Dissertation for Masters Degree in Bioengineering

Biological Engineering Branch

Bioprospection of antibiotics and biofilm inhibitors from under-exploited filamentous fungi

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"I would rather have questions that can't be answered than answers that can't be questioned."

Richard Feynman

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Abstract

Antibiotics revolutionized the way infectious diseases were treated, saving millions of lives. However, bacteria responsible infectious diseases may develop resistance to antibiotics, which has become more problematic since the speed of development of new drugs has significantly decreased. Filamentous fungi have a vast, largely unexplored metabolome, giving them great potential for novel antimicrobial production. The One Strain MAny Compounds (OSMAC) approach serves as a valuable tool to obtain a wide range of metabolites by varying various culture conditions.

This study tested eight under-explored filamentous fungal species for antimicrobial production. They all grew in three distinct culture media, under deep fermentation, and the resulting supernatants were tested for their activity against a Gram-positive and a Gram-negative bacteria using the disk diffusion method and a co-culture test. It was shown that one of the supernatants of *Coprinopsis spilospora* inhibited *Staphylococcus aureus* growth, and it has antibiofilm properties, reducing its mass by up to 78%, metabolic activity by 100%, and culturable cells count by 1.8 log. Supernatants of other strains had a more modest activity, although considered significant. Changes in agitation speed of the fermentation were shown to heavily influence the activity of the resulting supernatant. It was also found that the effect of a supernatant of *C. spilospora* is synergistic with ciprofloxacin when used against a *S. aureus* biofilm. An analysis using liquid chromatography (LC) coupled with high resolution mass spectrometry (HRMS) and UV-Vis spectroscopy showed the presence of dihydroilludin M and other illudins in the supernatant of *C. spilospora*. Dihydroilludin M has been found in prior literature to be produced by closely related species, but not *C. spilospora*. This compound has a known activity against both bacterial species tested, but its synergy with antibiotics has not been reported in prior literature.

The pioneer results obtained in the present study highlight the potential of filamentous fungi for bioprospection for antibiotic discovery.

Keywords: Antibiotic bioprospection; Biofilm control; Bioprocess; Filamentous fungi.

Resumo

Os antibióticos revolucionaram a forma como as doenças infecciosas eram tratadas, salvando milhões de vidas. No entanto, bactérias responsáveis por doenças infecciosas podem desenvolver resistência a antibióticos, o que se tornou mais problemático, pois a velocidade de desenvolvimento de novos medicamentos diminuiu significativamente. Os fungos filamentosos possuem um metaboloma vasto e amplamente inexplorado, conferindo-lhes grande potencial para a produção de novos antimicrobianos. A abordagem Uma Estirpe Muitos Compostos (OSMAC) serve como uma ferramenta valiosa para obter uma ampla gama de metabolitos variando várias condições de cultura.

Este estudo testou oito espécies de fungos filamentosos pouco exploradas para a produção de antimicrobianos. Todos cresceram em três meios de cultura distintos, sob fermentação submersa, e os sobrenadantes resultantes foram testados quanto à sua atividade contra bactérias Gram-positivas e Gram-negativas usando o método de difusão em disco e um teste de co-cultura. Foi demonstrado que um dos sobrenadantes de *Coprinopsis spilospora* inibiu o crescimento de *Staphylococcus aureus* e possui propriedades antibiofilme, reduzindo sua massa em até 78%, atividade metabólica em 100% e contagem de células cultiváveis em 1,8 log. Sobrenadantes de outras estirpes tiveram uma atividade mais modesta, embora considerada significativa. As mudanças na velocidade de agitação da fermentação mostraram influenciar fortemente a atividade do sobrenadante resultante. Verificou-se também que o efeito de um sobrenadante de *C. spilospora* é sinérgico com ciprofloxacina quando usado contra um biofilme de *S. aureus*. Uma análise por cromatografia líquida (LC) acoplada à espectrometria de massas de alta resolução (HRMS) e espectroscopia UV-Vis mostrou a presença de dihidroiludina M e outras iludinas no sobrenadante de *C. spilospora*. Verificou-se na literatura anterior que a dihidroilludina M é produzida por espécies estreitamente relacionadas, mas não *C. spilospora*. Este composto tem atividade conhecida contra ambas as espécies bacterianas testadas, mas sua sinergia com antibióticos não foi relatada na literatura anterior.

Os resultados pioneiros obtidos no presente estudo destacam o potencial de fungos filamentosos para bioprospecção para descoberta de antibióticos.

Palavras-chave: Bioprospeção de antibióticos; controlo de biofilmes; Bioprocessos; Fungos filamentosos

Declaration

Declaro, sob compromisso de honra, que este trabalho é original e que todas as contribuições não originais foram devidamente referenciadas com identificação da fonte.

Declare, under oath, that this work is original and that all non-original contributions were properly referenced with the source identification.

June, 16th 2023 João Correia

(João Miguel da Costa Sousa Correia)

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Abbreviations

BCAR – Biofilm Cells' Activity Reduction

BEL – Biofilm Engineering Lab

BGC – Biosynthetic Gene Cluster

BMR – Biofilm Mass Removal

CFU – Colony Forming Units

CECT – Colección Española de Cultivos Tipo

COVID-19 – Corona Virus Disease 2019

CSL – Corn Steep Liquor

CYAS – Czapek Yeast Autolysate Salt

DNA – Deoxyribonucleic acid

EPS – Exopolysaccharides

HGT – Horizontal Gene Transfer

HPLC – High-Performance Liquid Chromatography

HRMS – High-Resolution Mass Spectrometry

IZD – Inhibition Zone Diameter

IJUP – Investigação Jovem da Universidade do Porto

ICCC 15 – 15th International Conference on Culture Collections

JGI - Joint Genome Institute

KEGG – Kyoto Encyclopedia of Genes and Genomes

LAMG – Laboratory of Applied Mycology

LB – Lysogeny Broth

LC – Liquid Chromatography

LECA - Lightweight Expanded Clay Aggregates

LED – Light Emitting Diode

MDR – Multi-Drug Resistant

MEA – Malt Extract Agar

MHB – Mueller-Hinton broth

MIC – Minimum Inhibitory Concentration

MME – Mercks Malt Extract

MRSA - Methicillin-Resistant *Staphylococcus. Aureus*

MUM – Micoteca da Universidade do Minho

NMR – Nuclear Magnetic Resonance

OCT – Optical Coherence Tomography

OD – Optical Density

OSMAC – One Strain Many Compounds

PDA – Potato dextrose Agar

PDB – Potato Dextrose Broth

PDC – Potato Dextrose + Corn steep

PCA – Plate Count Agar

PES – Poliethersulfone

UV Vis – Ultraviolet -Visible

SEM – Scanning Electron Microscopy

WATM – Wickerhams antibiotic test medium

YES – Yeast Extract Sucrose

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1. Contextualization

1.1. Thesis Objectives

Multi-drug resistant (MDR) bacteria are a growing problem, estimated to soon be able to cause millions of deaths annually [1]. They are especially dangerous when associated with a biofilm, that enhances such resistance and makes infections much harder to treat [2]. It is thus very important to promote drug discovery efforts, even if they are not very profitable [3]. Filamentous fungi have great potential for antibiotic production, being valuable candidates for novel drug discovery [4]. However, new species are constantly discovered and many of them remain to be studied for their metabolome.

This study aimed to explore the potential of selected fungal species for the production of molecules with antibiotic and biofilm-inhibitor action, against two model pathogens, *Staphylococcus aureus* and *Escherichia coli.* This was tested through a co-culture technique, disk diffusion test of deep fermentation supernatants, and antibiofilm tests (crystal violet and Alamar Blue staining and counts of colony forming units - CFU). As it is a bioprospecting, exploratory study, each test was largely dependent on the results of each previous one. Once an active supernatant was found, the optimization of its fermentation was pursued by changing the culture medium, agitation speed, and aeration. The active component of that supernatant was also analyzed to identify active molecules. Given the promising results obtained regarding antibacterial and antibiofilm activities, the synergy between that supernatant and commercial antibiotics was also evaluated.

1.2. Thesis Outline

This thesis is divided into 6 sections. Section 1 presents the context, relevance of the work and main objectives, as well as thesis organization. Section 2 contains a literature review on the topic, with a description of the problem of antibiotic resistance and stagnation of drug development in this area, along with presenting several strategies involving filamentous fungi as valuable contributions to the field. Section 3 describes the materials and methodologies used in this study and section 4 follows, by presenting and discussing the related results: From the cultivation of the fungal species to the evaluation of their antibiotic and antibiofilm properties and the identification of bioactive molecules. In section 5, the main conclusions are reiterated. Lastly, in Section 6, some suggestions for follow-up work are expressed.

2. State of the Art

2.1. The rise of bacterial resistance

During most of human history, infectious diseases such as smallpox, cholera, diphtheria, pneumonia, typhoid fever, plague, tuberculosis, typhus, and syphilis were the leading cause of mortality, even after the world industrialized. Antibiotics revolutionized the way infections were treated, saving millions of lives, and shifting the leading causes of death to non-communicable diseases like cardiovascular disease, cancer, and stroke [5]. Antibiotics also allowed for other procedures of great importance in modern medicine, lowering the risks of organ transplants, childbirth, and chemotherapy [6].

Even in the early days of readily available antibiotics, it was observed that bacteria could develop resistance to these drugs, a phenomenon that has become a significant public health concern in modern times. Sir Alexander Fleming warned that when antibiotics are used too often or in improper amounts may induce modifications in bacteria, rendering them resistant to these drugs [7]. However, from 1944 until the 1980s, the discovery of new molecules occurred at a rapid pace, which effectively rendered bacterial resistance less of a concern. This is because new resistant strains appeared at a much slower rate compared to the frequent discovery of new antibiotic molecules, leading to the "golden age" of antibiotics [8]. More recently, drug development has been much slower, and more focused on making alterations to previously known molecules, instead of new modes of action. In circa 50 years, only two classes of antibiotics were discovered and advanced the clinical use: oxazolidinones and lipopeptides [2,9-11]. Bacteria had thus time to become resistant to antibiotics, sometimes belonging to more than one class. These MDR bacteria have been of increasing concern and in some cases, they may accumulate resistance to almost all available antibiotics, the so-called superbugs or pandrug-resistant [5]. Tigecycline, colistin, daptomycin, vancomycin, and linezolid are last resource antibiotics, due to their potent antimicrobial activity. However, nowadays the alarming increase in antibiotic-resistant bacteria has rendered these drugs ineffective in a growing number of cases [12-15]. According to an influential study done in 2014, it was estimated that altogether bacterial infections will kill 10 million people annually by 2050, surpassing the number of deaths currently caused by cancer [1]. The assumptions made to calculate this number are often overlooked: It is a prediction of what would happen if no immediate action took place, which it did. However, even if this number of victims is no longer likely in this time frame, antibiotic resistance is a growing problem that requires an active, continuous effort to avoid a critical scenario in just a few decades [16].

The growing incidence of MDR bacteria has many causes. Antibiotics are found in the wild, so some bacteria were naturally resistant to them, even before the modern era of antibiotics. If they do not have that resistance, they may develop it through contact with individuals, possibly from another species, that are already resistant, by horizontal gene transfer (HGT), or through direct exposure to an antibiotic that induces selective pressure [2,5,17]. Documented examples of HGT include, in Gram-positive bacteria, the vancomycin resistance encoding gene *vanA* that originated from *Enterococcus* species and was transferred to *S. aureus*. In Gram-negative bacteria, the carbapenemase-associated gene $bla_{\text{OXA-48}}$ found in a hospital isolate of *Proteus mirabilis* was discovered to have been transmitted from *Klebsiella pneumoniae* from the same hospital [17,18]. The misuse of antibiotics in a medical context helps in the selection of resistant strains by exposing them to sub-lethal doses of antibiotics. This can happen by not taking the antibiotic for the recommended time, selecting individuals with higher tolerance; or by using antibiotics too often and even when not necessary, exposing bacteria to the antibiotic without a clear therapeutic benefit [5]. The wastewater of pharmaceutical factories can contain high concentrations of antibiotics, further exposing environmental microorganisms to a selective pressure that induces resistance. Globalization has made it increasingly easy for those pathogens to spread across the world [19]. Also, some antibiotics like colistin have historically been used as feed growth promoters, making them ubiquitous. MDR bacteria have become so widespread to be present in wildlife that has minimal contact with humans [20].

Biofilms are an additional problem related to antibiotic resistance, as they significantly increase the risk associated with an infection and decrease susceptibility to antibiotics [14]. Biofilms are complex structures composed of microbial cells enclosed in hydrated extracellular and non-crystalline polymers, adhered to a surface, and used as a survival strategy. They form on various surfaces, including industrial equipment, consumer products, foods, and the human body, causing increased energy costs, food contamination and spoilage, and equipment damage [21,22]. They may also form on medical surfaces like catheters, heart valves, orthopedic devices, and contact lenses. Clinically, they can cause persistent infections that are very hard to treat such as chronic lung infections in cystic fibrosis patients, osteomyelitis, prostatitis, rhinosinusitis, otitis media, recurrent urinary tract infection, endocarditis, periodontitis, and dental caries [22]. It is estimated that over 80% of all human infections are associated with a biofilm [23]. The mutation rates and horizontal transmission of genes are more frequent in these structures, increasing the likelihood of the accumulation of resistance to several antibiotics by the same bacteria, further hindering the treatment of the infection. Depending on the biofilm structure, they may limit the molecular diffusion to their inner-most cells, making only a small portion of the antibiotic available rapidly. Further penetration thus

occurs through much more circuitous or impeded avenues, which reduces the maximum concentration at which cells are exposed and gives them time to produce resistance factors. Even if the exposure to antibiotics kills many cells, after some time, when the antibiotic is no longer present, the biofilm can regrow from the cells least affected. The biofilm does not even have to be very thick, where 15-30 μm might have a very significant clinical impact. A great deal is known about the resistance mechanisms bacteria use in the planktonic state but not so much in biofilms. In this state, some cells develop a biofilm-specific, spore-like phenotype known as persister cells, whose only objective is to survive. Unlike their active counterparts, they become resistant to antibiotics even without the genetic basis typically associated with said resistance, and their decreased growth rates and metabolism allow them to reduce the exposure effect to antibiotics. This helps explain their critical resistance, but the cause is usually multifactorial [5, 17, 18].

Ultimately, antibiotic resistance is not a critical challenge for humanity but rather a series of interconnected problems involving several areas that require action from microbiologists, ecologists, health care specialists, educators, policymakers, legislative bodies, agricultural and pharmaceutical industry workers, and the general public [24].

