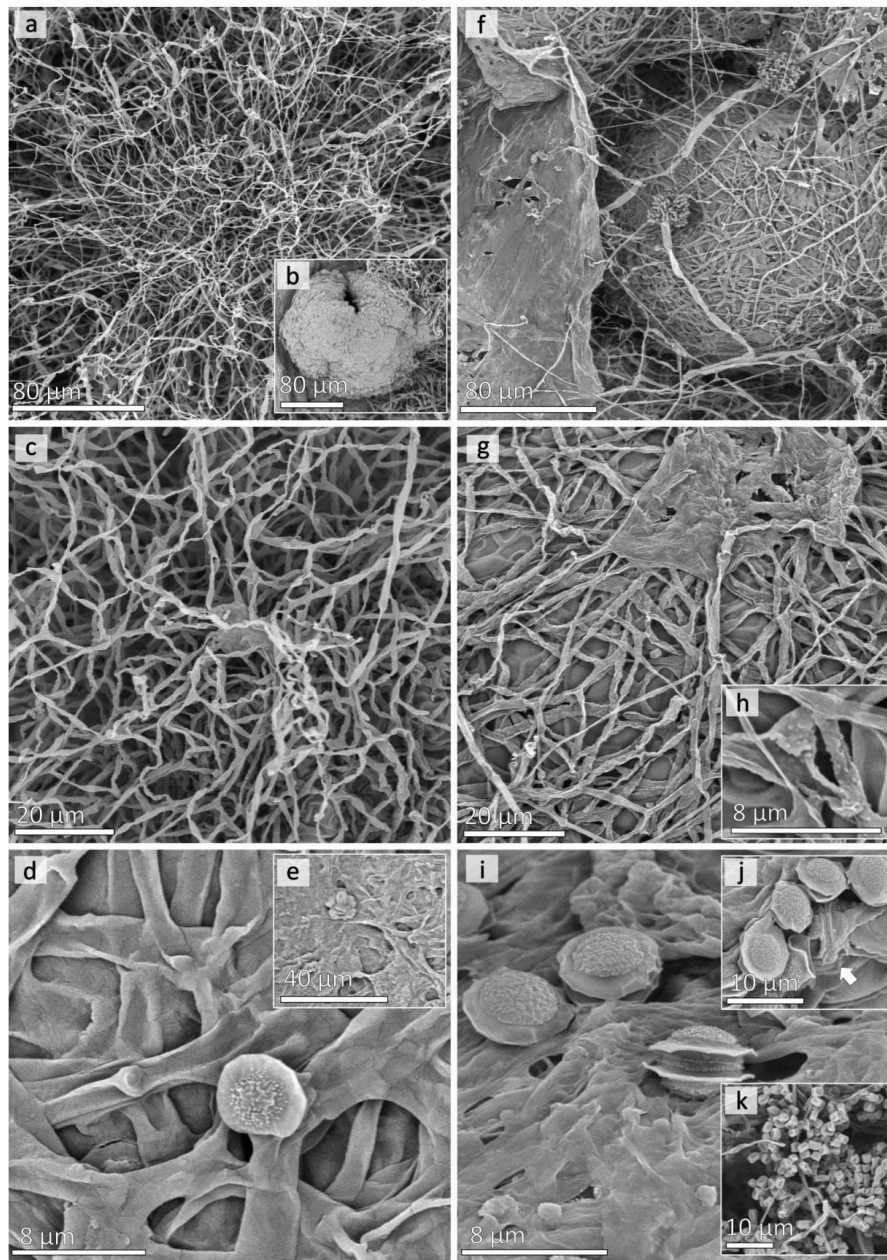
354 sodium metabisulfite, regardless of isolate or time of incubation. This indicates that the medium composition
355 significantly influences the metabolic profiles of the isolates.

356 Next, to visualise global metabolic variability of samples, PCA analyses were performed for all samples
357 incubated for 10 (Fig. SI5a) and 30 (Fig. SI5b) days. As expected, isolate G150/14 clustered in distance from
358 other isolates, regardless of cultivation conditions and duration. After 10 days of incubation, clusters of G127/14
359 on PDA and on PDA with natural extract exhibited similar positions in the PCA space. The highest similarity
360 was observed for G135/14 on preservative-enriched media, G132/14 on PDA, and G135/14 on PDA. Some
361 similarity was also observed for G127/14 and G130/14 on sodium metabisulfite. Following the 30 day
362 incubation period, the more pronounced separation of study variants was revealed. The strongest similarity was
363 found for G135/14 on PDA, G127/14, G130/14 and G150/14 on sodium metabisulfite.

364 3.3 Morphology Characterisation via SEM

365 Isolate G127/14 cultivated on PDA media for 10 days presented highly filamentous and branched aerial
366 hyphae (Fig. 5a). A single, ruptured cleistothecium (avg. Ø170.5 µm) was observed after 10 days of cultivation
367 on PDA (Fig. 5b). Hypha is of rather smooth texture (Fig. 5c) and a width varying between 0.7-2.7 µm. Fully
368 developed ascospores were lenticular and coarse, attaining 5.0-6.0 µm in diameter with two distinct equatorial
369 crests (Fig. 5d). Subglobose asci of approximately Ø10.6 µm were present (Fig. 5e). No conidia were detected.
370 After 30 days of incubation, the general mycelium was covered with a loose and tattered membranous peridium
371 made of what appeared to be pseudoparenchyma (Fig. 5f). Visible hyphae (0.5-1.9 µm wide) were dimorphic,
372 either smooth (Fig. 5g) or reticulated (Fig. 5h), dotted with tuberculate projections (avg. Ø0.4 µm) that gave
373 these structures a verrucose texture. After 30 days of incubation, more sporulation was detectable. Compact
374 spherical cleistothecia (Fig. 5f; avg. Ø202.3 µm) were observed, as well as loose mature ascospores (Fig. 5i;
375 Ø4.7-6.4 µm) and asci (Fig. 5j; avg. Ø6.0 µm). Present were loose globose conidiophores (Fig. 5k) with vesicles
376 of approximately 5.4 µm in width produced cubical conidiospores (avg. 1.7 µm length × 1.6 µm wide) with
377 shallow concave depressions on each side.

378 G127/14 on PDA with *Calendula officinalis* extract after 10 days of incubation possessed dense
379 filamentous hyphae (Fig. 6a), often covered with loose peridium (Fig. 6b), and characterized by closely
380 intertwined dimorphic textures which were either smooth or verrucose with protrusions (Fig. 6c; avg. Ø0.6 µm).



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382 Fig. 5 *Neosartorya* sp. G127/14 on PDA media observed via SEM after 10 (a, b, c, d, e) or 30 (f, g, h, i,

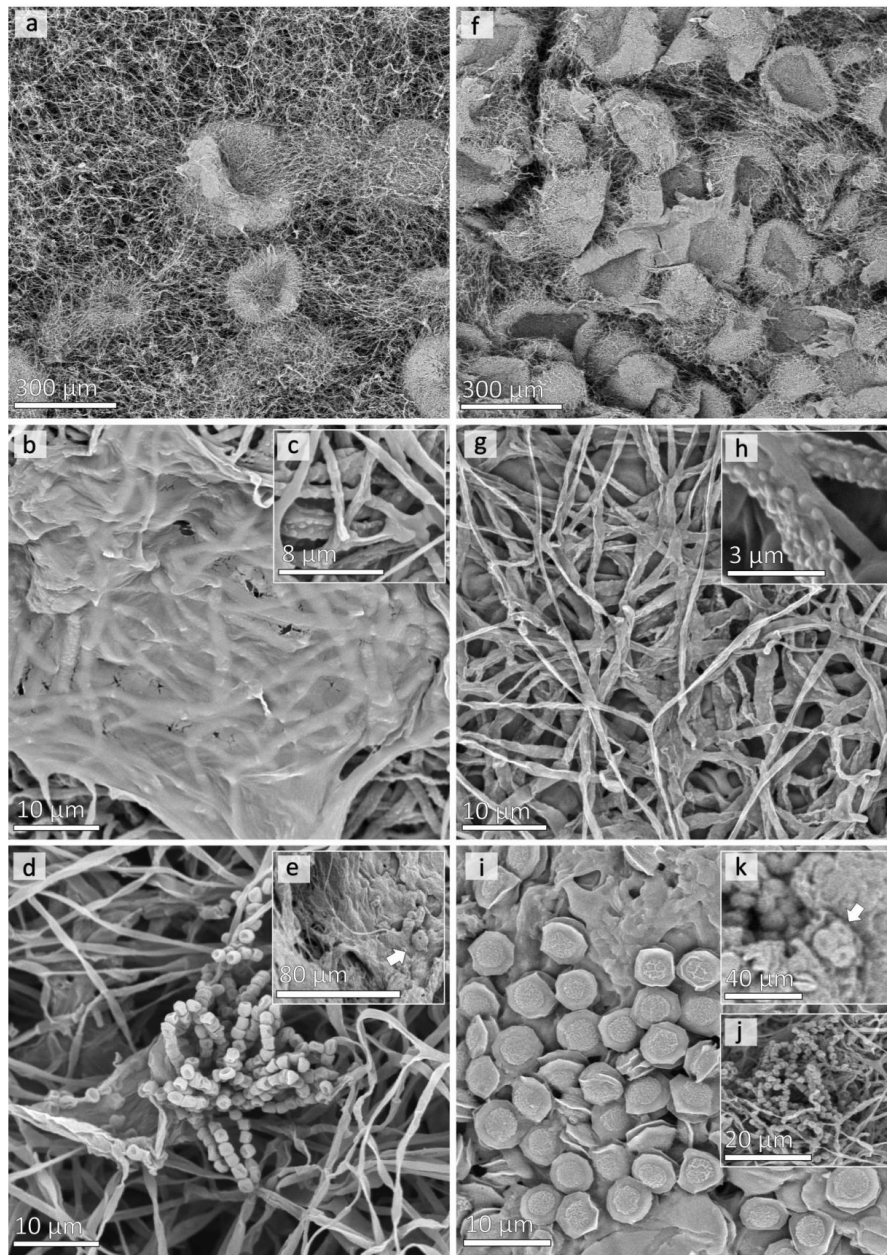
383 j, k) days of incubation. General mycelium structure (a, f), cleistothecium (b), hyphae surface (c, g), hyphal

384 protrusions (h), ascospores (d, i), asci (e, j – marked with an arrow), conidiospores (k)

385 A single hypha measured between 0.7-1.4 μm in width. Numerous non-ostiolate cleistothecia (avg.
 386 $\varnothing 348.9 \mu\text{m}$) of inwardly collapsed spherical shape were observed (Fig. 6a). Numerous conidiophores (vesicle
 387 avg. $\varnothing 13.2 \mu\text{m}$ in width) were identified, presenting well-developed chains of smooth-walled cubical conidia
 388 (avg. $1.4 \times 1.5 \mu\text{m}$) (Fig. 6d). Globose asci with an approximate diameter of $10.1 \mu\text{m}$ were present (Fig. 6e).
 389 Fully developed ascospores were not found. Following an incubation period of 30 days, the mycelium had a
 390 multi-layered structure (Fig. 6f) with coriaceous peridium fragments, a multitude of cleistothecia
 391 (avg. $\varnothing 217.5 \mu\text{m}$) in various stages of development, and an airy network of flexuous hyphae
 392 (Fig. 6g; $0.8\text{-}1.6 \mu\text{m}$ wide) of mostly verrucose surface dotted with slight bumps (Fig. 6h; avg. $\varnothing 0.4 \mu\text{m}$). The
 393 preponderance of cleistothecia had inwardly sunken walls. Fully developed ascospores ($\varnothing 5.4\text{-}6.6 \mu\text{m}$) displayed
 394 a finely roughened convex surface, with two distinct equatorial crests (Fig. 6i). Several asci were spotted
 395 (Fig. 6j; avg. $\varnothing 11.4 \mu\text{m}$). Conidiophore vesicles measuring approximately $6.7 \mu\text{m}$ in width produced irregularly
 396 shaped conidiospores (Fig. 6k; avg. $1.7 \times 1.8 \mu\text{m}$).

397 Incubation of G127/14 on PDA media with sodium metabisulfite for 10 days permitted observation of
 398 fibrous mycelium, characterized by varying levels of growth orientation (Fig. 7a). Irregularly shaped
 399 cleistothecia (avg. $\varnothing 226.5 \mu\text{m}$) were present, many of which appeared collapsed. Hyphae ($0.9\text{-}2.0 \mu\text{m}$ wide)
 400 displayed a serpentine morphology, and the majority was adorned with small, tuberculate projections (Fig. 7b;
 401 avg. $\varnothing 0.4 \mu\text{m}$). A substantial quantity of conidiophores (Fig. 7c; vesicle avg. $10.5 \mu\text{m}$ wide) and conidia
 402 (avg. $1.9 \times 1.5 \mu\text{m}$) in the form of elongated cubes was observed, most with at least the opposing sides exhibiting
 403 concave depressions. Numerous globular asci (Fig. 7d; avg. $\varnothing 9.7 \mu\text{m}$) produced ascospores of similar structure
 404 to those of the control isolate (Fig. 7e; $\varnothing 4.8\text{-}7.3 \mu\text{m}$). After 30 days (Fig. 7f) of incubation on media enriched
 405 with sodium metabisulfite, G127/14 developed even more dotted (avg. $\varnothing 0.5 \mu\text{m}$) and denticulated hyphae (Fig.
 406 7g; $0.5\text{-}2.7 \mu\text{m}$ in width). Numerous spherical cleistothecia (avg. $\varnothing 244.3 \mu\text{m}$) were observable, as well as fully
 407 developed ascospores (Fig. 7h; $\varnothing 5.4\text{-}6.1 \mu\text{m}$) and subglobose asci (Fig. 7i; avg. $\varnothing 4.7 \mu\text{m}$). No conidia were
 408 visible.

409 Isolate G130/14 cultivated on PDA media for 10 days developed an airy network (Fig. SI6a) of floccose
 410 smooth-textured hyphae with slightly pronounced septa (Fig. SI6b; $1.2\text{-}2.2 \mu\text{m}$ wide). A ruptured ellipsoidal
 411 cleistothecium was recorded (avg. $\varnothing 211.4 \mu\text{m}$), as well as fully developed globose ascospores (Fig. SI6c;
 412 $\varnothing 3.9\text{-}6.3 \mu\text{m}$) of convex, roughened surfaces, equipped with two well-separated equatorial crests, and loose
 413 ovoid asci (Fig. SI6d; avg. $\varnothing 9.2 \mu\text{m}$). Columnar conidiophores were visible with clavate vesicles of avg. $7.4 \mu\text{m}$
 414 width and flask-shaped phialides producing cylindrical conidia measuring avg. $1.7 \times 1.5 \mu\text{m}$ (Fig. SI6e).



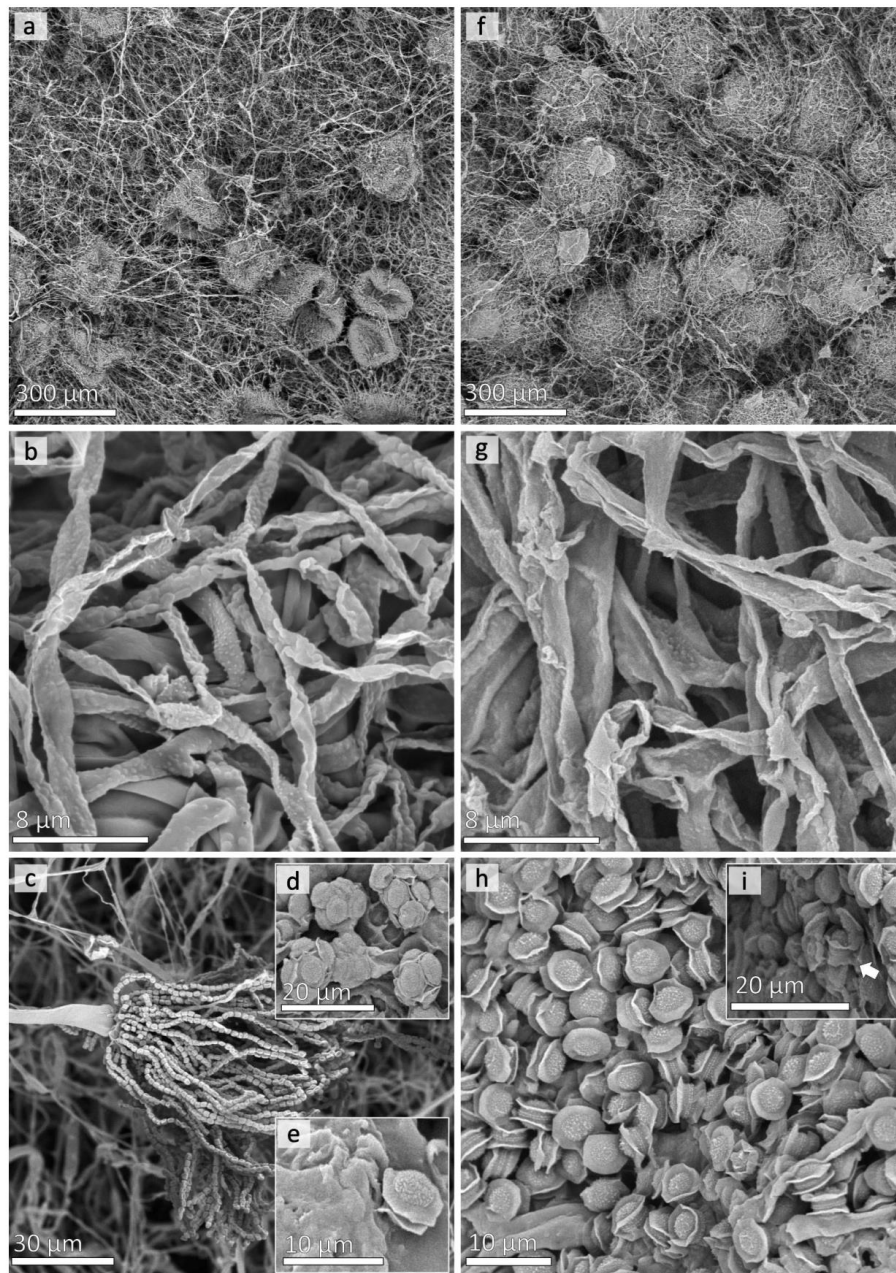
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Fig. 6 *Neosartorya* sp. G127/14 on PDA with *Calendula officinalis* media observed via SEM after 10 (a, b, c, d, e) or 30 (f, g, h, i, j, k) days of incubation. General mycelium structure (a, f), hyphae surface (b, g), hyphal protrusions (c, h), conidiospores (d, k), asci (e, j – marked with an arrow), ascospores (i)



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Fig. 7 *Neosartorya* sp. G127/14 on PDA with sodium metabisulfite media observed via SEM after 10 (a, b, c, d, e) or 30 (f, g, h, i) days of incubation. General mycelium structure (a, f), hyphae surface (b, g), conidiospores (c), asci (d, i – marked with an arrow), ascospores (e, h)

423 After 30 days of growth, the mycelium retained a similar structure (Fig. SI6f) with hyphae measuring
424 between 1.3 to 2.3 μm in width (Fig. SI6g). No cleistothecia were observed, as well as conidiospores and
425 conidiophores. However, some loose mature ascospores ($5.9\text{-}10.6\text{ }\mu\text{m}$) were present with similar morphology
426 to those coming from a 10-day-old sample (Fig. SI6h).

427 Cultivation of G130/14 with *Calendula officinalis* extract resulted in denser mycelium (Fig. SI7a) and
428 the hyphal width ranging from 0.6 to 2.1 μm , with singular smooth hypha frequently anastomosing under minute
429 regions of taut peridium (Fig. SI7b). Several globular cleistothecia were present, all in the early developmental
430 stage (Fig. SI7a; avg. $62.2\text{ }\mu\text{m}$). No observable asci or ascospores were detected, however the peridial wall
431 congregated at the edges of sampled material exhibited small protrusions similar to the very beginning of ascus
432 development (Fig. SI7c). Columnar conidiophores were present, bearing vesicles with an average width of 12.5
433 μm and subglobose or slightly cubical conidia (avg. $2.4 \times 2.0\text{ }\mu\text{m}$) of smooth surface (Fig. SI7d). The sample
434 undergoing a 30-day incubation period developed a reticulated network with more areas of hyphal fusion (Fig.
435 SI7e) and more coarse-surfaced with protrusions (Fig. SI7f; avg. $0.6\text{ }\mu\text{m}$) hyphae ($0.6\text{-}2.5\text{ }\mu\text{m}$ wide).
436 Significantly more cleistothecia (Fig. SI7e; avg. $247.4\text{ }\mu\text{m}$) were observed, varying in shape (globose to
437 irregular and sunken) and development (small globules to fully developed). Numerous globose asci (Fig. SI7g;
438 avg. $9.0\text{ }\mu\text{m}$) produced well-developed ascospores ($4.3\text{-}6.3\text{ }\mu\text{m}$) of similar morphology to those present in the
439 control sample. Conidiophore vesicles were of avg. $11.2\text{ }\mu\text{m}$ width, whereas conidia measurements were $1.7 \times$
440 $1.6\text{ }\mu\text{m}$ on average (Fig. SI7h).

441 Addition of sodium metabisulfite to the media permitted observation of abundant cleistothecia
442 formation, with numerous globules (avg. $216.8\text{ }\mu\text{m}$) appearing after the 10-day incubation period (Fig. SI8a).
443 Mycelium was of filamentous, heavily branched structure with mostly verrucose (avg. $0.5\text{ }\mu\text{m}$) hyphae (Fig.
444 SI8b; $0.9\text{-}3.4\text{ }\mu\text{m}$ in width). Visible were mature ascospores (Fig. SI8c; $4.7\text{-}6.4\text{ }\mu\text{m}$) and globose to subglobose
445 asci (Fig. SI8d; avg. $10.1\text{ }\mu\text{m}$) and, as well as conidiophores with vesicles (avg. $6.0\text{ }\mu\text{m}$ in width) and linked
446 subglobose conidia with somewhat sunken walls (avg. $1.8 \times 1.9\text{ }\mu\text{m}$). After 30 days of incubation, the general
447 sample structure was dominated by a multitude of cleistothecia (Fig. SI8e; avg. $214.9\text{ }\mu\text{m}$), mostly fully
448 developed and coated in coriaceous peridium. Hyphae (Fig. SI8f; $1.1\text{-}2.8\text{ }\mu\text{m}$ wide) retained the largely
449 verrucose structure with small protuberances (avg. $0.7\text{ }\mu\text{m}$). Ascospores were varying between 5.5 to $6.8\text{ }\mu\text{m}$ in
450 diameter (Fig. SI8g). Asci were of mostly globose configuration (Fig. SI8h; avg. $7.9\text{ }\mu\text{m}$). Multitudinous
451 conidiophores (Fig. SI8i) were present with vesicles of avg. $10.2\text{ }\mu\text{m}$ width and isodiametric conidia of approx.
452 $2.1\text{ }\mu\text{m}$ length and $2.0\text{ }\mu\text{m}$ width.

453 Isolate G132/14, after 10 days of incubation on PDA media, developed an airy, multi-layered structure
 454 of mycelium (Fig. SI9a) consisting of smooth-textured hyphae (Fig. SI9b). Individual hypha reached 0.7-1.8 μm
 455 in width. Observation detected no apparent cleistothecia; however, among lower sample layers were hidden
 456 loose asci (avg. $\varnothing 9.0 \mu\text{m}$) of irregular shape and mature globose ascospores ($\varnothing 4.5\text{-}6.1 \mu\text{m}$) marked with two
 457 equatorial ridges and roughened surfaces (Fig. SI9c). Abundant conidiation was observed, with conidiophore
 458 vesicles averaging approximately 13.7 μm in width (Fig. SI9d). Conidia (avg. $2.0 \times 1.7 \mu\text{m}$) were of ovoid
 459 shape, catenulate, and slightly folded. After 30 days, the mycelium remained of similar structure (Fig. SI9e),
 460 with hyphae measuring 0.8-1.9 μm in width (Fig. SI9f). No cleistothecia or conidiophores were visible, yet the
 461 sample presented loose subglobose conidiospores with walls more sunken than after 10 days of incubation (Fig.
 462 SI9g; avg. $2.5 \times 1.8 \mu\text{m}$), mature globose ascospores (Fig. SI9g; $\varnothing 5.5\text{-}6.9 \mu\text{m}$), and globular asci (Fig. SI9h;
 463 avg. $\varnothing 10.5 \mu\text{m}$).

464 After 10 days, incubation on PDA media enriched with *Calendula officinalis* extract caused changes in
 465 G132/14. The mycelium began to form subtle aggregations (Fig. SI10a). Many hyphae (0.9-2.7 μm in width)
 466 began to exhibit more prominent septation and developed small, globular protrusions (avg. $\varnothing 0.4 \mu\text{m}$) on their
 467 surface (Fig. SI10b). Although no cleistothecia could be seen, singular mature ascospores ($\varnothing 4.1\text{-}5.7 \mu\text{m}$) of
 468 convex shape were found at the sample's borders, close to the media (Fig. SI10c). A single subglobular asci (Fig.
 469 SI10c; $\varnothing 7.2 \mu\text{m}$) formation was seen, enmeshed in the media. Numerous columnar conidiophores were visible,
 470 with vesicles averaging 6.8 μm in width (Fig. SI10d). Conidiospores (avg. $2.5 \times 1.8 \mu\text{m}$) were mostly ovoid,
 471 smooth-textured, with indented walls. When incubation time was elongated to 30 days, mycelium of G132/14
 472 seemed more tightly-woven, with numerous areas concealed beneath a layer of loose, membranous, plicate
 473 peridium (Fig. SI10e). Hyphae (0.8-2.7 μm wide) were ramified, dichotomous in texture – either smooth or
 474 tuberculate (Fig. SI10f; avg. $\varnothing 0.4 \mu\text{m}$). Numerous cleistothecia (Fig. SI10e; avg. $\varnothing 175.5 \mu\text{m}$) were present,
 475 some exhibiting a globose morphology, while others presented indents and depressions in outer walls. Stages of
 476 development varied – from the beginning of formation to rupture. Mature ascospores ($\varnothing 5.6\text{-}6.6 \mu\text{m}$) were
 477 present in abundance (Fig. SI10g). Visible were subglobose asci (Fig. SI10h; avg. $\varnothing 5.7 \mu\text{m}$). The sample
 478 presented no asexual morphological characteristics.

479 The incorporation of sodium metabisulfite in the media caused a rapid change in the growth pattern of
 480 G132/14. After 10 days of incubation, the sample presented a fibrous texture (Fig. SI11a), filled with hyphae
 481 (1.1-2.4 μm) of mostly rugose texture rich in protrusions (Fig. SI11b; avg. $\varnothing 0.5 \mu\text{m}$). Copious amounts of loose,
 482 tattered peridia were situated on the top layer of the mycelium, especially dense in occurrence when near

483 globular cleistothecia (Fig. SI11a; avg. Ø220.9 µm). Globose asci (Ø12.7 µm) and mature ascospores
 484 (Ø5.6-7.0 µm) of convex shape could be observed near the medium (Fig. SI11c). Visible were conidiophores
 485 (avg. vesicle width: 5.8 µm) with chained cuboid conidia (avg. 2.0 × 1.7 µm) (Fig. SI11d). After 30 days of
 486 incubation, the mycelium retained a similar structure to that of the 10 days old sample, albeit with less numerous
 487 loose peridium occurrence (Fig. SI11e). Cleistothecia (avg. Ø178.7 µm) were globose or subglobose (Fig.
 488 SI11e). Hyphae measured between 0.9 to 2.1 µm in width and possessed subtle ovoid protuberances (Fig. SI11f;
 489 avg. Ø0.4 µm). Ascospores were spherical (Ø5.5-6.9 µm) with well-established central ridges (Fig. SI11g).
 490 Visible were ellipsoid asci (Fig. SI11h; avg. Ø6.2 µm). The isolate displayed populous conidiation, with vesicles
 491 measuring 12.5 µm in width on average (Fig. SI11i). Each conidiophore was dense with cuboid or subglobose
 492 conidia (avg. 1.9 × 1.8 µm) bound in chains.

493 Isolate G135/14, cultivated on PDA media for a duration of 10 days, developed a dense, floccose, and
 494 flexuous mycelial structure (Fig. SI12a) with smooth-textured hyphae varying from 0.6 to 1.3 µm in width
 495 (Fig. SI12b). No visible cleistothecia were present; however, near the medium, several mature ascospores
 496 (Fig. SI12c; Ø4.5-7.3 µm) and ovoid asci (Fig. SI12d; avg. Ø7.4 µm) could be found. These spores were of
 497 rough, papillate texture and mostly convex shape, placed level with the media's surface. Although two equatorial
 498 crests were easily discernible, the folds' tissue seemed flaccid. Numerous conidiophores were visible, with an
 499 average vesicle width of 18.1 µm (Fig. SI12e). Despite some of them being bare, some carried chains of ovoid
 500 conidia (avg. 2.1 × 1.6 µm) with slightly sunken walls. After 30 days of incubation, the general mycelium
 501 exhibited a denser reticulated interweaving (Fig. SI12f), characterized by numerous sinuous hyphae (0.5-1.4 µm)
 502 of smooth texture (Fig. SI12g). The sample was rather monomorphic, not exhibiting an occurrence of any
 503 fruiting bodies. Only near the media surface, attached to peridium, were some well-developed globular
 504 ascospores
 505 (Ø5.3-6.6 µm) found (Fig. SI12h). The spores were verrucose, with two taut equatorial ridges.

506 Cultivation on media with *Calendula officinalis* altered the sample's morphology. After 10 days of
 507 incubation, the mycelium took the form of a clumped, compact network of aggregating hyphae
 508 (Fig. SI13a; 0.7-1.4 µm). Multitudinous cleistothecia (Fig. SI13a; avg. Ø96.4 µm) could be seen, most still in
 509 early phases of development and heavily interconnected with dimorphic hyphae of either smooth or deeply
 510 verrucous texture with ovoid obtrusions (Fig. SI13b; avg. Ø0.5 µm). Subglobose asci (avg. Ø8.8 µm) and fully
 511 developed, globular ascospores (Ø5.1-7.1 µm) could be detected (Fig. SI13c). Visible were columnar
 512 conidiophores with vesicles averaging 18.7 µm in width and short chains of subglobular conidia with depressed

513 walls (Fig. SI13d; avg. $2.2 \times 1.7 \mu\text{m}$). After 30 days, the general mycelium structure was dominated by the
 514 appearance of numerous mature cleistothecia (Fig. SI13e; avg. $\text{Ø}143.3 \mu\text{m}$), many of which appeared ruptured.
 515 Filamentous hyphae ($0.3\text{-}0.9 \mu\text{m}$) spanned from one cleistothecium to another, with many intertwined with
 516 sections of taut coriaceous peridium (Fig. SI13f). Hyphae were dimorphic: presenting as thin, smooth, and
 517 thread-like, or as wider filaments with coarse texture in the form of minuscule protrusions (avg. $\text{Ø}0.4 \mu\text{m}$).
 518 Multifaceted ascospores (Fig. SI13g; $\text{Ø}5.5\text{-}6.4 \mu\text{m}$) presented diverse phenotypes: globular and denticulate with
 519 no crests; globular and rugose, with two distinct equatorial ridges; lenticular, decorated with several considerably
 520 undulate folds. No asexual reproductive structures could be detected.

521 Sodium metabisulfite caused mycelial clustering and mass cleistothecia formation in G135/14, even in
 522 the sample with a short, 10-day-long incubation period (Fig. SI14a). Ascocarps (avg. $\text{Ø}169.1 \mu\text{m}$) were globular,
 523 non-ostiolate, covered with multiple layers of prosenchymatous peridium (Fig. SI14a). Hyphae ($0.8\text{-}2.3 \mu\text{m}$)
 524 were dimorphic: diaphanous of smooth texture, or opaque and dotted with minuscule projections (Fig. SI14b;
 525 avg. $\text{Ø}0.4 \mu\text{m}$). Visible were globose asci (Fig. SI14c; avg. $\text{Ø}10.4 \mu\text{m}$) and globular ascospores
 526 (Fig. SI14d; $\text{Ø}5.2\text{-}6.4 \mu\text{m}$) with rough texture and double central ridges. Abundant conidiation was present, with
 527 tufty conidiophore vesicles measuring $15.2 \mu\text{m}$ on average (Fig. SI14e). Plentiful smooth-textured conidia (avg.
 528 $2.2 \times 1.7 \mu\text{m}$), joined in numerous chains, were subglobose or cubical with walls slightly folded inwards.
 529 Following 30 days of incubation, an increased presence of peridial structures was observed on the upper layers
 530 of the sample (Fig. SI14f). Hyphae ($0.5\text{-}2.8 \mu\text{m}$) retained their dimorphism, although the protrusions seemed
 531 more denticulate (Fig. SI14g; avg. $\text{Ø}0.4 \mu\text{m}$). Abundant reproductive structures were observable: globular
 532 cleistothecia (Fig. SI14f; avg. $\text{Ø}171.4 \mu\text{m}$); subglobular, almost ready to disperse asci (Fig. SI14h; avg. $\text{Ø}6.4$
 533 μm); multifaceted ascospores ($\text{Ø}5.0\text{-}6.5 \mu\text{m}$). Ascospores of globular shape and distinct equatorial crests were
 534 the most common, although some lenticular builds with undulate folds were also present. Conidiophores were
 535 sub-erect, with vesicles averaging $8.18 \mu\text{m}$ in width and subglobular conidia of $2.7 \mu\text{m}$ length and $2.2 \mu\text{m}$ width
 536 on average (Fig. SI14i).

537 Isolate G150/14, after a 10-day-long cultivation on PDA, developed a floccose structure (Fig. SI15a)
 538 with filamentous, smooth hyphae (Fig. SI15b; $0.8\text{-}2.4 \mu\text{m}$). Some early fruiting body development was
 539 perceivable, with clumps of hyphae possibly forming early stirrings of cleistothecia (avg. $\text{Ø}186.1 \mu\text{m}$). On the
 540 sample's margins, close to the media, mature ascospores (Fig. SI15c; $\text{Ø}5.2\text{-}6.6 \mu\text{m}$) of convex bivalved build
 541 with double midline ridges and coarse surface were present, as well as some subglobular asci (Fig. SI15d; avg.
 542 $\text{Ø}10.1 \mu\text{m}$). No asexual reproductive structures could be recorded. After 30 days of incubation, G150/14

543 developed slightly more flexuous (Fig. SI15e), smooth-textured hyphae (0.6-3.1 μm), which appeared to be
544 relatively homogenous, as no large fruiting bodies of sexual or asexual nature were present (Fig. SI15f).
545 However, at the sample's edge, a multitude of ascospores ($\varnothing$ 4.9-6.4 μm) and some asci (avg. $\varnothing$ 5.4 μm) could be
546 discovered (Fig. SI15g). The spores retained the morphology presented during the 10-day-long sampling,
547 whereas asci exhibited a more globular build than previously observed, probably due to the late-stage
548 development. No conidiospores could be recorded.

549 Supplementing *Calendula officinalis* extract to the media seemed to stimulate the creation of protective
550 structures in G150/14. The hyphae coalesced to form a loosely organized peridium, which was superficially
551 situated atop the sample (Fig. SI16a). After 10 days of incubation, an individual, smooth-textured hypha attained
552 between 1.1 to 2.3 μm in width (Fig. SI16b). Numerous globose to subglobose cleistothecia (Fig. SI16a; avg.
553 $\varnothing$ 180.3 μm), spheroidal asci (Fig. SI16c; avg. $\varnothing$ 9.4 μm), and fully developed, globose ascospores with verrucose
554 surfaces and two equatorial crests (Fig. SI16c; $\varnothing$ 3.8-5.9 μm) were visible. Elongating the incubation time to 30
555 days resulted in a formation of spherical cleistothecia (Fig. SI16d; avg. $\varnothing$ 253.4 μm) that were mostly separated
556 and only occasionally connected to long strings of branched out hyphae. Certain regions were covered by a
557 loosely attached peridium. The fungus' hyphae (0.6-2.8 μm) were dimorphic, exhibiting a texture that was either
558 smooth or dotted with irregular protrusions (Fig. SI16e; avg. $\varnothing$ 0.3 μm). Loose ascospores (Fig. SI16f; $\varnothing$ 5.7-6.2
559 μm) could be found near the medium, all presenting similar morphology to that attained after 10 days of
560 incubation. Observable were globose asci (Fig. SI16g; avg. $\varnothing$ 5.5 μm). No conidiation was evident after either of the
561 incubation periods.

562 Subsequent to a 10-day incubation period, the growth of G150/14 on PDA with sodium metabisulfite
563 was visibly altered. The mycelium was layered, fibrous, and airy (Fig. SI17a). Globular cleistothecia (avg.
564 $\varnothing$ 198.2 μm) were extensively interwoven with hyphae (Fig. SI17a). Hyphae (1.1-2.8 μm) were sinusoidal,
565 undulate, almost hyaline, and smooth-textured (Fig. SI17b). Loose globose asci (Fig. SI17c; avg. $\varnothing$ 10.1 μm) and
566 mature ascospores (Fig. SI17d; $\varnothing$ 4.8-6.1 μm) were prevalent near the medium. Sexual spores were globular,
567 rough in texture, and decorated with double equatorial crests. The isolate developed numerous erect
568 conidiophores (vesicles of avg. 6.8 μm in width) with lengthy strings of vaguely cubical conidiospores (avg. 1.6
569 $\times$ 1.5 μm) presenting walls slightly folded inwards (Fig. SI17e). As a consequence of the 30-day incubation
570 period, the mycelium lessened in density (Fig. SI17f). Cleistothecia (avg. $\varnothing$ 220.2 μm) appeared to be twofold as
571 bountiful as with the 10-day-old sample, with some additionally covered by segmented peridium (Fig. SI17f).
572 Hyphae (0.7-1.3 μm in width) developed a texture dimorphism, with some strands gaining a verrucose texture

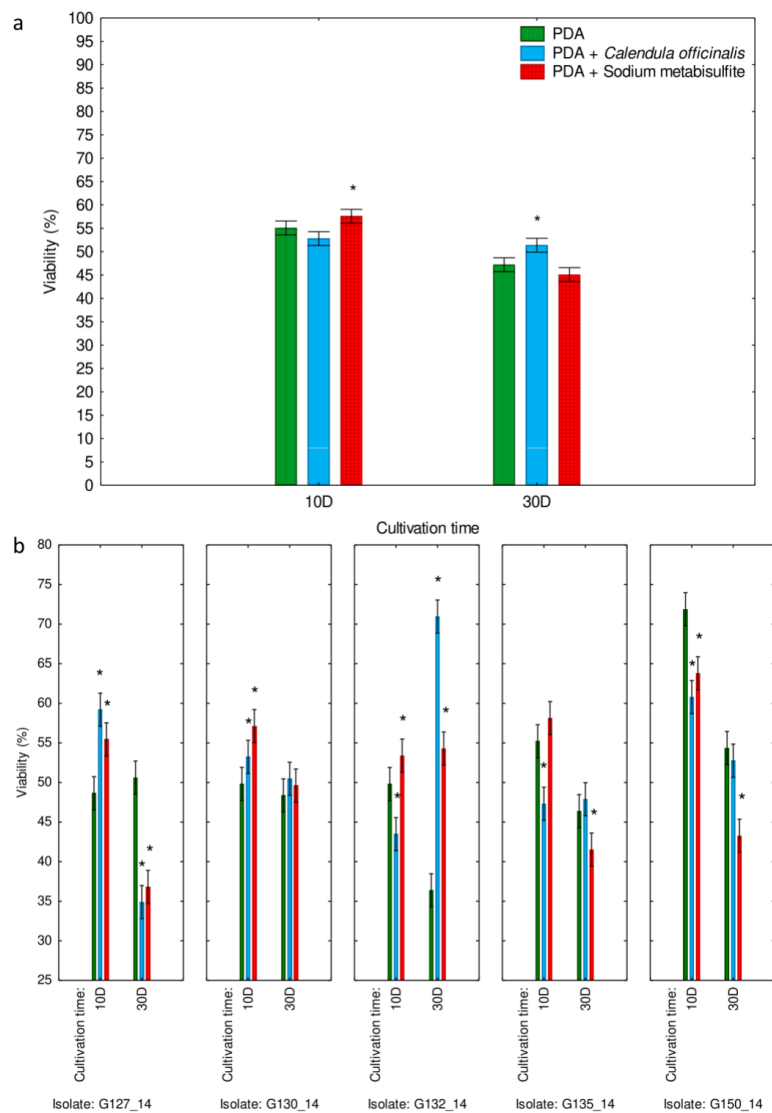
573 made up of tuberculate obtrusions (Fig. SI17g; avg. Ø0.3 µm). Subglobose asci (avg. Ø6.3 µm) were
574 infrequently observed, as the majority of ascospores (Ø5.5-6.4 µm), resembling those from the 10-day-old
575 sample, were fully developed (Fig. SI17h). The 30-day-long incubation period did not result in conidiation.

576 3.4 Viability Assessment via Confocal Fluorescence Microscopy and FUN-1 Staining

577 Fungal viability (%) assessment was conducted using the method of multi-colour fluorescence
578 microscopy for each of the studied isolates: G127/14 (Fig. SI18), G130/14 (Fig. SI19), G132/14 (Fig. SI20),
579 G135/14 (Fig. SI21), G150/14 (Fig. SI22). The evaluation revealed distinct effects of the cultivation media on
580 the five isolates over time (Fig. 8a). After 10 days of incubation, isolates grown on PDA supplemented with
581 sodium metabisulfite exhibited significantly higher viability compared to those grown on PDA alone, where
582 *Calendula officinalis* induced no observable impact (Fig. 21a). However, after 30 days of incubation, the trend
583 reversed: isolates cultivated on PDA with *Calendula officinalis* demonstrated increased viability, while the
584 sodium metabisulfite treatment no longer influenced viability. These findings suggest that the effects of sodium
585 metabisulfite and *Calendula officinalis* on isolate viability are time-dependent.

586 Viability comparisons for each isolate revealed more treatment-specific and time-dependent effects
587 (Fig. 8b). For isolate G127/14, viability was significantly higher for both *Calendula officinalis* and sodium
588 metabisulfite treatments after 10 days, while both treatments led to reduced viability after 30 days. Isolate
589 G130/14 exhibited higher viability for both treatments at 10 days but showed no significant difference for any of
590 the treatments after 30 days. Isolate G132/14 had lower viability with *Calendula officinalis* and higher viability
591 with sodium metabisulfite than the control on PDA after 10 days, but both treatments enhanced viability after 30
592 days. Isolate G135/14 displayed reduced viability with *Calendula officinalis* at 10 days, however this effect did
593 not occur after 30 days of incubation. However, after this time, G135/14 presented lower viability with sodium
594 metabisulfite. Finally, for isolate G150/14, viability was lower for both treatments at 10 days, while only sodium
595 metabisulfite caused a reduction in viability at 30 days.

596



597

598 Fig. 8 Effect of *Calendula officinalis* extract and sodium metabisulfite on *Neosartorya* spp. viability,
 599 according to confocal fluorescence microscopy. Influence of media composition on all isolates cumulatively
 600 after either 10 or 30 days of cultivation (a). Influence of media composition on each isolate separately after either
 601 10 or 30 days of cultivation (b). Asterisks indicate statistically significant differences between the viability of a
 602 group affected by one of the substances and the corresponding control during a particular experiment day

603 4. Discussion

604 Although *Aspergillus* genus genomes were recently studied, databases lack fully sequenced genomes
605 belonging to all species of this group of fungi (Machida et al., 2005; Arnaud et al., 2012). Here we provide
606 further insights into *Aspergillus/Neosartorya* spp. genomes composition and gene functions, with an emphasis on
607 defensive functions encoded by obtained sequences. Our whole-genome sequencing (WGS) results reveal genes
608 associated with both heat resistance and low-temperature tolerance, suggesting an adaptive resilience to
609 fluctuating environmental conditions—a characteristic that could have important implications for their control in
610 industrial and food storage settings. Among the genes associated with heat tolerance, several contain domains
611 such as STI1_HS-bd (a chaperonin-binding domain found in stress-inducible proteins like STI1; Lässle et al.,
612 1997), Hikeshi-like, N-terminal domain, and HS1-bd. The presence of these domains indicates the isolates’
613 potential reliance on robust stress-response mechanisms. STI1, which acts as a co-chaperone for HSP90, binds
614 heat shock proteins at both its N- and C-terminal ends, likely enhancing protein stabilization and repair under
615 high-temperature stress (Lässle et al., 1997). This aligns with studies in other fungi, like *Saccharomyces*
616 *cerevisiae* or *Aspergillus fumigatus* that describe the role of STI1 in assisting cells to endure sudden temperature
617 increases by heat-shock protein binding (Nicolet & Craig, 1989; Tiwari et al., 2015). Additionally, Hikeshi
618 domain acts as a specific nuclear import carrier for HSP70 proteins following heat-shock stress (Kose et al.,
619 2012). Lastly, the HS1-bd domain, which interacts with heat shock factor 1 (HSF1), functions to suppress HSF1
620 transcriptional activity following heat stress, contributing to thermotolerance and further supporting the thermal
621 resilience of *Neosartorya* spp. isolates in heat-stressed environments (Santoro, 2000; Muthusamy et al., 2023).

622 In addition to heat resilience, our results identify genes associated with low-temperature tolerance,
623 including Low temperature viability protein Ltv1, Low temperature requirement A, and Defect at low
624 temperature protein 1. These genes suggest that the isolates are not only adapted to heat but also capable of
625 enduring cold environments, which may enhance their persistence in refrigerated storage. The Ltv1 protein,
626 essential for 40S ribosomal subunit biogenesis in yeast, also facilitates growth and 40S subunits production at
627 low temperature (Merwin et al., 2014). The Defect at low temperature protein 1 (Dlt1) family proteins are known
628 to support growth under high-pressure and low-temperature environments (Abe & Minegishi, 2008). Finally, the
629 presence of Low temperature requirement A proteins, which support growth in cold conditions in organisms like
630 *Listeria monocytogenes*, implies that *Neosartorya* spp. could retain metabolic activity even at low temperatures,
631 thus evading typical refrigeration measures since it could potentially survive cultivation at 4°C (Zheng &
632 Kathariou, 1995). Since genes pertaining to low temperature survival mechanisms are found in all studied

isolates, it may be indicative of a broader tolerance range than previously anticipated. Such reasoning is further upheld by the presence of other abiotic stress survival genes. *Neosartorya* spp. seem to have innate alkaline pH signalling pathways based on the occurrence of pH-response regulator protein palH/rim21 which acts as a pH sensor in *Aspergillus nidulans* (Herranz et al., 2005). Often found is the hypoxia induced gene 1 (HIG1) which is a hypoglycaemia/hypoxia inducible mitochondrial protein (HIMP1), a protein up-regulated by stresses of the microenvironment such as low oxygen or low glucose conditions (Gracey et al., 2001). It is speculated that these proteins may have a regulatory role for the Cytochrome C in fungi (Garlich et al., 2017). *Neosartorya* spp. also possess genes encoding plant stress-response proteins, such as stress responsive alpha-beta barrel domain which may have its role in molybdopterin uptake and degradation of plant alkaloid nicotine (Cornilescu et al., 2004). Furthermore, UV radiation resistance protein/autophagy-related protein 14 found in the obtained sequences may influence response to starvation or UV radiation resistance (Kametaka et al., 1998; Liang et al., 2006). According to literature, substances such as fischerose, trehalose, mannitol, neosartose, and isobemisiose are responsible for heightened *Neosartorya* spp. survival and resilience in adverse conditions (Fujimoto et al., 1993; Kuenstner et al., 2020; Wyatt et al., 2014, 2015). This concurs with our findings as in tested isolates, genes TPS3_1, TPS1_1, TPS1_2, TPS2, TPS3_3 were found coding for Trehalose-6-P synthase/phosphatase (Avonce et al., 2006). Several mannitol-related gene sequences can also be found, mostly regarding mannitol synthesis via fermentation of fructose with mannitol dehydrogenase (Saha & Racine, 2011). As neosartose, fischerose and isobemisiose are trehalose-based tri-, tetra- and pentaoligosaccharides, another approach is needed to determine their occurrence (Walmagh et al., 2015; Kuenstner et al., 2020).

Besides an array of tools permitting overcoming the possible detrimental abiotic factors, *Neosartorya* spp. are equipped with genes enabling strong resistance to pathogens and bacteria. As such, studied isolates of *Neosartorya* spp. exhibit strong resistance to bacterial antibiotics by expressing genes for the production of tetracycline resistance protein TetA and multidrug resistance protein MdtG (Kimura et al., 1998; Kunihiko & Akihito, 2001). Isolates retain multiple copies of genes encoding beta-lactamase that catalyses beta-lactam antibiotics such as penicillins and cephalosporins (Gholap et al., 2021). A similar situation can be observed with genes coding for aminoglycoside phosphotransferase, which inactivates aminoglycoside antibiotics such as kanamycin and streptomycin (Shao et al., 2013). For further protection, isolates commonly use a typical for *Ascomycetes* mechanism of heterokaryon incompatibility to recognize and respond to pathogens (Espagne et al., 2002). Common are genes encoding toxins like aerolysin similar to bacterial toxin ETX, and cyanovirin, a toxin active against viruses (Botos et al., 2002; Cole et al., 2004). All isolates possess genes coding for defensin, a

663 cysteine-rich peptide with antibacterial properties (Contreras et al., 2018). *Neosartorya* spp. are also highly
 664 specialised in engaging with antifungal substances. Detection of genes coding for acriflavin resistance denotes
 665 resistance to disinfectants (Rasheed et al., 2021). Present are also genes coding MFS transporter superfamily and
 666 multi antimicrobial, multidrug and toxic compound extrusion proteins – all regarding cell protection from
 667 antifungal agents (Moriyama et al., 2008; Segato et al., 2008; Tanaka et al., 2017). *Neosartorya* spp. seems to
 668 present an array of genetic mechanisms for azole resistance, including genes CDR1_1, CDR1_2 and MDR1_1
 669 which relate to fluconazole resistance (Butassi et al., 2021). Studied isolates possess ERG11_1 and ERG11_2
 670 genes, which are involved in ergosterol biosynthesis, a crucial component of fungal cell membranes. Ergosterol
 671 is a key component of the fungal cell membrane, playing a critical role in both growth and ability to adapt to
 672 stress due to the remodelling of the cell wall and membrane, which reduces drug permeability (Hu et al., 2017;
 673 Robbins et al., 2017). Moreover, WGS detection of SOD1, SOD2, and SOD4, which code for superoxide
 674 dismutase, may be associated with the response to oxidative stress, as these genes play a crucial role in the
 675 removal of free radicals, helping the fungus survive under toxic conditions (Maurya & Namdeo, 2021).
 676 Interestingly, studied isolates seem to also possess genes relating to natural plant extract resistance. According to
 677 literature, pleiotropic drug resistance genes, the ATP-binding cassette superfamily (e.g. Pdr5p, Snq2p, Yor1p) as
 678 well as its transcriptional activators (Pdr1p, Pdr3p) have an important role in the detoxification of the antifungal
 679 constituents of medicinal plants e.g. *Artemisia annua* (Kolaczowski et al., 2009). Here, several ATP-binding
 680 cassette transporter genes are present (e.g. SNQ2_5, SNQ2_6) as well as genes partially related to pleiotropic
 681 drug resistance (CDR1_1, CDR1_2) which suggests innate plant natural extract resistance of *Neosartorya* spp.
 682 isolates. Other genes found in obtained sequences and possibly responsible for low susceptibility to plant-derived
 683 substances could be ERG4_2, ERG4_3, ERG4_4, (sterol reductases) and ERG6 (sterol methyltransferase), as
 684 they seem to be linked to tomatidine resistance in *Saccharomyces cerevisiae* and *Candida albicans* (Stéphane et
 685 al., 2017). Furthermore, previously detected *Neosartorya* spp. isolates resistance to some food preservatives may
 686 be explained by the occurrence of genes coding ABC transporters and multi drug resistance proteins, as both of
 687 these products take part in efflux of hazardous chemicals (Kaur et al., 2021; Maj et al., 2024b). Studied isolates
 688 also possess an innate defence mechanism against carboxylate preservatives in a form of glycerol channels coded
 689 by FPS1 gene which are too narrow to allow permeability of this type of substances into the cell in
 690 *Saccharomyces cerevisiae* (Piper, 2011). Additionally, previously mentioned genes coding superoxide
 691 dismutases (e.g. SOD1, SOD2, SOD4, but also CCS1) can be attributed to sorbate resistance, as seen in *S.*
 692 *cerevisiae* (Mollapour et al., 2004). All these resilience mechanisms not only equip *Neosartorya* spp. to

693 withstand diverse abiotic and pathogen stresses but also underscore their potential for adaptation to environments
694 containing natural extracts and preservatives, challenging efforts to control fungal growth under such conditions.

695 Although *Neosartorya* spp. are mostly thought to be involved in food spoilage and environmental
696 contamination, our WGS results suggest virulence and necrosis induction capabilities exist in the wide array of
697 *Neosartorya* spp. defence mechanisms (Yaguchi et al., 2012). Among such genes, Egh16-like virulence factor 1
698 was detected. This virulence factor acts by manipulating host resistance responses in *Phakopsora pachyrhizi*, the
699 causative agent of soybean rust (Castanho et al., 2024). Present are also pectin lyase fold/virulence factors that
700 degrade the pectic components of the plant cell wall to extract saccharides in *Rhizoctonia solani* associated with
701 banded sheath blight (Patro et al., 2024). *Neosartorya* spp. hold genes regarding the development of membrane
702 attack complexes and perforin which aid in pore formation towards infections (Sannigrahi & Chattopadhyay,
703 2022). All isolates present genes linked to NPP1-like necrosis inducing proteins responsible for witches' broom
704 in cacao by *Moniliophthora perniciosa*, litchi downy blight caused by *Phytophthora litchii* and infection of
705 grapevines by *Plasmopara viticola* (Garcia et al., 2007; Sun et al., 2017; Schumacher et al., 2020). The general
706 consensus derived from the occurrence of such genes may be that *Neosartorya* spp. poses a much greater risk to
707 crops and plants than previously thought, as it has all capability to infect and degrade plant tissues rather than
708 merely residing in the rhizosphere.

709 In the next part of the study, we explored the metabolic profiles of five *Neosartorya* spp. isolates cultivated
710 on three media: PDA, PDA supplemented with marigold extract, and PDA with sodium metabisulfite. Utilizing
711 liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (LC-QTOF MS), we
712 implemented an untargeted metabolomics approach to evaluate metabolic changes across studied conditions. We
713 focused on metabolomic fingerprinting - the detection of broad range of metabolites.. The metabolic data,
714 visualized through mass profiling and analysed using Principal Component Analysis (PCA), provided insights
715 into the differential metabolic responses induced by the varying media compositions. The significant differences
716 in metabolite profiles between the isolates as well the strong effect of cultivation media on the metabolism of
717 *Neosartorya* spp. were discovered. The general metabolic alterations associated with environmental factors in
718 *Neosartorya* species, were thus proved, paving the way for further in depth study aiming at the identification of
719 differentiating metabolites and affected biochemical pathways.

720 Scanning electron microscopy permitted the visualization of isolates morphology. Despite genomic and
721 metabolic differences, morphologically the isolates presented a very close resemblance. Conidiophore and

ascospore ornamentations resemble that of *Neosartorya laciniosa* or *Neosartorya fischeri* var. *glabra* (Hong et al., 2006; Samson et al., 1990). Despite a thorough analysis of SEM results, an unambiguous species identification was not possible for the studied isolates due to high visual analogy between *Neosartorya* spp., a challenge also encountered in other studies where closely related species are often misidentified due to their striking morphological similarity (Horie et al., 1995; Mead et al., 2019). However, certain recurring characteristics were studied further, such as an appearance of surface protuberances. Although, under normal growth conditions hyphae were of smooth texture, application of fungicides or development time elongation often changed hyphal surfaces to verrucose, dotted with small oval protrusions. Therefore, such mycelial adornments may be connected to stress response in studied fungi. The shape of abovementioned protrusions slightly resembles early stirrings of Hülle cells - unique formations of many functions connected with survival, such as storage of genetic back-ups and germination (Troppens et al., 2020). These globose-thick walled multinuclear cells are commonly found in specimens of the *Aspergillus* genus and their occurrence could explain the high degree of branching present in observed isolates, as they cause hyphal development (Bayram & Braus, 2012). However no fully developed Hülle cells are observable in any of the samples and the size of the protrusions is severely lacking, usually spanning under 0.5 μm in diameter, whereas adult Hülle cells measure 10-15 μm on average (Bayram & Braus, 2012). Observed morphology also does not match that of appressoria since presented protrusions are dot-like, situated on hypha surface rather than swelling of hyphal tips (Ryder et al., 2022). The observed protrusions could possibly be Woronin bodies, previously described in *Ascomycota* as miniscule safeguards varying from 100 nm to over 1 μm in diameter, that protect the mycelium from catastrophic injury following damage to one or more cell compartments (Money, 2016). The occurrence of Woronin bodies is not constant; instead, they are part of a dynamic pathway that involves response to stimulus (Jedd, 2011). Their occurrence may allude to the fact that applied fungicides either influence cell wall stability from the inside-out, making it brittle, or that particles of these substances evaporate from the medium and come in contact with mycelium suspended above, damaging its structure, as media vapor escape is an unavoidable occurrence (Esser et al., 2011; Jedd, 2011). Woronin bodies are known to play a role in septal sealing and can also contribute to the cellular response to environmental stress, however as their role is mostly internal it is doubtful they could be so perfectly observed on cell periphery (Jedd, 2011). Though protrusions observed due to the application of sodium metabisulfite and *Calendula officinalis* seem to closely match the morphology of Woronin bodies, further tests involving TEM or confocal microscopy with antibodies are required to confirm this as the verrucose surfaces

751 may be simply caused by the ingredients of antifungal substances attaching to the fungal walls or modifications
752 in cell wall build.

753 Looking back at the viability results, a clear pattern emerges, with sodium metabisulfite initially enhancing
754 early viability across the isolates. This effect, however, seems to diminish over time, suggesting that while
755 sodium metabisulfite may initially create favourable conditions or stimulate growth due to oxidative stress
756 tolerance mechanisms such as superoxide dismutase activation, prolonged exposure might lead to cellular stress
757 or toxicity accumulation, overwhelming fungal defence systems. Analogous situation can be observed with 14-
758 methylated sterols that inhibit fungal growth over time, with initial resistance triggered by the upregulation of
759 drug transporters (Ivanov et al., 2022). The accumulation of reactive species or gradual interference with
760 metabolic pathways could contribute to this delayed decline in viability, as the fungi may be unable to fully
761 detoxify or adapt to these conditions over extended periods, similarly to accumulation of intracellular squalene
762 (Elewski, 1993). In contrast, the impact of *Calendula officinalis* appears to be more transient. After 10 days, it
763 significantly stunted the vitality of three fungal isolates, indicating initial antifungal effects likely stemming from
764 bioactive compounds such as saponins, flavonoids and phenolic acids, which can disrupt fungal cell membranes
765 or inhibit essential metabolic enzymes (Doligalska et al., 2021; Al-Huqail et al., 2023; Tavallali et al., 2024).
766 However, after 30 days, only one isolate remained inhibited, suggesting that while *Calendula officinalis* extract
767 may impose antifungal pressure, its effectiveness is not sustained over longer durations. This reduced efficacy
768 could be due to the degradation or inactivation of active compounds over time or the development of adaptive
769 mechanisms in the fungi, allowing them to mitigate the extract's effects similarly as in *Candida* species
770 developing resistance to fluconazole during prolonged contact (Vanden Bossche et al., 1998). Thus, while
771 *Calendula officinalis* shows promise as an antifungal agent, its short-lived effectiveness suggests it may need to
772 be used in conjunction with other control measures to achieve sustained inhibition.

773 5. Conclusions

774 This study presents a comprehensive evaluation of the resilience mechanisms in *Neosartorya* spp.,
775 focusing on basal genome characterization, as well as metabolic, and morphological responses to antifungal
776 agents, which are critical for understanding fungal biodiversity and adaptability in challenging environments.
777 The study utilized WGS to create a foundational genome map for five distinct isolates, enabling detailed
778 comparisons and establishing a reference for evaluating their natural resilience mechanisms. Between over 10
779 000 to over 14 000 genes were characterized for each of the 5 genomes with one or more of the 25 COG

780 functions assigned for each individual gene. Furthermore, genes regarding defensive mechanisms were studied
781 more in-depth, with additional classification of 15 more detailed functions.

782 Other methods of fungi examination involved controlled exposure to sodium metabisulfite and *Calendula*
783 *officinalis* extract, followed by a series of advanced profiling techniques. Metabolic profiling via LC-QTOF MS
784 provided preliminary insights into alterations in metabolite production, indicating that antifungal agents induced
785 significant changes in *Neosartorya* spp metabolomes. These findings highlight the significant impact that
786 antifungal agents have on fungal metabolic pathways, emphasizing the importance of the growth medium in
787 shaping metabolic responses. Morphological changes were tracked through SEM imaging, showing structural
788 modifications, notably increased sporulation, denser mycelial composition and the appearance of structures akin
789 to Woronin bodies, which might indicate a stress response at the cellular level. Confocal fluorescence
790 microscopy further illustrated antifungal impacts on cell viability, suggesting that these agents affect viability
791 differently over time and for each of the isolates. The viability data revealed that while sodium metabisulfite may
792 initially promote viability, prolonged exposure potentially induces metabolic stress or toxicity, diminishing its
793 efficacy over time. Conversely, *Calendula officinalis* showed a temporary inhibitory effect, initially reducing the
794 viability of multiple isolates, yet with effects that diminished over extended incubation, leaving only one isolate
795 significantly affected. This transient efficacy emphasizes the importance of duration in antifungal applications
796 and the need to account for the adaptive responses of fungi over time.

797 Our findings highlight that *Neosartorya* spp. exhibit complex adaptive responses involving genetic,
798 metabolic, and morphological dimensions, contributing to the broader knowledge of fungal diversity and
799 adaptability under antifungal stressors. The observed resistance mechanisms, including metabolic adjustments
800 and structural adaptations, are key to understanding *Neosartorya* spp.'s resilience and their potential role as
801 spoilage agents in food. Additionally, discovery of virulence and necrosis induction genes highlights a
802 possibility of fungal infections due to *Neosartorya* spp. occurrence. These results underscore the need for
803 continued exploration into specific resilience pathways within fungi, providing a foundation for future antifungal
804 strategies that could enhance food safety and address fungal spoilage issues more effectively. Further research
805 will be required to pinpoint especially specific metabolic traits linked to antifungal resistance, allowing for the
806 development of more targeted, sustainable antifungal applications.

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1231 **Statements and Declarations**

1232 **Funding**

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1235 **Competing Interests**

1236 The authors have no relevant financial or non-financial interests to disclose.

1237 **Data Availability**

1238 The datasets generated during and/or analysed during the current study are available from the corresponding
1239 author on a reasonable request and WGS data were submitted to The National Center for Biotechnology
1240 Information (NCBI) under the following accession numbers: JBKOSS000000000, JBKOST000000000,
1241 JBKOSU000000000, JBKOSV000000000, JBKOSW000000000.

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7.4.3 Materiały dodatkowe

1 **Genome-Wide Analysis, Metabolomic Profiling and Microscopic Evaluation of *Neosartorya* spp.**
2 **(*Aspergillus* spp.) Diversity and Adaptations to Natural and Synthetic Antimicrobials –**
3 **SUPPLEMENTARY INFORMATION**

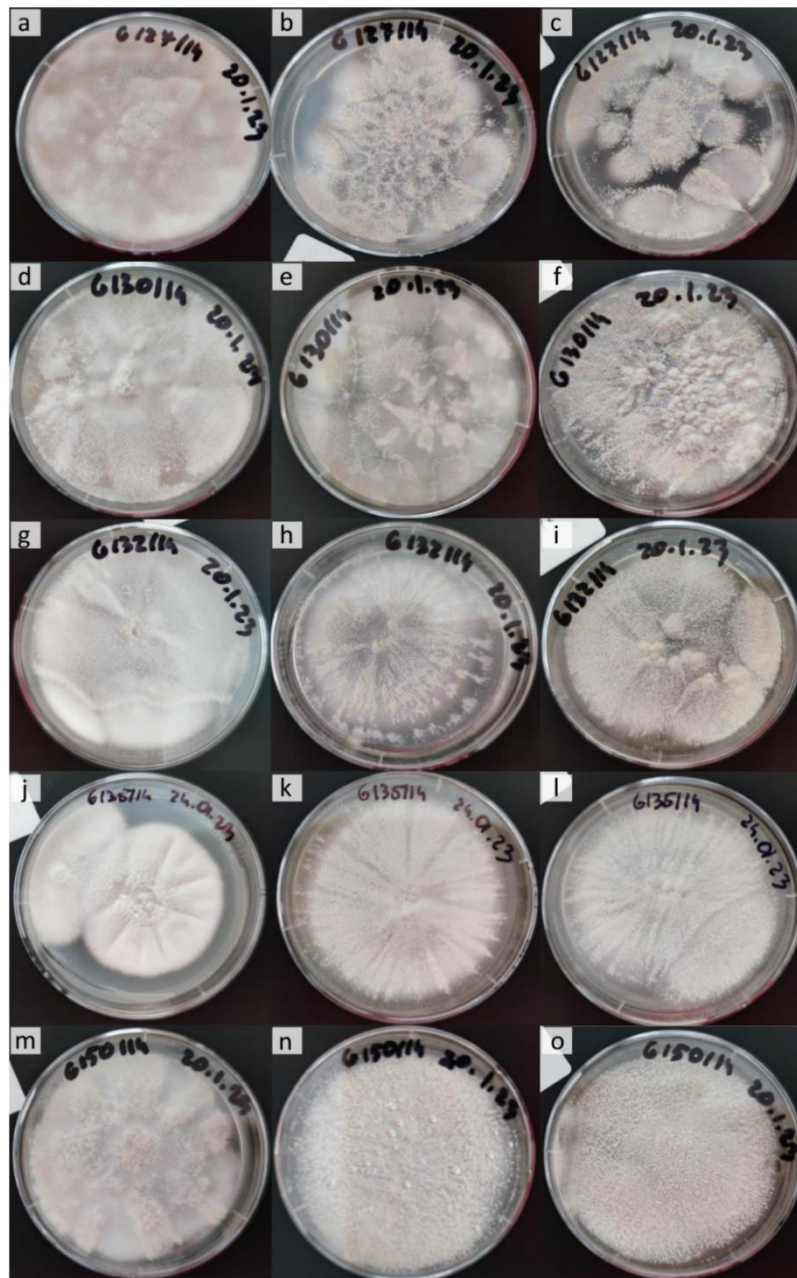
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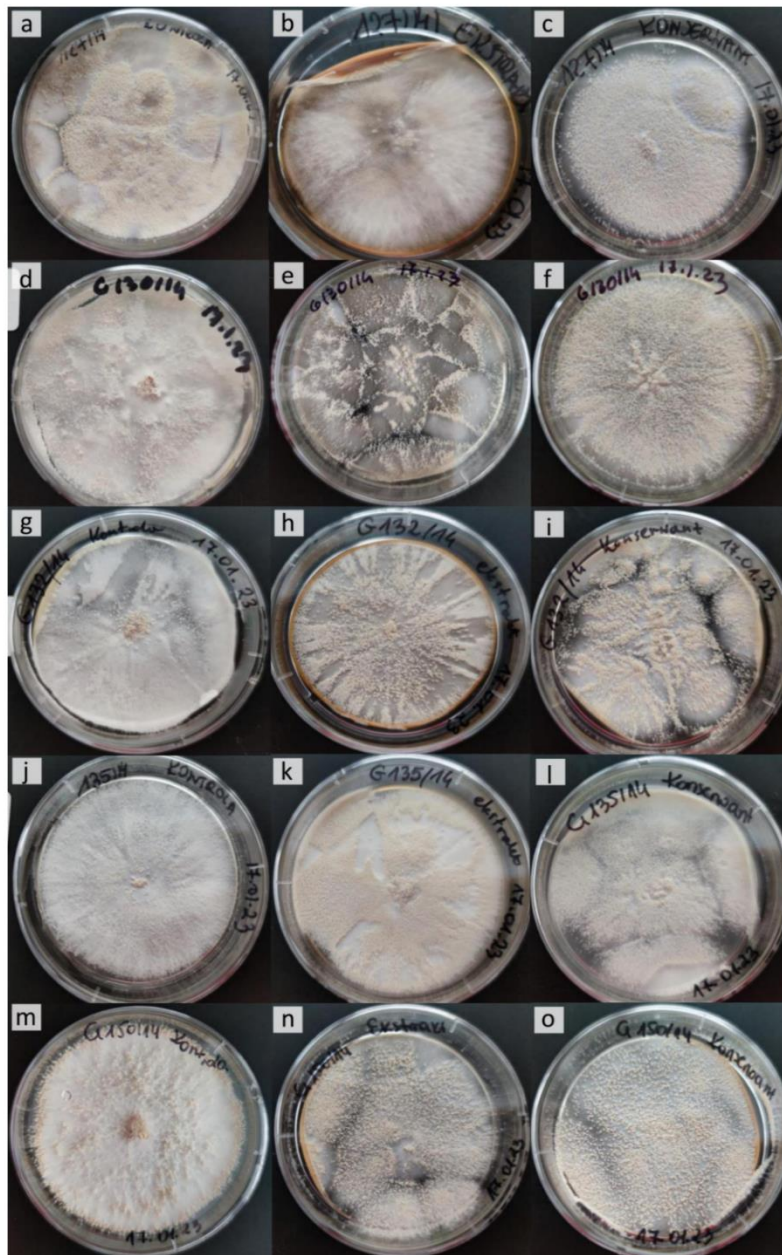
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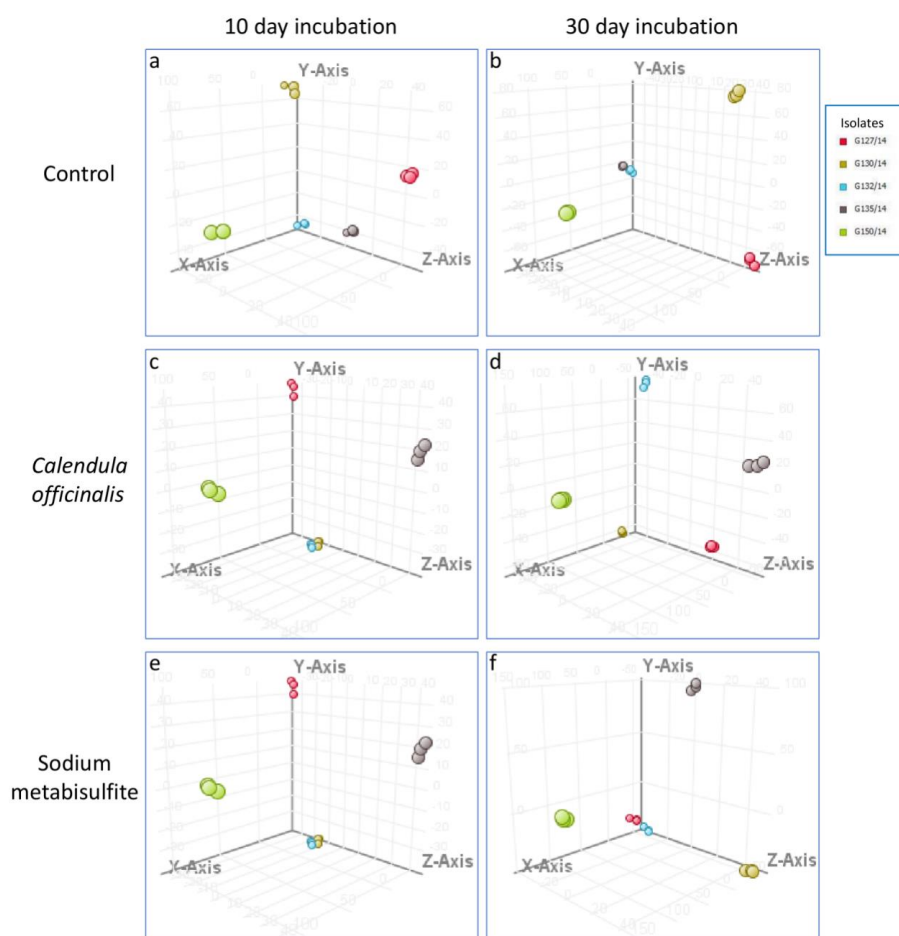


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 12 Fig. S11 *Neosartorya* spp. isolates G127/14 (a-c), G130/14 (d-f), G132/14 (g-i), G135/14 (j-l), and G150/14 (m-o)
 13 after 10 days of incubation on PDA (a, d, g, j, m), PDA with *Calendula officinalis* extract (b, e, h, k, n), and PDA
 14 with sodium metabisulfite (c, f, i, l, o)



15

16 Fig. S12 *Neosartorya* spp. isolates G127/14 (a-c), G130/14 (d-f), G132/14 (g-i), G135/14 (j-l), and G150/14 (m-o)
 17 after 30 days of incubation on PDA (a, d, g, j, m), PDA with *Calendula officinalis* extract (b, e, h, k, n), and PDA
 18 with sodium metabisulfite (c, f, i, l, o)



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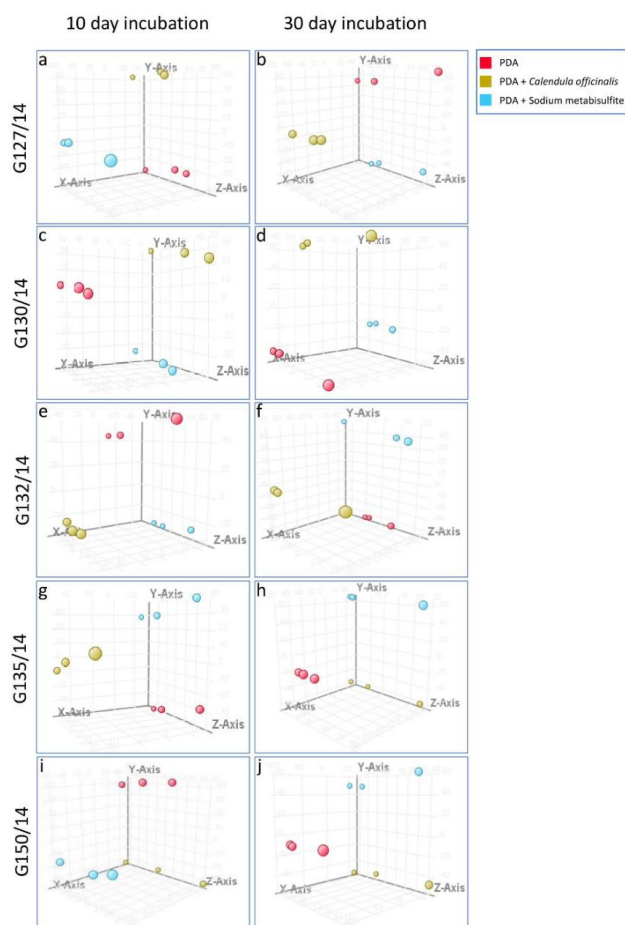
20 Fig. S13 Principal component analysis (PCA) 3D score plot based on LC-QTOF MS metabolic profiles of 5

21 *Neosartorya* spp. isolates. Plots were created for each cultivation variant separately:

22 - control on PDA after 10 (a) and 30 (b) days of incubation,

23 - PDA media with *Calendula officinalis* after 10 (c) and 30 (d) days of incubation,

24 - PDA media with sodium metabisulfite after 10 (e) and 30 (f) days of incubation



25

26 Fig. S14 Principal component analysis (PCA) 3D score plots discriminating for cultivation variants influence on
 27 LC-QTOF MS metabolic profiles for each isolate:

28 - G127/14 after 10 (a) or 30 (b) days of incubation;

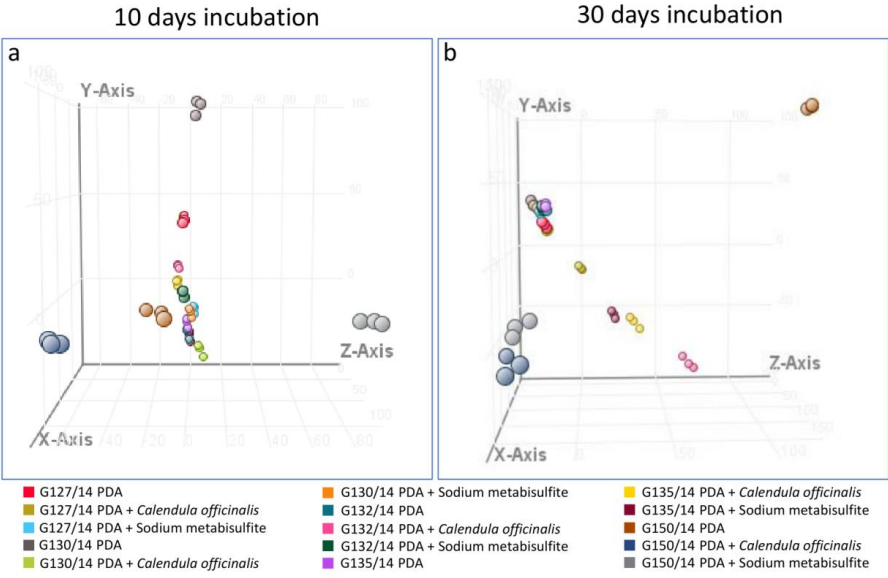
29 - G130/14 after 10 (c) or 30 (d) days of incubation;

30 - G132/14 after 10 (e) or 30 (f) days of incubation;

31 - G135/14 after 10 (g) or 30 (h) days of incubation;

32 - G150/14 after 10 (i) or 30 (j) days of incubation

33

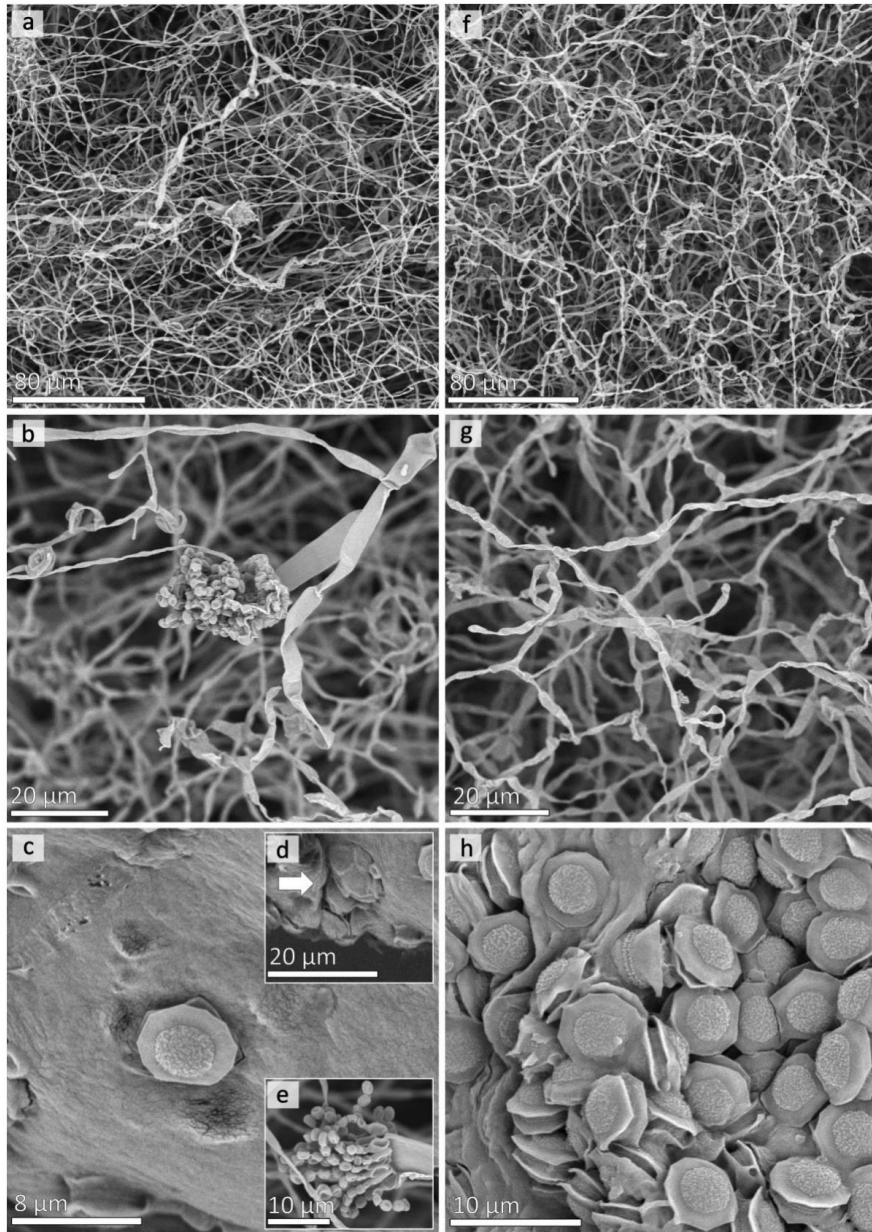


34

35 Fig. SI5 Principal component analysis (PCA) 3D score plot for all isolates and cultivation variants. After 10 (a)

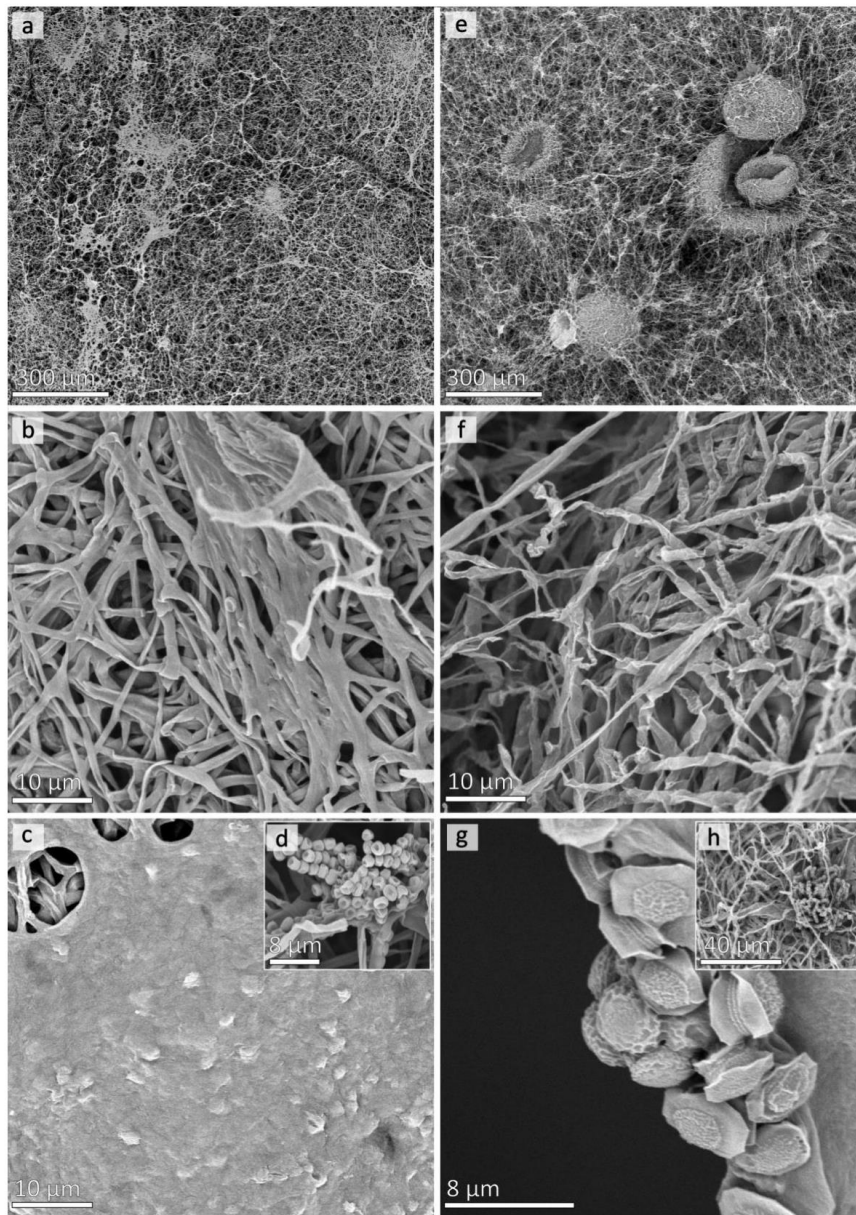
36 and 30 (b) days of incubation.