2.2. The search for new/natural antimicrobial drugs – a crisis

One of the most compelling things about the use of antibiotics is the relatively low price that enables them to be readily available to most people around the world. Newly discovered antibiotics, on top of having to compete with the ones already on the market like every other drug, are usually reserved for the cases when those do not work, to slow down eventual bacterial resistance to the new molecules. Although such an approach makes sense from a medical and public health point of view, it discourages the development of new drugs from for-profit organizations, as it substantially decreases their revenue in the limited time they have until the intellectual property becomes public domain. Zemdri (plazomicin), an aminoglycoside, was reported to have cost 750 M $USD to enter the market in 2018 but made less than 800 k $USD in sales in the first six months. Shortly after, the pharmaceutical company that developed it declared bankruptcy, and the intellectual property was sold for only 16 M $USD [25]. In recent years, new financial models have started to emerge, to incentivize the research and development of new antibiotics. However, despite the recognition of this problem, the implementation of incentives like government-funded market entry rewards has been gradual and restricted. After so much time neglected, the market is in dire need of new products [25]. Recent events including the COVID-19 pandemic and its aftermath as well as the war between Russia and Ukraine have introduced a new layer to the problem in Western countries, provoking shortages of staple, first-line antibiotics like amoxicillin, that are often

produced as generic medicine in China. This single country has cornered the antibiotic market which, given its importance, has become more problematic to Western policymakers that are now starting to try to take that industry back [26,27].

Natural products have always been a crucial part of the drug discovery pipeline. Tetracycline, an example of a natural antibiotic, was detected in ancient skeletal remains from the Roman period in Egypt. This particular antibiotic is easily preserved due to its strong chelator activity of the hydroxyapatite mineral portion of bones and tooth enamel. Other sources of antibiotics from the pre-antibiotic era have also been reported, such as those found in the red soils of Jordan and traditional Chinese medicine practices, but physical evidence is harder to collect [24]. Prontosil (sulfamidochrysoidine), Salvarsan (arsphenamine), and Neosalvarsan (arsphenamine methylenesulfoxylic acid sodium salt) were very successful antibiotic drugs from the beginning of the 20th century, but the antibiotic era truly started in the 1940s with the widespread availability of a penicillin medicine isolated from *Penicillium rubens* . In the next decades, until the 1970s, in large part due to the development of a systematic screening approach, many more antibiotics were discovered, and most are still in-use today, although the increased incidence of MDR bacteria has limited their use frequency [33]. After this period, the "me-too" drugs became more frequent. Those take an already existing drug and make a slight alteration that does not change its mode of action but makes it less toxic or more effective [34]. Importantly, they only slightly delay the resistance of bacteria to a given drug class. For example, methicillin entered the market as a penicillin derivative invulnerable to degradation by penicillinases, one of the main pathogenic bacteria defense mechanisms. Unfortunately, less than two years after its introduction, the first methicillin-resistant *S. aureus* (MRSA) was reported, rapidly becoming the reference for hospital-acquired infections and spreading elsewhere [35,36]. We are currently living at the beginning of the post-antibiotic era, where antibiotics are much less effective than before [2].

The discovery of natural antibiotic molecules is a well-established process that involves multiple steps, including the search for a promising source for bioactive molecule, fermentation, its optimization and up-scaling, fractionation, and identification of active molecules if the activity prevails. These steps are summarized in Figure 1.

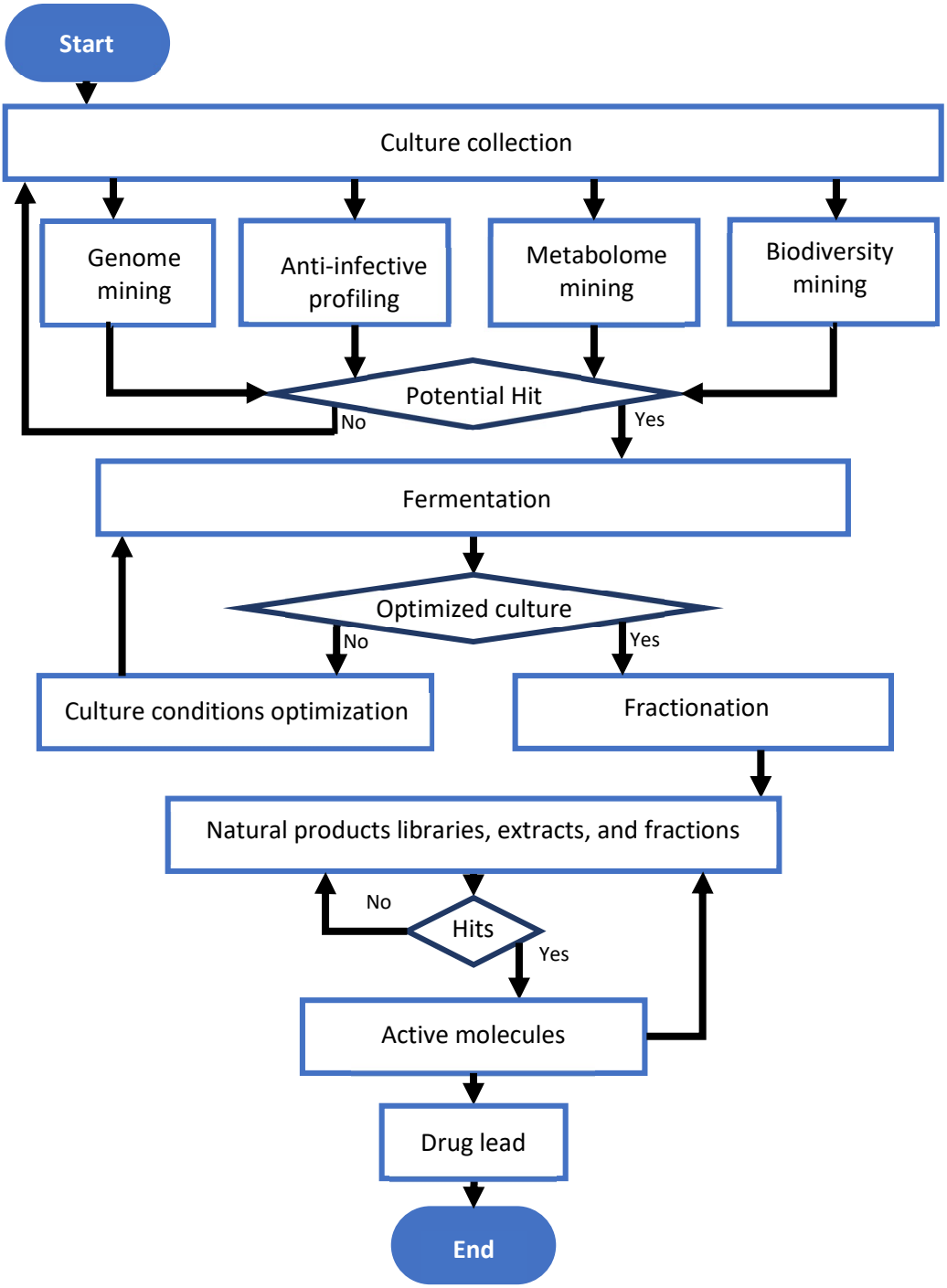


Figure 1. Flow chart of natural antibiotic discovery pipeline. Adapted from [37].

Since the 1990s, natural drug discovery programs have slowed down due to a combination of technical limitations and legal hurdles. Technical limitations stem from the belief that natural drugs are incompatible with high-throughput screening approaches, which have become a cornerstone of modern drug discovery. Legal hurdles have emerged from the United Nations Convention on Biological Diversity, which aims to regulate international access to natural products and their derivatives [38,39]. These factors have made it increasingly challenging to develop new natural drugs, which has led to a decline in the number of natural

products being used in drug discovery, in favor of synthetic molecules. This strategy was not very successful at finding new drug leads in major therapeutic areas, including antibiotics. Almost all classes of commercial antibiotics used are natural or semi-synthetic: the exceptions are the sulfa drugs, quinolones, and oxazolidinones, and only the last of those is somewhat recent. Natural drugs are thus resurfacing, as they have a better track record [11,40]. They are more chemically diverse and often have a larger molecular weight than synthetic drugs. Unlike those synthetic, natural drugs are not discovered by testing combinatorial-derived libraries of small molecules. They often do not follow Lipinski's rule of five, which should affect absorption, distribution, metabolism, excretion, and toxicity (ADMET), despite being viable oral drugs [11,38]. The relative complexity and consequent specificity of certain biological drugs, like peptide therapeutics, even allow them to reach targets previously considered "undruggable" [40].

Secondary metabolites, which are molecules that increase the competitiveness of an organism without being essential to its survival, constitute the majority of relevant natural products. Historically, although penicillin is very famous for being a revolutionary discovery, most natural antibiotics are produced by actinobacteria [37]. Microbes play a growing role in the discovery of these products as have provided effective drugs against viruses, bacteria, fungi, and even cancer [41]. The rediscovery of previously identified molecules poses a major bottleneck in natural product research, resulting in an increased workload that fails to yield meaningful results. Fortunately, there are newly available techniques like high-resolution mass spectrometry (HRMS) coupled with already existent ones like nuclear magnetic resonance (NMR) spectroscopy and pre-fractionation methods like C_{18} high-performance liquid chromatography (HPLC) or liquid- and solid-state extractions. These techniques have allowed the identification of replicated molecules at an early stage, thus mitigating this problem [39,42,43]. With such powerful tools increasingly available for molecule identification finding new sources of bioactive compounds becomes ever more fruitful.

2.3. Fungal secondary metabolites

The secondary metabolome of fungi is vast and largely unexplored, especially from Ascomycetes and Basidiomycetes, which contain filamentous fungi [42]. They are the source of many compounds with medical, industrial, and agricultural importance [44].

Filamentous fungi provide several ecosystem functions: the symbiotic ones have a close, mutually beneficial relationship with a nonfungal organism; the saprobic ones utilize the nutrients released when breaking down debris; and parasitic fungi feed by taking away nutrients from another organism [45]. One strategy employed by several fungi to stay ecologically relevant is to produce antibiosis molecules, and some of them can be used as

medicine [46]. The niches where they belong are very competitive so on top of a pure weaponization of their secondary metabolome, they also produce a multitude of signal molecules that may have other valuable properties. For example, 5-methyl-phenazine-1-carboxylic acid is an antifungal drug but in the wild, it is used in smaller quantities as a signal to induce asexual sporulation [42,47]. Other fungal metabolites have different, but potentially interesting effects, like emodin, which only has a moderate antibacterial effect but significantly enhances the efficacy of oxacillin against MRSA when combined with it [48]; and avellanin C, which has anti-quorum sensing properties in *S. aureus* [49].

The three most important classes of secondary metabolites from fungi are polyketides, derived from acyl CoA precursors; non-ribosomal peptides that condense amino acids in pathways involving enzymes instead of ribosomes; and terpenoids formed by isoprene units [50-52].

Many antibiotics have been discovered from filamentous fungi. Penicillins and cephalosporines were the first and second classes discovered, respectively. They are chemically very similar as both are non-ribosomal peptides that have a β-lactam ring (four-atom cyclic amide) that binds to peptidoglycans and prevents them from cross-linking, compromising the stability of cell walls, especially from Gram-positive bacteria [5,45]. Pleuromutilins have taken a long time to enter the human market, despite being discovered in the "golden age" of antibiotics. They block the start of protein synthesis by interfering with the 50S bacterial ribosomal unit [11]. Enniatins are produced by mycotoxin-producing *Fusarium* spp. They interfere with the cell wall and disrupt the cell membrane, but show other effects such as inhibition of drug efflux pumps activity and MDR phenotype expression [33,53]. Fusidic acid is the only member of its class and is a narrow-spectrum antibiotic commonly used against *S. aureus*. It binds to an elongation factor and the corresponding ribosome during protein synthesis, which does not allow it to continue. While eukaryotes have other elongation factors, bacteria become unable to synthesize proteins [54].

Figure 2 contains a timeline of the discovery and market introduction of the antibiotic classes isolated from fungi.

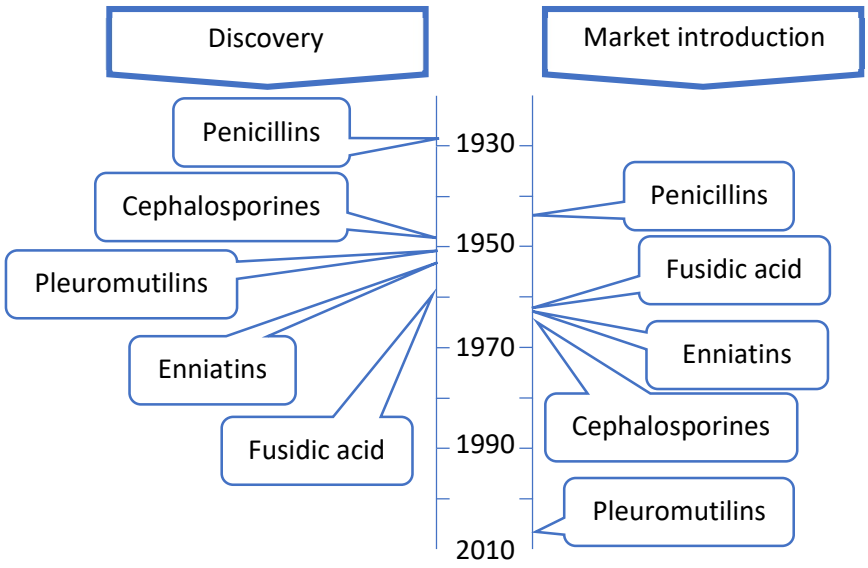


Figure 2. Timeline of the discovery and market entry of the first antibiotic of each class isolated from fungi (Adapted from [33]).

One of the most challenging aspects of discovering new drugs from fungi is their production at a large scale, as often standard laboratory conditions are not suitable for that purpose [4].

2.4. Approaches to secondary metabolite production from fungi

2.4.1. OSMAC approach

Culture conditions are of the utmost importance in determining which compounds will be synthesized and at which quantities. There are countless examples of effects, but a relevant one could be an extract of *Bulgaria inquinan*s which accumulated four new metabolites only when a mixture of inorganic salts was added to the culture [55]. Even the harmless change from tap water to distilled water in the making of the culture media promoted the production of six new secondary metabolites in a *Paraphaeosphaeria quadriseptata* culture [56]. The "One Strain MAny Compounds" (OSMAC) approach may be incredibly successful at enhancing the chemical diversity obtained, as changing culture conditions like the pH, temperature, aeration, and carbon and nitrogen sources, can alter the metabolite profile of the fungi. Potato dextrose or malt extract are both great to maintain fungal cultures but often fail in terms of secondary metabolite production. Adding enzyme inducers/inhibitors or chemical elicitors can also change what is produced [4].