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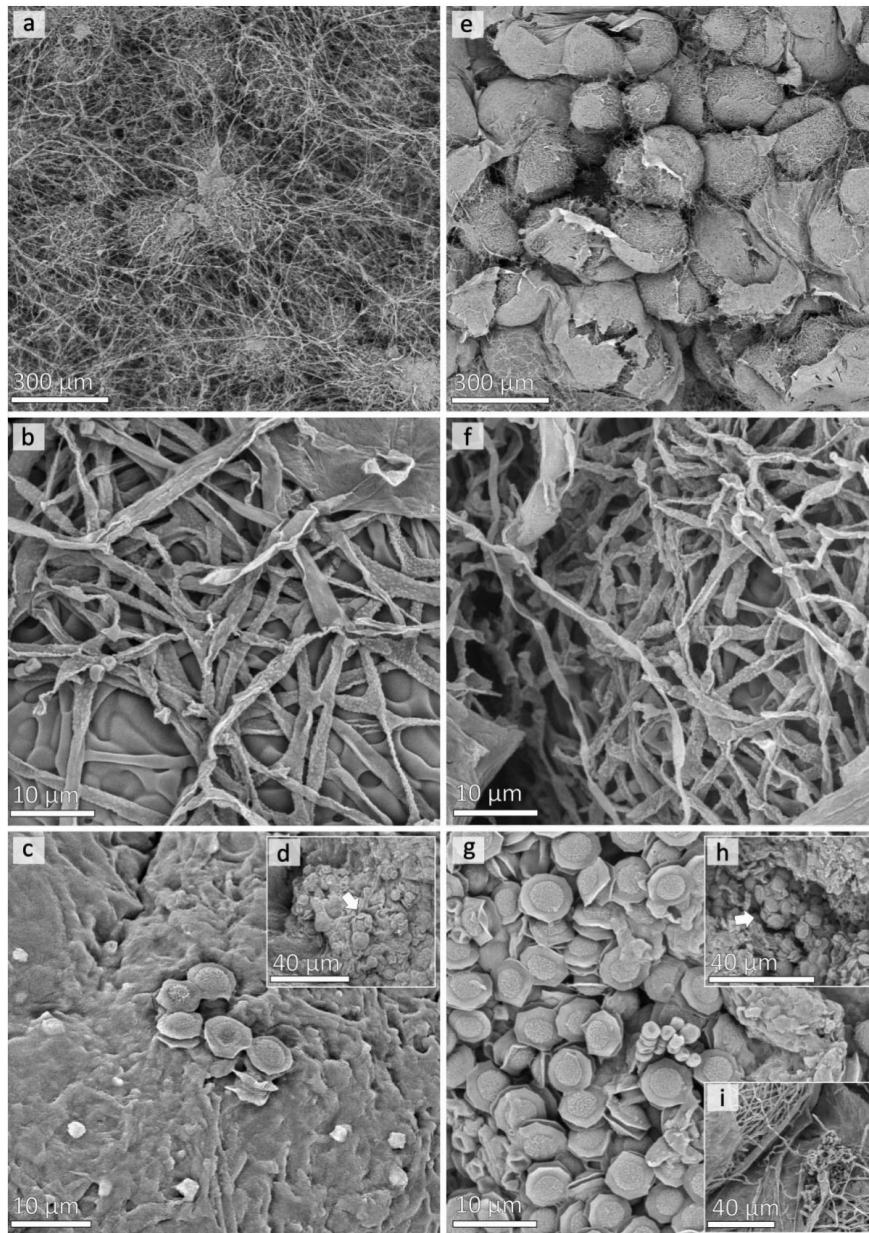
38

39 Fig. S16 *Neosartorya* sp. G130/14 on PDA media observed via SEM after 10 (a, b, c, d, e) or 30 (f, g, h) days of
 40 incubation. General mycelium structure (a, f), hyphae surface (b, g), ascospores (c, h), conidiospores (e), asci (d –
 41 marked with an arrow).



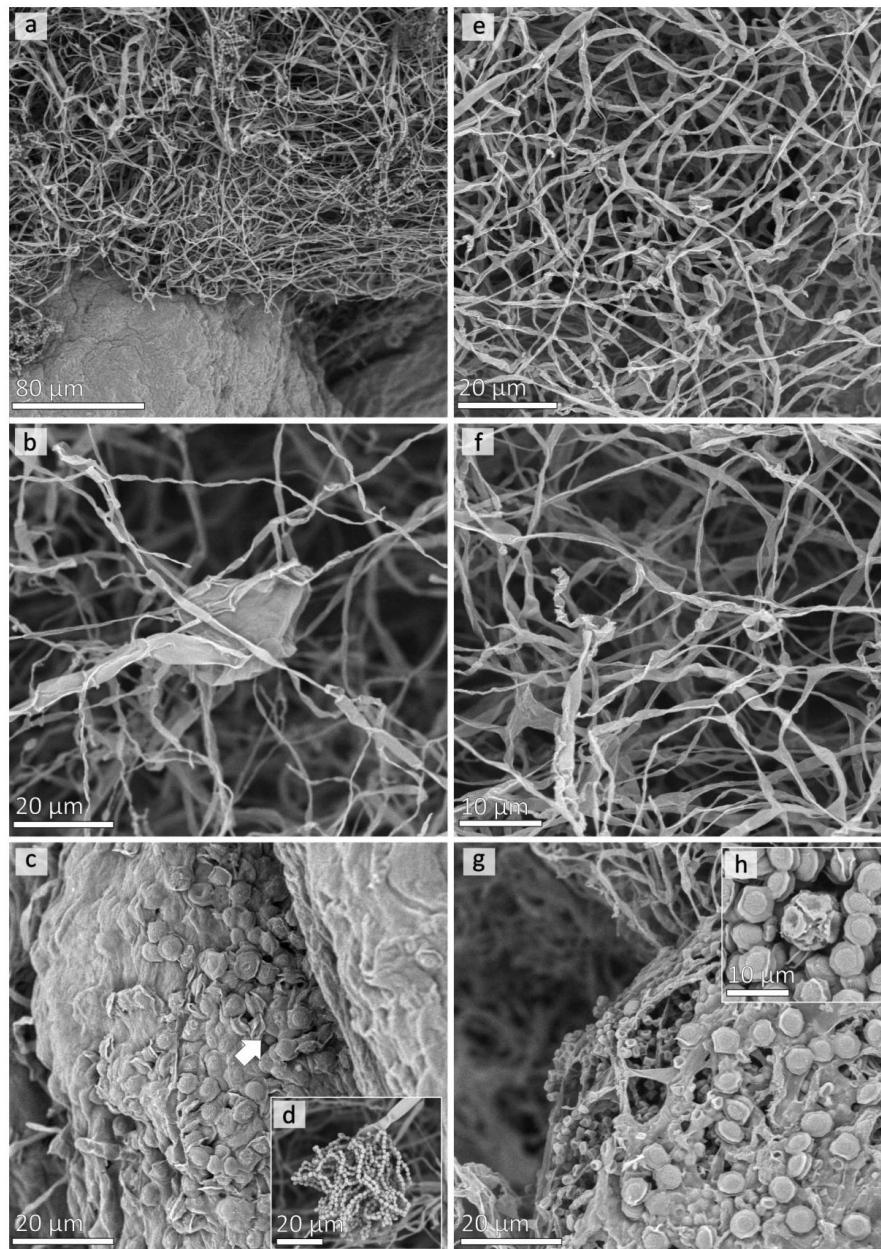
42

43 Fig. SI7 *Neosartorya* sp. G130/14 on PDA with *Calendula officinalis* media observed via SEM after 10 (a, b, c,
 44 d) or 30 (e, f, g, h) days of incubation. General mycelium structure (a, e), hyphae surface (b, f), asci (c, g),
 45 conidiospores (d, h), ascospores (g)



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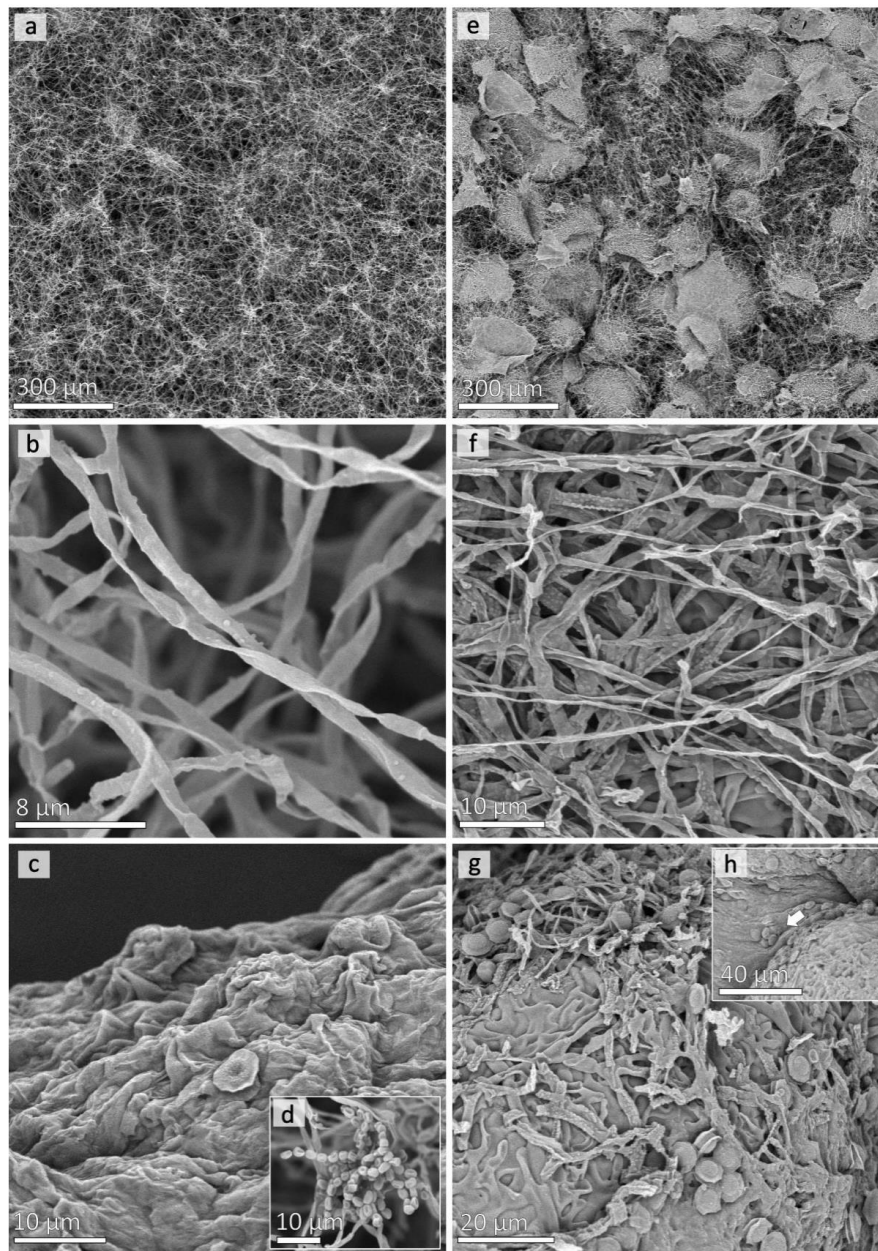
47 Fig. S18 *Neosartorya* sp. G130/14 on PDA with sodium metabisulfite media observed via SEM after 10 (a, b, c,
 48 d) or 30 (e, f, g, h) days of incubation. General mycelium structure (a, e), hyphae surface (b, f), ascospores (c, g),
 49 asci (d, h – marked with an arrow), conidiospores (b, i)



50

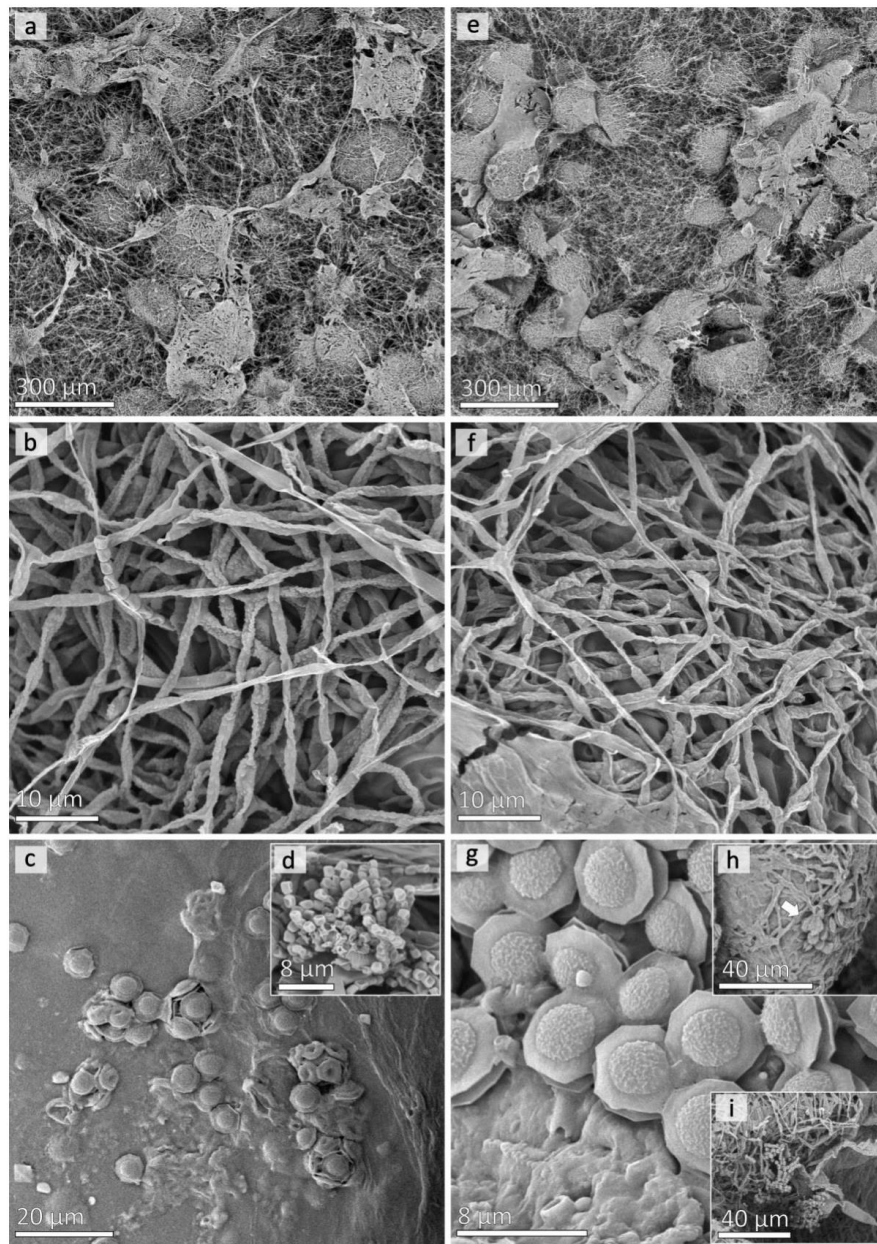
51 Fig. S19 *Neosartorya* sp. G132/14 on PDA media observed via SEM after 10 (a, b, c, d) or 30 (e, f, g, h) days of
 52 incubation. General mycelium structure (a, e), hyphae surface (b, f), ascospores (c, g), conidiospores (d, g), asci (c
 53 – marked with an arrow, h)

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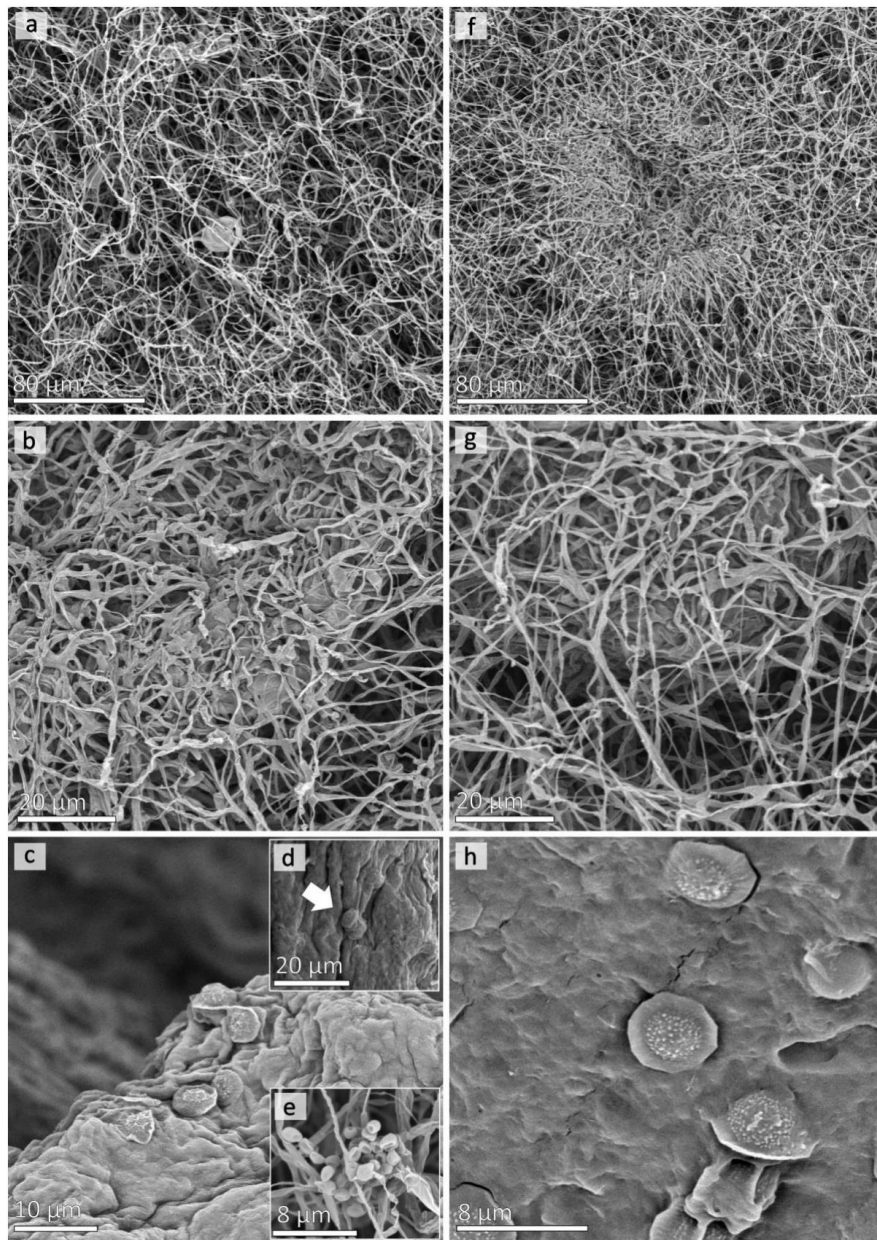
55 Fig. S110 *Neosartorya* sp. G132/14 on PDA with *Calendula officinalis* media observed via SEM after 10 (a, b, c,
 56 d) or 30 (e, f, g, h) days of incubation. General mycelium structure (a, e), hyphae surface (b, f), asci (c, h – marked
 57 with an arrow), conidiospores (d), ascospores (c, g)



58

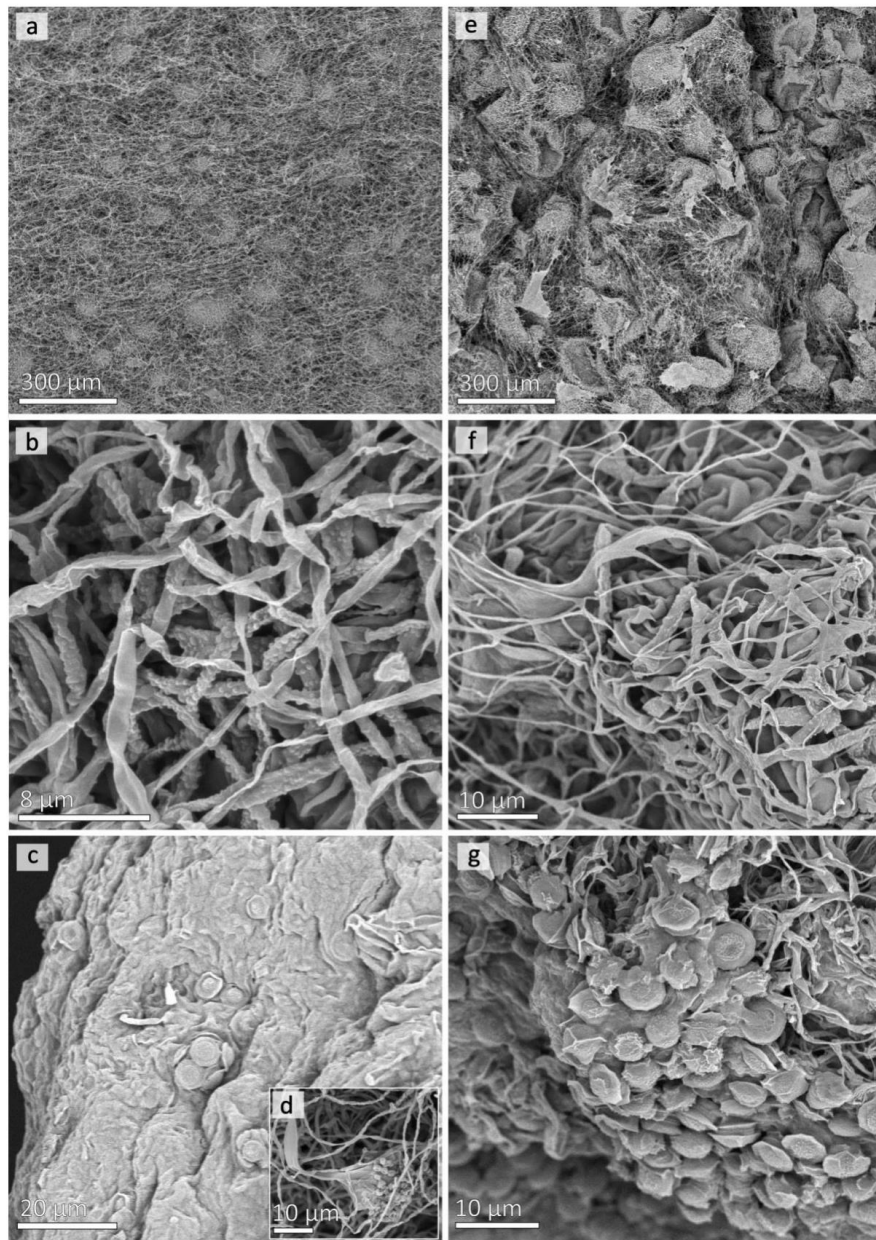
59 Fig. SI11 *Neosartorya* sp. G132/14 on PDA with sodium metabisulfite media observed via SEM after 10 (a, b, c,
60 d) or 30 (e, f, g, h) days of incubation. General mycelium structure (a, e), hyphae surface (b, f), ascospores (c, g),
61 asci (c, h – marked with an arrow), conidiospores (d, i)

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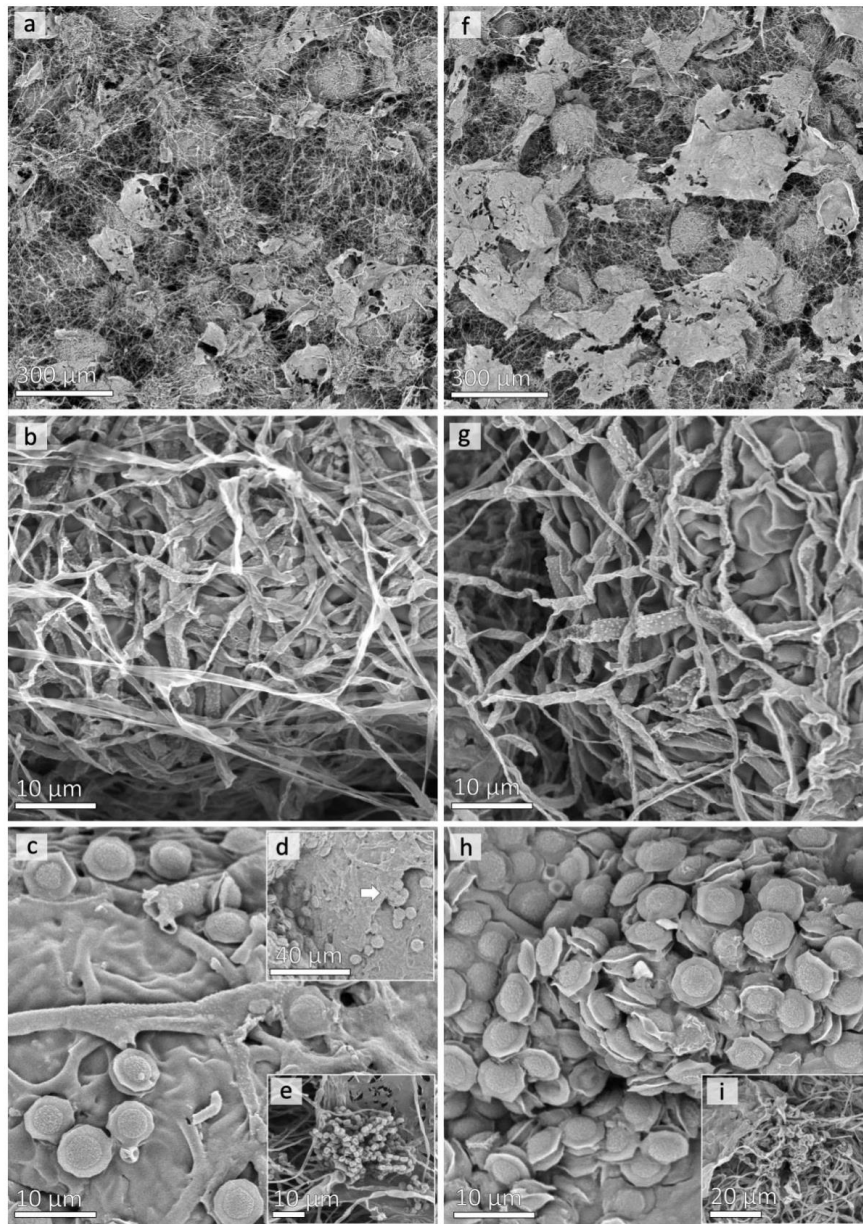
63 Fig. SI12 *Neosartorya* sp. G135/14 on PDA media observed via SEM after 10 (a, b, c, d, e) or 30 (f, g, h) days of
 64 incubation. General mycelium structure (a, f), hyphae surface (b, g), ascospores (c, h), asci (d – marked with an
 65 arrow), conidiospores (e)



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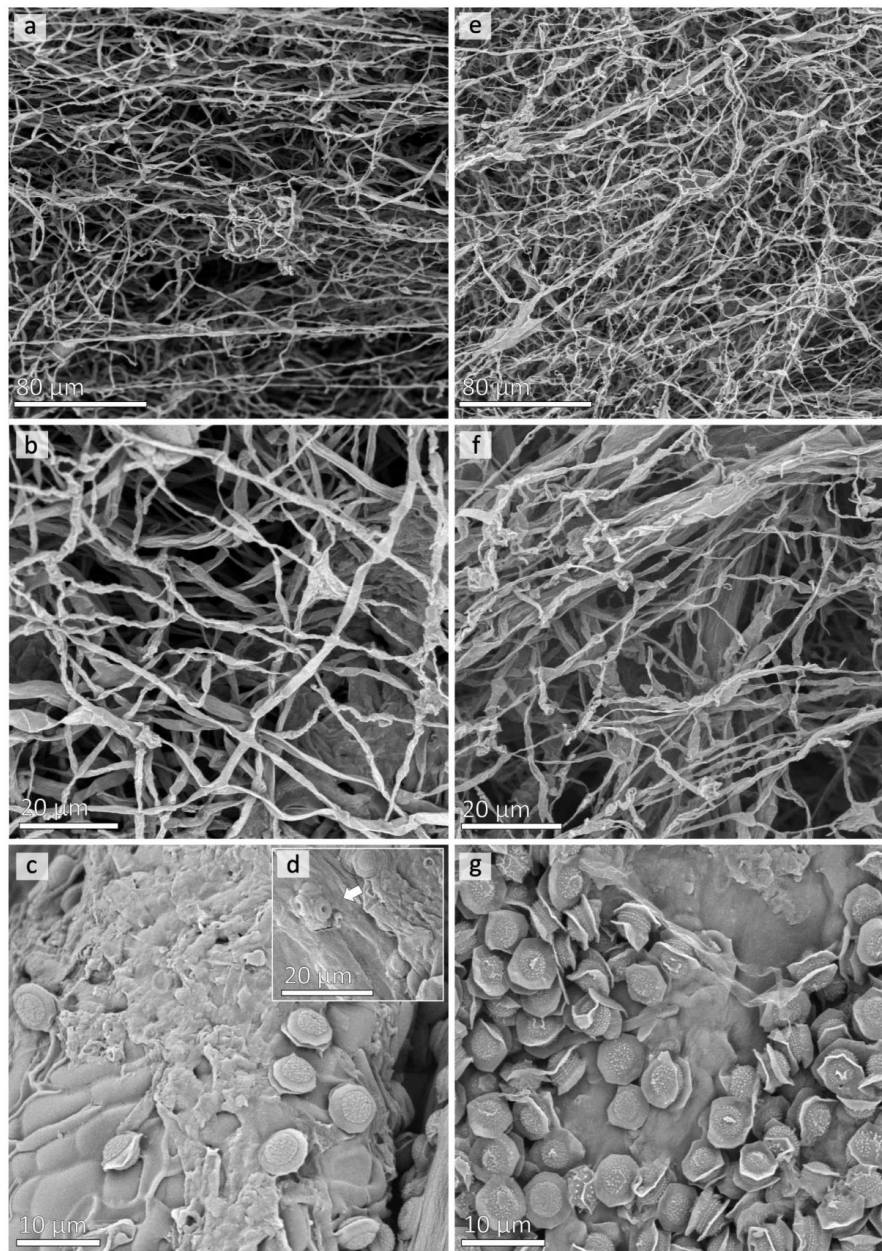
67 Fig. S113 *Neosartorya* sp. G135/14 on PDA with *Calendula officinalis* media observed via SEM after 10 (a, b, c,
 68 d) or 30 (e, f, g) days of incubation. General mycelium structure (a, e), hyphae surface (b, f), asci (c), conidiospores
 69 (d), ascospores (c, g)

14



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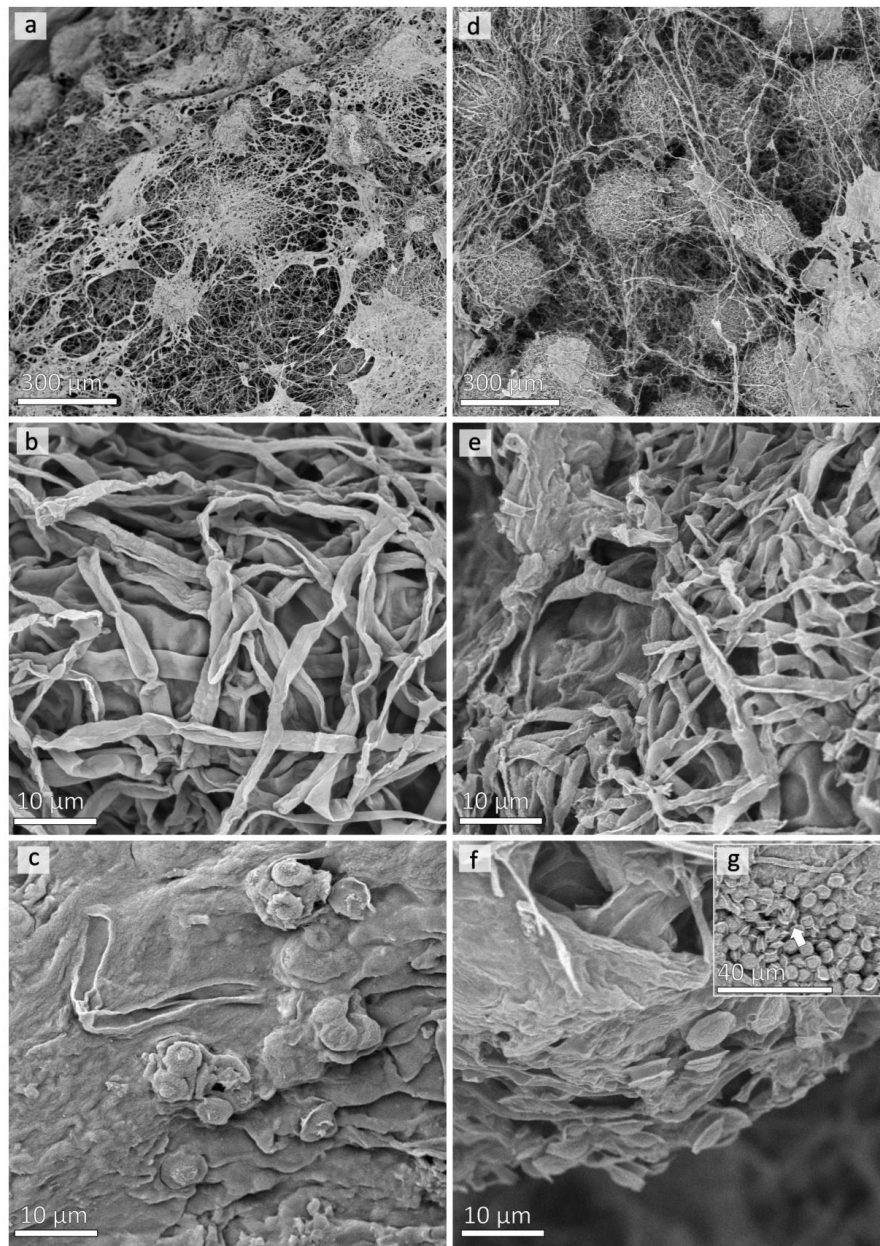
71 Fig. SI14 *Neosartorya* sp. G135/14 on PDA with sodium metabisulfite media observed via SEM after 10 (a, b, c,
 72 d, e) or 30 (f, g, h, i) days of incubation. General mycelium structure (a, f), hyphae surface (b, g), ascospores (c,
 73 h), asci (d – marked with an arrow, h), conidiospores (e, i)



74

75 Fig. SI15 *Neosartorya* sp. G150/14 on PDA media observed via SEM after 10 (a, b, c, d) or 30 (e, f, g) days of
 76 incubation. General mycelium structure (a, e), hyphae surface (b, f), ascospores (c, g), asci (d – marked with an
 77 arrow, g)

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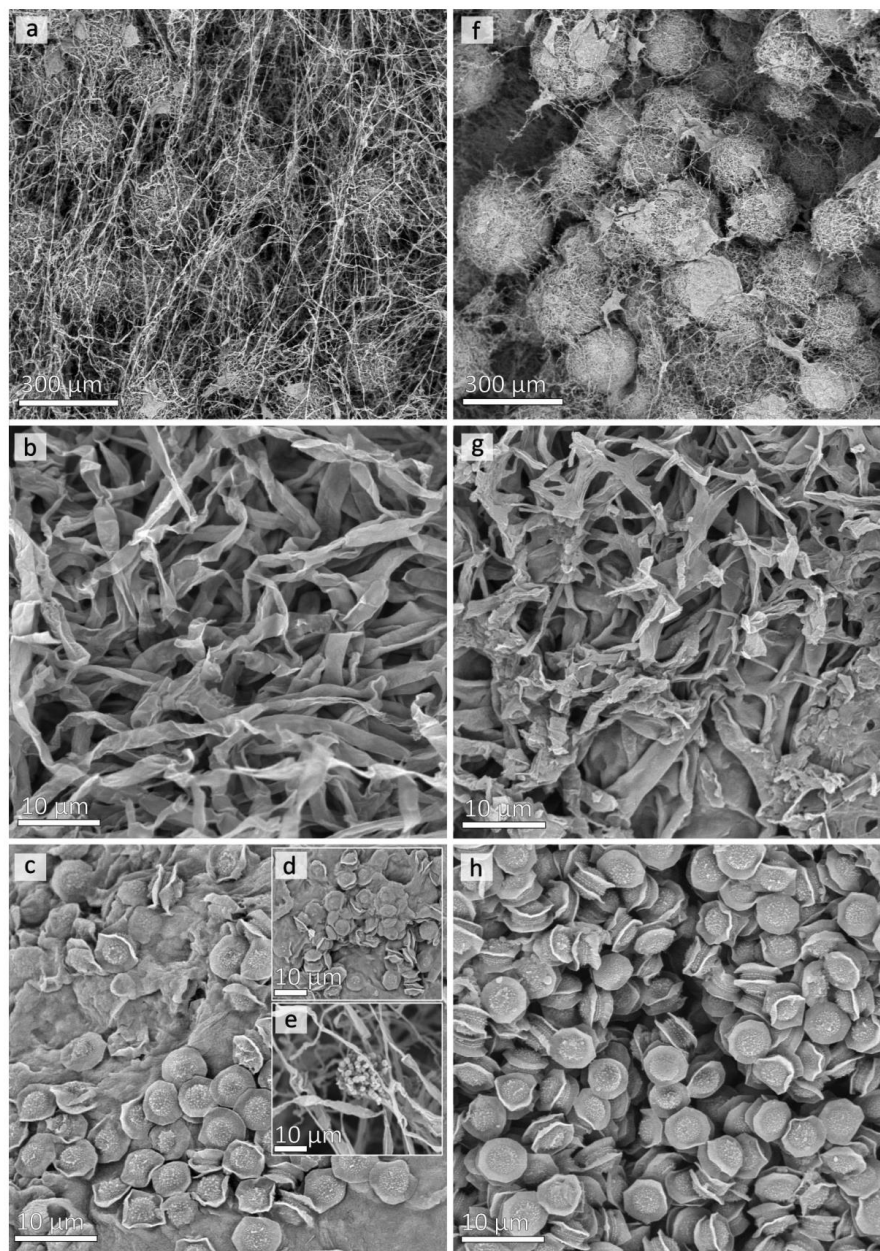


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79 Fig. SI16 *Neosartorya* sp. G150/14 on PDA with *Calendula officinalis* media observed via SEM after 10 (a, b, c)

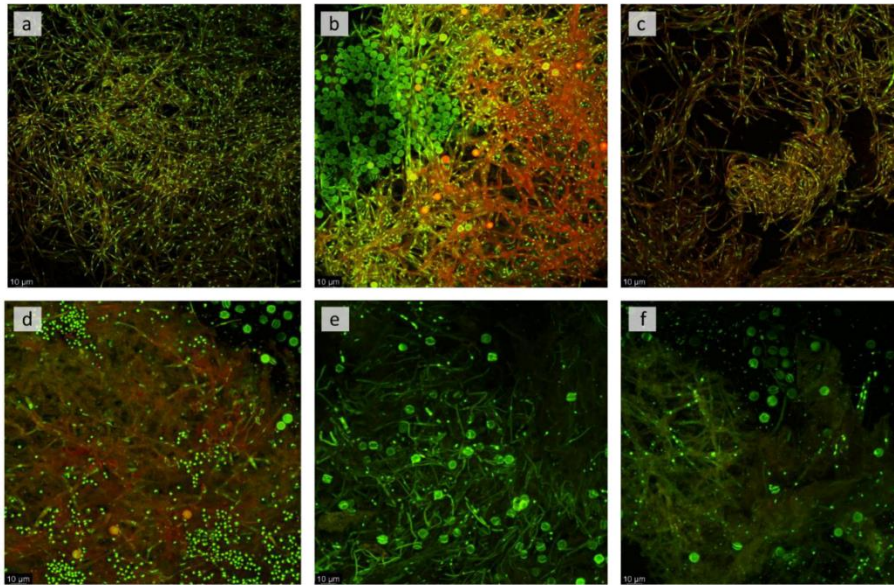
80 or 30 (d, e, f, g) days of incubation. General mycelium structure (a, d), hyphae surface (b, e), asci (c, g – marked

81 with an arrow), ascospores (c, f)



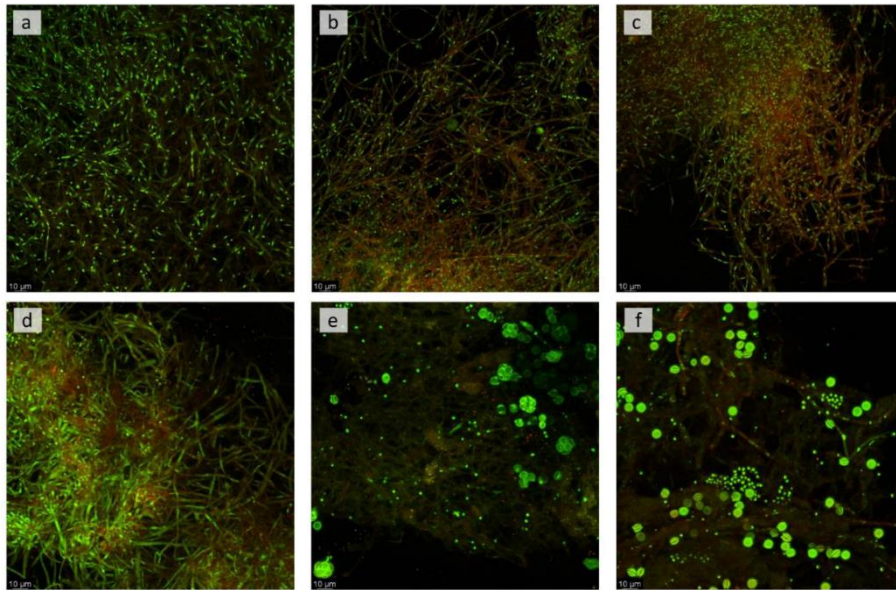
82

83 Fig. S117 *Neosartorya* sp. G150/14 on PDA with sodium metabisulfite media observed via SEM after 10 (a, b, c,
 84 d, e) or 30 (f, g, h) days of incubation. General mycelium structure (a, f), hyphae surface (b, g), ascospores (c, h),
 85 asci (d, h), conidiospores (e)



86

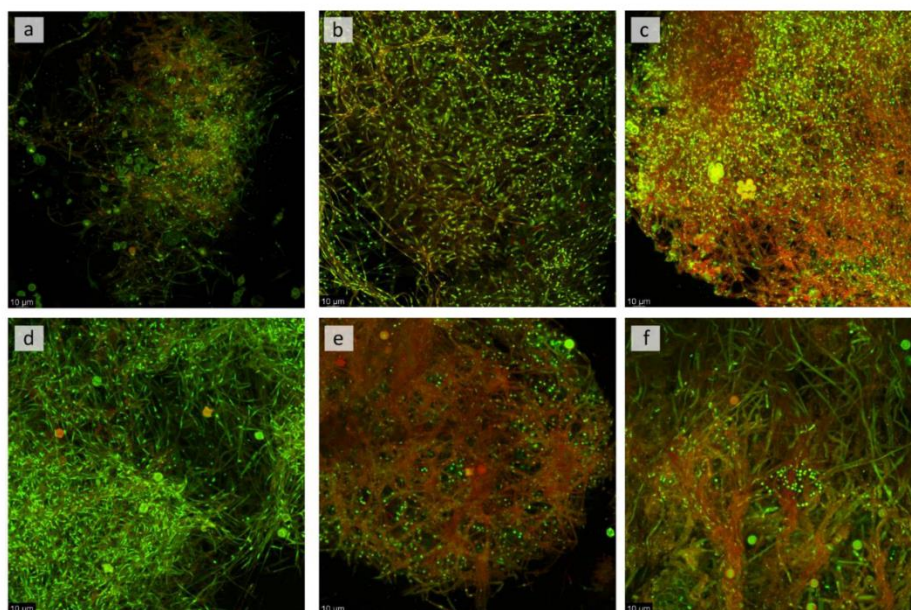
87 Fig. S18 Observation of *Neosartorya* sp. isolate G127/14 viability under different conditions using confocal
 88 fluorescence microscopy. Control on PDA at 10 days (a) and 30 days (d); PDA with *Calendula officinalis* extract
 89 at 10 days (b) and 30 days (e); PDA with sodium metabisulfite at 10 days (c) and 30 days (f)



90

91 Fig. SI9 Observation of *Neosartorya* sp. isolate G130/14 viability under different conditions using confocal
 92 fluorescence microscopy. Control on PDA at 10 days (a) and 30 days (d); PDA with *Calendula officinalis* extract
 93 at 10 days (b) and 30 days (e); PDA with sodium metabisulfite at 10 days (c) and 30 days (f)

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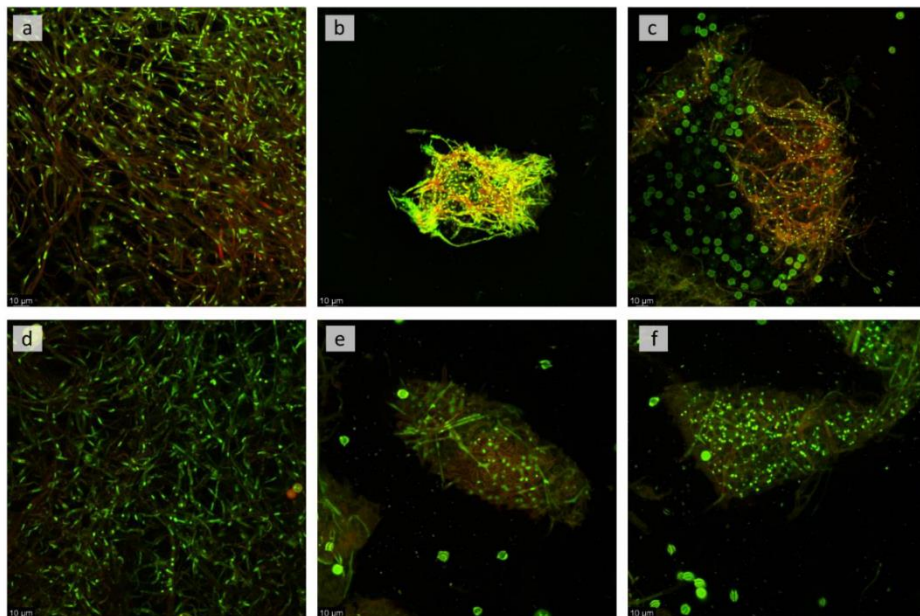
95

96 Fig. S20 Observation of *Neosartorya* sp. isolate G132/14 viability under different conditions using confocal

97 fluorescence microscopy. Control on PDA at 10 days (a) and 30 days (d); PDA with *Calendula officinalis* extract

98 at 10 days (b) and 30 days (e); PDA with sodium metabisulfite at 10 days (c) and 30 days (f)

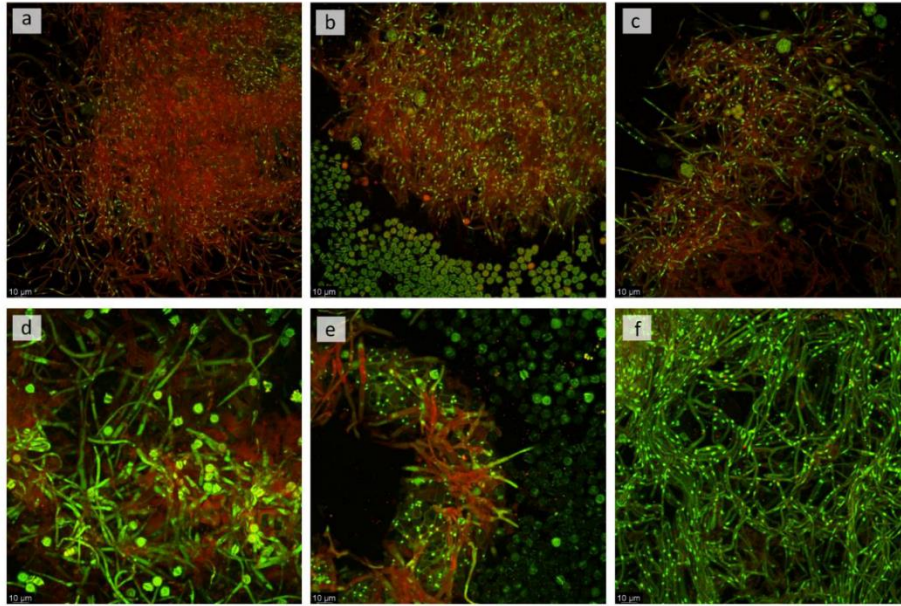
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100

101 Fig. SI21 Observation of *Neosartorya* sp. isolate G135/14 viability under different conditions using confocal
 102 fluorescence microscopy. Control on PDA at 10 days (a) and 30 days (d); PDA with *Calendula officinalis* extract
 103 at 10 days (b) and 30 days (e); PDA with sodium metabisulfite at 10 days (c) and 30 days (f)

104



105

106 Fig. SI22 Observation of *Neosartorya* sp. isolate G150/14 viability under different conditions using confocal
 107 fluorescence microscopy. Control on PDA at 10 days (a) and 30 days (d); PDA with *Calendula officinalis* extract
 108 at 10 days (b) and 30 days (e); PDA with sodium metabisulfite at 10 days (c) and 30 days (f)

7.5 Manuskrypt publikacji P5

7.5.1 Tekst manuskryptu publikacji naukowej

1 **Antifungal Agents Alter Strawberry Microbial and Metabolic Profiles During**
2 ***Neosartorya* spp. (*Aspergillus* spp.) Contamination**

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7 **Abstract**

8 Strawberries are highly susceptible to fungal spoilage, but the impact of antifungal
9 treatments on their native microbiome remains unclear. Here, we investigate microbial
10 communities responses in strawberries artificially contaminated with *Neosartorya* spp. and
11 treated with a natural extract, or a food preservative and water as a control. Amplicon
12 sequencing revealed that *Pseudomonas* genus remained dominant across bacterial
13 communities, with Burkholderiales enriched under the natural extract, while
14 Microbacteriaceae declined under the preservative. Despite these shifts, bacterial communities
15 exhibited high ASV overlap, suggesting resilience to environmental modifications. In
16 contrast, fungal communities showed significant treatment-induced shifts, with α -diversity
17 changes and altered core taxa composition. In the control Ascomycota and Basidiomycota
18 were well represented, but the natural extract promoted Basidiomycota. The preservative,
19 however, maintained higher Ascomycota levels, including spoilage-associated *Aspergillus* and
20 *Botrytis* genera. Functional predictions of bacterial communities indicated stable core
21 metabolic pathways, but treatment-driven shifts in amino acid and secondary metabolite
22 metabolism. Meanwhile, fungal trophic mode analysis revealed that the natural extract
23 increased the pathotroph-saprotroph guild over time, whereas the preservative sustained
24 elevated levels of the tripartite pathotroph-saprotroph-symbiotroph guild. Our study highlights

25 the intricate interplay between antifungal treatments and microbial communities, underscoring
26 the need to consider taxonomic and functional consequences when designing preservation
27 strategies.

28 Keywords: strawberry fruit microbiome, mycobiome, plant extract, food preservative, trophic
29 mode, functional diversity of microbes

30

31

32 1. Introduction

33 Fresh strawberries (*Fragaria x ananassa*), exhibit a high degree of perishability
34 attributed to their elevated water content and nutrient profile, rendering them vulnerable to
35 fungal contamination in the postharvest storage phase^{1,2}. *Neosartorya* spp., the teleomorphic
36 state of *Aspergillus* spp., represent a notable challenge among spoilage fungi responsible for
37 strawberry produce contamination³. Their resilience to food preservation techniques and
38 capacity to produce heat-resistant ascospores enable them to endure thermal processing and
39 refrigeration^{4,5}. The presence of these fungi not only affects the quality of fruit but also has
40 the potential to generate secondary metabolites that could pose toxicological risks, thereby
41 raising important food safety concerns⁶.

42 Food preservation strategies employ various agents to mitigate fungal contamination,
43 including synthetic preservatives and natural plant extracts. Chemical preservatives, such as
44 sodium metabisulfite and organic acids, are widely used in the food industry to inhibit fungal
45 growth^{7,8}. However, concerns regarding consumer safety and regulatory restrictions have
46 increased interest in natural antifungal alternatives, such as plant-derived compounds with
47 bioactive properties^{9,10}. Extracts from medicinal plants, including *Calendula officinalis*, have
48 demonstrated antifungal efficacy against multiple fungal pathogens, offering a promising
49 approach for reducing spoilage while meeting consumer demand for natural food preservation
50 solutions¹¹.

51 While antifungal agents effectively suppress fungal growth, their broader impact on
52 fruits' microbial and metabolic profiles remains largely unexplored, especially when coupled
53 with active contamination. The fruit microbiome is crucial in maintaining postharvest
54 stability, influencing pathogen interactions, and modulating fruit metabolism¹². Changes in
55 bacterial and fungal communities induced by preservation treatments could have unintended

56 consequences on fruit quality and storage potential¹³. Next-generation sequencing (NGS)
57 approaches, such as Illumina MiSeq-based amplicon sequencing of 16S rRNA and ITS
58 regions, provide a powerful tool for assessing taxonomic shifts in microbial communities
59 under different preservation conditions^{14,15}. Similarly, metabolic fingerprinting using
60 Biolog™ EcoPlates allows for evaluating microbial functional responses, offering insights
61 into how antifungal agents influence metabolic activity in complex food systems¹⁶.

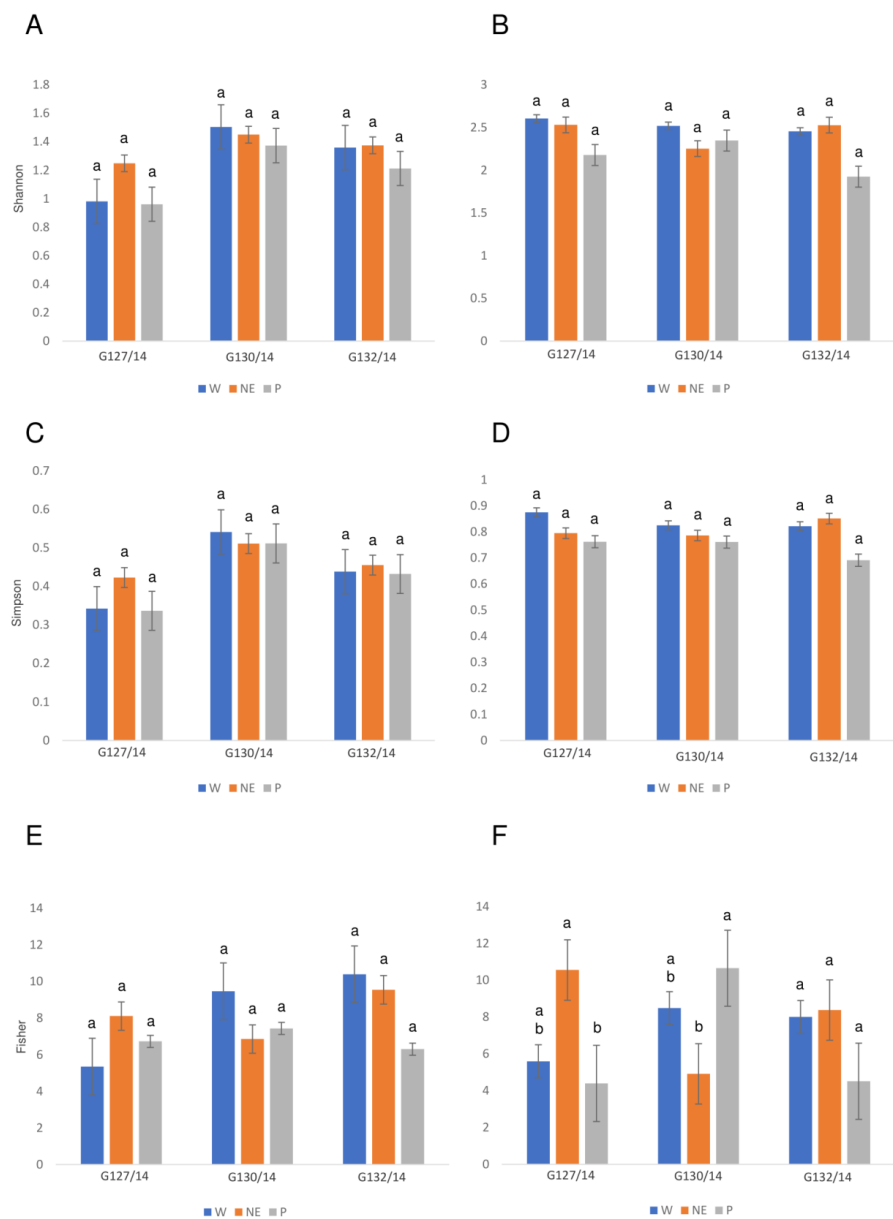
62 Recent studies have highlighted the need to assess the interactions between treatments
63 and the microbiome in real food systems rather than in artificial laboratory conditions^{17,18}.
64 While in vitro experiments have provided insights into the effectiveness of antifungal
65 compounds against *Neosartorya* spp., their usefulness in multifactorial food environments
66 remains largely unexplored^{19,20}. Furthermore, the impact of antifungal treatments on bacterial
67 and fungal diversity and metabolic activity has not been systematically evaluated in the
68 context of *Neosartorya* spp. contamination. This study aimed to investigate the influence of
69 antifungal agents on the native microbial and metabolic profiles of strawberries artificially
70 contaminated with *Neosartorya* spp. isolates. The experimental design included the analysis
71 of bacterial and fungal communities using NGS sequencing of V4 16S rRNA and ITS2 gene
72 amplicons, along with metabolic profiling through Biolog™ EcoPlates. The study focused on
73 three *Neosartorya* spp. isolates representing varying levels of sensitivity to antifungal
74 treatments (high, medium, and low) to assess their resistance and impact on fruit-associated
75 microbial communities over a seven-day refrigeration period. By integrating microbiome and
76 metabolome analyses, this study provides novel insights into the complex interactions
77 between fungal spoilage organisms, preservation strategies, and microbial ecosystems in
78 postharvest fruits. It underscores the need to understand antifungal treatments' ecological and
79 metabolic impacts, aiming to optimize preservation strategies while preserving fruit quality
80 and safety. The research highlights the importance of considering microbial and metabolic

81 responses, offering insights for developing sustainable methods to control fungal spoilage and
82 maintain a beneficial microbiome in fresh produce.

83 **2. Results**

84 **2.1 Microbial Diversity (α and β Diversity Indices)**

85 Alpha diversity indices were used to gauge the microbial composition within samples
86 treated with *Neosartorya* spp. contaminants and antifungals. No significant differences in
87 bacterial α -diversity were observed between treatments within any of the tested isolates,
88 regardless of the diversity index applied, indicating a general stability of bacterial
89 communities under the influence of water, natural extract, or food preservative (Fig. 1A, 1C,
90 1E). Similarly, no significant differences were observed in fungal communities for either the
91 Shannon or Simpson indices, regardless of isolate contaminant or treatment (Fig. 1B, 1D).
92 However, fungal communities demonstrated limited treatment-dependent variation in the
93 Fisher index. For isolate G127/14, the Fisher index was significantly higher under the natural
94 extract treatment compared to the preservative, while neither treatment differed from the
95 water control (Fig. 1F). A comparable pattern was observed for isolate G130/14. In this case,
96 a significant difference was detected between the preservative and the natural extract, with the
97 preservative yielding a higher Fisher score. However, neither treatment differed significantly
98 from the water control (Fig. 1F). No differences in fungal α -diversity were detected for isolate
99 G132/14 across treatments.

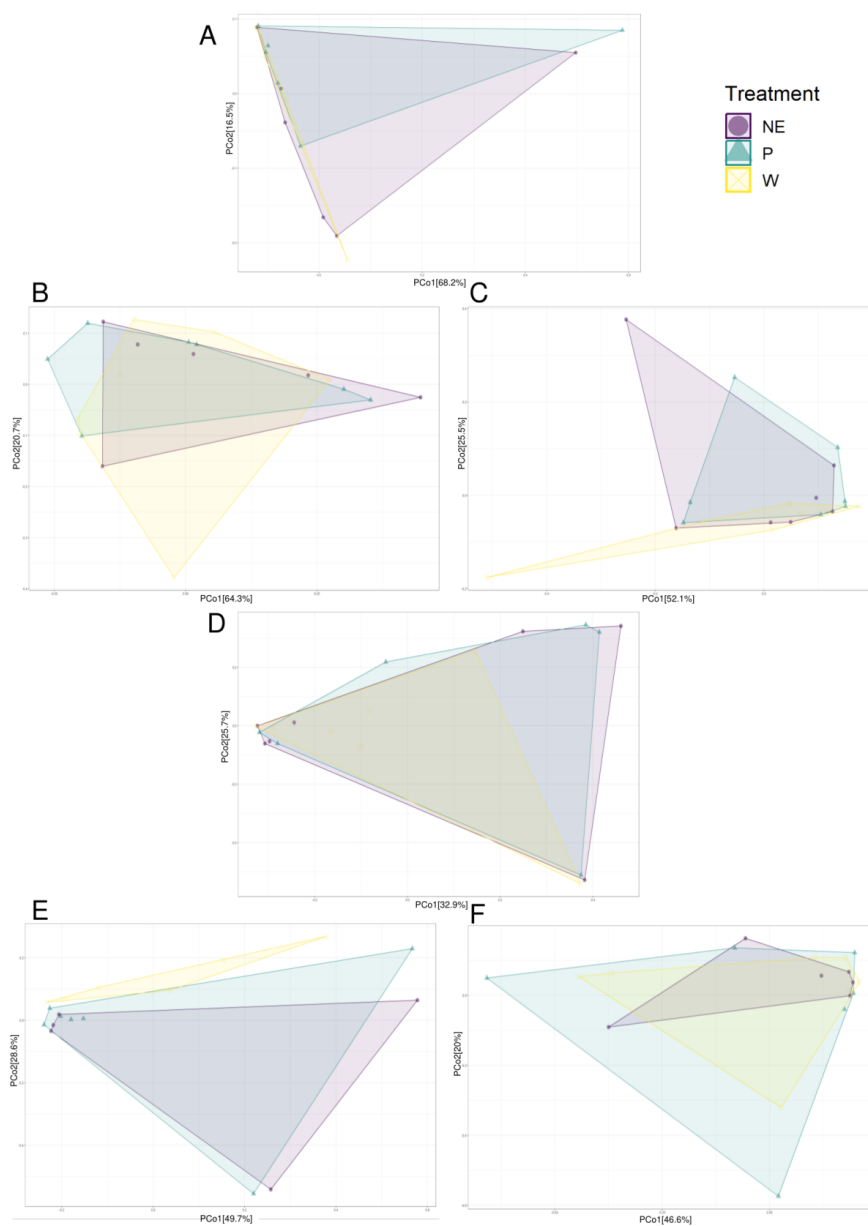


100

101 Fig. 1. α -diversity indices for bacterial (A, C, E) and fungal (B, D, F) communities of
102 strawberry fruits contaminated with isolates G127/14, G130/14 and G132/14 and under the

103 influence of water (W), natural extract (NE) and food preservative (P). Shannon (A, B),
104 Simpson (C, D), Fisher (E, F) indices were applied. Letters indicate statistical differences.

105 To further elaborate the results, β -diversity analysis was performed to assess the
106 differences in community composition of bacterial and fungal communities in strawberry
107 fruits contaminated with isolates G127/14, G130/14, and G132/14, treated with water (W),
108 natural extract (NE), and food preservative (P). For bacterial communities, PCo1 explained
109 68.1% and PCo2 explained 18.5% of the variance in samples contaminated with G127/14
110 (Fig. 2A). The PCoA plot shows close clustering of all treatments. For G130/14-contaminated
111 samples, PCo1 explained 64.3% and PCo2 explained 20.7%, with separation of bacterial
112 communities based on natural extract and preservative treatments, which cluster together,
113 while water treatment is more distinct (Fig. 2B). For G132/14-contaminated samples, PCo1
114 explained 52.1% and PCo2 explained 25.5%, with the water cluster positioned more
115 separately than natural antifungal treatments (Fig. 1C). For fungal communities, in the
116 G127/14-contaminated sample, PCo1 explained 32.9% and PCo2 explained 25.7% of the
117 variance, with all treatments following a similar pattern of clustering (Fig. 2D). For the
118 G130/14-contaminated samples, PCo1 explained 49.7% and PCo2 explained 28.6%, with
119 natural extract and preservative treatments clustering together while the water control seemed
120 to be more separate (Fig. 2E). Finally, for G132/14-contaminated samples, PCo1 explained
121 46.6% and PCo2 explained 20% of the variance, with three treatment groups forming
122 triangular clusters nested within each other, indicating a gradation or hierarchical separation
123 between the samples (Fig. 2F).



124

125 Fig. 2. β -diversity of bacterial (A, B, C) and fungal (D, E, F) communities of strawberry fruits
 126 contaminated with isolates G127/14 (A, D), G130/14 (B, E) and G132/14 (C, F) under the
 127 influence of water (W), natural extract (NE) and food preservative (P).

2.2 Structure of Bacterial and Fungal Communities

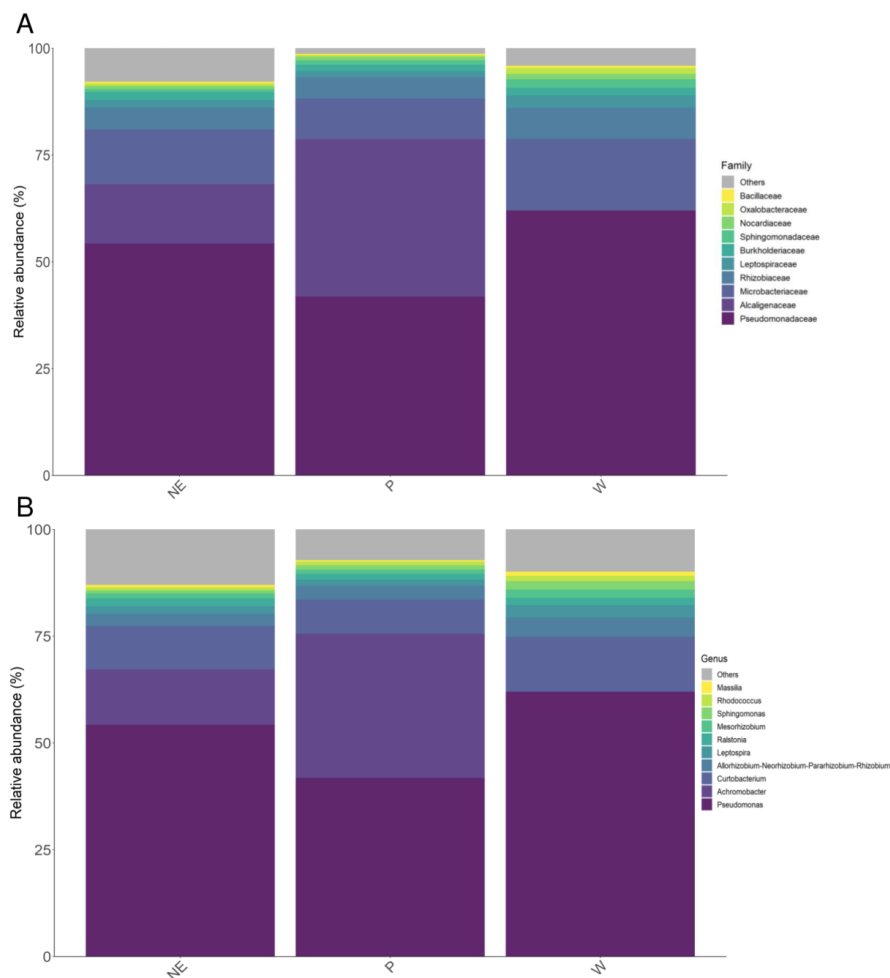
Bacterial communities in the control water-treated samples were predominantly composed of Proteobacteria (88.79%), followed by Actinobacteriota (9.39%) and *Spirochaetota* (1.29%), while other phyla, including Firmicutes, Bacteroidota, and Nitrospirata, were present in minor proportions (Fig. S1A). At the class level, Gammaproteobacteria (86.05%) was the dominant group, with notable contributions from *Actinobacteria* (9.37%) and *Alphaproteobacteria* (2.74%) (Fig. S1B). The most abundant orders included Pseudomonadales (77.74%), Micrococcales (8.70%), and Burkholderiales (6.44%, Fig. S1C), reflecting a strong dominance of Pseudomonadaceae (77.73%) at the family level (Fig. S2A). Within this family, *Pseudomonas* (77.73%) was by far the most abundant genus, followed by *Curtobacterium* (7.13%) and *Achromobacter* (4.29%) representatives (Fig. S2B). Bacterial phyla in samples treated with the natural extract showed a clear dominance of Proteobacteria (89.59%), similar to the water-treated control, but with a slightly increased presence of Actinobacteriota (8.36% vs. 5.92% in water, Fig. S3A). Spirochaetota (1.11%) appeared at a comparable level, whereas Firmicutes (0.55%) and Bacteroidota (0.24%) showed a slight decline. At the class level, Gammaproteobacteria remained the most abundant (86.45%), but there was an increase in Alphaproteobacteria (3.14% vs. 1.87% in water, Fig. S3B). The Actinobacteria class also increased slightly, reflecting the overall rise in Actinobacteriota. A slight shift was observed at the order level, where Pseudomonadales (71.24%) remained dominant but decreased compared to the water control (74.80%, Fig. S3C). In contrast, Burkholderiales increased (14.16% vs. 8.92%), indicating that the natural extract may have permitted a more abundant growth of these bacteria. The increase in Rhizobiales (2.14% vs. 1.05%) also supports this trend. At the family (Fig. S4A) and genus (Fig. S4B) levels, Pseudomonadaceae and *Pseudomonas* spp. still dominated but showed a slight reduction (71.20% vs. 74.77% in water), while Alcaligenaceae (*Achromobacter* spp.) expanded

153 (11.84% vs. 7.88%). Additionally, *Curtobacterium* spp. (Microbacteriaceae) increased (5.64%
 154 vs. 2.98%), suggesting that Actinobacteria were more resilient to the treatment. Bacterial
 155 communities in samples treated with the preservative showed an even stronger dominance of
 156 Proteobacteria (92.44%) compared to the water-treated control (87.23%) and the natural
 157 extract (89.59%) (Fig. S5A). In contrast, Actinobacteriota (6.28%) decreased relative to the
 158 natural extract (8.36%) but remained slightly higher than in water (5.92%). Other phyla, such
 159 as Firmicutes, Bacteroidota, and Spirochaetota, were present in minor amounts, similar to
 160 previous treatments. At the class level (Fig. S5B), Gammaproteobacteria (89.78%) further
 161 increased compared to water (84.95%) and the natural extract (86.45%). However,
 162 Alphaproteobacteria (2.67%) showed a notable rise from the water treatment (1.87%) but was
 163 still slightly lower than in the natural extract treatment (3.14%). At the order level (Fig. S5C),
 164 Pseudomonadales (75.26%) remained the most dominant but showed a slight increase
 165 compared to both water (74.80%) and the natural extract (71.24%). Burkholderiales (13.96%)
 166 increased significantly compared to water (8.92%), aligning closely with the natural extract
 167 (14.16%). However, within this order, different families responded differently to the
 168 preservative. At the family (Fig. S6A) and genus (Fig. S6B) levels, *Pseudomonas* spp.
 169 (75.26%) continued to dominate, slightly increasing over water (74.77%) and the natural
 170 extract (71.20%). However, while Alcaligenaceae (*Achromobacter* spp.) increased compared
 171 to water (11.38% vs. 7.88%), its abundance remained lower than in the natural extract
 172 (11.84%). Meanwhile, Microbacteriaceae (*Curtobacterium* spp.), an Actinobacteriota family,
 173 decreased from 5.64% in the natural extract to 4.44%, indicating that the preservative may
 174 have been more inhibitory to these bacteria.

175 The greatest variation in community composition occurred on day 4 of the experiment, so
 176 results from this day were analysed separately to highlight treatment differences. At the
 177 phylum level (Fig. S7A), Proteobacteria dominated all treatments (water: 77.03%, natural

178 extract: 78.06%, preservative: 87.88%), with the highest proportion in the preservative-treated
 179 samples. Actinobacteriota were the second most abundant (water: 18.27%, natural extract:
 180 16.54%, preservative: 10.21%), but their relative abundance decreased in both antimicrobial
 181 treatments, particularly with the preservative. Spirochaetota were reduced in treated samples
 182 (natural extract: 1.70%, preservative: 1.48%) compared to water (3.01%). Firmicutes (water:
 183 0.42%, natural extract: 2.36%, preservative: 0.25%) and Bacteroidota (water: 0.28%, natural
 184 extract: 0.98%, preservative: 0.16%) were enriched in the natural extract-treated samples but
 185 decreased with the preservative. Verrucomicrobiota appeared in natural extract samples
 186 (0.23%) and minimally in the water control (0.02%), while Cyanobacteria (0.01%) were
 187 found only in the preservative-treated samples. At the class level (Fig. S7B),
 188 Gammaproteobacteria dominated across all treatments, with their relative abundance
 189 increasing from 68.00% in the water-treated samples to 72.26% following application of the
 190 natural extract, and reaching 81.35% after treatment with the preservative. In contrast,
 191 Actinobacteria, the second most abundant class, showed a consistent decline in abundance in
 192 the presence of both the natural extract (16.34%) and the preservative (10.20%) compared to
 193 the water control (18.26%). Alphaproteobacteria (water: 9.03%, natural extract: 5.80%,
 194 preservative: 6.53%) and Leptospirae (water: 3.01%, natural extract: 1.7%, preservative:
 195 1.48%) also decreased in appearance after antifungal treatment application. Minor classes
 196 such as Bacilli (water: 0.42%, natural extract: 2.36%, preservative: 0.24%), and Bacteroidia
 197 (water: 0.28%, natural extract: 0.98%, preservative: 0.16%) also tended to decrease under
 198 sodium metabisulfite treatment, while at the same time increasing in occurrence under plant
 199 extract influence. At the order level (Fig. S7C), Pseudomonadales dominated in all treatments
 200 but decreased from 61.45% in water to 53.77% with the natural extract and 41.95% with the
 201 preservative. In contrast, Burkholderiales increased progressively (4.17%, 16.35%, and
 202 38.80%, respectively). Micrococcales, Rhizobiales and Leptospirales declined with treatment.

203 Overall, the preservative promoted a shift from Pseudomonadales towards Burkholderiales.
 204 At the family level (Fig. 3), Pseudomonadaceae dominated across all conditions but declined
 205 from 61.43% in water to 53.77% in the extract-treated samples and further to 41.94% with the
 206 preservative. In contrast, Alcaligenaceae increased markedly, rising from only 0.02% in water
 207 to 13.41% with the extract and reaching 36.80% under preservative treatment. The relative
 208 abundance of Microbacteriaceae, Rhizobiaceae, Leptospiraceae and Oxalobacteraceae
 209 decreased after antifungal substances application. At the genus level, *Pseudomonas*
 210 dominated across all treatments (water: 61.43%, natural extract: 53.77%, preservative:
 211 41.94%), although its abundance declined with both antimicrobial applications.
 212 *Curtobacterium* followed a similar trend, decreasing under both treatments (water: 13.32%,
 213 natural extract: 10.34%, preservative: 8.02%). In contrast, *Achromobacter* was markedly
 214 enriched by the natural extract (12.72%) and even more so by the preservative (33.61%),
 215 compared to near absence in the control (0.02%). Genera such as *Allorhizobium*-
 216 *Neorhizobium*-*Pararhizobium*-*Rhizobium*, *Leptospira*, *Ralstonia*, and *Sphingomonas* were
 217 present at lower levels and generally declined in treated samples.



218

219 Fig. 3. Relative abundance of top 10 bacterial families (A) and genera (B) during day 4 of the
220 experiment for each treatment: natural extract (NE), preservative (P), water control (W).