The discovery and commercialization of penicillin had a significant time gap, mostly due to the time it took to develop an effective large-scale production method. The first versions of this method used a broth consisting of lactose 3–4%, corn steep liquor (CSL) 4%, $CaCO_3$ 1%, KH_2PO_4 0.4%, and antifoam 0.25%. Over time, it has been improved, but the main difference

is the replacement of lactose by glucose or molasses at 10%, which are cheaper. Phenylacetic acid, or another molecule that may serve as a side chain, is also added, as its synthesis is the limiting step. CSL is advantageous for its price and chemical diversity, containing many micronutrients, nitrogen, and side chain precursors [57,58]. The maximum volume of the reactors used increased, as new technologies allowed better oxygen diffusion, which is essential for the production of most secondary metabolites, including penicillin, and is a challenge as the fungus significantly increases the viscosity of the medium [57]. The morphology acquired by the cells influences the production of secondary metabolites and this aspect needs to be optimized. It has been shown that it does not affect the ability of *Penicillium chrysogenum* to produce penicillin directly, but the formation of pellets reduces the medium viscosity, which in turn increases the diffusion rate of respiratory gases, reducing energy costs and increasing productivity. The morphology can be influenced by the spore density of the inoculum, agitation speed, or CO_2 concentration, for example [57,59,60]. The strain used also changed significantly, and all factors are important. This took the concentration of penicillin obtained in the broth from 0.001 g/L in the year 1939 to the current concentrations of over 50 g/L [51,57].

2.4.2. Liquid and solid-state fermentations

Secondary metabolites can be produced by fermentation in a liquid broth, or solid support, like the Lightweight Expanded Clay Aggregates (LECA) "nuts" or most often by the use of a gelling agent like agar media, which consistently gives good results [4].

Submerged fermentation is often preferred in industrial processes because it is easier to scale up, monitor and automate. Large scale is often essential for industrial scale viability, and contrary to solid-state fermentation, it avoids the build-up of temperature, pH, and O_2 concentration gradients in large reactors [61]. The purification of the products also becomes easier, but it ends up generating a great quantity of wastewater [62]. Liquid fermentation is notably fast, usually lasting only 1-2 weeks and so requires diverse equipment to manage in real-time. Frequently, the constant influx of nutrients supplied by a fed-batch stream is required, as nutrients in a liquid medium are easy to access, and thus rapidly consumed by the microorganisms [57,62]. In mushroom fermentations, it does not allow the formation of fruiting bodies that sometimes contain bioactive molecules [63].

Submerged fermentation at an industrial scale must carefully consider several physical, chemical, and biological parameters that can severely influence the success of the operation. Among them, agitation and aeration can significantly increase energy consumption. This

energy adds to that released from the fungal metabolism and so requires an effective cooling system to maintain an appropriate temperature for the microorganism [57,64,65]. At a laboratory scale, it is easier to maintain an adequate temperature and aeration, given that the volume of a reactor in relation to its walls and bottom is much smaller than in an industrial fermenter. Besides, a small pump can provide a relatively large volume of air. However, even these reactors may create concentration gradients and agitation dead zones [65]. These parameters must be carefully considered before scaling-up operations. It is common to express the agitation used in these experimental setups in rotations per minute (rpm), but this fails to precisely express the forces undergone by the broth. The use of the agitation Reynolds number or the mixing time is thus easier to compare between independent experiments, especially at different scales. It is important to consider that the fermentation broth of filamentous fungi is usually a viscous, non-Newtonian fluid, so its viscosity depends on the shear rate, and more heavily so when the mycelium does not grow in pellets [59,65,66]. In an *Aspergillus niger* culture, it was found that the optimal rotation frequency for pectinase production was 150 rpm. While a slower agitation did not promote as much mixture, higher speeds increased the shear force experienced by the cells and decreased enzyme activity, while still increasing biomass production, up to 200 rpm [67]. Another study on the same species directed for the production of tannase demonstrated that the optimal agitation speed was 130 rpm for enzyme activity and 100 rpm for growth optimization [68].

Solid-state fermentation may be described as occurring in a solid matrix that contains adequate moisture for the microorganism to develop, but no additional free water, which makes it optimal for organisms that thrive in low-water activity environments. It can be performed in either an inert carrier or on insoluble substrates that supply the nutrients. It resembles many valuable fungi's native environment allowing them to adapt the best. Both Basidiomycetes and Ascomycetes have evolved on land, and only later have some species migrated to the marine environment. Marine microorganisms of these phyla thus often prefer solid media, as most of them are isolated from sediments or solid structures and are not freely swimming in the water. It also removes the need for conditions that the fungi did not evolve to be adapted to, like high shear stresses and the presence of anti-foam products. This type of culture promotes secondary metabolite production - for example, conoicetin, a potential antibiotic from *Coniochaeta ellipsoidea* could only be produced in solid-state despite the mycelium also growing well in liquid media [61,69].

Solid state fermentation is sometimes considered a low technology approach, best suited to "high volume low cost" applications that require low purity like industrial enzymes – amylase, cellulase, protease, etc. However, this has been changing and more valuable products

have been produced this way, although bacteria and yeast are more commonly employed in such systems [69,70]. On a laboratory scale, it has many advantages such as higher productivity, concentration of products and their stability (due to lower water activity), and lower catabolic repression and sterility requirements, but its use is hindered by the difficulty in scaling up [61].

2.4.3. Co-culture strategies

Co-culture or mixed fermentation strategies may be of great value to unlock cryptic pathways expression, producing molecules absent in pure-strain cultures such as pheromones, defense molecules, or metabolites related to symbiotic associations. It consists in growing different microorganisms in the same confined environment, allowing them to interact with each other [71]. Such interactions may even alter the core metabolism of the cells, for example, by increasing the production of ergosterol [72]. In addition to a yield increase in common molecules, this type of strategy can increase the yield of previously unidentified or undetected molecules, facilitating their characterization [73]. It can also help overcoming some problems that fermentative processes undergo, including excessive metabolic burden, lack of active enzyme expression, and by-product formation [74]. This makes sense from an ecological perspective as the production of antibiosis molecules by a fungus is most important when exposed to a competitor [37].

To co-cultivate bacteria and fungi for drug discovery, the most common strategy is similar to regular monoculture fungi fermentation, but the liquid medium is inoculated with a small number of bacteria, often not at the same time as the fungi. Similarly, solid media like commercial rice or wheat can also be used. The bacteria should not dominate the culture but, at a small scale, that can be regulated by the inoculum ratio [71,75,76]. An alternative proposed method grows the fungi in glass beads submerged in a culture medium. After the fungus has developed, the medium is replaced with a bacterial suspension, but the mycelium remains adhered to the beads, establishing a mixed culture [77].

These methods, however, have some limitations. Firstly, they are not employed more often because they require laboratory expertise in the cultivation of both fungi and bacteria [71]. Then, the induced metabolites produced are quite random. For example, when the antimicrobial activity of a culture of *Streptomyces* sp. (that despite being a bacterium, has many similarities with fungi in terms of antibiotic production) was tested against human pathogens, no significant effect was found against *P. aeruginosa*. Co-culture with *P. aeruginosa* increased 4-fold the activity against all Gram-positive bacteria tested, but, unexpectedly, did not increase activity against that pathogen [78]. In the production steps of the drug development pipeline, especially while scaling up the reactor, it can also be found difficult to keep the co-culture

stable. In this regard, some methods allow it, like a pulsating feed of glucose that gives a fitness advantage to alternating species [79].

2.4.4. Other optimization strategies

Many other conditions can be varied to optimize the production of a metabolite, which are often limited by the knowledge of the mechanisms involved.

Surfactants can have an interesting effect on liquid fermentation. It was found that the yield of lovastatin production by *Monascus purpureus* was increased by 84.5% by the addition of Triton X-100, a nonionic surfactant. This surfactant increases membrane permeability facilitating the export of the product of interest and increasing the expression of genes related to lovastatin synthesis [80,81]. The addition of Tween 80 on a *Ganoderma lucidum* culture, which produces bioactive exopolysaccharides (EPS), or on a *Coprinopsis cinerea* culture, which produces cellulase also increased their yield for the same reasons [82,83].

The inclusion of polymers like polyacrylic acid or sodium polyacrylate modifies the spores' surfaces to avoid the formation of clumps and makes the mycelia grow in dispersed hyphal filaments [84]. Another exhaustive study used either XAD-16N or styrene-divinylbenzene, two resins, to induce the production of many compounds that were not present otherwise in fermentation with 349 different fungal strains [85]. Adsorptive polymeric resins can increase biomass growth and drastically change the color of the fermentation broth. They interfere with the fermentation by sequestrating components from the medium. Those might be microelements or toxic products, which is especially relevant if the product of interest is extracellular and toxic or degrades easily [86,87].

Some small molecules may serve as epigenetic modifiers by interfering with DNA methyltransferase, like 5-azacytidine and 5-aza-20-deoxycytidine; or with histone deacetylase, like compounds with hydroxamic acid or cyclic peptides (e.g., trichostatin A and trapoxin B) [86]. This may induce the expression of metabolic pathways that are cryptic in standard laboratory conditions, as those enzymes are responsible for regulation of gene expression. Although the production of secondary metabolites is enhanced, this method usually is not specific to certain secondary metabolites. However, it was found that hydrazine boosted the production of some specific metabolites in a *Dothiora* sp. culture, including the antibiotic fusidic acid, and inhibited others [88]. Similarly, while reducing histone deacetylase activity using a mixture of compounds, *Aspergillus nidulans* overexpressed and under-expressed similar numbers of secondary metabolites, by at least two orders of magnitude [89].

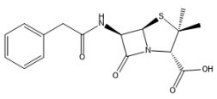
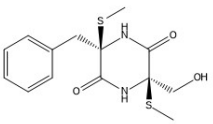
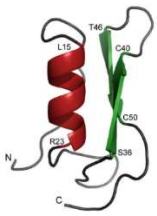
Microparticles of talc (e.g., magnesium silicate), aluminum oxide, titanium oxide, or silver nanoparticles are another strategy to enhance the production of fungal products, specifically enzymes [90]. Talc microparticles have been shown to increase lovastatin

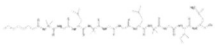
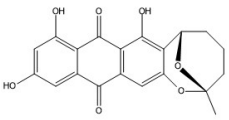
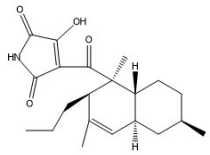
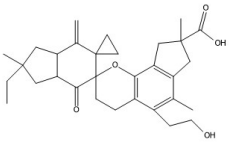
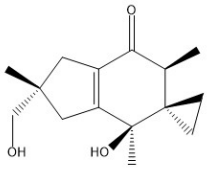
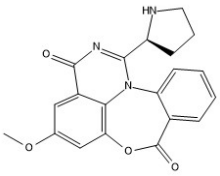
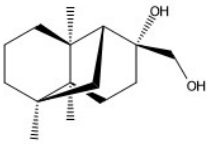
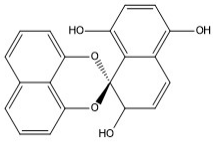
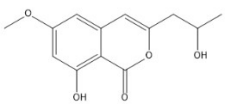
production by *Aspergillus terreus* by 60%, although this effect is due to their ability to morphologically engineer the fungus. By reducing the pellet diameter, they increased oxygen availability in the inner part of the pellets and consequently the product yield [91,92]. Aluminum oxide microparticles increased amylase production by 114% in an *Aspergillus oryzae* fermentation through a similar mechanism [90].

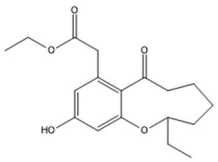
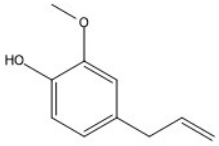
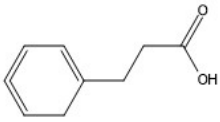
There are even other parameters that can be changed to improve the production of antibiotics. Starving the cells of a nutrient can be effective in specific cases, for example, in the production of the polyketide bikaverin by *Fusarium oxysporum*, nitrogen, phosphorous and sulfate limitations increase yield, while industrial production of penicillin by *P. chrysogenum* must have phosphates as the limiting nutrient [57,93]. Secondary metabolites are produced in the stationary phase of cellular growth, which usually happens when there is a sugar limitation [94,95]. pH can play a part in the production of secondary metabolites by regulating the expression of enzymes involved in their production. *A. nidulans* favors the production of penicillins in alkaline environments because the gene encoding the isopenicillin N synthetase, which is responsible for the production of an intermediate in the penicillin biosynthetic pathway, is up-regulated in these conditions [96]. This effect is observed in *P. chrysogenum*, but to a lesser extent [95]. Bikaverin production is, on the other hand, favored by acidic pH [93].

Table 1 contains examples of fungal metabolites with antibacterial properties, their structure, the producing species, and the culture media used.

Table 1. Examples of fungal metabolites with antibacterial properties.