221 Fungal communities of contaminated strawberry fruits treated with water were
222 dominated by Ascomycota (64.29%) and Basidiomycota (35.27%), with minor contributions
223 from Mortierellomycota (0.16%), Mucoromycota (0.02%), and Chytridiomycota (0.003%)
224 (Fig. S8A). At the class level, the most abundant groups were Tremellomycetes (33.54%),

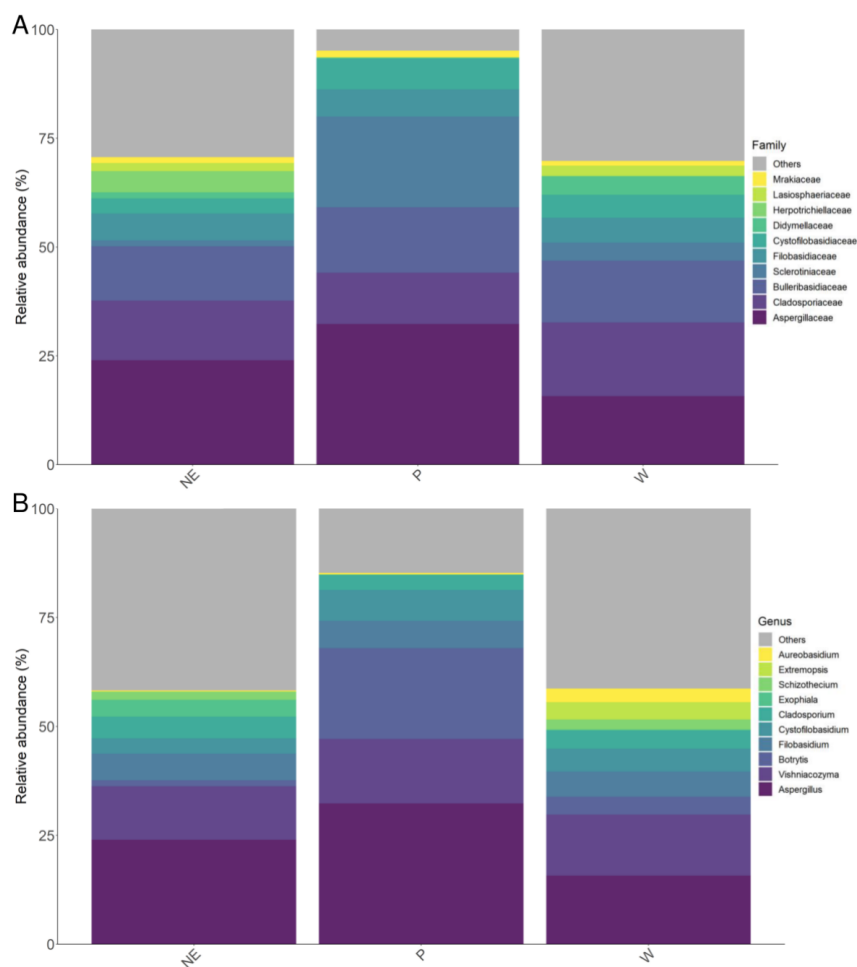
225 Eurotiomycetes (24.55%), Dothideomycetes (19.76%), and Leotiomyces (17.32%) (Fig.
 226 S8B). The predominant orders included Eurotiales (23.37%), Helotiales (17.32%),
 227 Capnodiales (16.46%), and Tremellales (15.24%) (Fig. S8C). At the family level (Fig. S9A),
 228 Aspergillaceae (23.37%), Sclerotiniaceae (16.98%), and Cladosporiaceae (16.45%) were most
 229 abundant, while at the genus level (Fig. S9B), *Aspergillus* spp. (23.37%), *Botrytis* spp.
 230 (16.98%), and *Vishniacozyma* spp. (14.36%) were the main constituents of fungal
 231 communities. In samples treated with the natural extract, Ascomycota remained the dominant
 232 fungal phylum (69.2% vs. 72.1% in water), while Basidiomycota increased slightly (Fig.
 233 S10A). At the class level, Eurotiomycetes (30.2%) and Dothideomycetes (19.7%) remained
 234 prevalent, while Tremellomycetes increased (28.6% vs. 24.3% in water), driven by
 235 *Vishniacozyma* spp. (12.9% vs. 10.8%) (Fig. S10B). Leotiomyces was also abundant
 236 (16.1%). Order Eurotiales (Aspergillaceae, 29.0%) remained dominant, while Tremellales
 237 (Bulleribasidiaceae, 13.8% vs. 10.2% in water) and Filobasidiales (Filobasidiaceae, 6.9% vs.
 238 5.4%) increased (Fig. S10C). Family Aspergillaceae (29.0%) was the most abundant,
 239 followed by Sclerotiniaceae (15.1%), while Bulleribasidiaceae (13.0%) and Filobasidiaceae
 240 (6.8%) showed increases (Fig. S11A). Cladosporiaceae (*Cladosporium*, 4.4%) decreased
 241 slightly. At the genus level, *Aspergillus* spp. (29.0%) remained the most abundant, while
 242 *Vishniacozyma* spp. and *Filobasidium* spp. increased (Fig. S11B). In contrast, *Cladosporium*
 243 spp. and *Alternaria* spp. showed slight reductions. After treatment with the preservative, at the
 244 phylum level, Ascomycota (72.5%) and Basidiomycota (27.0%) remained dominant, with
 245 minor contributions from Chytridiomycota (0.008%) and other fungi (0.4%) (Fig. S12A). At
 246 the class level, Leotiomyces (18.9%) increased compared to water (16.1%), while
 247 Sordariomycetes (2.6% vs. 3.5%) declined (Fig. S12B). At the order level, Eurotiales
 248 (Aspergillaceae, 29.6%) was the most abundant, followed by Helotiales (18.8% vs. 16.1%)
 249 and Capnodiales (17.0% vs. 15.5%). Tremellales (13.8%) remained similar to water treatment

250 (13.0%), while Filobasidiales (5.9% vs. 6.8%) and Pleosporales (1.9% vs. 2.3%) showed
 251 slight declines (Fig. S12C). Within families, Aspergillaceae (29.6%) was the most abundant,
 252 followed by an increase in Sclerotiniaceae when compared to water (18.2% vs. 16.1%), as
 253 well as Cladosporiaceae (17.0% vs. 15.5%) (Fig. S13A). Filobasidiaceae (5.9% vs. 6.8%) and
 254 Cystofilobasidiaceae (4.8% vs. 5.2%) decreased slightly. At the genus level, *Aspergillus* spp.
 255 (29.3%) remained dominant, with *Botrytis* spp. (18.2% vs. 16.1%) and *Cladosporium* spp.
 256 (5.0%) increasing in comparison to the water control (Fig. S13B). *Vishniacozyma* spp.
 257 (13.0%) showed a slight decrease compared to water (13.8%) but remained more abundant
 258 than in natural extract treatment (10.8%).

259 During the 4th day of the experiment, at the phylum level (Fig. S14A), Ascomycota
 260 dominated across all samples and slightly increased under both treatments (water: 61.94%,
 261 natural extract: 67.23%, preservative: 68.38%). Basidiomycota showed a slight shift,
 262 decreasing in the extract-treated samples (27.59%) but increasing in preservative-treated ones
 263 (31.52%) compared to the control (29.44%). Mortierellomycota appeared exclusively in plant
 264 extract-treated samples (0.41%). At the class level (Fig. S14B), the fungal communities were
 265 primarily composed of Tremellomycetes, Eurotiomycetes, and Dothideomycetes, which
 266 together accounted for over 70% of the total relative abundance in each treatment group.
 267 Tremellomycetes, the dominant Basidiomycota class, were slightly reduced under extract
 268 treatment (24.54%) but increased in preservative-treated samples (30.68%) compared to the
 269 control (28.50%), aligning with the phylum-level trend. Eurotiomycetes markedly expanded
 270 under both treatments, particularly with preservatives (32.45%) versus 15.92% in the control,
 271 suggesting strong treatment tolerance. In contrast, Dothideomycetes declined progressively
 272 with increasing antifungal pressure (water: 28.74%, natural extract: 21.28%, preservative:
 273 13.37%). Notably, Leotiomycetes were highly enriched in the preservative group (20.99%),
 274 while remaining low in other conditions (water: 4.85%, extract: 3.52%). Sordariomycetes and

275 Microbotryomycetes were more prevalent in natural extract treated samples than in water
 276 control and diminished in the presence of preservative. Lecanoromycetes class was only
 277 detectable in the control samples, whereas Orbiliomycetes were present in water control and
 278 natural extract treated samples but not in samples affected by preservative. Agaricomycetes,
 279 were only detectable in natural extract treated samples. Taphrinomycetes increased in number
 280 in both treatments in comparison to control (water: 0.14%, natural extract: 0.33%,
 281 preservative: 0.39%). At the order level (Fig. S14C), fungal communities were dominated by
 282 Eurotiales, which relative abundance steadily increased under both treatments (water: 15.73%,
 283 natural extract: 23.96%, preservative: 32.32%). Similarly, Helotiales were strongly enriched
 284 in preservative-treated samples (20.99%) compared to the extract (3.52%) and control
 285 (4.85%). In contrast, members of Capnodiales (water: 16.93%, extract: 13.72%, preservative:
 286 11.76%) declined under antifungal pressure. Pleosporales (7.27% in extract vs. 4.61% in
 287 control and only 1.18% in preservative). Orders affiliated with Tremellomycetes exhibited
 288 mixed trends: Tremellales decreased under the influence of natural extract (12.87% vs
 289 15.62% in the control), while Cystofilobasidiales increased under preservatives (8.61% vs.
 290 6.38% in the control) and Filobasidiales increased under both treatments (water: 5.74%,
 291 nature extract: 6.16%, preservative: 6.25%). Pleosporales, Hypocreales, Chaetothyriales and
 292 Sordariales showed a marked peak in the extract-treated samples and decreased under
 293 preservative exposure, with Sordariales entirely absent. At the family level (Fig. 4A),
 294 Aspergillaceae dominated the communities and steadily increased under both treatments
 295 (water: 15.73%, extract: 23.96%, preservative: 32.32%). Filobasidiaceae and Mrakiaceae
 296 followed a similar pattern. In contrast, Cladosporiaceae declined progressively under
 297 antifungal exposure (water: 16.93%, extract: 13.72%, preservative: 11.76%). The same trend
 298 was observed for Didymellaceae and Lasiosphaeriaceae, the latter of which could not be
 299 detected under preservative influence. Bulleribasidiaceae decreased slightly under the extract

300 (12.47%) but increased under preservative treatment (15.04%). Sclerotiniaceae followed a
 301 similar pattern, showing a sharp increase under preservative treatment (20.85%) compared to
 302 both the control (4.13%) and extract (1.36%). Similar situation was also true for
 303 Cystofilobasidiaceae which increased under preservative treatment (7.08%) but declined
 304 under the extract (3.47%). A marked enrichment under the extract was observed for
 305 Herpotrichiellaceae (4.86%) relative to both control (0.19%) and preservative (0.13%),
 306 suggesting selective tolerance to plant-derived compounds. At the genus level (Fig. 4B),
 307 *Aspergillus* dominated the communities and steadily increased under both treatments (water:
 308 15.73%, extract: 23.96%, preservative: 32.32%). *Filobasidium* followed a similar pattern,
 309 increasing slightly under both treatments. *Cystofilobasidium*, *Vishniacozyma* and *Botrytis*
 310 showed a notable rise under preservative exposure but a decline with the extract. In contrast,
 311 *Cladosporium* (water: 4.22%, natural extract: 4.97%, preservative: 3.45%) and *Exophiala*
 312 (water: 0.19%, natural extract: 3.89%, preservative: 0.13%) showed a mild peak under the
 313 extract but declined under preservative. Finally, *Schizothecium*, *Extremopsis* and
 314 *Aureobasidium* were substantially reduced under both treatments, with *Schizothecium*
 315 disappearing in the presence of the preservative and *Extremopsis* not occurring under both
 316 treatments.



317

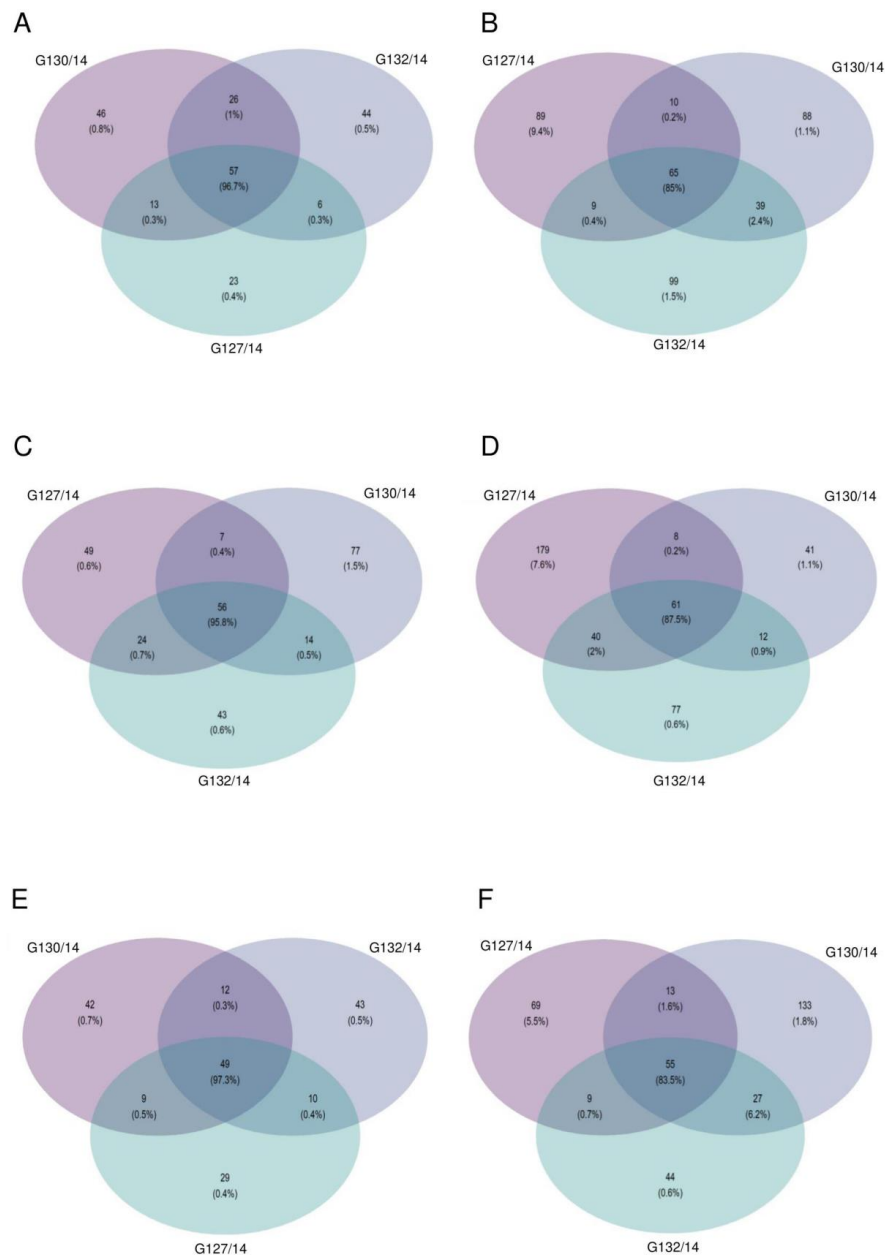
318 Fig. 4. Relative abundance of top 10 fungal families (A) and genera (B) during day 4 of the
 319 experiment for each treatment: natural extract (NE), preservative (P), water control (W).

320

321

322 **2.3 Shared and Unique Microbial Taxa Under Different Treatments**

323 Under water treatment, bacterial communities (Fig. 5A) showed a high proportion of
324 shared ASVs (57 ASVs, 96.7% of total sequences), with unique ASVs contributing
325 marginally to each isolate's composition (23 ASVs for G127/14, 46 for G130/14, and 44 for
326 G132/14). Shared ASVs between pairs of isolates were limited, with the highest overlap
327 observed between G130/14 and G132/14 (26 ASVs, 1.0% of sequences). In fungal
328 communities (Fig. 5B), the proportion of shared ASVs was lower (65 ASVs, 85% of total
329 sequences), with G127/14 harbouring the highest number of unique ASVs (89), followed by
330 G132/14 (99) and G130/14 (88). The overlap between isolate pairs was minimal, with
331 G130/14 and G132/14 sharing the most ASVs (39 ASVs, 2.4% of sequences).



332

333 Fig. 5. Venn diagrams representing core taxa for bacterial (A, C, E) and fungal communities

334 (B, D, F), for samples contaminated with isolates G127/14, G130/14 and G132/14, presented

335 individually for each treatment type: water (A, B), natural extract (C, D), preservative (E, F).
336 The integer is the ASV number, while the percentage data is the total sequence number.

337 In samples treated with the natural extract, bacterial communities (Fig. 5C) again
338 displayed a high proportion of shared ASVs (56 ASVs, 95.8% of sequences), with unique
339 ASVs being slightly more prominent in G130/14 (77) than in G127/14 (49) or G132/14 (43).
340 Paired isolate overlaps remained low, with G127/14 and G132/14 sharing 24 ASVs (0.7% of
341 sequences). In fungal communities (Fig. 5D), the shared proportion was slightly higher than
342 in water-treated samples (61 ASVs, 87.5% of sequences), with G127/14 having the largest
343 number of unique ASVs (179). The overlap between pairs remained low, with G127/14 and
344 G132/14 sharing 40 ASVs (2.0% of sequences).

345 For preservative-treated samples, bacterial communities (Fig. 5E) had the highest
346 proportion of shared ASVs (49 ASVs, 97.3% of sequences), with individual isolates
347 contributing fewer unique ASVs (29 for G127/14, 42 for G130/14, and 43 for G132/14). The
348 overlap between isolate pairs remained consistent, with G127/14 and G132/14 sharing 10
349 ASVs (0.4% of sequences). In fungal communities (Fig. 5F), the shared proportion was
350 lowest (55 ASVs, 83.5% of sequences), with G130/14 containing the highest number of
351 unique ASVs (133). Pairwise overlaps remained low, with the greatest overlap observed
352 between G130/14 and G132/14 (27 ASVs, 6.2% of sequences).

353 Changes in the core bacterial (Fig. S11) and fungal (Fig. S12) taxa across treatments over
354 time were also investigated. In bacterial communities, the majority of ASVs were shared
355 among all treatments at each time point, with preservative-treated samples consistently
356 exhibiting the highest proportion of shared taxa ($\geq 95\%$ of sequences). Unique ASVs
357 contributed marginally, though they were slightly more prominent in natural extract-treated
358 samples on earlier days. Fungal communities displayed greater variability. The proportion of
359 shared ASVs fluctuated across time points, with the lowest values observed under

360 preservative treatment on day 1 (59.9% of sequences) and day 7 (73.4% of sequences).
361 Unique ASVs were more pronounced, particularly in water-treated samples on day 5 and
362 preservative-treated samples on day 7.

363 **2.4 Predicted Functional Traits of the Bacterial Microbiome**

364 PICRUSt predictions showed no substantial differences in the relative abundances of
365 main functional classes across isolates and treatments, with metabolism-related sequences
366 consistently dominating (~75–76%) and other categories remaining stable (Fig. S13A).
367 However, under closer examination, some differences could be seen between treatments and
368 days for certain subclasses. For water control, Metabolism of cofactors and vitamins and
369 Amino acid metabolism consistently had the highest functional sequences, with 222,181.06
370 on day 6 and 318,421.43 on day 3, respectively. In contrast, Nucleotide metabolism and
371 Biosynthesis of other secondary metabolites displayed the lowest values, peaking at 26,769.2
372 and 24,069.42, respectively. (Fig. 6A). Energy, Carbohydrate, and Lipid metabolism showed
373 a gradual increase in activity, peaking around day 6, followed by a slight decline on day 7.

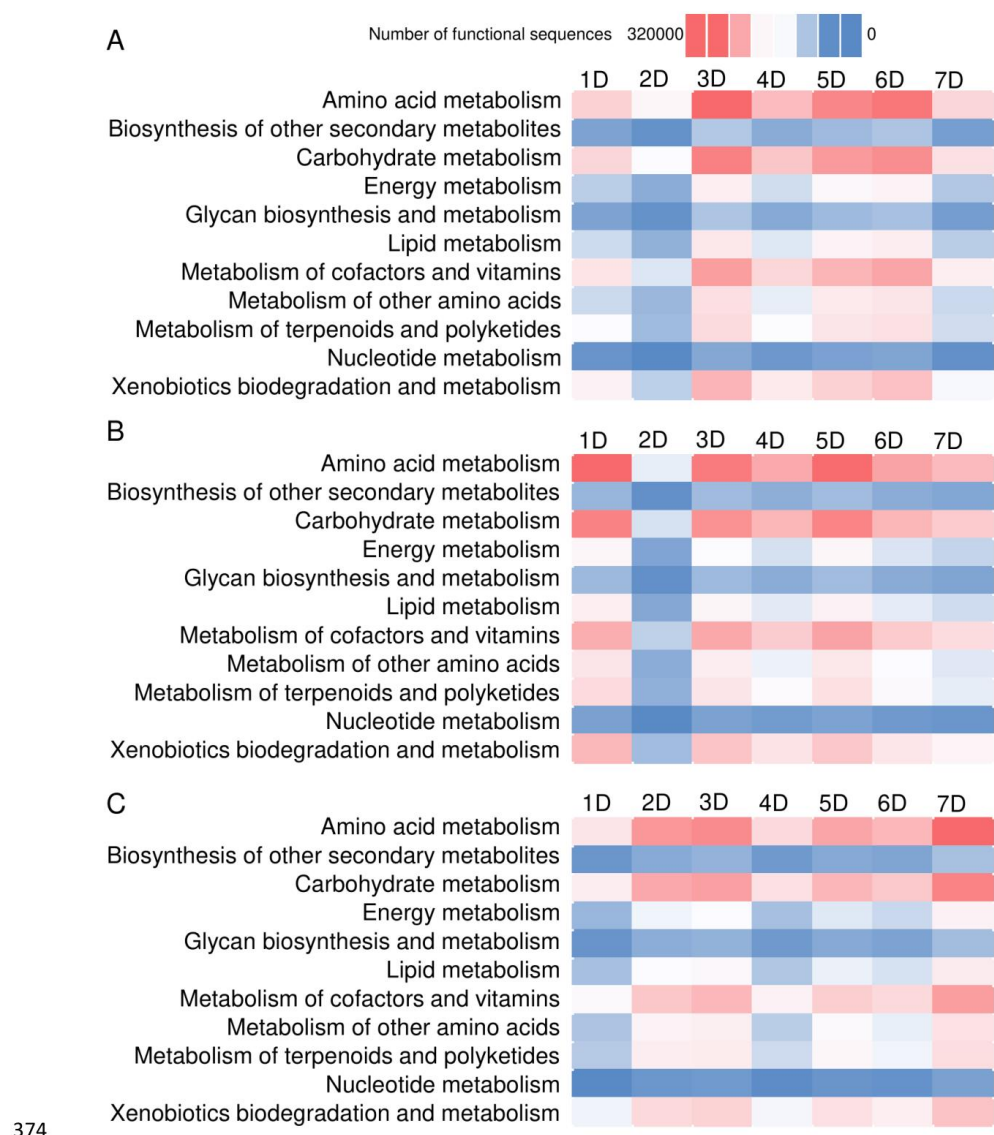


Fig. 6. PICRUSt prediction of the bacterial functional profile with the number of functional sequences associated with the metabolism of various compounds. Data visualised for each experiment day for each treatment separately: water (A), natural extract (B), preservative (C).

378 The data for the natural extract treatment showed several key differences compared to
379 the water control. Amino acid metabolism was higher on day 1 in the natural extract treatment
380 (270,779.78) but declined more sharply over time, while the control fluctuated gradually.
381 Biosynthesis of other secondary metabolites also peaked higher on day 1 (34,615.96) in the
382 natural extract, but showed a more rapid decline by day 7, contrasting with the control's more
383 stable pattern. In Energy metabolism, the natural extract treatment initially had higher values
384 on day 1 (88,551.11) but decreased more quickly after day 3, reaching 54,183.72 by day 7.
385 Meanwhile, the control remained stable. Similarly, the Metabolism of cofactors and vitamins
386 peaked at 195,854.88 on day 5 in the natural extract, with a sharper decline by day 7
387 (122,028.20), while the control showed consistent levels.

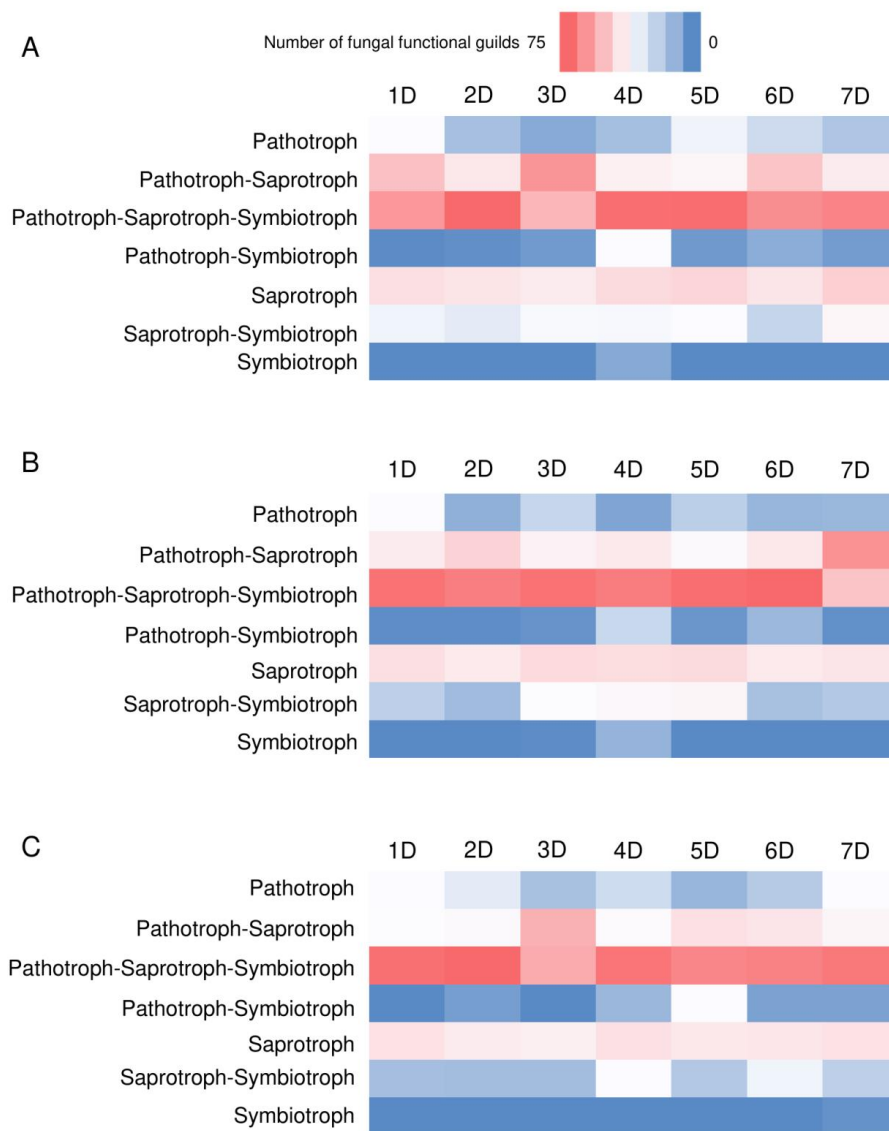
388 The preservative treatment showed several differences compared to the control.
389 Amino acid metabolism began lower (116,877.50) but increased significantly by day 7
390 (298,188.36), surpassing the control's final value of 143,261.47. Similarly, Biosynthesis of
391 other secondary metabolites peaked at 45,277.90 on day 7, compared to the water control's
392 maximum of 24,069.42. In Carbohydrate metabolism, the preservative treatment increased
393 from 104,150.00 on day 1 to 260,833.02 on day 7, exceeding the control's peak of 240,530.43.
394 Energy metabolism showed a similar trend, rising from 38,121.23 to 97,970.62, higher than
395 the control's final value of 47,257.06. The Metabolism of cofactors and vitamins peaked at
396 221,464.01 on day 7, the highest in this dataset, slightly higher than the control's peak of
397 222,181.06. Metabolism of terpenoids and polyketides also increased, reaching 126,715.33 on
398 day 7, higher than the control's peak of 119,005.38.

399 **2.5 Fungal Trophic Modes and Ecological Roles**

400 The FUNGuild database classified fungi into six main trophic modes (Fig. S13B). The
401 analysis revealed that the dominant fungal trophic mode across samples is the tripartite
402 pathotroph-saprotroph-symbiotroph, with percentages ranging from approximately 42% in

403 some G127 treatments to nearly 78% in the G130 preservative treatment. Notably, isolate
404 G127 under water control exhibited a markedly higher saprotroph fraction (~30%) compared
405 to its natural extract and preservative treatments (~12–14%), whereas isolates G130 and
406 G132 consistently showed higher proportions of the tripartite mode. For all isolate
407 contaminants, pure pathotrophs remained below 2%, indicating a minor role relative to
408 combined trophic strategies. Overall, both treatment type and isolate identity appear to
409 influence the relative distribution of fungal trophic modes, underscoring distinct community
410 shifts under different conditions.

411 The results were also visualised for each treatment and day of the experiment (Fig. 7).
412 Under the water treatment, fungal communities were dominated by the pathotroph-
413 saprotroph-symbiotroph guild, which ranged from 35.8% to 73.7% across the sampling days
414 (Fig. 7A). The pathotroph-saprotroph group also contributed significantly, though it was more
415 variable (4.5–52.3%), while pure saprotrophs consistently ranged between 10% and 24%. In
416 contrast, pure pathotrophs, pathotroph-symbiotrophs, and saprotroph-symbiotrophs remained
417 below 2%, and symbiotrophs were nearly absent except for a minor presence on day 4. Under
418 natural extract treatment, the pathotroph-saprotroph-symbiotroph guild dominated at first
419 (~71–76%) but declined to 30% by day 7, while pathotroph-saprotroph increased in later
420 stages (55%). Other guilds showed minor mid-experiment fluctuations, resembling control
421 trends (Fig. 7B). Under preservative treatment, the pathotroph-saprotroph-symbiotroph guild
422 was consistently elevated (ranging from 46% to 79%) relative to the water control, while the
423 pathotroph-saprotroph fraction was markedly reduced on most days (notably, 2–5% versus up
424 to 52% in the control). Saprotroph percentages were similar early on but decreased at later
425 time points, and the saprotroph-symbiotroph guild was substantially lower compared to the
426 control (Fig. 7C).

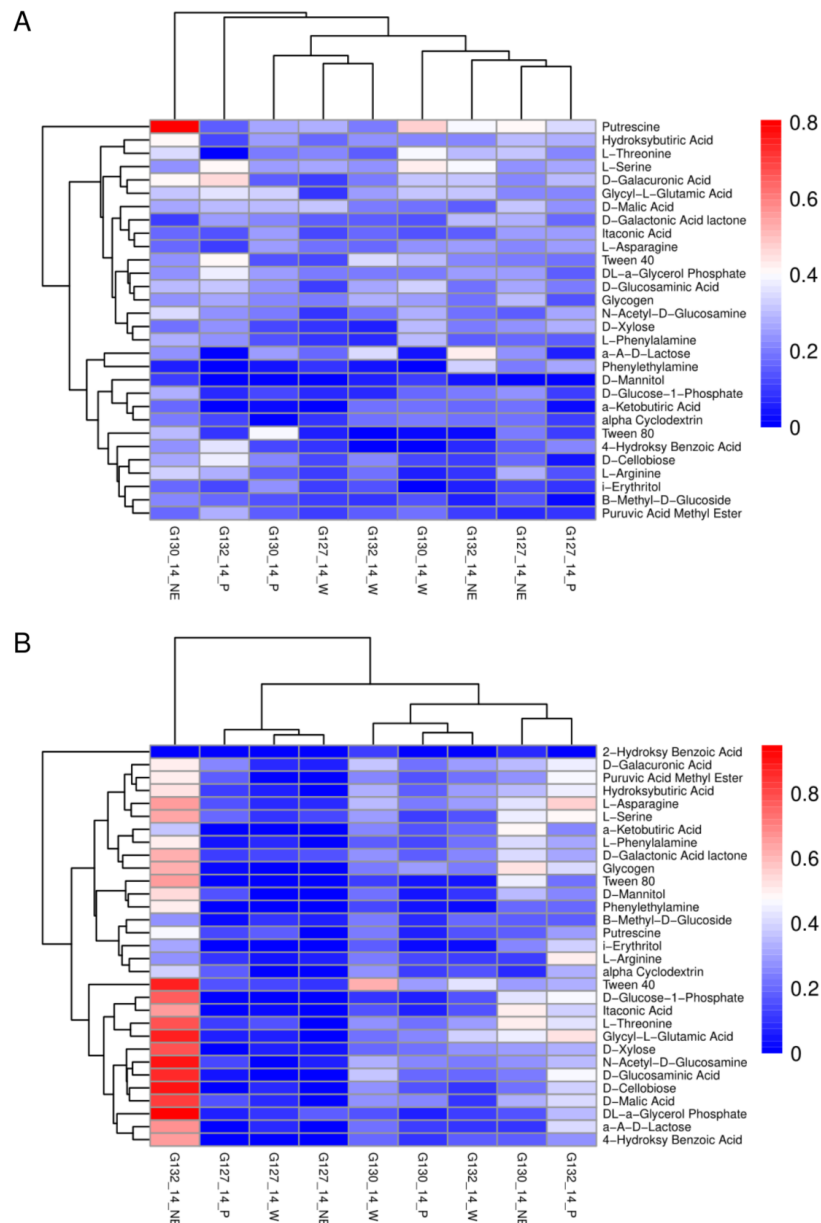


427

428 Fig. 7. Fungal functional profile inferred by FUNGuild with the number of fungal functional
 429 guilds. Data visualised for each experiment day for each treatment separately: water (A),
 430 natural extract (B), preservative (C).

431 **2.6 Metabolic Activity in Artificially Contaminated Strawberry-Originated**
432 **Microbial Populations**

433 For Biolog™ EcoPlate data, 120 h of incubation was chosen (Fig. S14) for each plate
434 batch to generate heatmaps which reveal distinct biomass production profiles which vary with
435 refrigeration time and contaminant treatment. After 1 day (Fig. 8A), substrate utilization was
436 largely similar for all samples—showing elevated biomass production on putrescine, D-
437 galacuronic acid and L-serine while D-mannitol, D-glucose phosphate and pyruvic acid
438 methyl ester were not metabolized. With 7 days of refrigeration (Fig. 8B), treatment and
439 isolate-specific differences emerged and were more distinct rather than differences between
440 individual carbon sources. Samples contaminated with isolate G127/14 generally produced
441 lower biomass, yet displayed transient activity spikes on day 3 (Fig. S15B), day 4 (Fig.
442 S15C), and day 5 (Fig. S15D) under preservative treatment, with an additional spike on day 6
443 (Fig. S15E) when treated with water. In contrast, communities contaminated with G130/14
444 and G132/14 showed broadly similar growth trends from day 2 (Fig. S15A), with nuanced
445 deviations—a day 4 surge (G132/14 with water, G130/14 with preservative, Fig. S15C), a day
446 5 increase for G130/14 under natural extract and preservative (despite lower values for
447 G132/14, whose control exceeded that of G130/14, Fig. S15D), and elevated day 6 scores for
448 G132/14 under preservative (Fig. S15E). By day 7, G127/14 samples consistently
449 underperformed, while G130/14 and G132/14 treatments converged, with G132/14 under
450 natural extract achieving the highest biomass stimulation (Fig. 8B).



451

452 Fig. 8. Biomass production (OD 750) of strawberry-originated microbial communities on
 453 different carbon sources in Biolog™ ECO plates. Data shown corresponds to microplates

454 incubated for 120 hours with inocula from samples refrigerated for 1 (A) or 7 (B) days.
 455 Communities were exposed to artificial contamination with *Neosartorya* spp. (isolates
 456 G127/14, G130/14, G132/14) and treated with water (W), natural extract (NE), or
 457 preservative (P).

458 For Biolog™ EcoPlate data, Richness (R) was also visualised (Fig. S16). On day 1,
 459 samples with G127/14 showed significantly lower R with water compared to natural extract or
 460 preservative; for G130/14, no significant treatment differences were observed; and for
 461 G132/14, natural extract increased R while preservative decreased it relative to water. Similar
 462 trends were seen on days 2 and 3. On day 4, treatment differences disappeared for G127/14
 463 and G132/14, although preservative increased R for G130/14. On day 5, both natural extract
 464 and preservative elevated R for G127/14, only natural extract increased R for G130/14, and
 465 preservative reduced R for G132/14. On day 6, water-treated samples had the highest R for
 466 G127/14; for G130/14, water exceeded preservative; and for G132/14, water surpassed natural
 467 extract. On day 7, water resulted in higher R than natural extract for G127/14, lower R than
 468 both natural extract and preservative for G130/14, with no significant differences for
 469 G132/14.

470 **3. Discussion**

471 The dynamics of bacterial-fungal interactions are deeply entwined with the diversity and
 472 composition of microbial communities, shaping the microbial landscape and playing a critical
 473 role in its functionality and resilience²¹. Since microbial diversity affects environmental
 474 stability, the analysis of α diversity indices (Shannon, Simpson, and Fisher) was conducted
 475 which revealed that bacterial communities remained relatively stable across all treatments,
 476 with no significant differences in species richness (Shannon, Fisher) despite contaminant
 477 variants²². However, slight divergence of dominance of species could be seen via Simpson
 478 index. This stability was further supported by the high proportion of shared bacterial ASVs

479 observed in the Venn diagrams, indicating that bacteria are largely resilient to environmental
480 modifications induced by natural and synthetic antifungal treatments. This opposes results
481 from other teams, where *Calendula officinalis* extract^{23,24} or sodium metabisulfite²⁵ displayed
482 antibacterial properties. However, this fact can be explained by attributing the usage of
483 abovementioned substances against singular pathogens rather than communities. This results
484 further supports the thesis that microbial communities can accomplish complex functions that
485 are unattainable by individual members alone²⁶. In contrast, studied fungal communities
486 exhibited greater variability in diversity, with differences observable in all α diversity indices
487 and in core taxa composition shown on Venn graphs. These differences suggest that, even as a
488 whole, fungal communities are more susceptible to sodium metabisulfite and *Calendula*
489 *officinalis* treatment-induced shifts, and that applied treatments have a varying degree of
490 influence on fungi. Such response difference between bacterial and fungal communities is
491 possible as it has been previously documented in studies focused on microbial responses to
492 environmental changes caused by soil disturbance and drought^{27,28}. Despite variations in α -
493 diversity, which measures the richness and evenness within a sample²⁹, the overall
494 composition of microbial communities remained similar across different samples, as reflected
495 by β diversity metrics³⁰. This similarity in β diversity could be attributed to mostly the same
496 species being present regardless of treatment but at different abundance scores. A similar
497 situation was observed in other research, where glyphosate-based herbicide affected the
498 individual taxa of strawberry rather than the microbial community composition as a whole³¹.