Antibiotic	Producing species	Structure	Media used	Observations	References
Penicillin G	*Penicillium chrysogenum*		Glucose or molasses, 100 g/L; CSL solids, 45 g/L; phenylacetic acid, 0.65 g/L (fed continuously); vegetable oil-antifoam, 0.5 g/L; ammonium sulfate (continuously kept at 275 g/L).	Media for industrial production	[57]
Bis-N-norgliovictin	*Asteromyces cruciatus*		Glucose, 30 g/L; arginine, 1 g/L; asparagine, 2.5 g/L; glutamate, 1.5 g/L; $FeSO_4$, 0.01 g/L; KCl, 0.5 g/L; $MgSO_4$, 0.5 g/L; K_2HPO_4, 1.0 g/L; in artificial sea water.		[97]
Copsin	*Coprinopsis cinerea*		Glucose, 5 g/L; asparagine, 2 g/L; adenine sulfate, 50 mg/L; KH_2PO_4, 1 g/L; Na_2HPO_4, 2.3 g/L; Na_2SO_4, 0.3 g/L; ammonium tartrate, 0.5 g/L; thiamine-HCl, 40 µg/L; $MgSO_4 \cdot 7H_2O$, 0.25 g/L; p-aminobenzoic acid, 5 mg/L.	Cultured in glass beads; Co-cultured with *Bacillus subtilis* or *Escherichia coli*	[77,98]

Trichogin GA IV	*Trichoderma longibrachiatum*		Glucose, 5 g/L; potassium dihydrogen phosphate, 0.8 g/L; potassium nitrate, 0.72 g/L; calcium phosphate 0.2 g/L; magnesium sulfate, 0.5 g/L; manganese sulfate, 0.01 g/L; zinc sulfate, 0.01 g/L; copper sulfate, 0.005 g/L; iron sulfate, 0.001 g/L.		[99,100]
Averufanin	*Aspergillus carneus*		Glucose, 10 g/L; mannitol, 20 g/L; sucrose, 20 g/L; yeast extract, 3 g/L; corn syrup, 1 g/L; peptone, 10 g/L; tryptophan, 0.5 g/L; K_2HPO_4, 0.5 g/L; $MgSO_4.7H_2O$, 0.5 g/L; $FeSO_4.7H_2O$, 0.1 g/L; agar, 15 g/L.	Extracted from the solid culture medium	[101]
Oxasetin	*Vaginatispora aquatica*		Extract from potato, 4 g/L; glucose, 20 g/L. pH adjusted to 7.		[102]
Bovistol D	*Coprinopsis strossmayeri*		Extract from potato, 4 g/L; glucose, 20 g/L.		[103]
Illudin I	*Coprinopsis episcopalis*		Sucrose, 80 g/L, yellow corn meal, 50 g/L yeast extract, 1 g/L.		[104]
Aspergicin	Two *Aspergillus* spp.		Glucose, 10 g/L; yeast extract 1 g/L; peptone, 2 g/L; crude sea salt, 3.5 g/L.	Yielded another antimicrobial compound	[73]
Emericellin A	*Emericella* sp.		Peptone, 1 g/L; malt extract, 20 g/L; sucrose, 20 g/L.	Another similar compound was also isolated	[105]
Palmarumycin C_8	*Lophiotrema* sp.		Glucose, 20 g/L; maltose, 10 g/L; yeast extract, 4 g/L; oatmeal, 20 g/L, 5-azacytidine, 12 mg/L.		[106]
Diaporthin	*Diaporthe terebinthifolii*		Malt extract, 20 g/L; glucose, 20 g/L; peptone, 1 g/L.	Other media also yielded this and another antimicrobial compound	[107]

Phomopsin A	*Phomopsis* sp. ZSU-H76		Glucose, 10 g/l; peptone, 2 g/l; yeast extract, 1 g/l; NaCl 3 g/l.	Other compounds were also isolated	[108]
Eugenol	*Neopestalotiopsis* sp. MFLUCC15-1130		Extract from potato, 4 g/L; glucose, 20 g/L.		[109,110]
3-phenylpro-pionic acid	*Cladosporium cladosporioides*		Extract from potato, 4 g/L; glucose, 20 g/L.	Other compounds with weaker antimicrobial effects were also identified	[111]

Unless otherwise stated, the studies collected were conducted in relatively small-scale, liquid medium, and monoculture fermentation.

The culture conditions optimization process can have an improved hit rate using the ever-increasing available information about the biosynthetic pathways involved in the production of the metabolites [112]. While some parameters should be optimized based on the producing species, others are specific to a particular pathway and must be established accordingly.

2.5. Biosynthetic pathways for antibiotic production

Computational tools have been of great utility as a first step in the search for new secondary metabolism pathways, especially with the developments of large-scale sequencing technologies [112]. One such tool is genome mining for biosynthetic gene clusters (BGC), which enables the identification of strains with the enzymatic machinery required to produce bioactive compounds. Unlike the sequences related to primary metabolites, which are spread through the genome, the ones related to the same secondary metabolite usually appear together, in a cluster, and can be identified with appropriate algorithms [42]. Enzymes encoded within these clusters can be used to predict the metabolites that they produce, based on known analogies [113]. However, traditional laboratory techniques are still required to properly identify the active molecule [114].

BGCs are very common among fungal species. For instance, in one study performed by de Vries *et al* [115], the genomes of 19 *Aspergillus* species were analyzed and it was verified that they contained between 21 and 66 BGCs each. Considering the millions of fungal species, many of which with a number of BGCs in that range, the potential for biosynthetic pathways is enormous [116,117].

The Kyoto Encyclopedia of Genes and Genomes (KEGG) provides a brief look at the potential of a microorganism for its biosynthetic potential. However, it lacks information about many less-common metabolites and fungal species. It is still a great, and easy-to-use tool, only when it is known which metabolite production is intended. If any fungal species is to be checked for penicillin or cephalosporin production, the analysis of "Biosynthesis of secondary metabolites" from *P. rubens* quickly points to the probable production of penicillin from L-cysteine, L-valine, and lysine. This approach is, however, very limited when two restrictions pile up: only antibiotics, from a specific fungus, is being investigated. This means that many compounds are not even present in the pathway. Many pathways are also incomplete for several reasons [118], even when the microorganism is widely reported to produce the end metabolite, like the penicillin synthesis by *P. rubens* [119]. Other pathways have an isolated hit because of an enzyme that is used for multiple substrates. Even if the pathway is accurate and complete, using its information to boost the production of a metabolite may be misleading. Taking penicillin again as an example, the use of the abovementioned amino acids to boost production will probably be unfruitful as the limiting step in its synthesis is related to the side chain, and not the penicillin scaffold [120]. The availability of L-cysteine is only an important factor for achieving high yields of penicillin production in industrial strains that have already been optimized for side-chain synthesis. For those, ensuring an adequate supply of L-cysteine is crucial for maximizing the efficiency of the production process [51]. This kind of metabolic engineering is required to obtain the desired product as economically viable.

2.6. Advances in metabolic and genetic engineering of filamentous fungi

The disparity between the number of BGCs identified by bioinformatic tools and the actual number of secondary metabolites produced, suggests that many of these clusters remain silent under the conditions typically used in laboratory settings. Other molecules may even be produced, but at such low titers that they cannot be analytically detected, and their bioactivity is masked by more abundantly produced metabolites [41,55]. Metabolic engineering of fungi is one of the most important research directions in modern industrial biotechnology.

Genetically modifying filamentous fungi is possible, but much harder than doing the same with bacteria or yeasts [51]. *E. coli*, *Saccharomyces cerevisiae*, and *Komagataella phaffii* have successfully been turned into efficient cell factories but the equivalent does not exist for molds nor mushrooms. Common industrial "workhorse" species include *A. niger*, *A. oryzae*, *P. chrysogenum*, *Trichoderma reesei,* and more recently *Myceliophthora thermophila.* They use cheap feedstock, have powerful secretory pathways, and can post-translationally modify proteins.

They are, however, still not as easy to engineer as bacteria or yeast because of their slow growth rate, low throughput of genetic transformation, and secretion of unwanted enzymes [121]. Bioprospecting new strains with optimized properties in which valuable BGCs can be inserted may be facilitated with the use of the fast-expanding genomics data, for example from the Joint Genome Institute (JGI) Mycocosm database. Even if imperfect, the ones currently available can already produce great results: heterologous production of tenellin in *A. oryzae* has a five-fold increased yield when compared with the native host of the biosynthetic pathway, *Beauveria bassiana* [121-123]. Alternatively, strain improvement through breeding tools that select desired traits or genetic engineering may provide a suitable strain [51,121]. A *A. niger* strain that produced 31 g/L of citric acid underwent UV-induced mutagenesis and the yield increased to 50 g/L. Further improvement using N-methyl, N-nitro-N-nitroso-guanidine, a chemical mutagenic, increased yield to 96 g/L, and substrate optimization made it reach 114 g/L [124]. A different strain of the same species was genetically engineered to overexpress a high-affinity glucose transporter. It boosted the production of citric acid from 162 g/L to 174 g/L by increasing glucose usage [125]. Genomics, transcriptomics, proteomics, metabolomics, and localizomics, in addition to proper identification of the biochemical pathways, are the key to inducing or inhibiting the expression of a secondary metabolite [51]. New algorithms using machine learning and artificial intelligence may help combining all the omics data obtained from ever-increasing throughput methods. They might discover ways to produce new industrially viable biological products by activating silent BCGs, recurring to an extensive knowledge of the genome of many species, or better ways to produce those that already exist, for example, by avoiding the formation of an inconvenient secondary product. The data required for all of this may even be already discovered. Having a comprehensive understanding of all the mentioned fields is essential to develop a new generation of filamentous fungal chassis that can serve as chemical cell factories in synthetic biology. Such a platform would offer precise control over regulation at all levels, providing significant potential for enhancement [51,126,127].

Industrially viable penicillin-producing strains contain many examples of the application of various engineering techniques, as the associated product has been under constant improvement of metabolic yield for many decades [57]. In penicillin production, the excess presence of the precursor phenylacetic acid up-regulates the expression of genes in the homogentisate pathway, involved in its degradation. Although that makes sense for the fungi, as it can reduce the concentration of this very toxic metabolite, it diminishes the amount of compound available to be incorporated into the penicillin molecule. Industrial strains have thus this catabolic pathway inactivated, to avoid the waste of a chemical that constitutes one

of the major costs of the process and to overproduce penicillin. Those strains have more copies of penicillin-associated BGCs; a higher transcription of these genes and the genes associated with the precursor amino acid production (L-cysteine, L-valine, and lysine); inactivation of pathways that compete with their synthesis, such as the tryptophane synthesis; increased number of peroxisomes, where one of the reactions take place; as well as inactivation of the synthesis of a pigment that is considered a contaminant in downstream processing, among other mutations [57,128,129]. The mutations in industrial penicillin-producing strains are the accumulation of many years of improvement by several methods and many lessons can be learned from them. They can be used as an inspiration for strain improvement in other processes, using modern technology [51].

3. Materials and methods

3.1. Filamentous fungi selection criteria

The number of fungal species prospected for bioactive molecules was limited by time and space constraints. In the end, eight strains of filamentous fungi were chosen based on four different criteria: availability at MUM; absence of mycotoxin production; limited published documents on the species; and record of other species in the same genus that produce antibiotics. Those were *Coprinopsis spilospora* MUM 20.36, *Penicillium tunisiense* MUM 17.80, *Trichoderma aestuarinum* MUM 19.05i, *Colletotrichum coccodes* MUM 20.94, *Talaromyces saxoxalicus* MUM 20.30, *Diaporthe phillipsii* MUM 19.28, *Cladosporium rubrum* MUM 19.39, and *Neopestalotiopsis scalabiensis* MUM 21.34

Additional information on the selected species is in Appendix A.

3.2. Filamentous fungi culture conditions and cell-free supernatant isolation

Either two 8 mm agar plugs were taken from the periphery of the fungal culture, or a 2 mL suspension of spores in a solution of 0.85% NaCl (Merck, Darmstad, Germany) and 0.05% Tween 80 (Merck, Darmstad, Germany) was used for inoculums [130]. *C. spilospora, T. aestuarinum, C. coccodes*, *D. phillipsii*, *C. rubrum*, and *N. scalabiensis* were kept in potato dextrose agar (PDA) (Merck, Darmstadt, Germany) while *P. tunisiense* and *T. saxoxalicus* were kept in malt extract agar (MEA) (Merck, Darmstadt, Germany), all at 25 °C. The deep fermentation occurred in 250 mL Erlenmeyer flasks on an orbital agitator (Sartorius certomat R shaker, B. Braun, Melsungen, Germany) set at 120 rpm, 25 °C in darkness. A volume of 100 mL of three different media were used: Yeast extract sucrose broth (YES), Potato dextrose broth (PDB), and Lysogeny Broth (LB) (Table 2).

Each combination of species and medium was performed twice, for 7 and 14 days to ensure the reproducibility of the results, while simultaneously widening the range of conditions tested [4,37]. On the 7th day, cultures with the same species in the same media should look identical, and when they did not, the experiment was discarded. When antibacterial activity was found on a supernatant, more culture conditions were tested to optimize such results. Four additional media were tested: Wickerhams antibiotic test medium (WATM), Mercks Malt Extract (MME), Czapek yeast autolysate salt (CYAS), and Potato dextrose + CSL (PDC). Some parameters related to these media are summarized in Appendix B. The agitation

speed was also increased to 180 rpm and decreased to 60 rpm, and the effect of increased aeration was tested with the use of an air pump with a small air flow, to avoid excessive evaporation.

Table 2. Components of the liquid culture media used.

Media	Components
YES	Sucrose (Merck, Darmstad, Germany) (150 g/L), yeast extract (Oxoid, Basingstoke, United Kingdom) (20 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $ZnSO_4 \cdot H_2O$ (0.01 g/L), $CuSO_4 \cdot H_2O$ (0.005 g/L).
PDB	Potato dextrose broth (HiMedia, Mumbai, India).
LB	Tryptone (HiMedia, Mumbai, India) (10 g/L), yeast extract (5 g/L), NaCl (10 g/L).
WATM	$NaNO_3$ (2 g/L), glucose (Merck, Darmstad, Germany) (2 g/L), sucrose (30 g/L), yeast extract (2 g/L), peptone (HiMedia, Mumbai, India) (3 g/L), CSL solids (Merck, Darmstad, Germany) (5 g/L), $KH_2PO_4 \cdot 3H_2O$ (1 g/L), KCl (0.2 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $FeSO_4 \cdot 7H_2O$ (0.01 g/L), $ZnSO_4 \cdot 7H_2O$ (0.01 g/L), $CuSO_4 \cdot 5H_2O$ (0.005 g/L).
MME	malt extract (Oxoid, Basingstoke, United Kingdom) (30 g/L), peptone (3 g/L), $ZnSO_4 \cdot 7H_2O$ (0.01 g/L), $CuSO_4 \cdot 5H_2O$ (0.005 g/L).
CYAS	NaCl (50 g/L), $NaNO_3$ (3 g/L), yeast extract (5 g/L), sucrose (30 g/L), $K_2HPO_4 \cdot 3H_2O$ (1.3 g/L), KCl (5 g/100 mL), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $FeSO_4 \cdot 5H_2O$ (0.01 g/L), $ZnSO_4 \cdot 7H_2O$ (0.01 g/L), $CuSO_4 \cdot 5H_2O$ (0.005 g/L).
PDC	PDB + CSL solids (20 g/L).

All salts were of analytical grade; based on **[4]**.

To facilitate the perception of the morphology and color of the suspension, when available, the flasks were photographed with an LED flashlight (from a smartphone) at the bottom, although only for a short period to avoid the potential degradation of any compound.

It was intended for the isolated supernatants to be sterile (cell-free supernatants), to increase their stability (if biomass was present, it could metabolize some components) and to avoid contamination of the following assays, so they were filtered using a sterile 0.2 μm poliethersulfone (PES) membrane (Merck, Darmstad, Germany). Some of the chosen fungi, by their filamentous nature, become very viscous, so sometimes this was a challenge. To facilitate

the filtration, the suspension was first manually decanted into 250 mL centrifuge tubes, centrifugated at 13180 G at 4 °C for 20 min when necessary, and only then filtrated. All sterile supernatants were frozen and protected from light. For simplicity, they were used directly, without an extraction step.

3.3. Coculture test

To test whether there was a significant and easily observable production of an antimicrobial compound, a simple test was made by growing each fungus with a Gram-positive and a Gram-negative bacterium [131]. A volume of 100 µL of a suspension of either *E. coli* CECT 434 or *S. aureus* CECT 976 was spread on a 90 mm PCA plate (Oxoid, Basingstoke, United Kingdom), along with two plugs with the fungus to be tested. The optical density of the suspensions at 600 nm (OD_{600}) was adjusted to 0.15. The controls consisted of growing only the bacteria and only the fungi in each plate. After three days of incubation at 25 °C, or when the fungi appeared grown, it was recorded whether there was an inhibition zone around the mycelium.

3.4. Disk diffusion test

Similarly to the coculture method, suspensions of *E. coli* and *S. aureus* with an OD_{600} of approximately 0.15 were spread with swabs on 90 mm PCA plates. Paper disks 6.25 mm in diameter were placed on the plates and soaked with 15 µL of cell-free supernatants. After an overnight incubation at 37 °C, inhibition zone diameters (IZD) were measured. The negative controls consisted of fresh culture media, and the positive ones in an aqueous solution of ciprofloxacin (0.333 g/L), a broad-spectrum antibiotic.

Four different measurements corresponding to two independent experiments were made.

3.5. Identification of the active molecules in bioactive cell-free supernatants

The identification of the active molecules in the bioactive cell-free supernatant was made in collaboration with the MEDINA Foundation (Av. Conocimiento, 34 - 18016 Granada). The samples were lyophilized (VirTis BenchTop "K", ATS, Warminster, USA) and sent to them along with fresh culture medium. They were fractioned using Liquid Chromatography (LC) and the absorbance of the outflow was read at 210 nm. Two main peaks in absorbance, corresponding to two retention times were identified and tested using High-Resolution Mass

Spectrometry (HRMS) and UV Vis spectroscopy. The molecular masses obtained were compared to the Dictionary of Natural Products 31.2 database. According to those results and the taxonomy of the fungi, the UV Vis spectrum and the retention time were compared to standard solutions in the foundation of expected metabolites. For a definitive structure elucidation of the compounds, a scaled-up culture would be necessary, as HRMS does not distinguish between isomers and the other methods are not as exact.

3.6. Antibiofilm assessment

Contrary to the previous experiments, not all supernatants were tested for their antibiofilm properties, for time and equipment limitations. The supernatants to be tested were chosen based on previous positive results, bibliographic information, and an apparent abundance of secondary metabolite production. At least one supernatant of each species was tested, against Gram-positive and Gram-negative bacteria. After the optimization assays, the best supernatant was also diluted in distilled water, in concentrations of 3%, 6%, 12%, 25%, 50% and 100% (no dilution), to assess the effect of concentration in its antibacterial properties on a pre-stablished 24 hours aged biofilm.

The supernatants have an unknown composition, so it is hard to choose a valid control experiment. Mueller-Hinton broth (MHB) was the media used to grow bacteria, so the control consists of using this solution instead of the supernatant. However, the supernatants may be more nutritious to the bacteria than MHB (especially the YES broth) or may be nearly depleted of nutrients, after the fungi have been growing on them.

The experiments started by incubating 96 well microtiter plates (VWR, Radnor, USA) (with 200 μL/well of a suspension of *S. aureus* in MHB with an OD_{620} of 0.04 for 24 hours at 37 °C, under agitation of 160 rpm (KS 130 basic, IKA, Staufen, Germany). After this time, the liquid content of the wells was discarded and 100 μL of supernatant were added along with 100 μL of fresh MHB. The plates were further incubated for 24 hours in the same conditions, the liquid content was once again removed, and the wells were washed with 200 μL of sterile saline solution to remove non-adherent and weakly adherent cells.

To simplify notation, the supernatants are identified with [fungus species]/[culture media]/[incubation time]. The agitation used is not indicated, but it was 120 rpm unless otherwise stated. The species name was also abbreviated: C.s. – *C. spilospora*; C.c. – *C. coccodes*; T.s. – *T. saxoxalicus*; P.t. – *P. tunisiense*; T.a. – *T. aestuarinum;* D.p. – *D. phillipsii;* C.r. – *C. rubrum*; N.s. – *N. scalabiensis.*

Biofilm mass – crystal violet staining

The biofilm mass was quantified by crystal violet (Merck, Lisbon, Portugal) staining and absorbance measuring at 570 nm [132]. The cells were fixed with 250 µL of ethanol (VWR International, Rosny-sous-Bois, France) 99% (v/v) for 15 min. The ethanol was then removed, and the plate was left to dry. After that, 200 µL of a solution of 1% crystal violet was added and incubated for 5 min before being removed. The excess coloring solution was removed with paper and the wells were filled with an aqueous solution of glacial acetic acid (VWR International, Rosny-sous-Bois, France) (at 33% (v/v)) to solubilize the remaining crystal violet. After some time, the optical density was measured at 570 nm in a microtiter plate reader (FLUOstar Omega, BMG Labtech, Champigny-sur-Marne, France).

Results are expressed in the form of a percentage of biofilm mass removal (BMR) and calculated according to (1) [133].

$$BMR\ (\%) = (1 - \frac{\overline{OD_S}}{\overline{OD_C}}) \times 100 \quad (1)$$

Where OD_S is the average optical density of the wells exposed to a given supernatant, and OD_C is the same, but for the negative control wells.

Biofilm cells metabolic activity – Alamar Blue staining

The metabolic activity of the biofilm cells was assessed based on the Alamar Blue assay [134]. The liquid in the microtiter wells was removed and 190 µL of fresh MHB medium was added along with 10 µL of a solution of resazurin sodium salt (Sigma-Aldrich, St. Louis, USA) 400 µM. The plate was incubated for 20 min at 37 °C and the fluorescence was read, with an excitation wavelength of 570 nm and an emission wavelength of 590 nm, in a microtiter plate reader.

Results are expressed as biofilm cells activity reduction (BCAR) and calculated according to (2) [133].

$$BCAR\ (\%) = (1 - \frac{\overline{FL_S}}{\overline{FL_C}}) \times 100 \quad (2)$$

Where FL is the average fluorescence and, similarly to (1), the indexes S and C correspond to the supernatant and the negative control.

Biofilm culturable cells

The number of culturable cells was quantified by scraping and washing the wells with sterile saline solution three times (1 min each, using 200 μL of NaCl 8.5% (w/v)) and collecting the suspension obtained. A volume of 400 μL of saline solution were further added and the suspension was vortexed for 3 minutes, to break potential cell aggregates. Then, 10-fold successive dilutions were performed.

Two 10 μL drops of each dilution were placed on PCA medium and the number of colony-forming units (CFU) was visually counted after an overnight incubation at 37 °C. The value used was the highest possible to count among the dilutions, preferably if 10 < CFU < 200 [135].

The results were expressed as a logarithmic reduction of CFUs, according to (3):

$$\log(CFU)\,reduction = \overline{\log\left(CFU_S \times 10^{Ds}\right)} - \overline{\log(CFU_C \times 10^{Dc})} \qquad (3)$$

Where *CFU* is each individual CFU count, and *D* is the dilution at which it was read.

3.7. Combinatorial effect of active supernatants and antibiotics

The antibacterial properties of the most effective supernatant were tested in combination with various commercial antibiotics, against *E. coli* and *S. aureus*. That supernatant was first lyophilized, and due to time constraints, its selection did not take into consideration the results obtained from WATM, MME, CYAS, or PDC, nor the effect of aeration and agitation, as they only later became available.

The IZD of the control group – a solution of antibiotic (IZD_a) – and its combination with the supernatant (IZD_{a+S}) were obtained according to the disk diffusion test method already described in 3.4. The control group consisted of solutions of eight antibiotics, from different classes. The antibiotic solutions used were ciprofloxacin (333 mg/L), tobramycin (667 mg/L), fusidic acid (667 mg/L), erythromycin (1 g/L), chloramphenicol (2 g/L), methicillin (333 mg/L), tetracycline (2 g/L), and mupirocin (13.3 g/L). Additionally, a solution of the supernatant at a concentration of 1 g/L was also tested. The combination test consisted of the disk diffusion assay of the same antibiotics, but in solid plates enriched with the supernatant, at 100 mg/mL [136].

The interaction between the combination was classified as potentiation ($IZD_{a+S} - IZD_a$ > 6 mm), additive (6 mm > $IZD_{a+S} - IZD_a$ > 4 mm), indifferent (4 mm > IZD_{a+S} - IZD_a > -6 mm) or negative (-4 mm > IZD_{a+S} - IZD_a) [137].

The most promising interaction, e.g., the combination of antibiotic and bacterial species, whose IZD increased the most when the supernatant was present, was further tested in a biofilm scenario, similarly to 3.6, with the only differences relating to the application of the solution intended to be tested. The effect of the antibiotic was tested at the MIC, while the supernatant was tested at 10 mg/L, 100 mg/L, and 1 g/L. The effect was also tested by combining both, at those same concentrations. In total, 20 μL of either, or 10 μL of each solution was added, instead of the previously mentioned 100 μL, as this allowed a closer comparison to the control group, and the use of the lyophilized supernatant allows changing the concentration without changing the volume.

3.8. Statistical analysis

All results are expressed as Average ± Standard error and *p*-values were calculated through the t-student two-tailed test, assuming heteroskedasticity. They were considered significant for $p < 0.05$.

4. Results and Discussion

4.1. Filamentous fungi cultivation

During the cultivation of the various species in solid appropriated culture media, some different features of the various fungal species became apparent, as is shown in Figure 3. For instance, *T. aestuarinum* grew much faster than the remaining species, but only produced mycelium; *P. tunisiense* barely grew outside the inoculation plug but produced many spores; *C. spilospora* developed better near the edges of the plate and also did not produce many spores; *C. coccodes* produced some very dark spores; *T. saxoxalicus* started to produce a red pigment; *N. scalabiensis* grew very heterogeneously, involving parts with and without spores; *C. rubrum* produced a red pigment that quickly diffused through the plate, but it faded over time; and *D. phillipsii* grew a bright white mycelium, with black spots slowly growing.

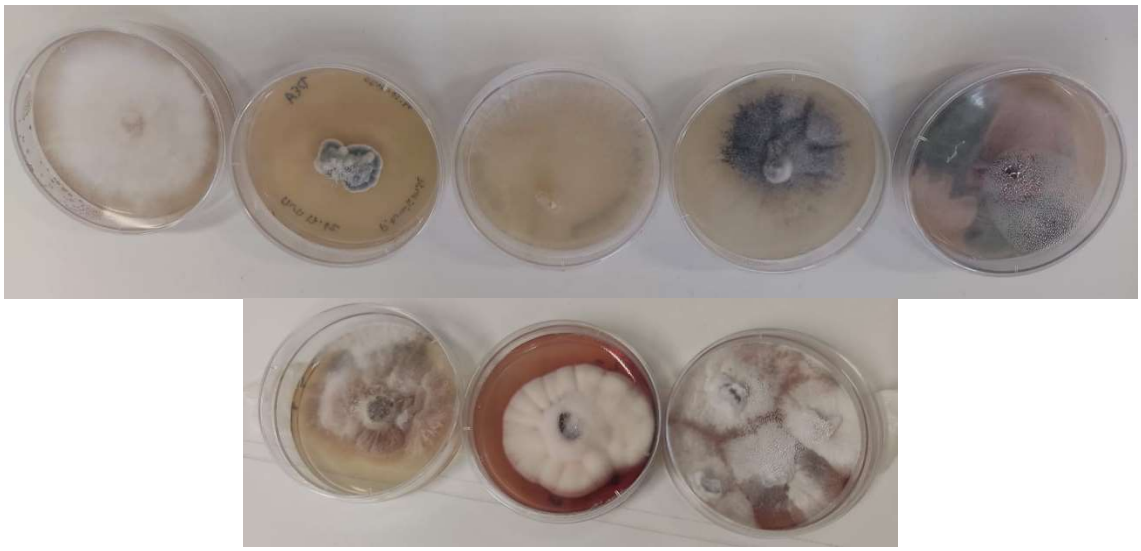
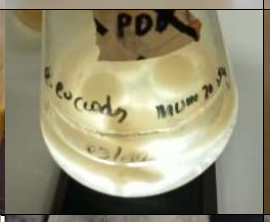
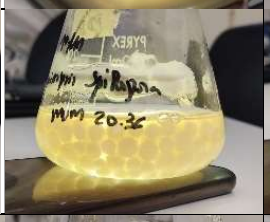
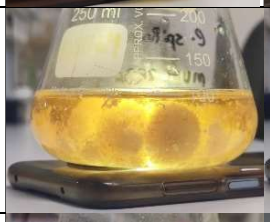
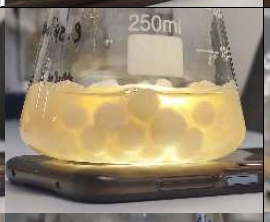
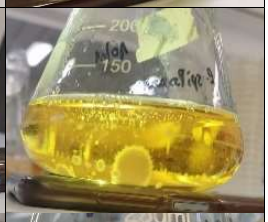
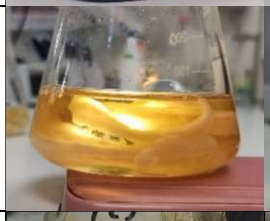
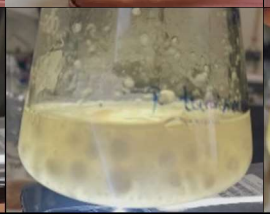


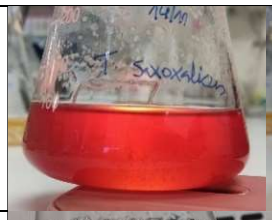
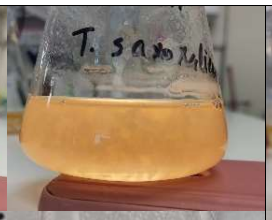
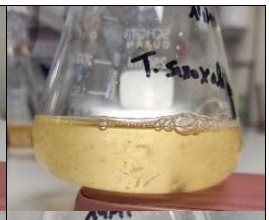
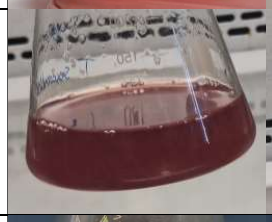
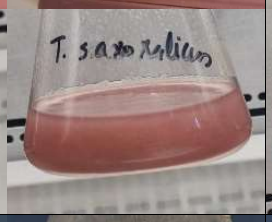
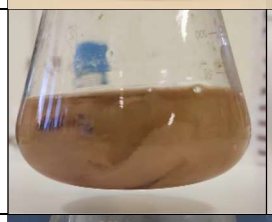
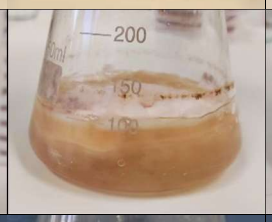
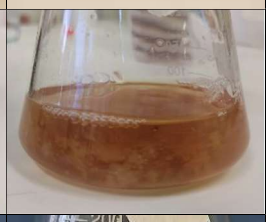
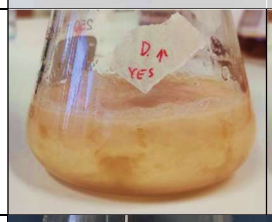
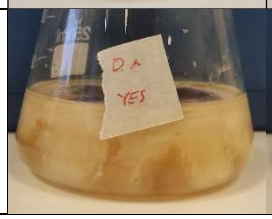
Figure 3. Solid state growth of the filamentous fungi used in appropriate culture medium. From left to right, on top: *T. aestuarinum, P. tunisiense, C. spilospora, C. coccodes,* and *T. saxoxalicus.* And on the bottom: *N. scalabiensis, C. rubrum,* and *D. phillipsii.*

4.2. Cell-free supernatant isolation

The different culture conditions generally induced different morphologies from the fungi, making it plausible that different secondary metabolites were produced. In Table 3, photographs of the Erlenmeyer flasks as a result of deep fermentations of several filamentous fungi in different culture media for two incubation times and biomass are presented.