499 In strawberry samples studied by others, bacterial families: Pseudomonaceae,
500 Yearsiniaceae, Hafniaceae¹⁴ and Alcaligenaceae³² were discovered to be most abundant. This
501 is partially mirrored by our results, where the families: Pseudomonaceae, Microbacteriaceae
502 and Alcaligenaceae tended to occur the most. At the phylum level, Proteobacteria dominated
503 the bacterial communities across all treatments, which has been reported before³³, though in

504 our study their relative abundance was further elevated under preservative treatment
 505 compared to the water control and natural extract. The natural extract treatment, however, was
 506 associated with a slight increase in Actinobacteriota (generally detectable in melons³⁴ and
 507 blueberries³⁵) and Alphaproteobacteria (especially active during an infection of strawberry
 508 plants³⁶), indicating that it may permit the growth of these groups more than the preservative.
 509 At the genus level, *Pseudomonas* spp. remained predominant across all treatments, but its
 510 relative abundance increased slightly under preservative treatment. In addition, members of
 511 the order Burkholderiales, particularly the family Alcaligenaceae (*Achromobacter* spp.), were
 512 more abundant in natural extract-treated samples. The ecological significance of
 513 Burkholderiales in the strawberry microbiome has been demonstrated in other studies³⁷, as
 514 well as its increased abundance being observed under phosphate and glyphosate-based
 515 treatments³¹. This suggests that members of this order may respond to environmental stressors
 516 and chemical exposures. Microbacteriaceae (*Curtobacterium* spp.) which promote plant
 517 growth³⁸, were reduced under the preservative. These observations suggest that the
 518 preservative reinforces the dominance of Proteobacteria, especially *Pseudomonas* spp., while
 519 exerting a stronger inhibitory effect on certain Actinobacteria compared to the natural extract.
 520 Interestingly, both of these bacterial groups display protective effects against strawberry plant
 521 diseases^{39,40}. Notably, these trends were generally consistent across isolate contaminants,
 522 although the magnitude of the changes appeared less pronounced in the most sensitive isolate,
 523 G127/14, compared to the less sensitive G130/14 and G132/14. Despite taxonomic shifts
 524 indicating probable microbiome restructuring under different treatments, PICRUST functional
 525 predictions show stable metabolic pathway composition overall. However, the natural extract
 526 and preservative treatments resulted in significant differences in bacterial community
 527 function, particularly in Amino acid and Secondary metabolite metabolism. The natural
 528 extract treatment induced an initial metabolic increase, followed by a more rapid decline

529 compared to the water control, suggesting a transient metabolic response⁴¹. In contrast, the
530 preservative treatment initially decreased metabolic activity, followed by a compensatory
531 increase over time, indicating an initial inhibitory effect followed by microbial adaptation⁴².

532 Fungal communities were markedly more dynamic in response to antifungal treatments.
533 Under water treatment, Ascomycota and Basidiomycota were both well represented, both
534 denoted as crucial for strawberry microbiomes⁴³; however, the natural extract treatment led to
535 a noticeable shift toward Basidiomycota, particularly increasing the proportion of yeasts such
536 as *Vishniacozyma* spp., which is linked to strawberry flavour formation⁴⁴. In contrast, the
537 preservative treatment maintained a higher relative abundance of Ascomycota, with spoilage
538 agents such as *Aspergillus* spp. and *Botrytis* spp.⁴⁵ remaining more abundant than when the
539 natural extract treatment was used. These differential effects suggest that the natural extract
540 may selectively favour specific yeasts and reduce pathogenic fungi, while preservatives tend
541 to sustain the populations of filamentous fungi. The fungal trophic mode analysis further
542 indicated that, in contrast to natural extract treatment, which showed a pronounced late
543 increase in the pathotroph-saprotroph guild, the preservative treatment consistently
544 maintained elevated levels of the tripartite pathotroph-saprotroph-symbiotroph guild while
545 effectively suppressing the saprotroph-symbiotroph fraction. The varying effects on fungal
546 functional guilds reflect the intricate ecological consequences of antifungal treatments and
547 emphasize that the type of intervention can substantially influence the equilibrium between
548 pathogenic and saprophytic fungi. Strawberry fruit samples infected with the G127/14 isolate,
549 the most sensitive to antifungal treatments, generally exhibited lower microbial diversity and
550 metabolic activity under antifungal interventions, particularly under preservative treatment. In
551 contrast, isolate G130/14 (the least sensitive) often maintained or even increased its diversity
552 and metabolic activity, especially under preservative conditions. G132/14 typically showed
553 intermediate responses. These patterns suggest that the intrinsic resistance of the contaminant

554 not only affects its survival but also influences the surrounding microbial community's
555 structure and function. This has important implications for designing targeted preservation
556 strategies, as antifungal agents' efficacy and broader ecological impacts may vary
557 significantly depending on the specific contaminant profile.

558 The Biolog™ EcoPlate data corroborated these findings, with biomass production and
559 richness measures showing treatment- and isolate-specific trends. Notably, microbial
560 communities contaminated with the sensitive isolate G127/14, consistently exhibited lower
561 biomass production than communities contaminated with the more resistant G130/14 and
562 intermediate G132/14, further emphasizing the influence of contaminant sensitivity on
563 metabolic outcomes.

564 Overall, our findings suggest that antimicrobial treatments may differentially influence
565 bacterial and fungal communities in strawberries, with fungal populations appearing more
566 responsive to certain interventions, though the extent of these effects may depend on both the
567 treatment applied and the characteristics of the microbial contaminants. The natural extract
568 treatment appears to induce a more transient metabolic response and a shift toward beneficial
569 yeast populations. In contrast, the preservative treatment reinforces the dominance of certain
570 filamentous fungi and leads to a delayed but substantial increase in metabolic activity. These
571 insights are crucial for developing sustainable and effective postharvest preservation
572 strategies that suppress spoilage organisms and maintain a balanced and beneficial
573 microbiome.

574 **4. Methodology**

575 **4.1 Fungal isolates and antifungal treatments**

576 Three isolates of *Neosartorya* spp. were selected from an internal culture collection of the
577 Laboratory of Molecular and Environmental Microbiology, Institute of Agrophysics, Polish

Academy of Sciences (Lublin, Poland). The selection was based on the isolates' innate resilience to natural extracts and food preservatives, as established in previous research: G127/14 being the most sensitive, G132/13 showing average resistance, and G130/14 demonstrating the highest resilience^{19,20}. In this experiment, antifungal effects of dry natural extract and food preservative against strawberry microbiomes infected with *Neosartorya* spp. were analysed. *Calendula officinalis* dry extract was produced by Zrób Sobie Krem Kosmetyki Naturalne (Prochowice, Poland), whereas sodium metabisulfite was manufactured by Sigma-Aldrich (St. Louis, MO, USA). The extract and the preservative were resuspended and dissolved in sterile water to achieve a concentration of 1000 µg mL⁻¹ and filtered via 0.22 µm filters (Millex®, Ireland). For sodium metabisulfite solution, the content of available sulphur dioxide (SO₂), calculated as sodium metabisulfite via the distillation method, was also measured (according to Polish Standard PN-A-75101-23:1990/Az2:2002), which yielded an insignificant score of less than 10 mg kg⁻¹ - an inconsequential value compared to the permissible range for various foods, which spans from 10 mg kg⁻¹ to 2000 mg kg⁻¹ depending on the product⁴⁶.

Prior to experimentation, the isolates were recultivated on potato dextrose agar (PDA, BioMaxima S. A., Lublin, Poland) at 30°C for 10 days to promote active growth and sporulation. Successively, the mycelium was harvested and suspended in sterile water to achieve a suspension of 75% transmittance equating to approximately 4.44 × 10⁴ ascospores mL⁻¹.

4.2 Artificial contamination and antifungal treatments of strawberries

Fresh, undamaged strawberry fruits (*Fragaria × ananassa*) were obtained from a local supplier and stored at 4°C before experimentation. Each strawberry was artificially inoculated by spraying with approximately 2.5 mL of the prepared *Neosartorya* sp. suspension using a fine-mist sprayer to ensure even distribution across the fruit surface. Inoculated strawberry

603 fruits were then subjected to one of three treatments: water (control), spraying with *Calendula*
604 *officinalis* extract (1 mg mL⁻¹), or spraying with sodium metabisulfite (1 mg mL⁻¹). After
605 treatment, fruits were placed on individual sterile trays and incubated at 4°C for 7 days.

606 **4.3 DNA extraction and metataxonomic sequencing (Illumina MiSeq)**

607 Every day of the incubation period, samples were collected for DNA extraction. A total of
608 36 strawberry fruits were used per day, with nine allocated per fungal isolate or control
609 condition. Within each treatment group, three strawberries were exposed to *Calendula*
610 *officinalis* extract, three to sodium metabisulfite, and three to sterile water. Each strawberry
611 was individually weighed before pooling into sterile filtering bags (three per bag, maintaining
612 biological replicates). Strawberry tissues were homogenized for 2 minutes using Smasher®
613 peristaltic blender (AES Laboratoire, Bruz, France) to obtain 0.25 g of homogenate from each
614 sample. Total DNA was extracted using the Fast DNA Spin Kit for Feces (MP Biomedical,
615 USA) following the manufacturer's protocol, with modifications to improve fungal DNA
616 yield, including an additional bead-beating step using a FastPrep-24 homogenizer (MP
617 Biomedical, USA) for 40 s at 6.0 m s⁻¹. The quality and quantity of extracted DNA were
618 assessed using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, West
619 Palm Beach, FL, USA) and Quantus fluorometer with QuantiFluor® ONE reagents (Promega,
620 Madison, WI, USA).

621 For microbial community profiling, the V4 region of the bacterial 16S rRNA gene was
622 amplified using primers 515F–806R⁴⁷, and the fungal ITS2 region was amplified with
623 ITS3/ITS4 primers⁴⁸. Libraries were prepared following the procedure described in Siegieda
624 et al.⁴³. The amplification of each specific region was performed using KAPA HiFi HotStart
625 ReadyMix (Kapa Biosystems, Cape Town, South Africa) under the following conditions: an
626 initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s,
627 and 72°C for 60 s, with a final extension at 72°C for 5 minutes. The resulting amplicons were

35

628 purified using CleanNGS magnetic beads (CleanNA, Gouda, Netherlands). Each sample was
629 indexed with a distinct Illumina sequencing adapter. After purification with magnetic beads,
630 DNA concentration was measured using the Quantus fluorometer with QuantiFluor® ONE
631 reagents (Promega, Madison, WI, USA). Library size was assessed with a D1000 DNA
632 ScreenTape on the TapeStation 4150 (Agilent Technologies, Santa Clara, USA), and all
633 samples were diluted to a uniform concentration using PCR-grade water before pooling at a
634 final concentration of 8 pM. Sequencing was performed on the Illumina MiSeq platform (2 ×
635 300 bp paired-end), with 5% PhiX added as an internal quality control (Illumina, San Diego,
636 CA, USA).

637 **4.4 Biolog™ EcoPlates assay**

638 To evaluate the metabolic activity of microbial communities contaminated with
639 *Neosartorya* spp. under different treatments, the Biolog™ EcoPlates (Biolog™, Hayward,
640 CA, USA) system was used⁴⁹. From each homogenized sample, 0.25 g was transferred to an
641 Eppendorf tube for DNA extraction, and another 1 g was placed into a sterile capped
642 container with 99 mL of saline peptone water for microplate analysis, as per manufacturer's
643 instructions. The suspension was subjected to 20 min of shaking at room temperature,
644 followed by 30 min incubation at 4°C. After incubation, 120 µL of the prepared inoculum was
645 transferred into each well of the Biolog™ EcoPlates, designed for microbial community
646 metabolic profiling by containing 31 different carbon sources. Plates were incubated at 30°C
647 for 216 h, with optical density (OD590) measurements taken every 24 h using a MicroStation
648 microplate reader (Biolog™, Hayward, CA, USA).

649 **4.5 Statistical analysis and bioinformatics**

650 The sequencing data was processed and analysed using QIIME2⁵⁰. Within QIIME2,
651 ITSxpress⁵¹ was employed to trim primers. At the same time, DADA2⁵² was utilized for

denoising, chimera removal (using the consensus method with a minimal fold parent-over-abundance threshold of 12), sequence merging, and the identification of Amplicon Sequence Variants (ASVs)^{53,54}. Taxonomic classification was performed with the Scikit-learn classifier⁵⁵. Fungal ASVs were classified into ecological guilds using FUNGuild, a platform-independent tool designed to assign ecological roles based on taxonomic identity⁵⁵. Bacterial functional potential was inferred from 16S rDNA sequences using the PICRUSt tool⁵⁶, which predicts gene content based on phylogenetic relationships. Functional annotation was performed regarding the KEGG database⁵⁷.

For metataxonomic data analysis, the following packages were used: DESeq2⁵⁸, tidyverse⁵⁹, microeco⁶⁰, phyloseq⁶¹, file2meco⁶², mecodev⁶⁰, btools⁶³, and vegan⁶⁴ for statistical analysis, data manipulation, and ecological modelling. Data visualization was performed using ggplot2⁶⁵ and viridis⁶⁶ for generating plots and visual representations. The dataset was rarefied to a sample size of 1,000 reads per sample for fungi and 2,000 reads per sample for bacteria using the rarefy_samples function from the microeco package. Successful rarefaction was verified by checking the sample sums. A diversity indices, including Shannon, Simpson, and Fisher, were calculated using the cal_αdiv() function to assess microbial richness and evenness, while β diversity was computed using the cal_βdiv() function. Beta diversity was further analysed using Bray-Curtis dissimilarity and visualized via Principal Coordinates Analysis with multidimensional scaling (PCoA). Statistical tests were used to assess the differences in α diversity indices between treatments and *Neosartorya* spp. isolates with statistical significance set at $p < 0.05$. Initially, the assumptions of ANOVA, including dataset normality and homoscedasticity of variance, were verified using the Shapiro–Wilk and Levene tests, respectively. Subsequently, data were analysed using ANOVA when assumptions were met to compare α diversity indices across treatments and isolates. For cases where normality was preserved but the assumption of homogeneity of variance was violated,

the Welch ANOVA was applied to account for unequal variances across groups. Taxonomic composition was determined at multiple levels using `cal_abund()`, and relative abundance data were saved; subsequently, boxplots were generated to visualize the relative abundance of the top 10 taxa across different taxonomic levels, ranging from phylum to genus, for both fungal and bacterial communities, with variation depending on the treatment used. Venn diagrams were created using `trans_venn$new()` to visualize the sequence ratio between treatment types or *Neosartorya* spp. contaminants, both for the average over 7 days and for each day separately, providing insights into shared and unique taxa between experimental groups. Biolog™ microassay results were visualised as heatmaps using `pheatmap` v. 2.6-8⁶⁷. Additionally, the results were presented as substrate richness (R) using `ggpubr` v. 2.6-8⁶⁸, with pairwise comparisons between treatments performed using the Wilcoxon test, and the significance of these comparisons annotated on the plot. The results were visualized with a facet-wrapped bar plot, showing the treatment effects on R values for each isolate and day separately.

5. References

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892 BPN/PRE/2022/1/00083/U/00001.

893 **7. Ethics declarations**

894 **7.1 Author contributions**

895 Wiktoria Maj: Formal Analysis, Investigation, Methodology, Visualization,
896 Conceptualization, Writing – Original Draft Preparation, Writing – Review & Editing.
897 Giorgia Pertile: Conceptualization, Investigation, Writing – Review & Editing. Jacek Panek:
898 Investigation, Methodology, Writing – Review & Editing. Magdalena Frąc: Funding
899 Acquisition, Project Administration, Resources, Conceptualization, Methodology,
900 Supervision, Writing – Review & Editing.

901 **7.2 Competing interests**

902 The authors declare no competing interests.

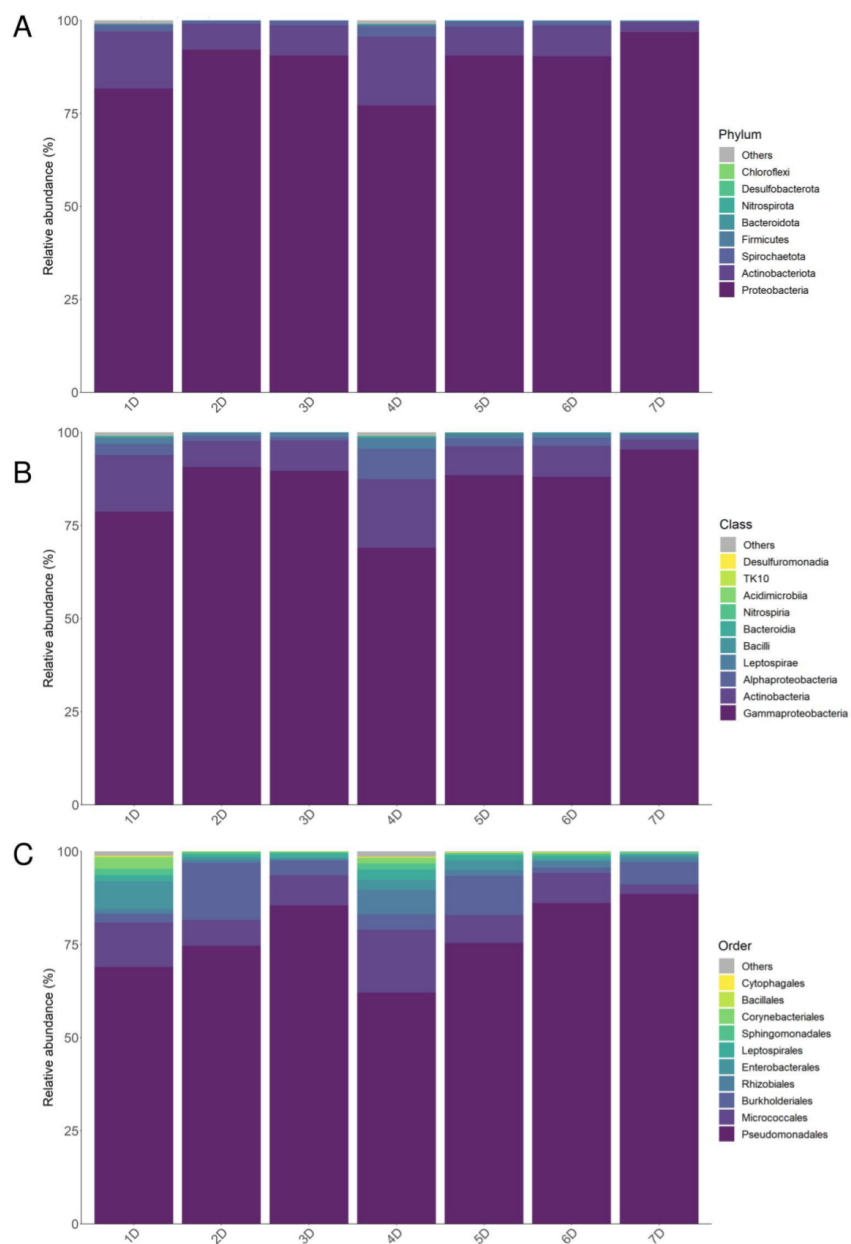
7.5.2 Materiały dodatkowe

- 1 **Supplementary Information: Antifungal Agents Alter Strawberry Microbial and**
2 **Metabolic Profiles During *Neosartorya* spp. (*Aspergillus* spp.) Contamination**

3 Wiktoria Maj¹, Giorgia Pertile¹, Jacek Panek¹, Magdalena Frąc^{1*}

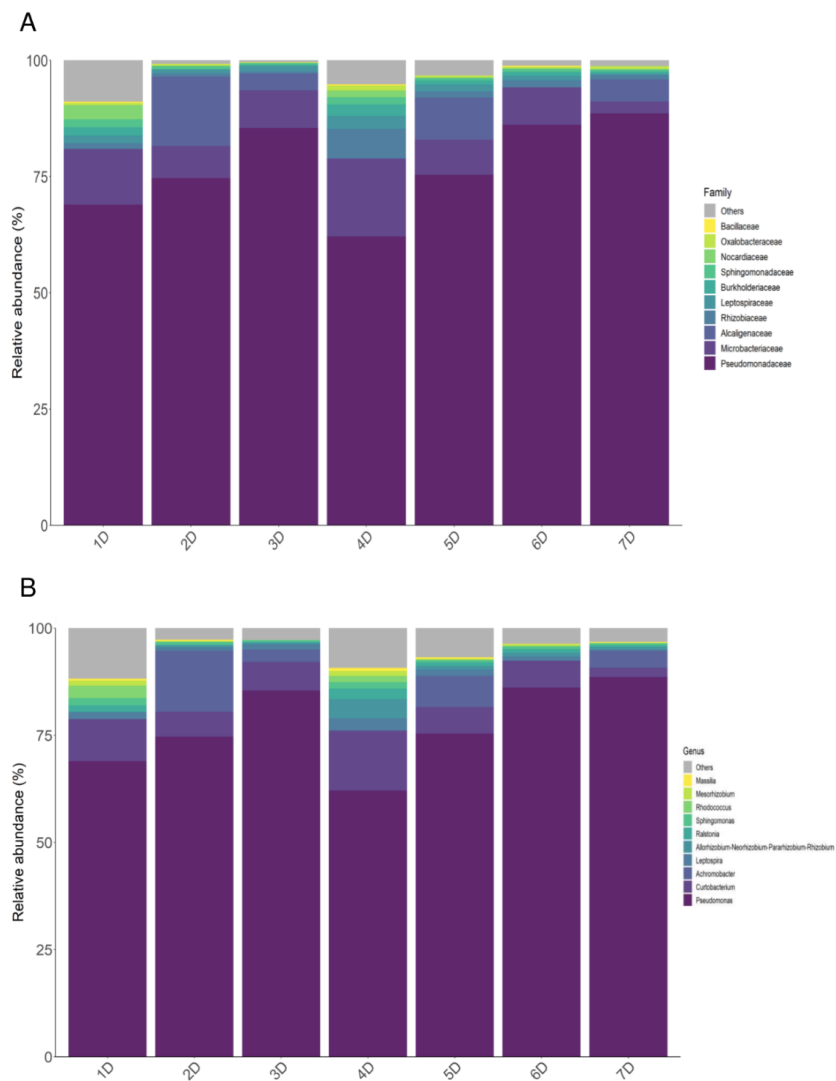
4 ¹Institute of Agrophysics, Polish Academy of Sciences, Doświadczalna 4, 20-290 Lublin,
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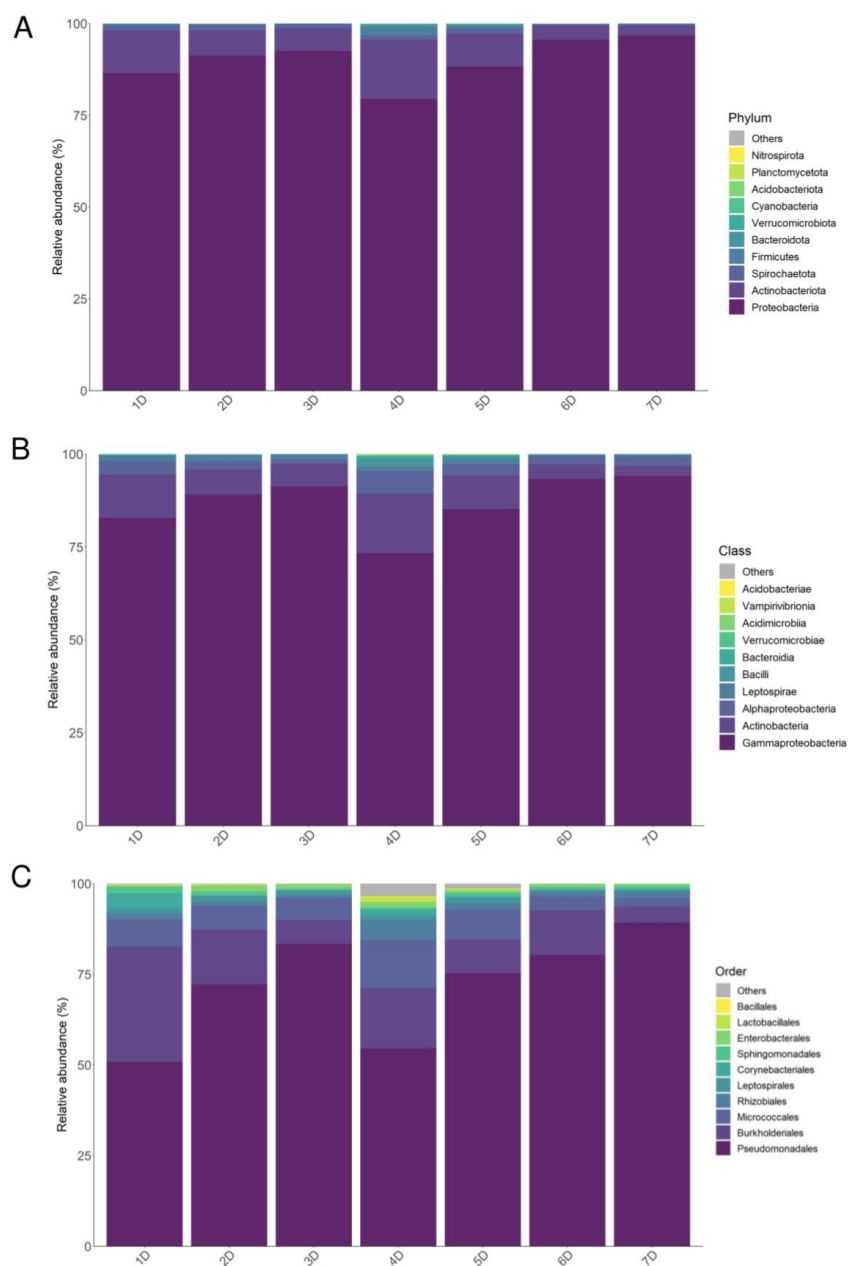
8 Fig. S1. Relative abundance of top 10 bacterial phyla (A), classes (B) and orders (C) in each
9 day of the experiment for samples treated with water.



10

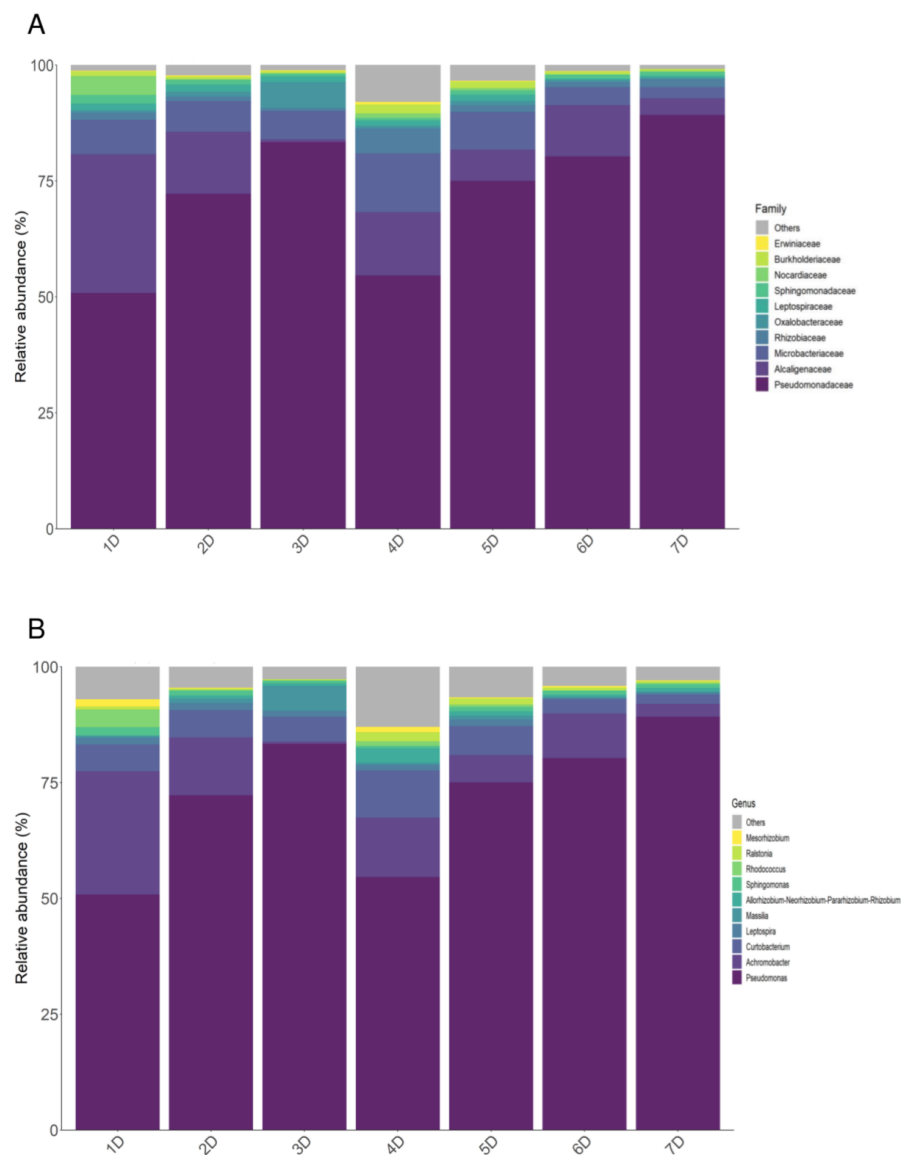
11 Fig.S2. Relative abundance of top 10 bacterial families (A) and genera (B) in each experiment
 12 day for control samples treated with water.

13



14

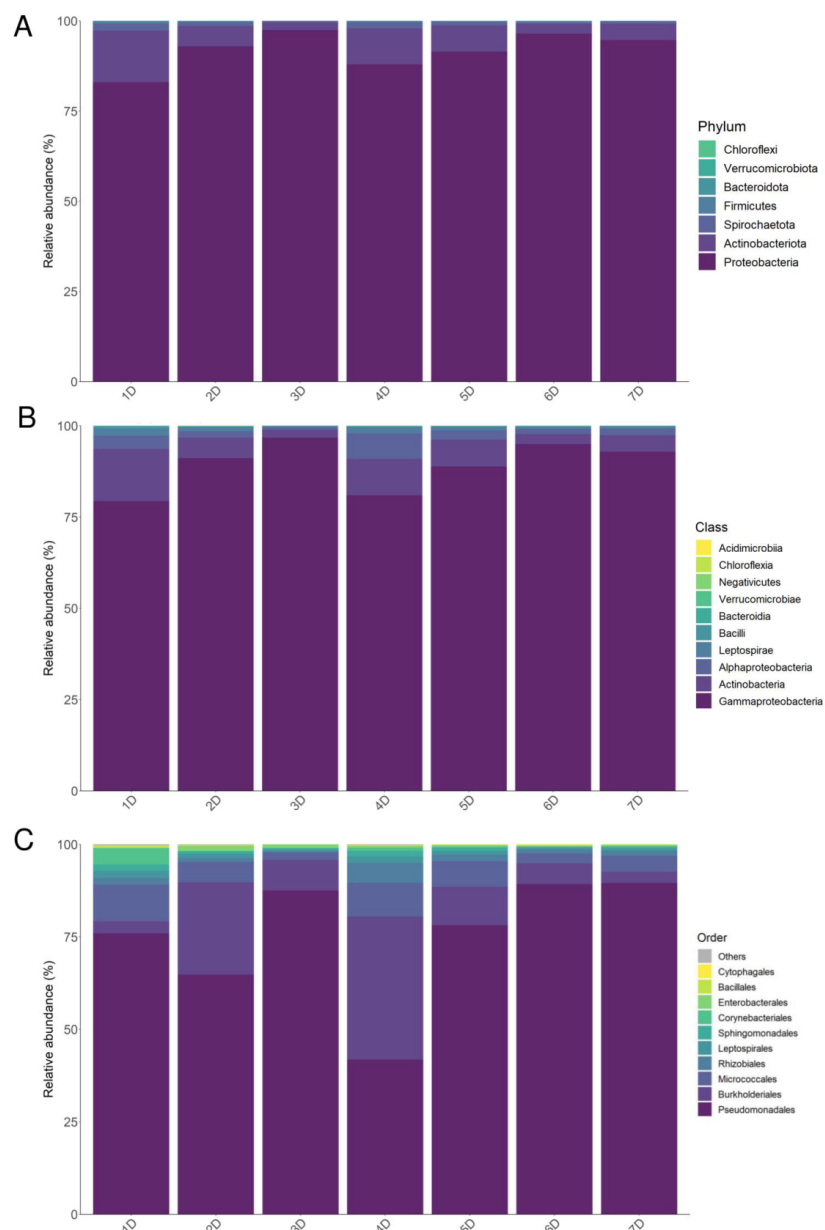
15 Fig. S3. Relative abundance of top 10 bacterial phyla (A), classes (B) and orders (C) in each
 16 day of the experiment for samples treated with natural extract.



17

18 Fig. S4. Relative abundance of top 10 bacterial families (A) and genera (B) in each day of the

19 experiment for samples treated with natural extract.



20

21 Fig. S5. Relative abundance of top 10 bacterial phyla (A), classes (B) and orders (C) in each

22 day of the experiment for samples treated with preservative.

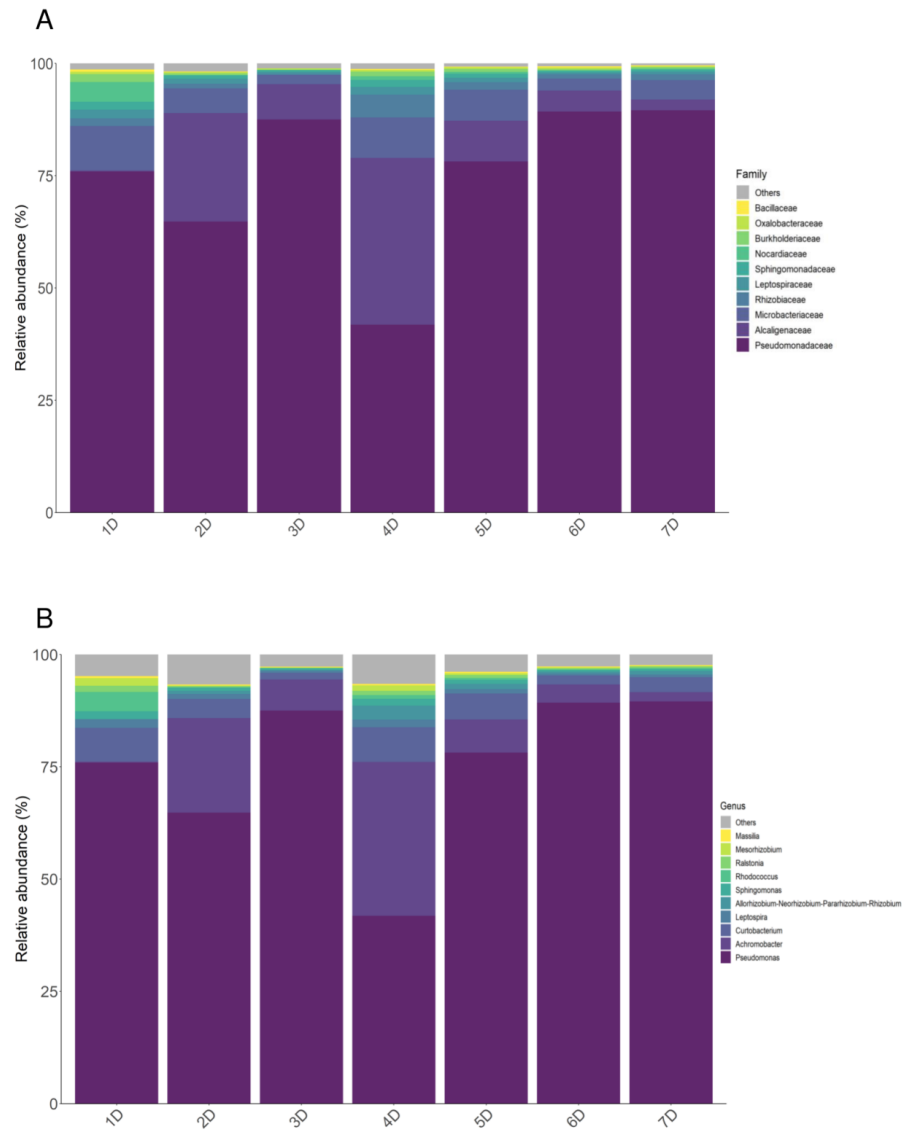
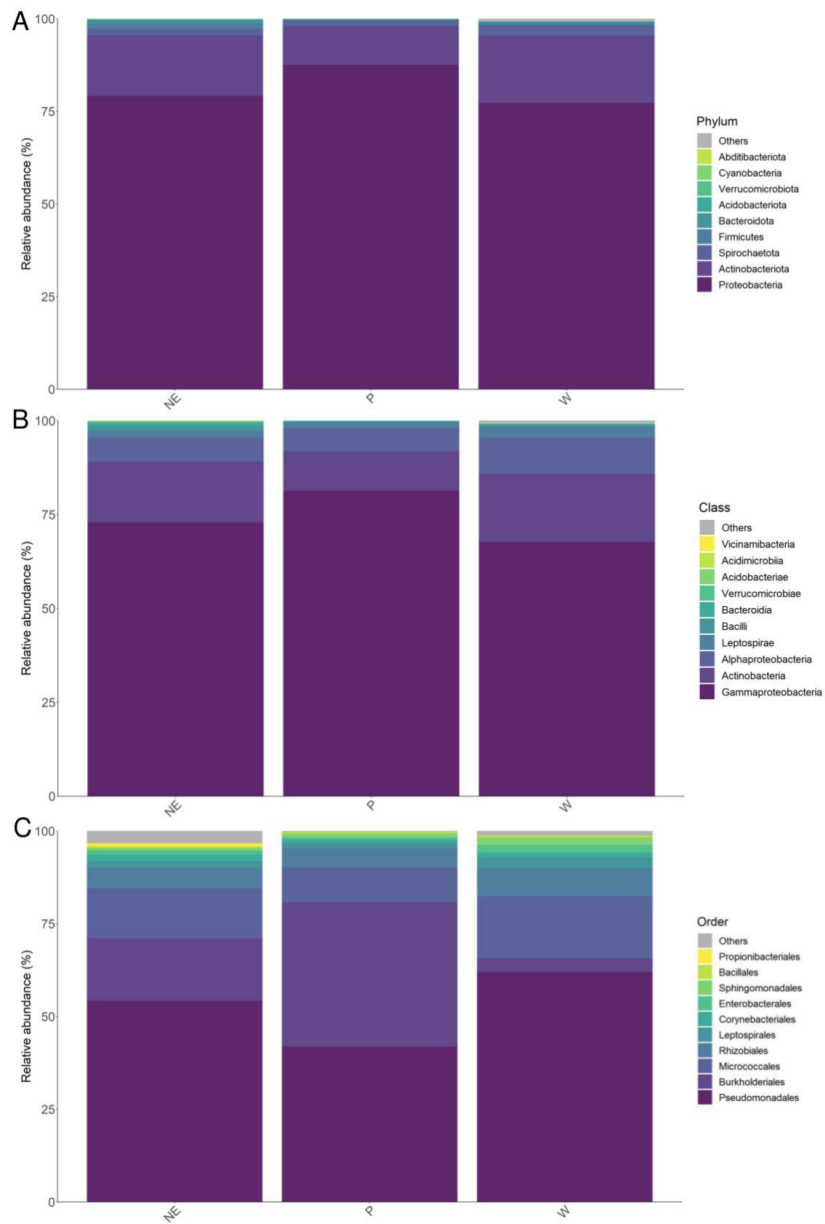
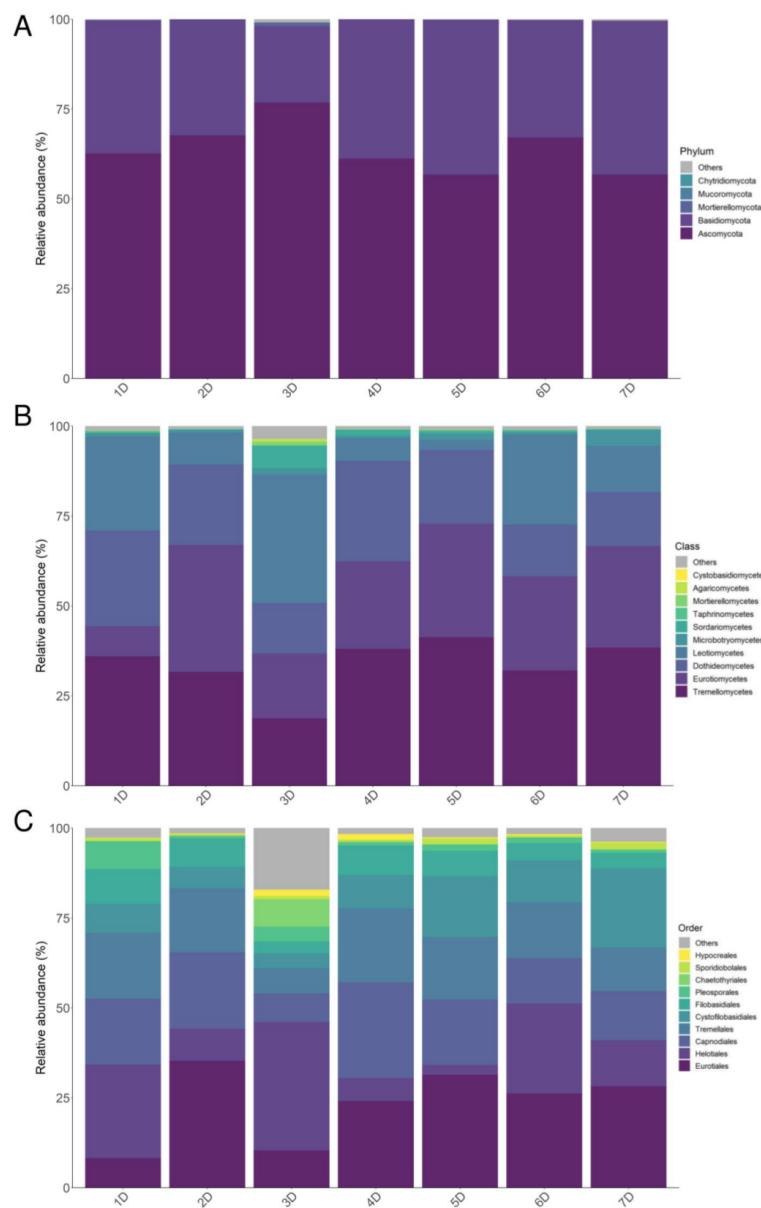


Fig. S6. Relative abundance of top 10 bacterial families (A) and genera (B) in each day of the experiment for samples treated with preservative.



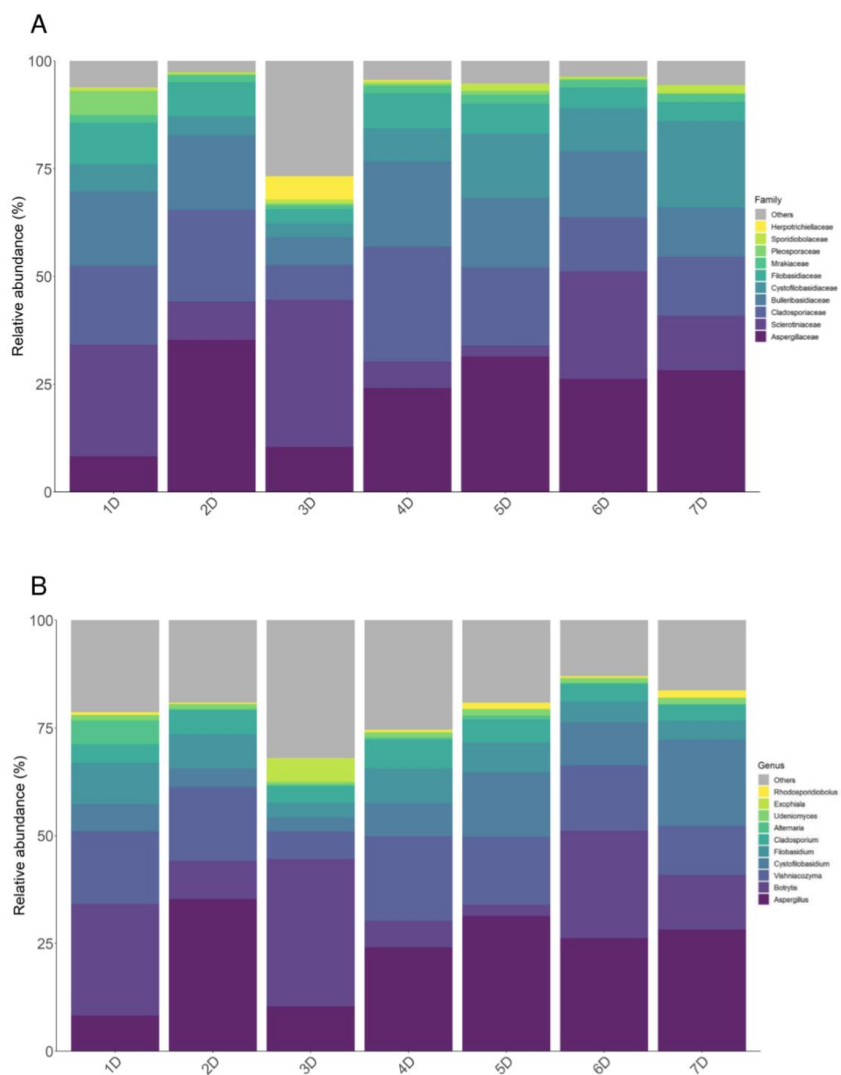
27

28 Fig. S7. Relative abundance of top 10 bacterial phyla (A), classes (B) and orders (C) during day
 29 4 of the experiment for each treatment: natural extract (NE), preservative (P), water control
 30 (W).



31

32 Fig. S8. Relative abundance of top 10 fungal phyla (A), classes (B) and orders (C) in each day
33 of the experiment for samples treated with water.

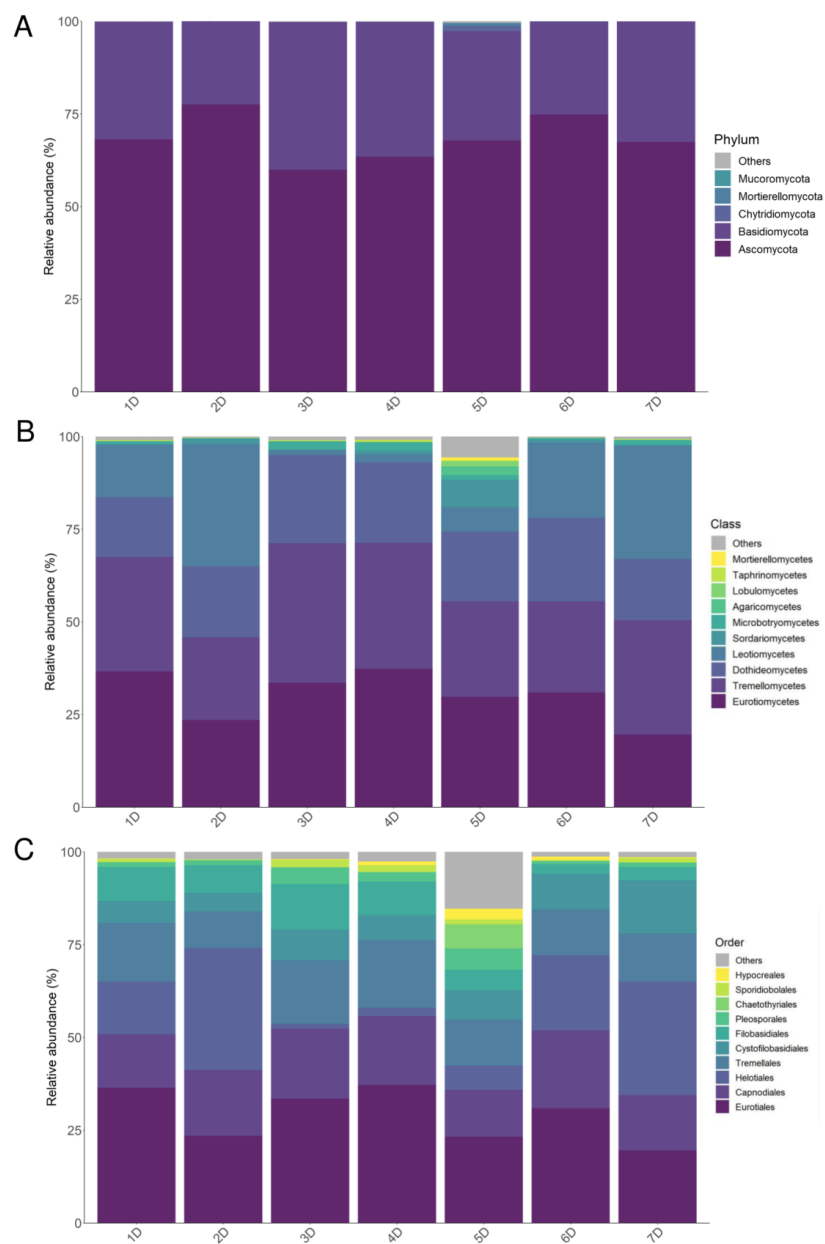


34

35 Fig. S9. Relative abundance of top 10 fungal families (A) and genera (B) in each experiment

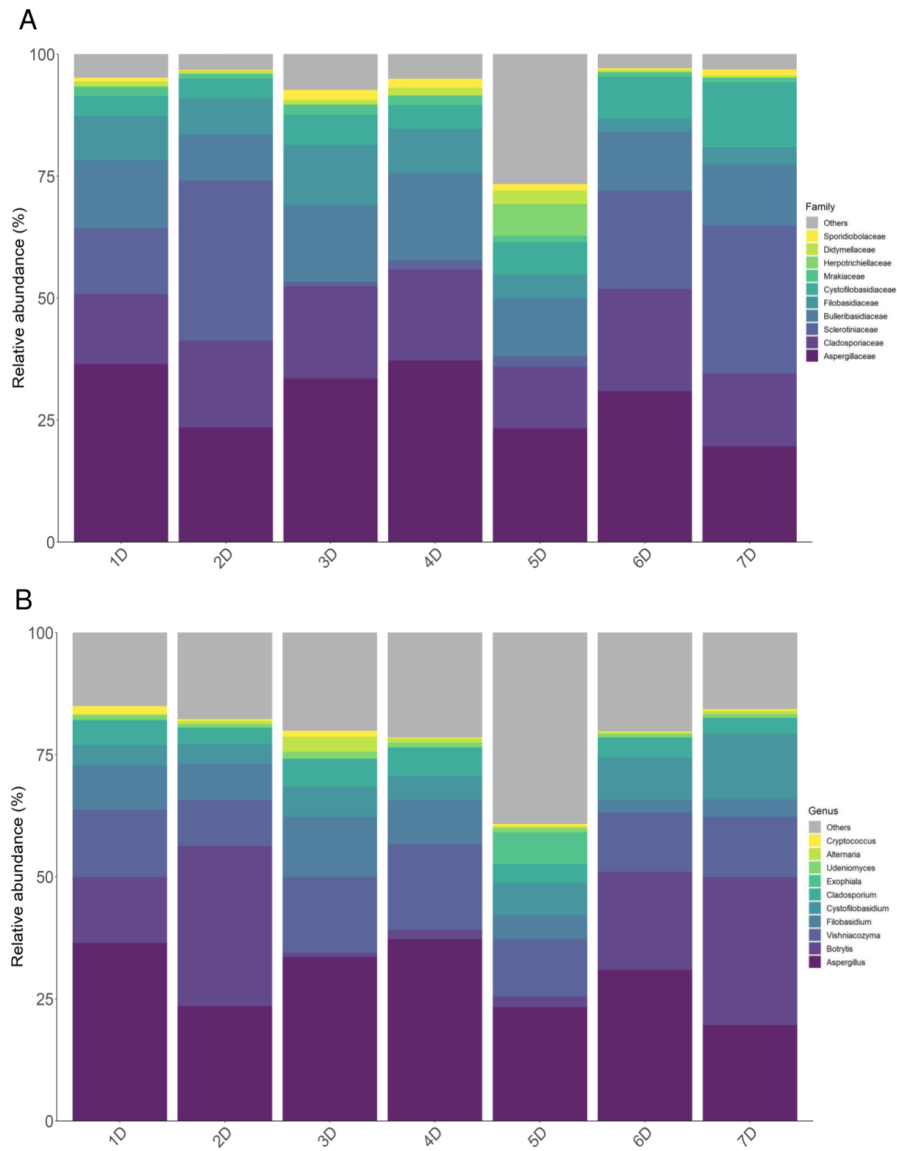
36 day for samples treated with water.

37



38

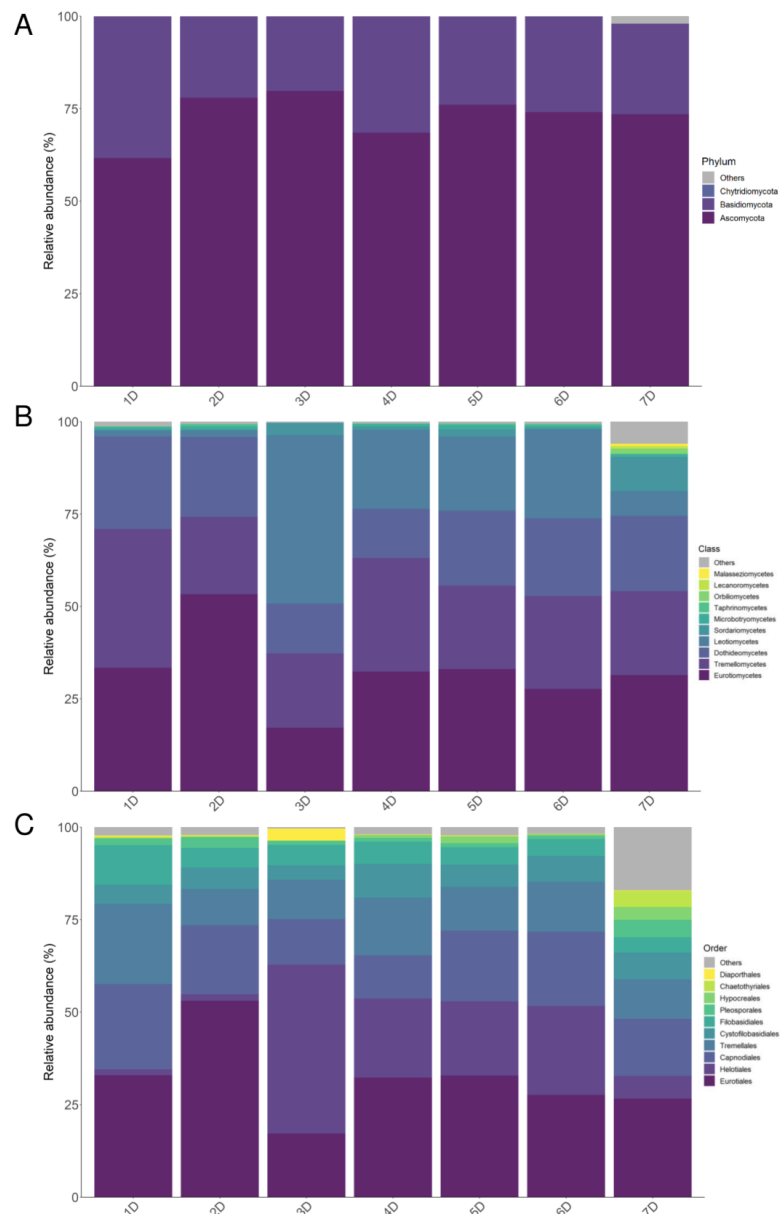
39 Fig. S10. Relative abundance of top 10 fungal phyla (A), classes (B) and orders (C) in each day
40 of the experiment for samples treated with natural extract.



41

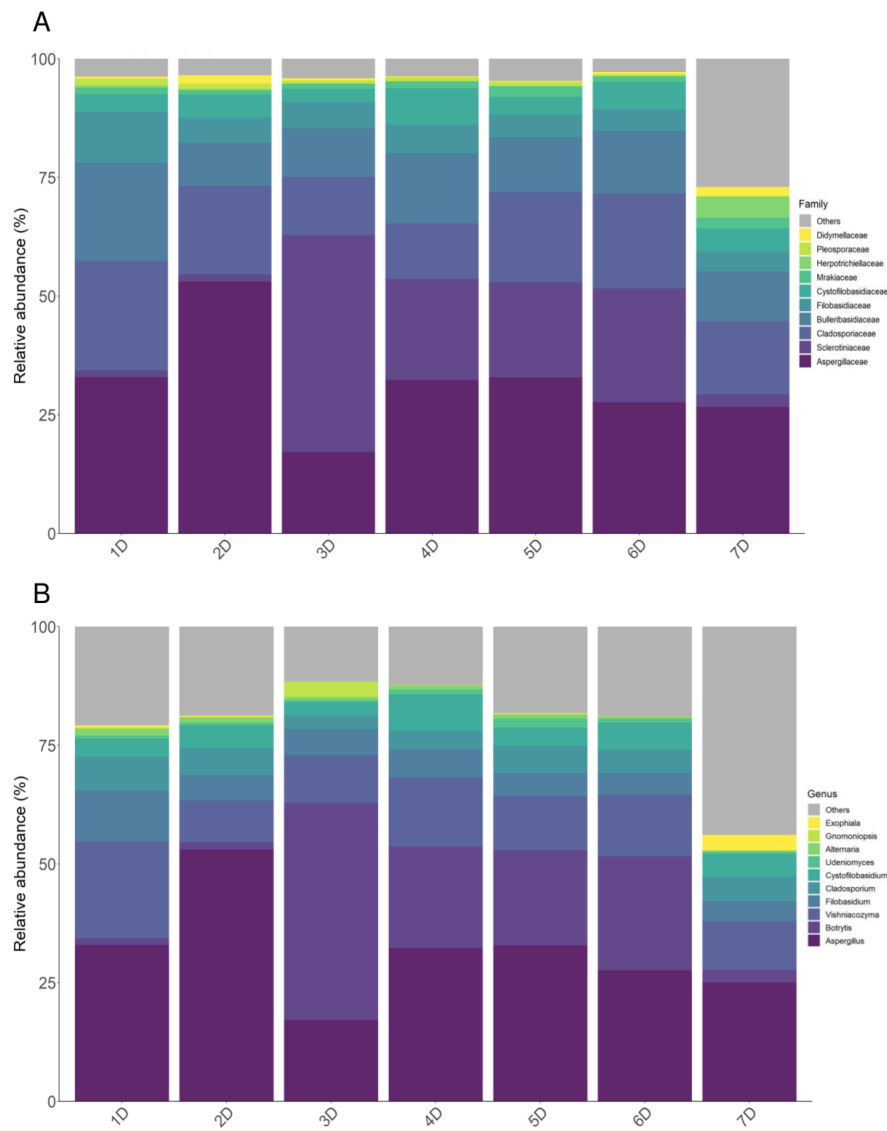
42 Fig. S11. Relative abundance of top 10 fungal families (A) and genera (B) in each day of the

43 experiment for samples treated with natural extract.



44

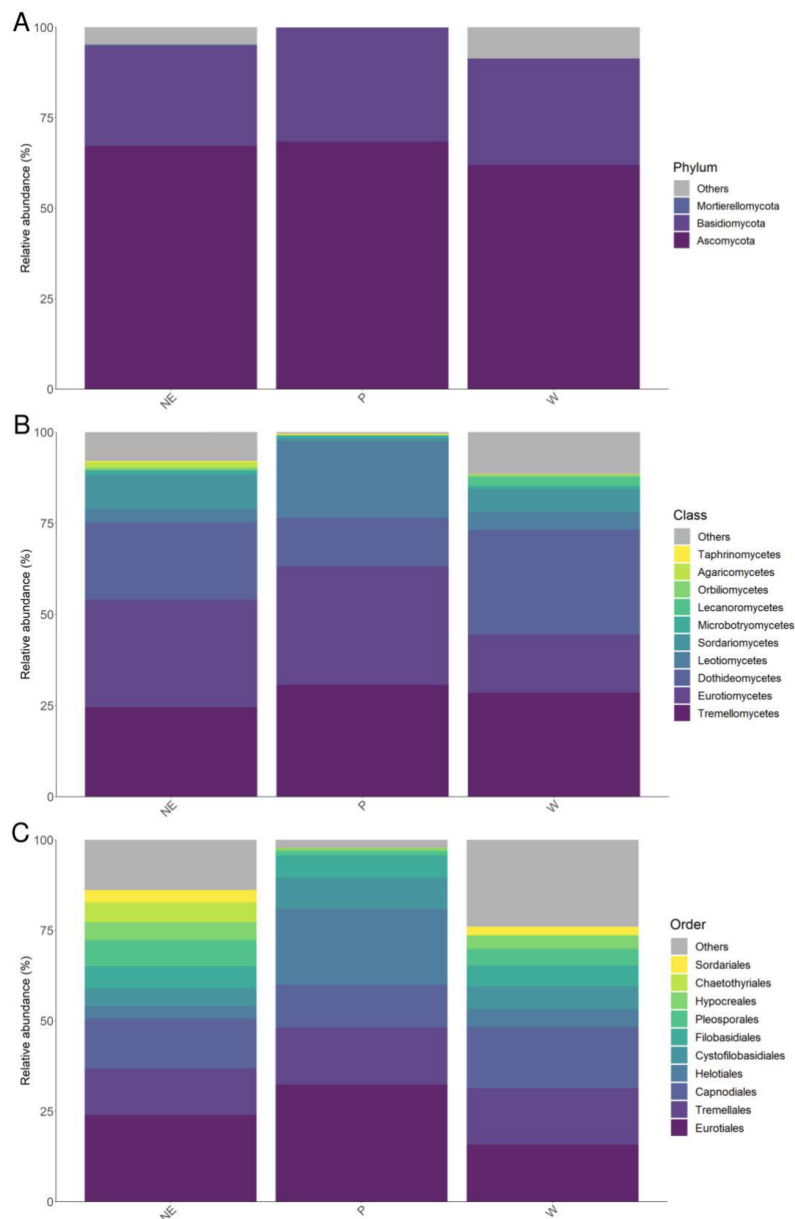
45 Fig. S12. Relative abundance of top 10 fungal phyla (A), classes (B) and orders (C) in each day
 46 of the experiment for samples treated with preservative.



47

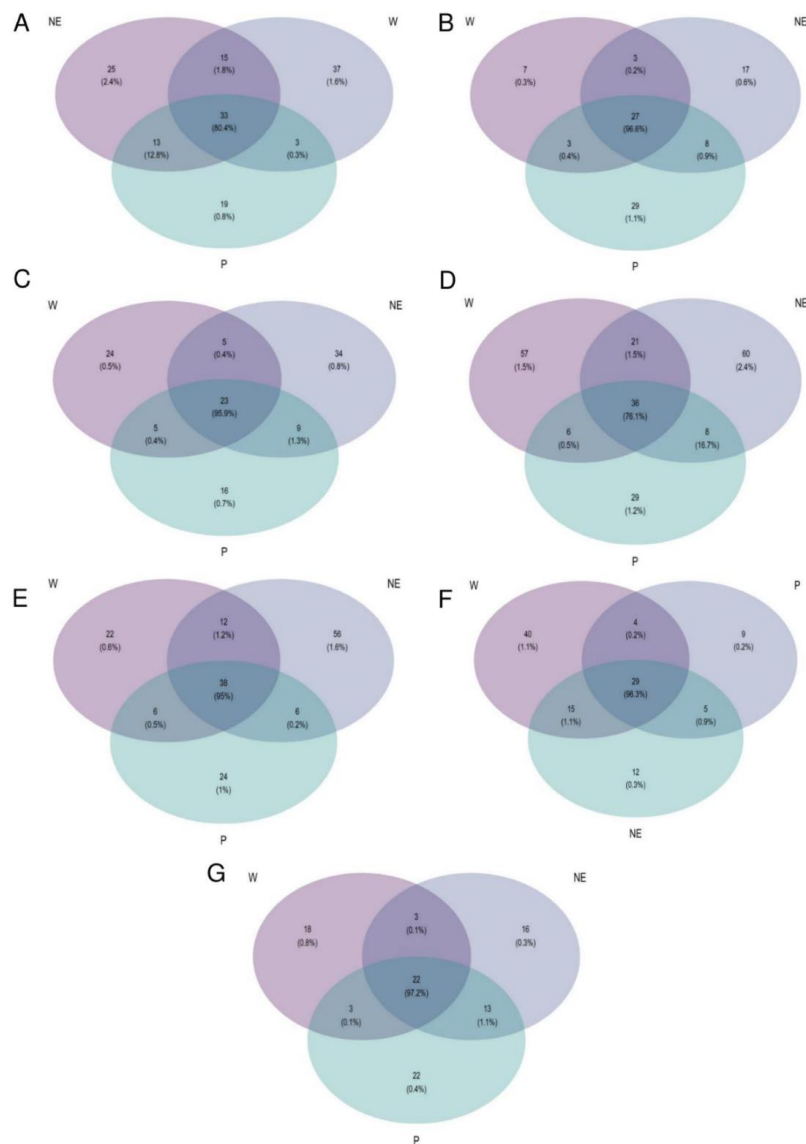
48 Fig. S13. Relative abundance of top 10 fungal families (A) and genera (B) in each day of the
 49 experiment for samples treated with preservative.

50



51

52 Fig. S14. Relative abundance of top 10 fungal phyla (A), classes (B) and orders (C) during day
 53 4 of the experiment for each treatment: natural extract (NE), preservative (P), water control
 54 (W).



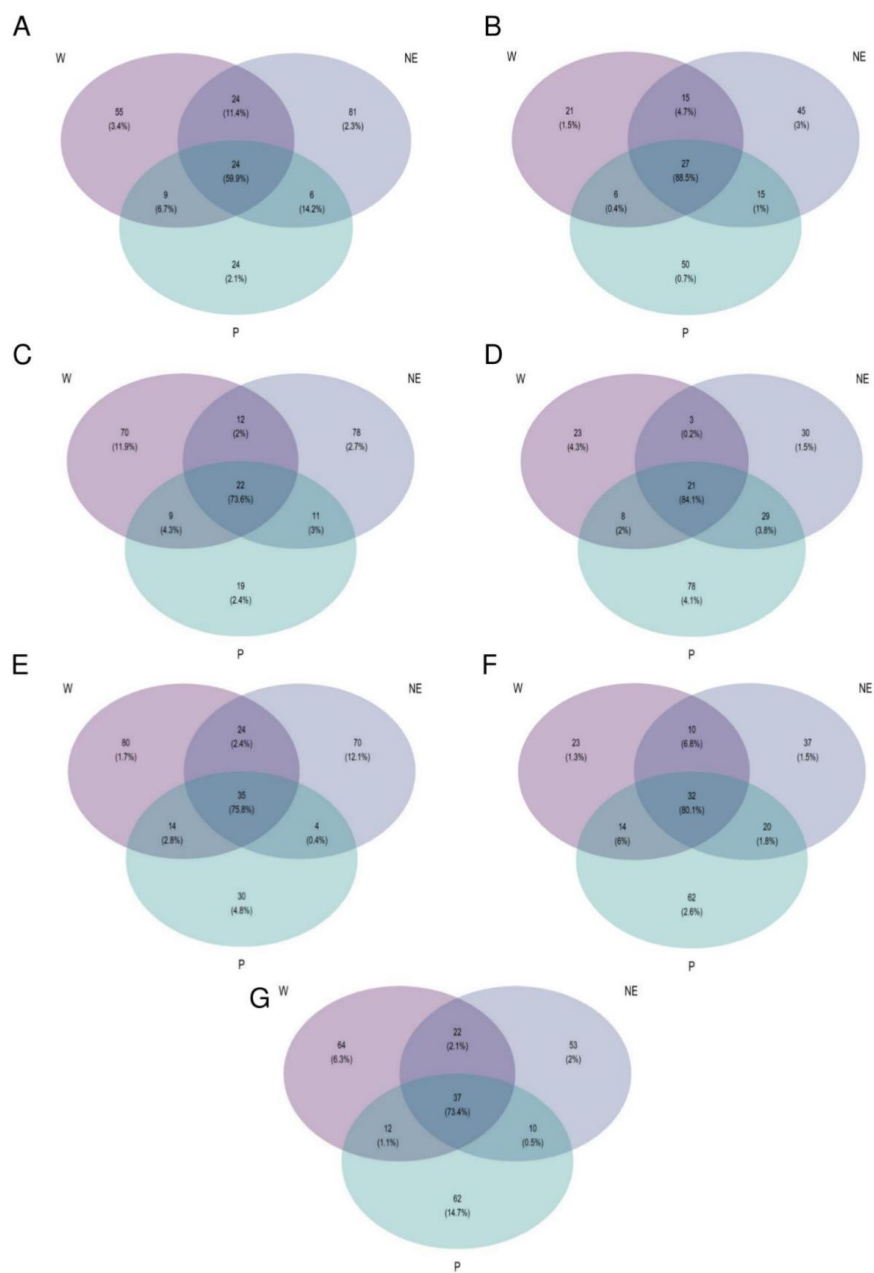
55

56 Fig. S15. Venn diagrams representing core taxa for bacterial communities for samples
 57 contaminated with isolates G127/14, G130/14 and G132/14, compared between each treatment
 58 type: water, natural extract, preservative. Data presented separately for each day of the

59 experiment: 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F), 7 (G). The integer is the ASV number, while

60 the percentage data is the total sequence number.

61

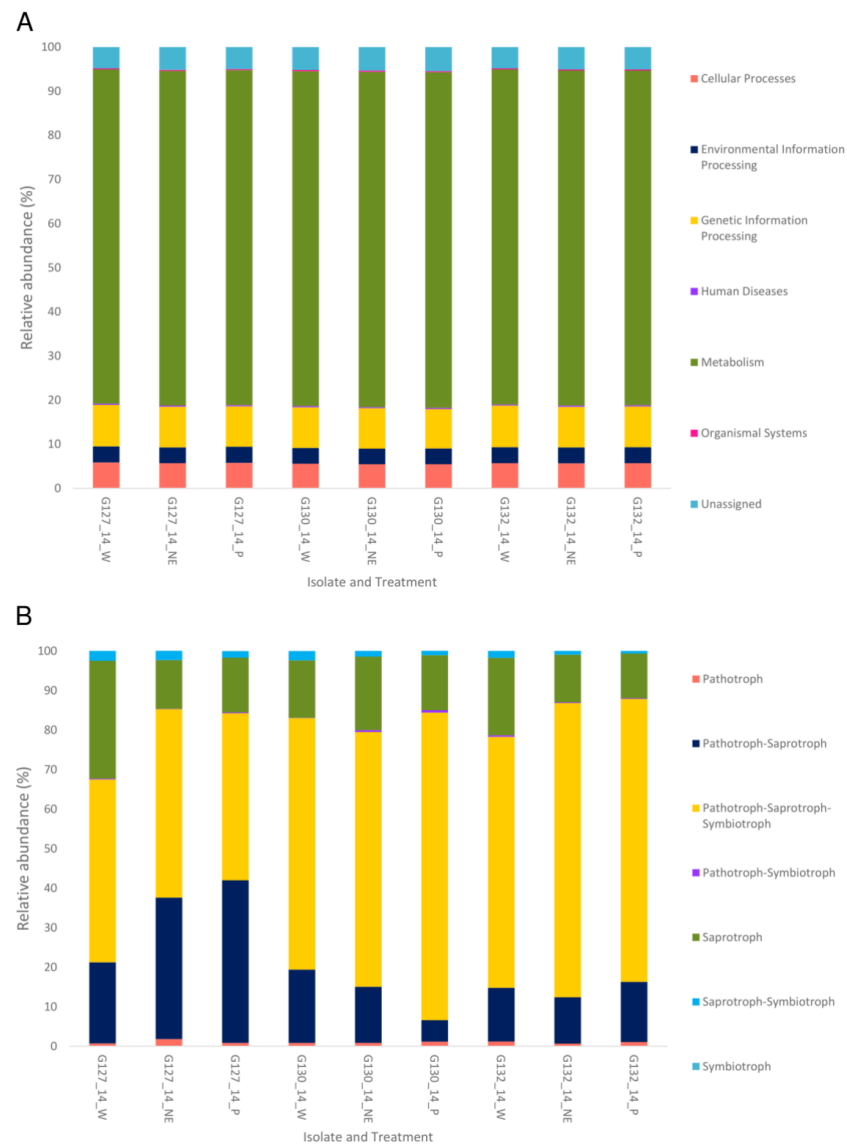


62

63 Fig. S16. Venn diagrams representing core taxa for fungal communities for samples
 64 contaminated with isolates G127/14, G130/14 and G132/14, compared between each treatment

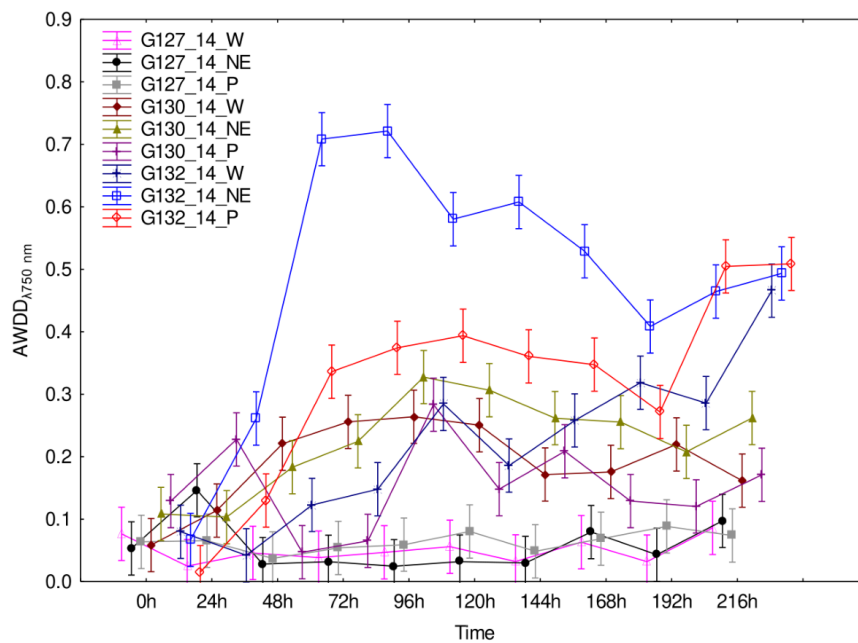
65 type: water, natural extract, preservative. Data presented separately for each day of the
66 experiment: 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F), 7 (G). The integer is the ASV number, while
67 the percentage data is the total sequence number.

68



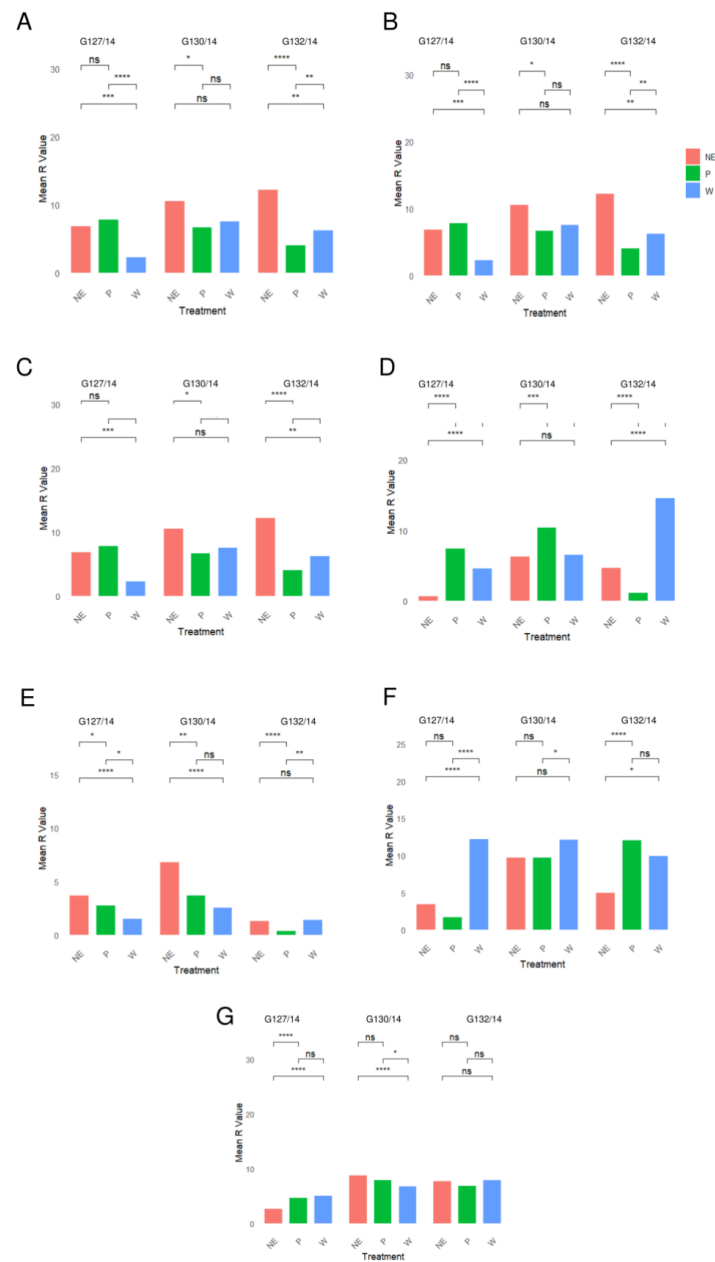
71 Fig. S17. PICRUSt prediction of the bacterial profile with the relative abundance of the main
72 KEGG classes (A). Fungal profile inferred by FUNGuild with the relative abundance of the

73 fungal trophic modes (B). Data presented for each combination of isolate contaminant
74 (G127/14, G130/14, G132/14) and treatment (W – water, NE – natural extract, P – preservative)
75 individually.
76



77

78 Fig. S18. Biomass production on the Biolog™ EcoPlate during all experiment hours for
 79 homogenate samples after 7 days of refrigeration. Samples contaminated with *Neosartorya* spp.
 80 isolates (G127/14, G130/14, G132/14) and cultured in the presence of either natural extract
 81 (NE), preservative (P), or water (W).



89

90 Fig. S20. Comparison of Richness (R) values by isolate (G127/14, G130/14, G132/14) and
 91 treatment (W – water, NE – natural extract, P – preservative). Data presented for each day

8. Oświadczenia współautorów



Lublin, 08.06.2025

mgr inż. Wiktoria Maj
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

OŚWIADCZENIE

Niniejszym oświadczam, że w niżej wymienionych pracach inicjatywa podjętych badań jest moim wkładem intelektualnym, a mój udział obejmował:

P.1: Maj, W., Pertile, G., Frąc, M. 2023. *Soil-Borne Neosartorya spp.: A Heat-Resistant Fungal Threat to Horticulture and Food Production—An Important Component of the Root-Associated Microbial Community*. International Journal of Molecular Sciences, 24(2), 1543. <https://doi.org/10.3390/ijms24021543>

- współudział w opracowaniu koncepcji manuskryptu,
- szczegółową analizę danych literaturowych,
- przygotowanie rysunków oraz tabel do manuskryptu,
- przygotowanie manuskryptu,
- korektę manuskryptu.

P.2: Maj, W., Pertile, G., Różalska, S. Skic, K., Frąc, M. 2024. *Comprehensive antifungal investigation of natural plant extracts against Neosartorya spp. (Aspergillus spp.) of agriculturally significant microbiological contaminants and shaping their metabolic profile*. Sci Rep 14, 8399. <https://doi.org/10.1038/s41598-024-58791-4>

- współudział w opracowaniu koncepcji badań,
- szczegółową analizę danych literaturowych,
- współudział w projektowaniu i przeprowadzaniu eksperymentów, w tym identyfikacji za pomocą sekwencjonowania Sangerowskiego, badań inhibicji metodą dyfuzyjno-krażkową i mikropłytkową (MT2 Biolog™), oceny profilu metabolicznego metodą mikropłytkową (FF Biolog™), obserwacji mikroskopowych technikami SEM i metodą konfokalną fluorescencyjną,
- analizę statystyczną i interpretację wyników badań,
- współudział w opracowaniu koncepcji manuskryptu,
- przygotowanie rysunków oraz tabel,
- przygotowanie manuskryptu,
- korektę manuskryptu.

Wiktoria Maj

P.3: Maj, W., Pertile, G., Różalska, S., Skic, K., Frąc, M. 2024. *The role of food preservatives in shaping metabolic profile and chemical sensitivity of fungi-an extensive study on crucial mycological food contaminants from the genus Neosartorya (Aspergillus spp.)*. Food Chemistry, 453, 139583. <https://doi.org/10.1016/j.foodchem.2024.139583>

- współudział w opracowaniu koncepcji badań,
- szczegółową analizę danych literaturowych,
- współudział w projektowaniu i przeprowadzaniu eksperymentów, w tym badań inhibicji metodą dyfuzyjno-krążkową i mikropłytkową (MT2 Biolog™), oceny wrażliwości chemicznej metodą mikropłytkową (PM Biolog™), oceny profilu metabolicznego metodą mikropłytkową (FF Biolog™), oceny aktywności metabolicznej metodą fluorescencyjną, obserwacji mikroskopowych technikami SEM i metodą konfokalną fluorescencyjną,
- analizę statystyczną i interpretację wyników badań,
- współudział w opracowaniu koncepcji manuskryptu,
- przygotowanie rysunków oraz tabel,
- przygotowanie manuskryptu,
- korektę manuskryptu.