Table 3. Appearance of deep fermentation flasks after the incubation times (7 and 14 d) for the culture media (YES, PDB and LB) for the eight filamentous fungi tested.

Fungal species	7 d	Medium		
		YES	PDB	LB
Trichoderma aestuarinum	7 d			
	14 d			
Colletotrichum coccodes	7 d			
	14 d			
Coprinopsis spilospora	7 d			
	14 d			
Penicillium tunisiense	7 d			
	14 d			

Talaromyces saxoxalicus	7 d			
	14 d			
Neopestalotiopsis scalabiensis	7 d			
	14 d			
Cladosporium rubrum	7 d			
	14 d			
Diaporthe phillipsii	7 d			
	14 d			

Although the observation of distinct characteristics of each fermentation is somewhat beyond the scope of this project, some of them are useful later, when choosing the most promising results, and potentially for future works.

- *T. aestuarinum*: This fungus grew at a very fast rate, especially in PDB and YES media. Before the 7th day, there was already a large amount of biomass and at the 14th day the medium was significantly clouded by possibly dead cells. The aeration may have been a limitation on the growth as there was a thick layer of cells near the air interface when using these two media.

- *C. coccodes*: This species generally grew in large spheres filled with liquid. Its most notable characteristic was the production of a sticky polymer made of very long filaments, even visible to the naked eye under certain conditions. It was also apparent when discarding the supernatant from a syringe. This was only noted clearly for the YES media but may have happened to a lesser extent in PDB.

- *C. spilospora*: The most notable observation made in this case was that the growth was very slow in LB media but was also not very good in YES media. Only in PDB fast, effective growth was observed. It is important to note that this fungus was growing on potato dextrose agar (PDA), which has the same composition as PDB except for the agar. Its growth on plate count agar (PCA) (required for the assay in 3.3) was not particularly slow either, suggesting this species either does not deal very well with most liquid media and prefers an agar support, or it is somewhat temperamental and there is an overlooked, relevant, experimental parameter influencing the growth.

- *P. tunisiense*: Despite its slow growth on solid media, this *Penicillium* grew very well in all three liquid media, exhibiting significantly different morphology in each. While in YES broth it grew in elongated shapes, in PDB it grew in a mixture of those same shapes and spheroids. In LB, it also grew as spheroids, but those had much more hair-like structures on the surface that significantly increased surface area. Other experiments developed turbidity on the medium and a flask even became black with the amount of spores produced, but at the time, it was wrongly assumed that it was a contamination, and the results, unfortunately, could not be later reproduced, and so were discarded. Most secondary metabolite production happens when the fungus is starting to produce spores [44], so the remaining results associated with this fungal species and medium could have been different if this supernatant was used instead.

- *T. saxoxalicus*: The most striking characteristic of the fermentation broths obtained with YES media was their bright red color. This is a well-known characteristic of many species in this genus [138], even making them potentially industrially competitive for dye production [139]. The color obtained on the 7th day in the PBD medium may derive from a different pigment, or it may be the same, just at a much lower concentration. In the 14th day result, there

is a much more similar color between the YES media and PDB. The LB did not appear colored at all. Copper is usually involved in the production of redox pigments [45] and the only medium enriched with cupric salts was YES. Another observation that may not be evident from the pictures is the large amount of small translucid spheres in all three mediums, although in LB they had less homogenous shapes. They were so small it could hardly be avoided for them to end up in the centrifuge flasks during decantation, but the centrifugation was effective at forming a solid pellet.

- *N. scalabiensis*: This fungus rapidly developed a large amount of biomass in all media, but especially in YES. There was essentially no supernatant available, even on the 7th day and most of the used flask's volume was occupied with a dense structure. In LB, this did not happen, although there still were large, hairy, clumps and a lot of smaller filamentous aggregates in suspension. The agitation proved itself insufficient, as the biomass was so compact that it stopped moving at that speed. This aggravated the problem, as the cell mass was not further broken up, and on the 14th day, in PDB, dry cells were visible at the surface, in the center of the flask, which is common when there is no agitation.

- *C. rubrum*: Although the production of large amounts of pigment was expected, given that's what happened in PDA, this was not observed in any of the tested media. Only a brownish red color was observed but it was not very intense. The fungus developed on the surface but unlike other species that did the same, it stuck there tightly, even after agitating the flask, especially after the second week on YES medium. It grew well, but not especially so, and so it is likely that it has a strong preference for solid media or fixed supports, rather than an unmet oxygen demand.

- *D. phillipsii*: Different than the other species which overgrew, the aggregates fused in a spongy mass that could not even pass through the flask's opening, in PDB and especially YES. This mass contained air bubbles and the boundaries of the original clumps were still perceptible after the first week. In those cases, agitation was not effective as the single clump was too big to move around much. Little liquid was able to be recovered as supernatant. The filtration was not particularly difficult, as most biomass was aggregated, and the suspension was mostly clear. In LB, the mycelium formed very irregular clumps, that collapsed once out of suspension.

4.3. Qualitative assessment of fungal species potential for antibiotic production

The qualitative assessment of fungal species potential for antibiotic production was done through the co-culture method. The only species tested that inhibited bacterial growth was *C. spilospora*. It showed a clear halo around the mycelium when cultured with *S. aureus*. In one of the assays, it also inhibited *E. coli* from growing very close to the outermost hyphae, but repetitions of the experiment were unable to reproduce these results. Similarly to the growth in deep fermentation, where this fungus has also shown itself to be relatively sensitive to different conditions, namely the culture medium, a small adjustment in an overlooked experimental parameter may have caused the production, even in small quantities of a different metabolite, or the same metabolite that inhibited the Gram-positive, just at a higher concentration. No prior published literature was found regarding the production of an antibiotic or antibacterial substance by *C. spilospora*, despite the fact that this species was described many decades ago [140]. However, it has been stated that many other species of this genus, are able to produce some molecules, like *C. strossmayeri* that produces bovistols and strossmayerin [103], *C. episcopalis* synthesizes illudins [141] and *C. cinerea*, that produces lagopodin B [46], coprinol [142,143] and copsin [77], among others. The results related to *T. aestuarinum*, *P. tunisiense*, *T. saxoxalicus*, *C. coccodes* and *C. rubrum* were relatively unremarkable, although *D. phillipsii* and *N. scalabiensis* eventually made the proximity of the inoculation plug slightly clearer than the remaining plate. Photographs of the cultures are in Appendix C.

4.4. Evaluation of antibacterial properties of fungal supernatants

The evaluation of the antibacterial properties of the supernatants followed the disk diffusion test protocol and showed results in line with the co-culture test ones, as presented in Table 4. In the initial set of conditions, only the supernatant of *C. spilospora* grown on PDB showed effect, probably because this medium was the most favorable for its growth and this is when larger amounts of secondary metabolites are produced [4]. Both incubation times yielded significant positive results against *S. aureus*: the inhibition zone diameter was 14 ± 2 mm for 7 days of incubation and 8.8 ± 1.0 mm for 14 days, compared to the 6.25 mm of the control.

Table 4. IZDs (mm) of cell free supernatants, after the incubation times, for the eight fungal species, fermented in the different media, against *S. aureus* and *E. coli.*

Fungal species	**Bacterial species**	***E. coli***		***S. aureus***	
	Day	**7 d**	**14 d**	**7 d**	**14 d**
Trichoderma aestuarinum	YES	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	PDB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	LB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
Colletotrichum coccodes	YES	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	PDB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	LB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
Coprinopsis spilospora	YES	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	PDB	6.25 ± 0	6.25 ± 0	**14 ± 2 ***	**8.8 ± 1 ***
	LB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
Penicillium tunisiense	YES	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	PDB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	LB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
Talaromyces saxoxalicus	YES	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	PDB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	LB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
Neopestalotiopsis scalabiensis	YES	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	PDB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	LB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
Cladosporium rubrum	YES	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	PDB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	LB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
Diaporthe phillipsii	YES	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	PDB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	LB	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
Unfermented Media	YES	6.25 ± 0		6.25 ± 0	
	PDB	6.25 ± 0		6.25 ± 0	
	LB	6.25 ± 0		6.25 ± 0	
Ciprofloxacin (0.333 g/L)		**27.5 ± 0.6 ***		**29.8 ± 0.5 ***	

* The IZD is significantly different from the control ($p < 0.05$)

The decrease in the inhibition zone diameter with the longer time may indicate that the active compound starts to degrade after some time. The yield of antimicrobial compounds produced by *Coprinopsis*, like illudins, is reported to decrease in older mushrooms [144]. Those same supernatants against *E. coli* did not produce a measurable effect. The inhibition in the other supernatants of *C. spilospora*, the other fungi species, and the negative controls was inexistent, against both bacterial species. Another important visual observation related to the negative control was that a single 15 μL drop of culture media increased the cellular density

around the diffusion disk, especially in the case of YES in *S. aureus*. Photographs of the inhibition halos are presented in Appendix D.

4.5. Identification of active molecules in bioactive cell-free supernatants

The supernatant of *C. spilospora* was the most interesting to be analyzed, according to the results obtained in 4.4. Two peaks were found in the LC trace of the *C. spilospora* supernatant: P1, at 1.46 min, and P2 at 2.00 min. Such trace is presented in Figure 4.

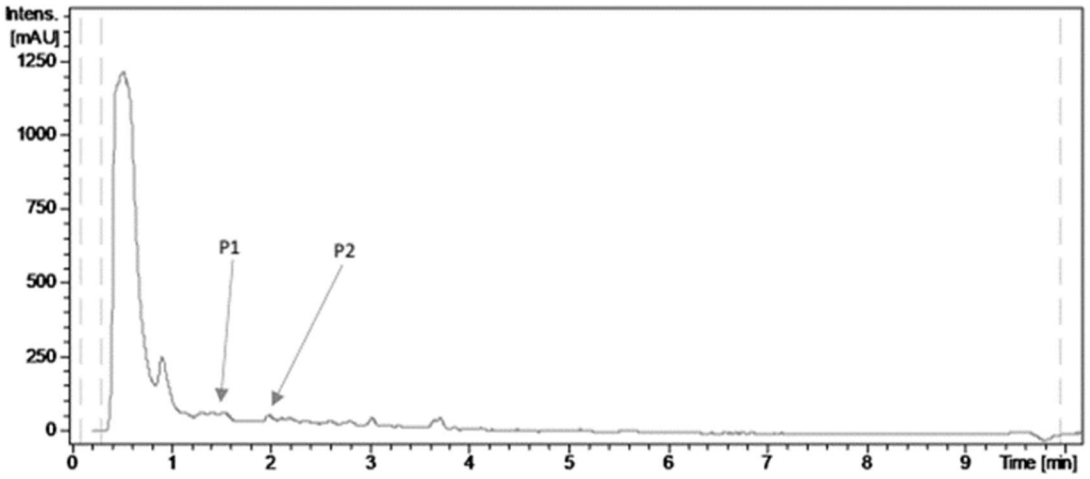


Figure 4. Absorbance reading of the LC outflow of the supernatant, at a wavelength of 210 nm, with the two peaks, P1 and P2 highlighted.

The absorbance spectra in the UV Vis range of those peaks were analyzed and are presented in Figure 5, along with the HRMS peaks.

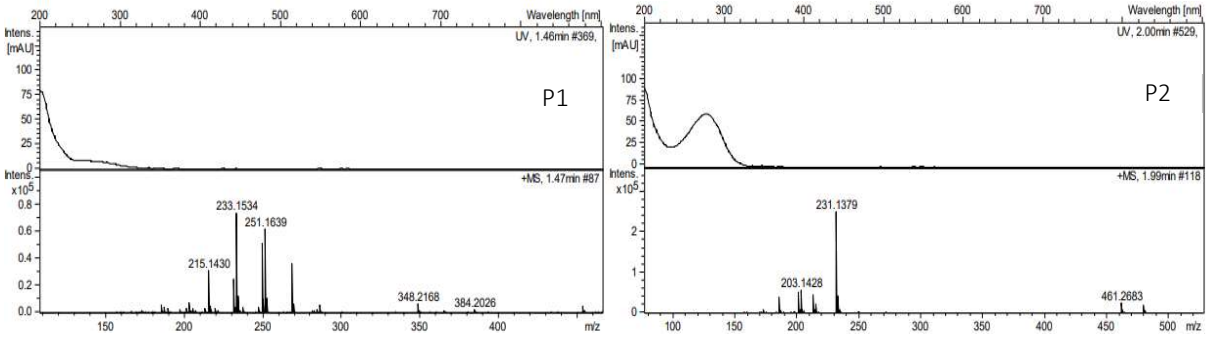


Figure 5. UV Vis absorption spectrum (top) of peaks P1 (left) and P2 (right) along with HRMS peaks (bottom).

The peak P1 most likely corresponds to dihydroilludin M, represented in Figure 6, as according to the MEDINA Foundation results, its retention time and UV spectra are similar to

a standard solution of this compound. The HRMS signal points to a molecular formula of $C_{15}H_{22}O_3$, which corresponds to 1304 known natural products, 7 of which are from the illudin family, and include dihydroilludin M, according to the Dictionary of Natural Products 31.2 database [43,145].[43,145]. Illudins have a known activity against Gram-positive and Gram-negative bacteria, as well as against tumor cell lines and viruses. They are believed to mainly target DNA synthesis and damage it in a very characteristic way. Dihydroilludin M is less toxic than other members of the class and shows an improved therapeutic index [144,146,147].[144,146,147]. The reported minimum inhibitory concentrations (MIC) of analytical grade illudin I_2 and illudin J_2 are 300 μg/mL and 150 μg/mL against *S. aureus* and *E. coli*, respectively [141]. Their use as antibiotics has been hindered mainly by their toxicity. The lethal dose of illudin S in mice was determined to be only 5 mg/kg, although other compounds of this class are less toxic, and their derivatives have entered clinical trials in other therapeutic areas [144,148].[144,148]. It is reported that a corn steep agar medium, when compared to PDA, substantially increases the activity of *Clitocybe illudens*, a species with antibacterial properties that produces illudins S and M [149]. This information goes against what was observed for the growth of *C. spilospora* in PDC, where it barely grew. Although there are many differences between both cases, this suggests that either that fermentation was unsuccessful because of an unpredicted factor (like the viability of the inoculum or the production of toxic compounds when combining CSL solids and PDB), or that other compounds are contributing to the antibacterial properties of the supernatant.

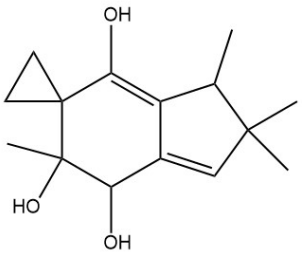


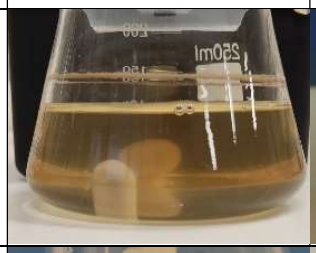
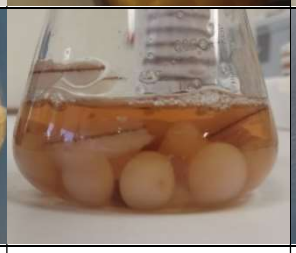
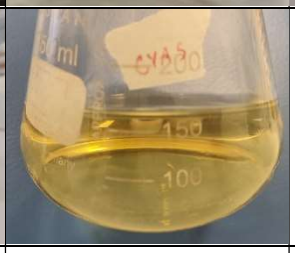
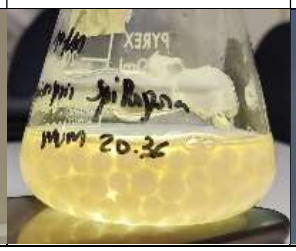
Figure 6. Dihydroilludin M.

4.6. Optimization of active cell-free supernatant production

C. spilospora

To increase the activity of the supernatant which showed antibacterial effect – *C. spilospora* grown on PDB, especially when grown for 7 days – additional supernatants were isolated, varying other culture conditions. Four additional culture media were tested, – WATM, MME, CYAS, and PDC – agitation speed was increased to 180 rpm and decreased to 60 rpm, and active aeration was performed. These results are presented in Table 5.

Table 5. Appearance of deep fermentation flasks after the incubation times (7 and 14 d) in different culture media (WATM, MME, CYAS and PDC), and after 7d in PDB under different agitation speeds (60, 120, and 180 rpm) and active aeration, for *C. spilospora*.

Condition		WATM	MME	CYAS	PDC
Culture media	7 d				
	14 d				-
Agitation (rpm)		60	120	180	120 (with aeration)
Agitation and aeration					

The fermentation in WATM was slower than the remaining. The medium became clearer over time indicating that the suspended solids of the CSL were progressively consumed, which is normal to happen at a slower rate when compared with solubilized nutrients [62]. The growth in MME was similar to that of PDB, the best of the original three media, although some aggregates had a hairier surface. In both PDC and CYAS, the fungus did not grow outside the inoculation plug. In PDC, a solid precipitate was formed while autoclaving. No definitive explanation was found for this, but the *Maillard* reaction does produce several brown-colored, toxic compounds by combining reducing sugars, such as dextrose, and amine nitrogen, abundant in CSL, and is favored by high temperatures [150,151].[150,151]. If it was the case, the mechanism by which it happened is still unclear as CSL and glucose are commonly used together [4]. The systematization of the effect of culture media on the growth and antibiotic production of *C. spilospora* is presented in Appendix E.

The increase in agitation intensity reduced the sphere's size and increased their number, which in turn increased the interface area of the aggregates, as it is reported in the literature [67]. This may have increased the concentration of relevant antibacterial metabolites in the supernatant. Lower agitations were observed to induce the production of much less biomass. The visual assessment is important but does not substitute activity comparisons, as it is common for the optimum agitation for biomass production to be higher than the optimum for metabolite production [68].

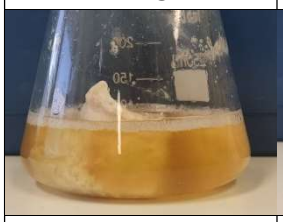
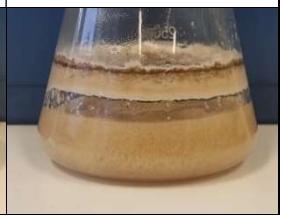
The aeration rate used was not measured but was kept low to avoid excessive evaporation. Excluding the foam formed, no significant differences could be observed between the aerated and unaerated flasks.

D. phillipsii

Optimization of *D. phillipsii* fermentation was more challenging than *C. spilospora*, as no initial set of conditions could be taken as an optimized starting point. As such, and because the initial agitation seemed insufficient, the fungi grew on all previous media, WATM, MME, CYAS, PDC, and PDB supplied with Tween 80 using an agitation speed of 180 rpm, for 14 days. These results are presented in Table 6.

The increase in agitation speed appeared to be beneficial for the fungus development. The spongy mass in YES medium was now split into a few angular clumps, although they were still large, and the liquid was still very clear. All media with a PDB base had a similar appearance, with very small clumps, unlike what happened at a lower agitation, and a light color. At this agitation speed, the addition of a surfactant was not needed, as the clump size was already very small, but it could perhaps have been helpful if it was added to YES medium instead, to further reduce the size of the clumps [80]. The growth in LB was not very much affected by agitation, but the clump size was slightly smaller using 180 rpm. The cultures grown in WATM and MME became very dark, but, after filtration, the supernatant was actually of a similar color to the initial culture medium. A lot of dispersed biomass was formed, and it had a black color, possibly due to sporulation. In CYAS medium, the mycelium was also dispersed, but kept its white color for the duration of the fermentation, as it had a slow growth in the first few days.

Table 6. Appearance of deep fermentation flasks after the incubation time (14 d) in different culture media (YES, PDB, LB, PDB + Tween 80, WATM, MME, CYAS and PDC), for *D. phillipsii*.

YES	PDB	LB	PDB + Tween 80
			
WATM	**MME**	**CYAS**	**PDC**
			

4.7. Antibiofilm properties assessment

The results of the planktonic tests were important tools when choosing which supernatants to test. Although testing all supernatants would be desirable, the assays are very time-consuming and that was not possible. The supernatants used were the *C. spilospora* grown on PDB, for 7 and 14 days, to assess how active they were against *E. coli* and *S. aureus* biofilms; *C. coccodes* on YES broth, as it also produced an interesting extracellular polymer that could be bioactive; *T. saxoxalicus* on YES broth and PDB, because of their evident pigment production, that is reported to have antibacterial properties in other species of the same genus [138]; *P. tunisiense* in all media as this species grew well in them, and to avoid missing on a metabolite that is antibiofilm but not antimicrobial, as is reported in other *Penicillium* species [152]; *D. phillipsii* on PDB as it grew better on it than on LB and the sample volume obtained from YES might have been insufficient for all assays, and for a 14 days fermentation, as the fungus still appeared healthy and the co-culture test pointed towards longer growth results; *N. scalabiensis* on LB as it appeared to have altered the cells in disk diffusion test, even without inhibiting their growth; *C. rubrum* on LB, because that is where its characteristic red pigment was most notable; and *T. aestuarinum,* to test at least one supernatant of each species, on YES broth, as this appeared to have been the media where it grew the best.

Biofilm mass removal quantification

Biofilm mass removal results are shown in Figure 7. For *S. aureus,* the *C. spilospora* supernatants resulted in a biomass removal of 78 ± 9%, when fermented for 7 days, by 68 ± 9%, for 14 days fermentation, and by 78 ± 20% for 7 days fermentation with increased agitation. The *D. phillipsii* supernatant also significantly decreased biofilm mass by 48 ± 10%. The *D. phillipsii* and *N. scalabiensis* supernatants removed E. *coli* biofilms mass by 22 ± 20% and 35 ± 18%, respectively. All other supernatants showed either no biomass removal effect or significant promotion of the biofilm mass.

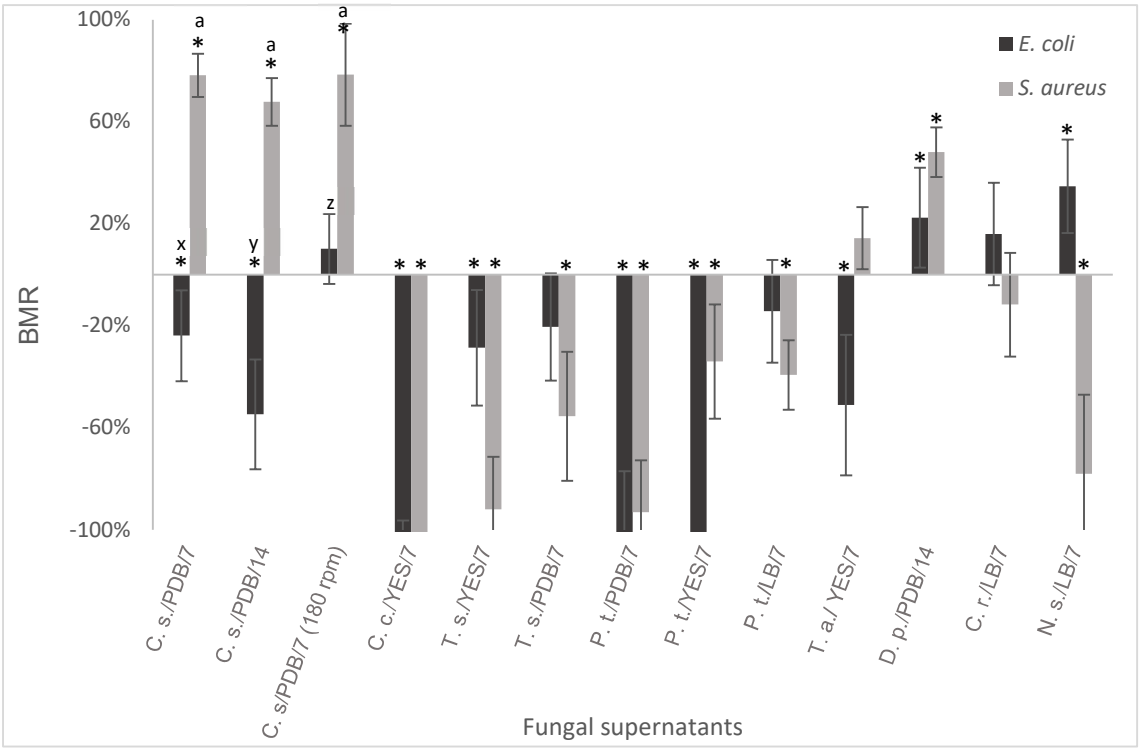


Figure 7. BMR (%) of the tested supernatants. Mean ± standard deviation are illustrated. An * denotes results significantly different from the control (MHB). For the *C. spilospora* supernatants, values marked with the same letter are not statistically significant different.

The cases where the biofilm mass increased were somewhat unexpected, but there is evidence of fungi that produce extracellular metabolites that promote bacterial growth [153]. Another explanation for these results is that the growth media (present in the fungi supernatants) was far from being depleted and it promoted a more effective biofilm growth, unrelated to any metabolites produced by the fungi. It is important to note that YES broth has a much higher nutrient content than MHB. This explanation is unlikely, however, because fresh PDB has an overall nutrient content similar to MHB and although it is described as suitable to cultivates bacteria by its producer, its low pH, 4.8 ± 0.5, should not favor bacterial growth [154]. LB media is also suitable to grow bacteria but is reported to not increase the bacterial biofilm-forming ability when compared to MHB [155]. The *C. coccodes* supernatant increased biofilm mass the most, with a BMR much lower than the other supernatants with a YES base (e.g., *T. saxoxalicus*, *P. tunisiense*, and *T. aestuarinum*) ($p < 0.05$). In fact, the results are probably even more extreme, as the reading assembled slightly outside the range of the measuring equipment. Extracellular polymers in the supernatant may be related to this effect, aggregating bacteria and thus increasing the biofilm mass [156]. The mass of the polymer itself may have made a significant difference, being a substantial part of the biofilm.

For the *C. spilospora* supernatant against *S. aureus,* increasing the fermentation time or the agitation speed did not produce a significant effect. The results, however, may be saturated, meaning that an increase in the activity of the supernatant would not have produced an increase in the measured biomass removal. For *E. coli*, the 7 days fermentation supernatant did not promote biofilm formation as much as the 14 days one. For increased agitation, the result was even better, promoting some biomass removal in comparison to the control. Although such reduction was not significant, the difference between the two agitation speeds can now be better evaluated. Indeed, it is evident that antibacterial activity is higher when the fermentation occurs at 180 rpm. No source of information has been found to report on this effect by a supernatant of this species. Other works where deep fermentation was used to produce antibacterial metabolites are also not a very good comparison, as the molecules produced and their concentration differs completely according to the species of fungi, and culture conditions. Comparing the reduction in biofilm mass tells nothing about what caused this effect. Another limitation is that many studies on the topic, instead of using the supernatant directly, make an ethyl acetate or methanolic extract out of it, significantly changing the concentration of active molecules [157,158].