Witold Maj

Lublin, 08.05.2025

mgr inż. Wiktoria Maj
Zakład Badań Systemu Gleba-Roślina
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OŚWIADCZENIE

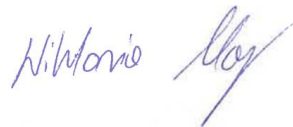
Niniejszym oświadczam, że w niżej wymienionych manuskryptach artykułów naukowych inicjatywa podjętych badań jest moim wkładem intelektualnym, a mój udział obejmował:

P4: Maj, W., Pertile, G., Panek, J., Różalska, S., Skic, K., Kozub-Pędrak, A., Wielgosz, A., Fornal, E., Frąc, M., *Genome-Wide Analysis, Metabolomic Profiling and Microscopic Evaluation of Neosartorya spp. (Aspergillus spp.) Diversity and Adaptations to Natural and Synthetic Antimicrobials*.

- współudział w opracowaniu koncepcji badań,
- szczegółową analizę danych literaturowych,
- współudział w projektowaniu i przeprowadzaniu eksperymentów, w tym sekwencjonowania następnej generacji WGS, charakterystyki metabolomu metodą LC-QTOF-MS, obserwacji mikroskopowych technikami SEM i metodą konfokalną fluorescencyjną,
- analizę statystyczną i interpretację wyników badań,
- współudział w opracowaniu koncepcji manuskryptu,
- przygotowanie rysunków oraz tabel,
- przygotowanie manuskryptu,
- korektę manuskryptu.

P5: Maj, W., Pertile, G., Panek, J., Frąc, M., *Antifungal Agents Alter Strawberry Microbial and Metabolic Profiles During Neosartorya spp. (Aspergillus spp.) Contamination*.

- współudział w opracowaniu koncepcji badań,
- szczegółową analizę danych literaturowych,
- współudział w projektowaniu i przeprowadzaniu eksperymentów, w tym analiz metataksonomicznych z wykorzystaniem platformy Illumina MiSeq oraz oceny aktywności metabolicznej techniką mikropłytkową EcoPlates Biolog™,
- analizę statystyczną i interpretację wyników badań,
- współudział w opracowaniu koncepcji manuskryptu,
- przygotowanie rysunków oraz tabel,
- przygotowanie manuskryptu,
- korektę manuskryptu.



Lublin, 03.06.25 r.

Miejscowość, data

PhD Giorgia Pertile

Zakład Badań Systemu Gleba-Roślina

Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN

ul. Doświadczalna 4, 20-290 Lublin

OŚWIADCZENIE

Niniejszym oświadczam, że w poniższych pracach inicjatywa podjętych badań jest wkładem intelektualnym mgr inż. Wiktorii Maj.

P.1: Maj, W., Pertile, G., Frąc, M. 2023. *Soil-Borne Neosartorya spp.: A Heat-Resistant Fungal Threat to Horticulture and Food Production—An Important Component of the Root-Associated Microbial Community*. International Journal of Molecular Sciences, 24(2), 1543. <https://doi.org/10.3390/ijms24021543>

P.2: Maj, W., Pertile, G., Różalska, S. Skic, K., Frąc, M. 2024. *Comprehensive antifungal investigation of natural plant extracts against Neosartorya spp. (Aspergillus spp.) of agriculturally significant microbiological contaminants and shaping their metabolic profile*. Sci Rep 14, 8399. <https://doi.org/10.1038/s41598-024-58791-4>

P.3: Maj, W., Pertile, G., Różalska, S., Skic, K., Frąc, M. 2024. *The role of food preservatives in shaping metabolic profile and chemical sensitivity of fungi-an extensive study on crucial mycological food contaminants from the genus Neosartorya (Aspergillus spp.)*. Food Chemistry, 453, 139583. <https://doi.org/10.1016/j.foodchem.2024.139583>

Mój wkład w powyższe prace polegał na:

- współudziale w opracowaniu koncepcji badań,
- współudziale w identyfikacji izolatów metodą sekwencjonowania Sangerowskiego,
- współudziale w pomiarach oraz analizie statystycznej testów inhibicji metodą dyfuzyjno-krążkową,
- współudziale w opracowaniu koncepcji manuskryptów,
- korekcie manuskryptów.

Jednocześnie wyrażam zgodę, aby prace zostały wykorzystane w rozprawie doktorskiej mgr inż. Wiktorii Maj.



Lublin, 03.06.25 r.

Miejscowość, data

PhD Giorgia Pertile

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Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN

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OŚWIADCZENIE

Niniejszym oświadczam, że w poniższych manuskryptach artykułów naukowych inicjatywa podjętych badań jest wkładem intelektualnym mgr inż. Wiktorii Maj.

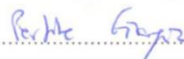
P4: Maj, W., Pertile, G., Panek, J., Różalska, S., Skic, K., Kozub-Pędrak, A., Wielgosz, A., Fornal, E., Frąc, M., *Genome-Wide Analysis, Metabolomic Profiling and Microscopic Evaluation of Neosartorya spp. (Aspergillus spp.) Diversity and Adaptations to Natural and Synthetic Antimicrobials.*

P5: Maj, W., Pertile, G., Panek, J., Frąc, M., *Antifungal Agents Alter Strawberry Microbial and Metabolic Profiles During Neosartorya spp. (Aspergillus spp.) Contamination.*

Mój wkład w powyższe prace polegał na:

- współudziale w opracowaniu koncepcji badań,
- współudziale w opracowaniu metodyki badań,
- współudziale w opracowaniu koncepcji manuskryptów,
- korekcie manuskryptów.

Jednocześnie wyrażam zgodę, aby manuskrypty artykułów naukowych zostały wykorzystane w rozprawie doktorskiej mgr inż. Wiktorii Maj.

.....

Lublin 08.06.25

Miejscowość, data

dr inż. Jacek Panek

Zakład Badań Systemu Gleba-Roślina

Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN

ul. Doświadczalna 4, 20-290 Lublin

OŚWIADCZENIE

Niniejszym oświadczam, że w poniższych manuskryptach artykułów naukowych inicjatywa podjętych badań jest wkładem intelektualnym mgr inż. Wiktorii Maj.

P4: Maj, W., Pertile, G., Panek, J., Różalska, S., Skic, K., Kozub-Pędrak, A., Wielgosz, A., Fornal, E., Frąc, M., *Genome-Wide Analysis, Metabolomic Profiling and Microscopic Evaluation of Neosartorya spp. (Aspergillus spp.) Diversity and Adaptations to Natural and Synthetic Antimicrobials.*

P5: Maj, W., Pertile, G., Panek, J., Frąc, M., *Antifungal Agents Alter Strawberry Microbial and Metabolic Profiles During Neosartorya spp. (Aspergillus spp.) Contamination.*

Mój wkład w powyższe prace polegał na:

- współudział w projektowaniu i przeprowadzaniu analiz sekwencjonowania całogenomowego (WGS) oraz analiz metataksonomicznych oraz złożeniu uzyskanych wyników do bazy danych GenBank,
- obróbce bioinformatycznej danych przed ich analizą,
- korekcie manuskryptów.

Jednocześnie wyrażam zgodę, aby manuskrypty artykułów naukowych zostały wykorzystane w rozprawie doktorskiej mgr inż. Wiktorii Maj.



Lublin, 03.06.2025r.

Miejscowość, data

prof. dr hab. Magdalena Frąc
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

OŚWIADCZENIE

Niniejszym oświadczam, że w poniższych pracach inicjatywa podjętych badań jest wkładem intelektualnym mgr inż. Wiktorii Maj.

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P.2: Maj, W., Pertile, G., Różalska, S., Skic, K., Frąc, M. 2024. *Comprehensive antifungal investigation of natural plant extracts against Neosartorya spp. (Aspergillus spp.) of agriculturally significant microbiological contaminants and shaping their metabolic profile*. Sci Rep 14, 8399. <https://doi.org/10.1038/s41598-024-58791-4>

P.3: Maj, W., Pertile, G., Różalska, S., Skic, K., Frąc, M. 2024. *The role of food preservatives in shaping metabolic profile and chemical sensitivity of fungi—an extensive study on crucial mycological food contaminants from the genus Neosartorya (Aspergillus spp.)*. Food Chemistry, 453, 139583. <https://doi.org/10.1016/j.foodchem.2024.139583>

Mój wkład w powyższe prace polegał na:

- określeniu koncepcji, problematyki i zakresu pracy,
- pozyskaniu finansowania,
- współudziale w opracowaniu koncepcji badań,
- współudziale w zaplanowaniu wszystkich eksperymentów i opracowaniu metodyki badań,
- zapewnieniu materiałów i aparatury umożliwiających realizację badań,
- kierowaniu przebiegiem badań zgodnie z założeniami projektu,
- współudziale w opracowaniu koncepcji manuskryptów i nadzorowaniu ich przygotowania,
- recenzji i korekcie manuskryptów,
- udzielaniu odpowiedzi na recenzje.

Jednocześnie wyrażam zgodę, aby prace zostały wykorzystane w rozprawie doktorskiej mgr inż. Wiktorii Maj.

Magdalena Frąc

Lublin, 03.06.2025r.

Miejscowość, data

prof. dr hab. Magdalena Frąc
Zakład Badań Systemu Gleba-Roślina
Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN
ul. Doświadczalna 4, 20-290 Lublin

OŚWIADCZENIE

Niniejszym oświadczam, że w poniższych manuskryptach artykułów naukowych inicjatywa podjętych badań jest wkładem intelektualnym mgr inż. Wiktorii Maj.

P4: Maj, W., Pertile, G., Panek, J., Różalska, S., Skic, K., Kozub-Pędrak, A., Wielgosz, A., Fornal, E., Frąc, M., *Genome-Wide Analysis, Metabolomic Profiling and Microscopic Evaluation of Neosartorya spp. (Aspergillus spp.) Diversity and Adaptations to Natural and Synthetic Antimicrobials.*

P5: Maj, W., Pertile, G., Panek, J., Frąc, M., *Antifungal Agents Alter Strawberry Microbial and Metabolic Profiles During Neosartorya spp. (Aspergillus spp.) Contamination.*

Mój wkład w powyższe prace polegał na:

- określeniu koncepcji, problematyki i zakresu pracy,
- pozyskaniu finansowania,
- współudziale w opracowaniu koncepcji badań,
- współudziale w zaplanowaniu wszystkich eksperymentów i opracowaniu metodyki badań,
- zapewnieniu materiałów i aparatury umożliwiających realizację badań,
- kierowaniu przebiegiem badań zgodnie z założeniami projektu,
- współudziale w opracowaniu koncepcji manuskryptów i nadzorowaniu ich przygotowania,
- nadzorowaniu przygotowania manuskryptów,
- recenzji i korekcie manuskryptów.

Jednocześnie wyrażam zgodę, aby manuskrypty artykułów naukowych zostały wykorzystane w rozprawie doktorskiej mgr inż. Wiktorii Maj.

Magdalena Frąc

Lublin... 09.05.2025

Miejscowość, data

dr Kamil Skic

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OŚWIADCZENIE

Niniejszym oświadczam, że w poniższych pracach inicjatywa podjętych badań jest wkładem intelektualnym mgr inż. Wiktorii Maj.

P.2: Maj, W., Pertile, G., Różalska, S., Skic, K., Frąc, M. 2024. *Comprehensive antifungal investigation of natural plant extracts against Neosartorya spp. (Aspergillus spp.) of agriculturally significant microbiological contaminants and shaping their metabolic profile*. Sci Rep 14, 8399. <https://doi.org/10.1038/s41598-024-58791-4>

P.3: Maj, W., Pertile, G., Różalska, S., Skic, K., Frąc, M. 2024. *The role of food preservatives in shaping metabolic profile and chemical sensitivity of fungi-an extensive study on crucial mycological food contaminants from the genus Neosartorya (Aspergillus spp.)*. Food Chemistry, 453, 139583. <https://doi.org/10.1016/j.foodchem.2024.139583>

Mój wkład w powyższe prace polegał na:

- wkładzie w opracowanie metodologii badań z wykorzystaniem skaningowej mikroskopii elektronowej (SEM),
- współudziale w przeprowadzeniu obserwacji mikroskopowych techniką SEM,
- korekcie manuskryptów.

Jednocześnie wyrażam zgodę, aby prace zostały wykorzystane w rozprawie doktorskiej mgr inż. Wiktorii Maj.

Kamil Skic

Lublin 08.05.2025

Miejscowość, data

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Instytut Agrofizyki im. Bohdana Dobrzańskiego PAN

ul. Doświadczalna 4, 20-290 Lublin

OŚWIADCZENIE

Niniejszym oświadczam, że w poniższym manuskrypcie artykułu naukowego inicjatywa podjętych badań jest wkładem intelektualnym mgr inż. Wiktorii Maj.

P4: Maj, W., Pertile, G., Panek, J., Różalska, S., Skic, K., Kozub-Pędrak, A., Wielgosz, A., Fornal, E., Frąc, M., *Genome-Wide Analysis, Metabolomic Profiling and Microscopic Evaluation of Neosartorya spp. (Aspergillus spp.) Diversity and Adaptations to Natural and Synthetic Antimicrobials*

Mój wkład w powyższe prace polegał na:

- wkładzie w opracowanie metodologii badań z wykorzystaniem skaningowej mikroskopii elektronowej (SEM),
- współudziale w przeprowadzeniu obserwacji mikroskopowych techniką SEM,
- korekcie manuskryptów.

Jednocześnie wyrażam zgodę, aby manuskrypt artykułu naukowego został wykorzystany w rozprawie doktorskiej mgr inż. Wiktorii Maj.

Kamil Skic

Lublin, 06.06.2025

Miejscowość, data

mgr Anna Kozub-Pędrak
Zakład Bioanalityki,
Uniwersytet Medyczny w Lublinie,
ul. Jaczewskiego 8b, 20-090 Lublin

OŚWIADCZENIE

Niniejszym oświadczam, że w poniższym manuskrypcie artykułu naukowego inicjatywa podjętych badań jest wkładem intelektualnym mgr inż. Wiktorii Maj.

P4: Maj, W., Pertile, G., Panek, J., Różalska, S., Skic, K., Kozub-Pędrak, A., Wielgosz, A., Fornal, E., Frąc, M., *Genome-Wide Analysis, Metabolomic Profiling and Microscopic Evaluation of Neosartorya spp. (Aspergillus spp.) Diversity and Adaptations to Natural and Synthetic Antimicrobials*.

Mój wkład w powyższą pracę polegał na:

- współudziale w opracowaniu metodyki i przeprowadzeniu oceny metabolomu metodą LC-QTOF-MS,
- korekcie manuskryptu.

Jednocześnie wyrażam zgodę, aby manuskrypt artykułu naukowego został wykorzystany w rozprawie doktorskiej mgr inż. Wiktorii Maj.

Anna Kozub-Pędrak

Lublin, 03.06.2025

Miejscowość, data

mgr Alicja Wielgosz
Zakład Bioanalitik,
Uniwersytet Medyczny w Lublinie,
ul. Jaczewskiego 8b, 20-090 Lublin

OŚWIADCZENIE

Niniejszym oświadczam, że w poniższym manuskrypcie artykułu naukowego inicjatywa podjętych badań jest wkładem intelektualnym mgr inż. Wiktorii Maj.

P4: Maj, W., Pertile, G., Panek, J., Różalska, S., Skic, K., Kozub-Pędrak, A., Wielgosz, A., Fornal, E., Frąc, M., *Genome-Wide Analysis, Metabolomic Profiling and Microscopic Evaluation of Neosartorya spp. (Aspergillus spp.) Diversity and Adaptations to Natural and Synthetic Antimicrobials.*

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- współudziale w opracowaniu metodyki i przeprowadzeniu oceny metabolomu metodą LC-QTOF-MS,
- korekcie manuskryptu.

Jednocześnie wyrażam zgodę, aby manuskrypt artykułu naukowego został wykorzystany w rozprawie doktorskiej mgr inż. Wiktorii Maj.

Alicja Wielgosz



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Londyn, 2 czerwca 2025

OŚWIADCZENIE

Niniejszym oświadczam, że w poniższym manuskrypcie artykułu naukowego inicjatywa podjętych badań jest wkładem intelektualnym mgr inż. Wiktorii Maj.

P4: Maj, W., Pertile, G., Panek, J., Różalska, S., Skic, K., Kozub-Pędrak, A., Wielgosz, A., Fornal, E., Frąc, M., *Genome-Wide Analysis, Metabolomic Profiling and Microscopic Evaluation of Neosartorya spp. (Aspergillus spp.) Diversity and Adaptations to Natural and Synthetic Antimicrobials.*

Mój wkład w powyższą pracę polegał na:

- współudziale w opracowaniu koncepcji badań,
- zapewnieniu materiałów i aparatury umożliwiających realizację badań,
- współudziale w opracowaniu metodyki i przeprowadzeniu oceny metabolomu metodą LC-QTOF-MS,
- korekcie manuskryptu.

Jednocześnie wyrażam zgodę, aby manuskrypt artykułu naukowego został wykorzystany w rozprawie doktorskiej mgr inż. Wiktorii Maj.

prof. dr hab. Emilia Fornal
/dokument podpisany elektronicznie/

Podpisano przez/ Signed by:
Emilia
Fornal
Data/ Date: 02.06.2025 15:42
mSzafir

Łódź, 3.06.2025

Miejscowość, data

dr hab. Sylwia Różalska, prof. uczelni
Katedra Mikrobiologii Przemysłowej i Biotechnologii,
Wydział Biologii i Ochrony Środowiska,
Uniwersytet Łódzki,
ul. Banacha 12/16, 90-237 Łódź

OŚWIADCZENIE

Niniejszym oświadczam, że w poniższych pracach inicjatywa podjętych badań jest wkładem intelektualnym mgr inż. Wiktorii Maj.

P.2: Maj, W., Pertile, G., Różalska, S., Skic, K., Frąć, M. 2024. *Comprehensive antifungal investigation of natural plant extracts against Neosartorya spp. (Aspergillus spp.) of agriculturally significant microbiological contaminants and shaping their metabolic profile*. Sci Rep 14, 8399. <https://doi.org/10.1038/s41598-024-58791-4>

P.3: Maj, W., Pertile, G., Różalska, S., Skic, K., Frąć, M. 2024. *The role of food preservatives in shaping metabolic profile and chemical sensitivity of fungi-an extensive study on crucial mycological food contaminants from the genus Neosartorya (Aspergillus spp.)*. Food Chemistry, 453, 139583. <https://doi.org/10.1016/j.foodchem.2024.139583>

Mój wkład w powyższe prace polegał na:

- opracowaniu metody fluorescencyjnej do oceny aktywności metabolicznej,
- opracowaniu metodologii i przeprowadzeniu obserwacji mikroskopowych techniką mikroskopii konfokalnej fluorescencyjnej,
- zapewnieniu materiałów i aparatury umożliwiających realizację badań,
- korekcie manuskryptów.

Jednocześnie wyrażam zgodę, aby prace zostały wykorzystane w rozprawie doktorskiej mgr inż. Wiktorii Maj.

Sylwia Różalska

Łódź, 3.06.2025

Miejscowość, data

dr hab. Sylwia Różalska, prof. uczelni
Katedra Mikrobiologii Przemysłowej i Biotechnologii,
Wydział Biologii i Ochrony Środowiska,
Uniwersytet Łódzki,
ul. Banacha 12/16, 90-237 Łódź

OŚWIADCZENIE

Niniejszym oświadczam, że w poniższym manuskrypcie artykułu naukowego inicjatywa podjętych badań jest wkładem intelektualnym mgr inż. Wiktorii Maj.

P4: Maj, W., Pertile, G., Panek, J., Różalska, S., Skic, K., Kozub-Pędrak, A., Wielgosz, A., Fornal, E., Frąc, M., *Genome-Wide Analysis, Metabolomic Profiling and Microscopic Evaluation of Neosartorya spp. (Aspergillus spp.) Diversity and Adaptations to Natural and Synthetic Antimicrobials.*

Mój wkład w powyższą pracę polegał na:

- opracowaniu metodologii i przeprowadzeniu obserwacji mikroskopowych techniką mikroskopii konfokalnej fluorescencyjnej,
- zapewnieniu materiałów i aparatury umożliwiających realizację badań,
- korekcie manuskryptu.

Jednocześnie wyrażam zgodę, aby manuskrypt artykułu naukowego został wykorzystany w rozprawie doktorskiej mgr inż. Wiktorii Maj.

Sylwia Różalska

9. Życiorys naukowy

ŻYCIORYS NAUKOWY

Dane osobowe:

Imię i nazwisko: Wiktoria Marta Maj
Obywatelstwo: polskie
Data i miejsce urodzenia: 30.12.1996 r., Lublin, Polska

Dane adresowe:

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21-040, Świdnik, Polska
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E-mail: wiktoria.marta.maj@gmail.com

WYKSZTAŁCENIE

2021 – 2025 Uczestnik Interdyscyplinarnej Szkoły Doktorskiej Nauk Rolniczych w dyscyplinie rolnictwo i ogrodnictwo. Jednostka Prowadząca: Instytut Agrofizyki im. Bohdana Dobrzańskiego Polskiej Akademii Nauk.

2021 Tytuł magistra na kierunku Biotechnologia, Uniwersytet Przyrodniczy w Lublinie.

2019 Tytuł inżyniera na kierunku Bioinżynieria, Uniwersytet Przyrodniczy w Lublinie.

PROJEKTY NAUKOWE

10.2021-09.2025 Doktorantka w ramach Projektu Preludium Bis 2 Narodowego Centrum Nauki, 2020/39/O/NZ9/03421, pt. **„Rola właściwości metabolicznych, morfologicznych i genetycznych grzybów *Neosartorya* spp. kształtowaniu ich odporności na związki konserwujące, chemiczne oraz naturalne ekstrakty roślinne”**. Kierownik projektu: prof. dr hab. Magdalena Frąc. Zakład Badań Systemu Gleba-Roślina Instytutu Agrofizyki Polskiej Akademii Nauk w Lublinie.

02.2019-08.2021 Wolontariuszka w Projekcie pt. **„Ochrona czynna aldrowandy pęcherzykowej (*Aldrovanda vesiculosa*) na terenie Lubelszczyzny”**, Wydziału Ogrodnictwa i Architektury Krajobrazu - Zakładu roślin ozdobnych i dendrologii Uniwersytetu Przyrodniczego w Lublinie. Numer POIS.02.04.00-00-0034/18, w ramach Programu Operacyjnego Infrastruktura i Środowisko 2014-2020, działania 2.4. Kierownik projektu: dr inż. Marzena Parzymies.

09.2019-2020 Uczestnik Projektu Ministerstwa Nauki i Szkolnictwa Wyższego **„Mistrzowie Dydaktyki”** w Instytucie Produkcji Ogrodniczej Uniwersytetu Przyrodniczego

w Lublinie – Realizacja zajęć indywidualnych pt. „**Metodologia prowadzenia badań z zakresu uprawy roślin ogrodniczych**” w ramach Działania 4.3 Programu Operacyjnego Wiedza Edukacja Rozwój współfinansowanego ze środków Europejskiego Funduszu Społecznego.

09.2019 Wykonawca projektu pt. „**Mikroorganizmy pomocne w ochronie środowiska**” w ramach XVI Lubelskiego Festiwalu Nauki pt. „Nauka Technika Innowacje”, Katedra Mikrobiologii Środowiskowej Wydziału Agrobioinżynierii Uniwersytetu Przyrodniczego w Lublinie.

STAŻE

2025 **Medical Mycological Research Center of Chiba University**, 03.2025-06.2025, opiekun stażu prof. Takashi Yaguchi, współpraca w ramach projektu Narodowej Agencji Wymiany Akademickiej Preludium Bis 2, BPN/PRE/2022/1/00083/U/00001, Chiba University, 1-8-1 Inohana, 260-8673, Chiba Chuo-ku, Japonia

2023 **Uniwersytet Medyczny w Lublinie**, 08/28.03.2023, 05/11/12/13/18/04.2023- opiekun stażu prof. dr hab. n. chem. Emilia Fornal; współpraca w ramach Projektu Preludium Bis-2, 2020/39/O/NZ9/03421; Zakład Bioanalitiky, Uniwersytet Medyczny w Lublinie, ul. Jaczewskiego 8b (Collegium Pathologicum), 20-090, Lublin

2022 **Uniwersytet Łódzki**, 16-22.10.2022, – opiekun stażu dr hab. Sylwia Różalska, prof. UŁ; współpraca w ramach Projektu Preludium Bis-2, 2020/39/O/NZ9/03421; Katedra Mikrobiologii Przemysłowej i Biotechnologii, Uniwersytet Łódzki, ul. Banacha 12/16, 90-237, Łódź

PRAKTYKI

09.2019 **Laboratorium Biochemiczne** w Zakładzie Mikrostruktury i Mechaniki Biomateriałów Instytutu Agrofizyki Polskiej Akademii Nauk w Lublinie

09.2019 **Klinika Chorób Zakaźnych i Katedra Epizootologii** Wydziału Medycyny Weterynaryjnej Uniwersytetu Przyrodniczego w Lublinie

08.-09.2019 **Katedra Mikrobiologii Środowiskowej** Wydziału Agrobioinżynierii Uniwersytetu Przyrodniczego w Lublinie

07.2019 **Biolive Innovation Sp. z o.o.**

NAGRODY I WYRÓŻNIENIA

2024 III miejsce w konkursie „Młodzi naukowcy w świecie nauki” – postery, organizowanego w ramach 56. Ogólnopolskiej Konferencji Mikrobiologicznej.

2021 Wyróżnienie wystąpienia ustnego „Understanding *Neosartorya* spp. fungi – a threat to fruit production” na IV. Konferencji Doktorantów „Cztery Żywioły – współczesne problemy w naukach o życiu”.

2021 Wyróżnienie pracy magisterskiej, pt. „Charakterystyka peroksydazy syntetyzowanej przez szczep *Trichoderma harzianum* BsIII33 w obecności barwnika antrachinonowego Alizarin Blue Black B”.

2021-2020	Stypendium naukowe prezydenta miasta Lublin z „Miejskiego programu stypendialnego dla studentów i doktorantów”.
2020-2019	Studenckie Stypendium Marszałka Województwa Lubelskiego.
2020-2019	Stypendium rektora Uniwersytetu Przyrodniczego w Lublinie za wybitne osiągnięcia naukowe.
2019	Wyróżnienie pracy inżynierskiej, pt. „Oznaczanie toksyczności wybranych pochodnych antrachinonów”.
2019	II miejsce w sesji referatowej Ochrony Środowiska i Biogospodarki podczas XVI Międzynarodowego Seminarium Studenckich Kół Naukowych “Środowisko - Zwierzę – Produkt”.

POPULARYZACJA NAUKI

2024	Zaangażowanie w prace recepcji konferencji naukowej – 23rd International Workshop for Young Scientists „BioPhys Spring 2024”, 23-24.05.2024, Lublin, Polska.
2023	Zaangażowanie w prace recepcji konferencji naukowej – 14th International Conference on Agrophysics, 11-13.09.2023, Lublin, Polska.
2023	Zaangażowanie w prace sekretariatu konferencji naukowej - Członek sekretariatu konferencji podczas VII Ogólnopolskiego Sympozjum Mikrobiologicznego „Metagenomy Różnych Środowisk”, 20-21.06.2023, Lublin, Polska.
2023	Zaangażowanie w prace komitetu organizacyjnego konferencji naukowej - Członek komitetu organizacyjnego IV edycji Konferencji MycoRiseUp! Youth in Mycology, 20-21.05.2023, Lublin, Polska.
2021-2017	Członek Studenckiego Koła Analityków Środowiska, Katedra Mikrobiologii Środowiskowej. Czynny udział w projektach badawczych Koła, organizacja wydarzeń naukowo-kulturalnych (Dni Otwarte Uczelni, XVI Lubelski festiwal Nauki, Dni Kariery 2019).

WYKAZ OSIĄGNIĘĆ W PRACY NAUKOWO-BADAWCZEJ

A. PUBLIKACJE

Maj, W., Pertile, G., Różalska, S., Skic, K., & Frąc, M. (2024). The role of food preservatives in shaping metabolic profile and chemical sensitivity of fungi - an extensive study on crucial mycological food contaminants from the genus *Neosartorya* (*Aspergillus* spp.). *Food Chemistry*, 453, 139583. <https://doi.org/10.1016/j.foodchem.2024.139583>

Maj, W., Pertile, G., Różalska, S., Skic, K., & Frąc, M. (2024). Comprehensive antifungal investigation of natural plant extracts against *Neosartorya* spp.(*Aspergillus* spp.) of agriculturally significant microbiological contaminants and shaping their metabolic profile. *Scientific Reports*, 14(1), 8399. <https://doi.org/10.1038/s41598-024-58791-4>

Maj, W., Pertile, G., & Frąc, M. (2023). Soil-borne *Neosartorya* spp.: A heat-resistant fungal threat to horticulture and food production—An important component of the root-associated microbial community. *International Journal of Molecular Sciences*, 24(2), 1543. <https://doi.org/10.3390/ijms24021543>

B. UDZIAŁ W KONFERENCJACH NAUKOWYCH

Wystąpienia ustne na międzynarodowych konferencjach naukowych:

- Maj W., Pertile G., Panek J., Frąc M., (2025). Innate resilience mechanisms of *Neosartorya* sp. (*Aspergillus* sp.) – Whole Genome Sequencing Insights, Wystąpienie ustne, 24th International Workshop for Young Scientists „BioPhys Spring 2025, 29-30.05.2025, Prague, Czech Republic (online), Książka Abstraktów Str. 112-113.
- Maj W., Pertile G., Frąc M., (2023). Exploring the Effects of Marigold Extract on the Morphological Alterations of *Neosartorya* spp. (Anamorph: *Aspergillus* spp.) Using Scanning Electron Microscopy, Wystąpienie Ustne, 22nd International Workshop for Young Scientists "Biophys Spring 2023", 15-16.06.2023, Gödöllő, Hungary, Książka Abstraktów Str. 47.
- Maj W., Pertile G., Frąc, M. (2023). Morphological Changes of *Neosartorya* spp. (Anamorph: *Aspergillus* spp.) Observed Under the Influence of a Food Preservative, Wystąpienie Ustne, 4th Edition of Mycorise Up! Youth in Mycology Conference, 20-21.05.2023, Książka Abstraktów Str. 26.
- Maj, W., Pertile, G., Frąc M., (2022). *Neosartorya* spp. - the Hidden Threat to Agriculture and Food Production, Wystąpienie Ustne, 3rd Edition of Mycorise Up! Youth in Mycology Conference, Warsaw 27-29.05.2022, Książka Abstraktów, Str. 13.
- Maj, W., Pertile, G., Frąc, M. (2022). The Effect of Conservative Substances on a Group of Fungi Belonging to the *Neosartorya* Genus. Wystąpienie Ustne, 21st International Workshop for Young Scientists Biophys Spring 2022 (BPS 2022), 30-31.05.2022, Książka Abstraktów, Str. 73.

Wystąpienia ustne na ogólnopolskich konferencjach naukowych:

- Maj W., Pertile G., Panek J., Frąc M., (2024). Analiza genetyczna izolatu *Neosartorya* sp. (*Aspergillus* sp.) w kontekście oporności na abiotyczne czynniki stresowe, Wystąpienie ustne, Warsztaty Dla Młodych Badaczy, 18 - 19.11.2024, Lublin, Książka Abstraktów Str. 25-26.
- Maj W., Pertile G., Frąc M., (2023). Wpływ Wybranych Kationów na Grzyby z Rodzaju *Neosartorya* spp. (Anamorfa: *Aspergillus* spp.), Wystąpienie Ustne, Warsztaty Dla Młodych Badaczy, 30.11-01.12.2023, Książka Abstraktów Str. 20-21.
- Maj W., Pertile G., Frąc M., (2023). Analysis of Chemical Sensitivity of Different Isolates of *Neosartorya* spp. (Anamorph: *Aspergillus* spp.), Wystąpienie Ustne, VI Konferencja Doktorantów "Cztery Żywioty – Współczesne Problemy W naukach O Życiu", 14.12.2023, Książka Abstraktów Str. 55-56.
- Maj, W., Pertile, G., Frąc, M. (2022). Wpływ Naturalnych Ekstraktów Roślinnych na Metabolizm Grzybów Termoopornych z Rodzaju *Neosartorya* spp., Wystąpienie Ustne, Warsztaty Dla Młodych Badaczy, 28-29.11.2022, Książka Abstraktów Str. 19.
- Maj, W., Pertile, G., Frąc, M. (2022). Chemical Substances and Their Effect on *Neosartorya* spp. Metabolism, Wystąpienie Ustne, V Konferencja Doktorantów Współczesne Problemy W Naukach O Życiu Cztery Żywioty, 14.12.2022, Książka Abstraktów Str. 30.
- Maj W., Pertile G., Frąc, M., (2021). Understanding *Neosartorya* spp. Fungi – a Threat to Fruit Production; Wystąpienie Ustne, Konferencja 4 Żywioty – Współczesne Problemy W naukach O Życiu, 14.12.2021, Książka Abstraktów, Str. 23.

Prezentacje posterów na międzynarodowych konferencjach naukowych:

- Maj W., Pertile G., Frąc M., (2024). Study of Antibiotics Influence on *Neosartorya* (*Aspergillus*) spp. via the Biolog™ PM21-25 Microplate Method, Poster, MycoRise Up! Youth in Mycology 5th edition 25–26.05.2024, Kraków, Książka Abstraktów Str. 45.

- Maj W., Pertile G., Frąc M., (2024). *Neosartorya* spp. (Teleomorph of *Aspergillus* spp.) Sensitivity on Chelators Available in Biolog™ PM21-25 Microplates, Poster, 23rd International Workshop for Young Scientists "Biophys Spring 2024", 23-24.05.2024, Lublin, Książka Abstraktów Str. 97.
- Maj W., Pertile G., Frąc M., (2023) Metabolic Changes of *Neosartorya* spp. (Anamorph: *Aspergillus* spp.) in Response to Incubation With Sodium Metabisulfite, Poster, 14th International Conference on Agrophysics, Lublin, Poland, 11-13.09.2023, Książka Abstraktów Str. 127.
- Maj W., Pertile G., Frąc M., (2021). Relevance and Biodiversity of *Neosartorya* spp. Fungi in Fruits Production, Poster, 13th International Conference on Agrophysics: Agriculture in Changing Climate, 15-16.11.2021, Książka Abstraktów, Str. 145.

Prezentacje posterów na ogólnopolskich konferencjach naukowych:

- Maj W., Pertile G., Frąc M., (2024). Chemical sensitivity of *Neosartorya* spp. (teleomorph of *Aspergillus* spp.) – the influence of organic compounds on fungal metabolism, Poster, IV Ogólnopolska Konferencja Polskiego Towarzystwa Mykologicznego, 20-22.09.2024, Lublin, Książka Abstraktów Str. 112-113.
- Maj W., Pertile G., Frąc M., (2024). Mikromacierze Biolog™ PM21-25 jako skuteczne narzędzie do oceny odpowiedzi *Neosartorya* spp. (teleomorfa *Aspergillus* spp.) na związki azotowe, Poster, 56. Ogólnopolska Konferencja Mikrobiologiczna 11-13.09.2024, Fojutowo, Książka Abstraktów Str. 68.
- Maj W., Pertile G., Frąc M., (2024). Wrażliwość *Neosartorya* spp. (teleomorf *Aspergillus* spp.) na związki o funkcjach membranowych dostępnych na mikropłytkach PM21-25 Biolog™, Poster, VI Konferencja Naukowa Bioróżnorodność środowiska glebowego, 05-06.09.2024, Puławy, Książka Abstraktów Str. 43.
- Maj W., Pertile G., Frąc M., (2023) Assessing the Effect of *Calendula officinalis* on *Neosartorya* spp. Mycelium Viability, Poster, 55 Jubileuszowa Konferencja Mikrobiologiczna, Puławy, 14-15.09.2023, Książka Abstraktów Str. 75.
- Maj W., Pertile G., Frąc M., (2023). Changes in the Metabolic Activity of Fungi From *Neosartorya* Genus (Anamorph: *Aspergillus*) Pre-Cultured on a Medium With Marigold Extract, Poster, VII Narodowe Sympozjum Naukowe Metagenomy Różnych Środowisk, 20-21.06.2023, Lublin, Książka Abstraktów Str. 159.
- Maj, W., Pertile, G., Frąc, M. (2022). Comparison of Antimicrobial Effects Caused By Plant Extracts, Brix/Ph Combinations, and Preservatives on *Aspergillus* spp. (Teleomorph *Neosartorya* spp.), Poster, Konferencja Z Okazji 10-Lecia Polskiego Towarzystwa Mykologicznego, 24-28.09.2022, Książka Abstraktów Str. 89.
- Maj, W., Pertile, G., Frąc, M. (2022). Acidity And Sweetness: Properties of Food and Their Importance in Control of a Spoilage Agent, *Aspergillus* spp. (Teleomorph: *Neosartorya* spp.), Poster, XXIX Zjazd Polskiego Towarzystwa Mikrobiologów, 15–17.09.2022, Warszawa, Książka Abstraktów Str. 58.
- Maj, W., Pertile, G., Frąc, M. (2022). Badanie Skuteczności Wybranych Ekstraktów Roślinnych Wobec Grzybów z Rodzaju *Neosartorya* z Wykorzystaniem Mikropłytek MT2 Biolog™ - Intensywność Oddechowa oraz Przyrost Biomasy, Poster, "Nowoczesne Spojrzenie na Fitopatologię" Polskiego Towarzystwa Fitopatologicznego Poznań, 7-8.09.2022, Książka Abstraktów Str. 166.
- Maj, W., Pertile, G., Frąc, M. (2022). Porównanie Wrażliwości Zidentyfikowanych Genetycznie Grzybów z Rodzaju *Neosartorya* na Wybrane Ekstrakty Roślinne, Poster, VI. Ogólnopolskie Sympozjum Mikrobiologiczne "Metagenomy Różnych Środowisk" Puławy, 23-24.06.2022, Książka Abstraktów, Str.45.

Uczestnictwo bierne:

- Annual Meeting of the Mycological Society of Japan & The Joined Symposia of Mycological Society of Japan (MSJ), Mycological Society of America (MSA) and the Korean Society of Mycology (KSM), uczestnictwo bierne, 16-18.05.2025, Chiba, Japonia.

SZKOLENIA I CERTYFIKATY

- Biegłość językowa potwierdzona certyfikatem Certificate in Advanced English, Grade A, British Council.
- Data Analytics with R, NobleProg.
- Bioinformatics Training Course, GreenFinch Research.
- Certyfikat z obsługi programu AutoCAD, Autodesk Authorized Training Center.
- Szkolenie „Narzędzia bibliometryczne - Web of Science, Incites”, Clarivate Analytics.
- Szkolenie „Oxford Academic Journals”, Certyfikat Oxford University Press.
- Szkolenie „Metodyka, wyszukiwanie i prezentowanie informacji naukowej”, Uniwersytet Przyrodniczy w Lublinie.
- Szkolenie „Rozwój Kompetencji Cyfrowych”, Sempre a Frente.
- Szkolenie z roli liderów w działaniach NGO, zarządzaniem projektami i komunikacji, Akademia Liderów Rzeczypospolitej.
- Szkolenie z umiejętności przywódczych i komunikacji medialnej, Akademia Liderów Rzeczypospolitej.
- Szkolenie z zakresu legislacji i udziału w procesie stanowienia prawa, Akademia Liderów Rzeczypospolitej.
- Certyfikat uczestnictwa „Warsztaty Interpersonalne”, Uniwersytet Przyrodniczy w Lublinie.
- Szkolenie „Ideas for a Better World: Leading Change Through Policymaking”, Future Leaders Connect, British Council.
- Certyfikat „Podstawy marketingu internetowego”, Google.
- Certyfikat „Nutrition - An Introduction”, POLHN.
- Certyfikat „BIO101: Introduction to Molecular and Cellular Biology”, Sailor Academy.