The determination of the concentration effect of the most effective supernatant, which was obtained from *C. spilospora* grown on PDB for 7 days under agitation of 180 rpm, is presented in Figure 8. The BMR results for *S. aureus* vary in a relatively narrow range (from 57 ± 12% to 74 ± 10%, for the supernatant concentrations of 3% and 100% respectively). This was probably caused by the water used to dilute the supernatant, that exposed the cells to a medium less nutritious than the control group of MHB. Even if the supernatant has no effect at low concentrations, the biofilm mass may thus apparently decrease. However, the fact that unfermented PDB promotes expressive biofilm growth and the undiluted supernatant showed significantly less biomass than the water proves that the supernatant can decrease *S. aureus* biofilm mass. The results obtained for *E. coli* are not as clear, but it appears that the supernatant was not very effective at removing the biofilm, as the obtained BMR increases with increased dilution.

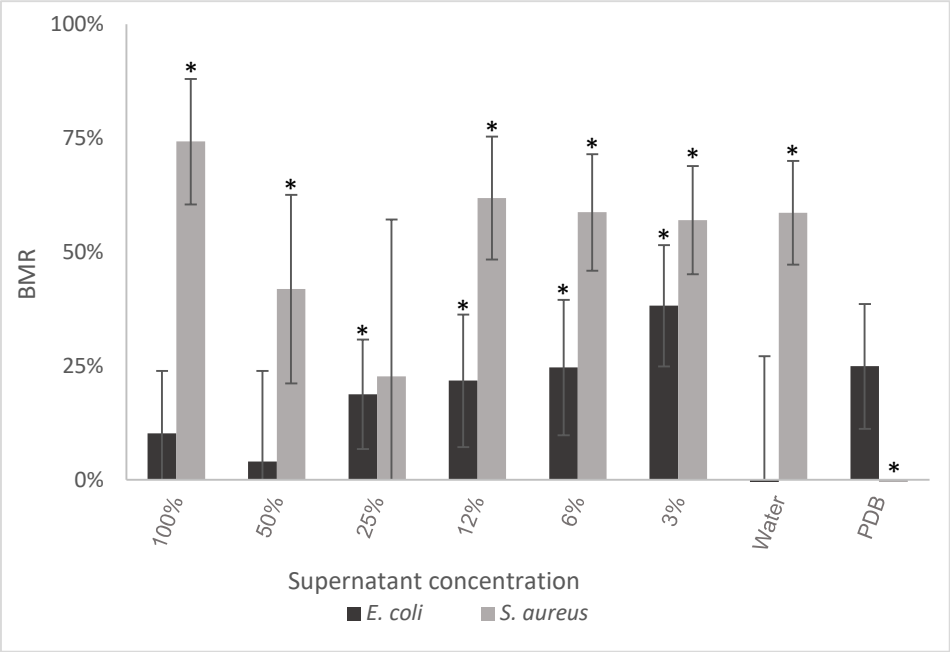


Figure 8. Effect of increased supernatant concentration in BMR, for *E. coli* and *S. aureus*. Mean ± standard deviation are illustrated. Controls include distilled water and unfermented PDB. An * denotes results significantly different from the control (MHB).

Biofilm cells metabolic activity reduction quantification

Biofilm cells metabolic activity reduction measured after 20 min of incubation is presented in Figure 9. The 7 days supernatant of *C. spilospora* grown on PDB had high BCAR for *S. aureus* 96 ± 7%; as well as the 14 days one 93 ± 8%. The higher agitation speed further increased metabolic activity inhibition, to 101 ± 8%. It should be impossible to reduce metabolic activity any more than 100%, which corresponds to no cells being present, but that difference is contained in the error associated to the value. No significant difference was found between the BCARs of that supernatant and the sterile culture medium ($p > 0.05$). Although the differences between the BCARs of the different *C. spilospora* supernatants are relatively small, they are all statistically significant. The *D. phillipsii* supernatant decreased metabolic activity by 60 ± 15%, which is still a very promising result, although more modest. In the *E. coli* biofilm, only one of the *C. spilospora* supernatants - the one produced at higher agitation speed - reduced the metabolic activity by 41 ± 9%. The supernatants of *D. phillipsii, C. rubrum,* and *N. scalabiensis* also caused reductions of 53 ± 9%, 30 ± 13%, and 54 ± 9%, respectively. All other supernatants either promoted bacterial metabolic activity or showed no effect.

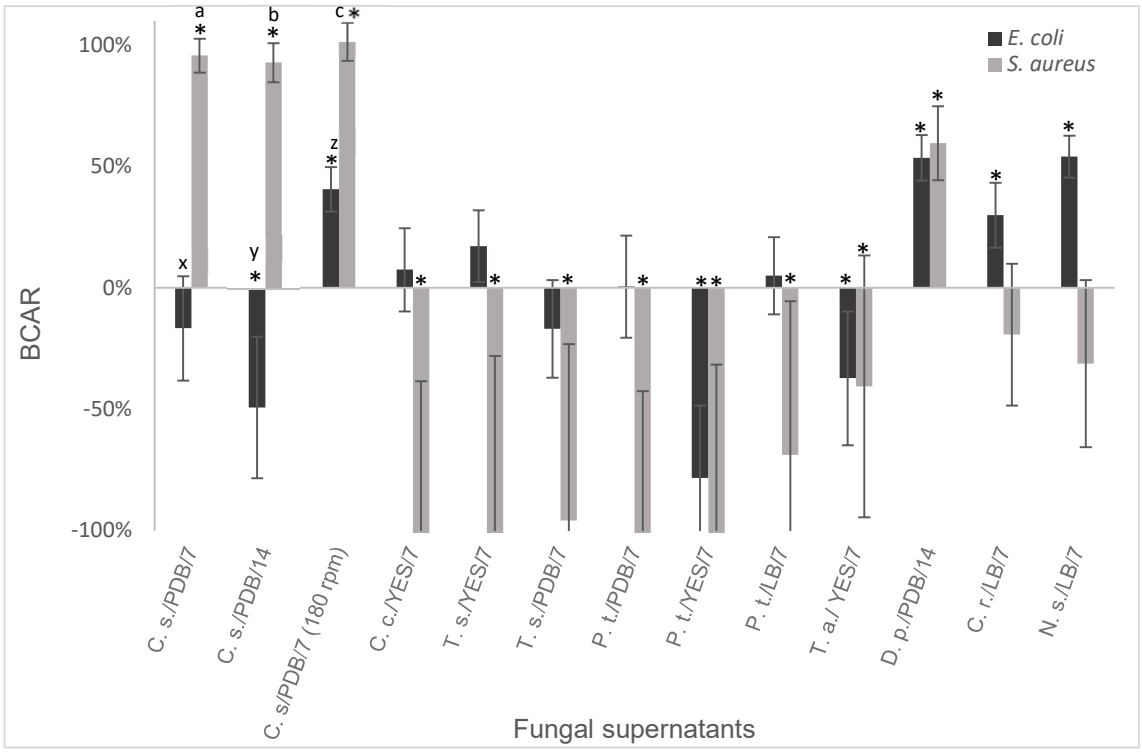


Figure 9. BCAR (%) of the tested supernatants. Mean ± standard deviation are illustrated. An * denotes results significantly different from the control (MHB). For the *C. spilospora* supernatants, values marked with the same letter are not significantly different.

Once again, the increase in effectiveness for higher agitations may be due to increased homogeneity of the culture media or higher surface area available for metabolite excretion from the fungal clumps [68]. The activity decrease for a longer fermentation may be due to decreased yield over time coupled with the degradation of the molecules [144].

The effect of the most effective *C. spilospora* supernatant at various concentrations is presented in Figure 10. Once again, the BCAR obtained for *S. aureus* varies in a somewhat narrow range (55 ± 17% to 101 ± 9% for the least and most diluted supernatants respectively), but it shows that the supernatant is indeed effective at inhibiting the biofilm cells metabolic activity. The effect of the supernatant in *E. coli* metabolic activity is not very meaningful, as BCAR essentially did not change with the supernatant concentration.

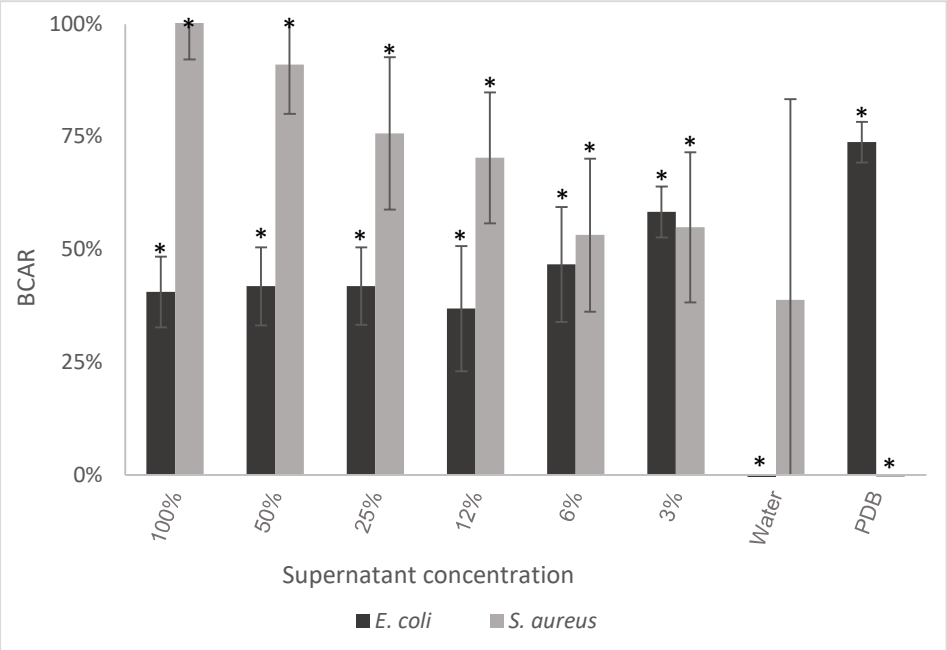


Figure 10. Effect of increased supernatant concentration in BCAR, for *E. coli* and *S. aureus*. Mean ± standard deviation are illustrated. Controls include distilled water and unfermented PDB. An * denotes results significantly different from the control (MHB).

Biofilm culturable cells

The logarithmic reduction values of the number of biofilm culturable cells is presented in Figure 11. Somewhat expectably considering the previous results, some supernatants increased the number of biofilm culturable cells, like the one from *C. coccodes*. The three *C. spilospora* supernatants significantly decreased the number of culturable *S. aureus* cells, with 1.5 ± 0.4 log (CFU) reductions, for the 7 days fermentation and 1.55 ± 0.12 log (CFU), for the 14 days one, and 1.8 ± 0.2 log (CFU) for the one obtained at higher agitation speed. No condition was found to be significantly different from the 7 days fermentation at a lower agitation speed. For *E. coli*, the 14 days fermentation supernatant did not reach statistical significance for culturable cell reduction, but the other two did, with reductions of 0.85 ± 0.06 log (CFU) and 1.5 ± 0.4 log (CFU) for the 120 rpm and 180 rpm, respectively.

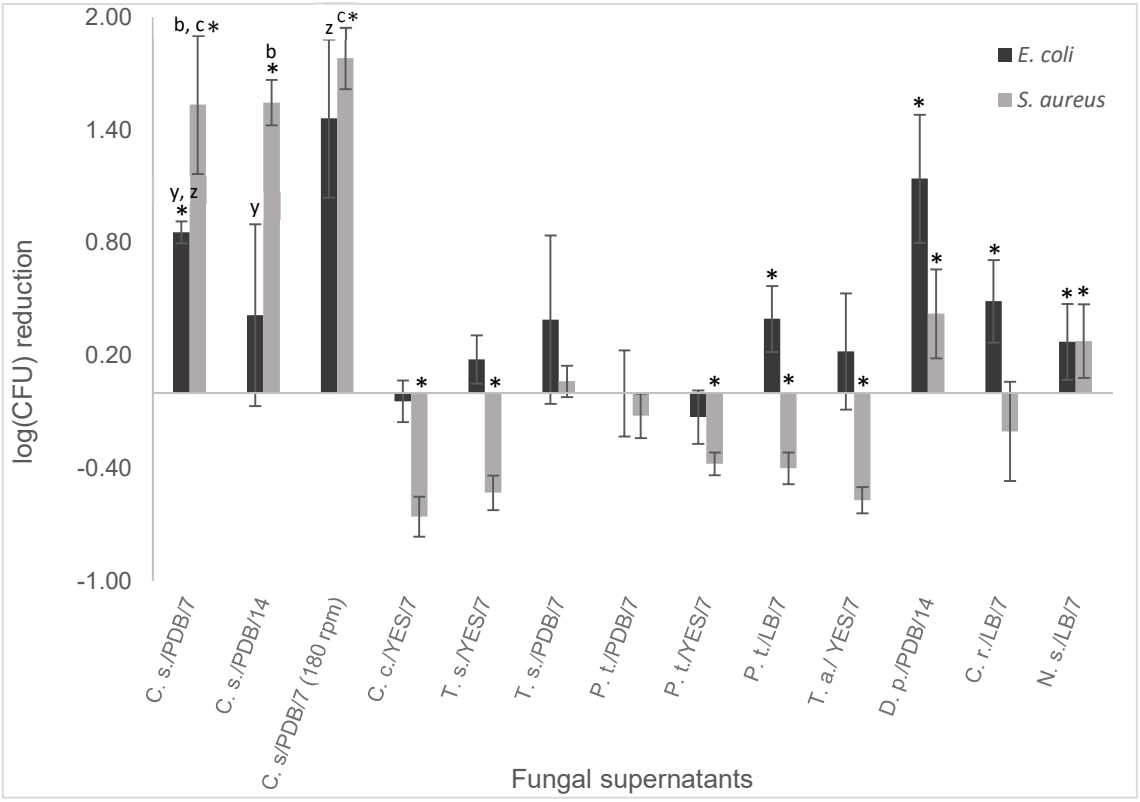


Figure 11. Log (CFU) reductions of *E. coli* and *S. aureus* biofilm cells after 24 h of exposure to the selected fungi supernatants. Mean ± standard deviation are illustrated. An * denotes results significantly different from the control (MHB). For the *C. spilospora* supernatants, values marked with the same letter are not significantly different.

The reduction in biofilm culturable cells was, overall, according the biofilm mass reduction and the cells metabolic activity reduction results, as essentially the same supernatants were effective against the biofilm cells.

The effect of increased concentration on the culturable cell count is presented in Figure 12. The effect of the supernatant concentration does not change linearly, but that may be due to competing effects, like a molecule that promotes biofilm growth in addition to the antibacterial one [153,159]. Those molecules may produce measurable effects at different concentrations, so an in-depth study of the effect of the supernatant concentration may require smaller dilution intervals. For the most diluted supernatant, the inhibition obtained is in line with the inhibition for distilled water, especially in the quantification of biofilm culturable cells, the most reproducible biofilm assay. This indicates that further dilutions would probably be pointless.

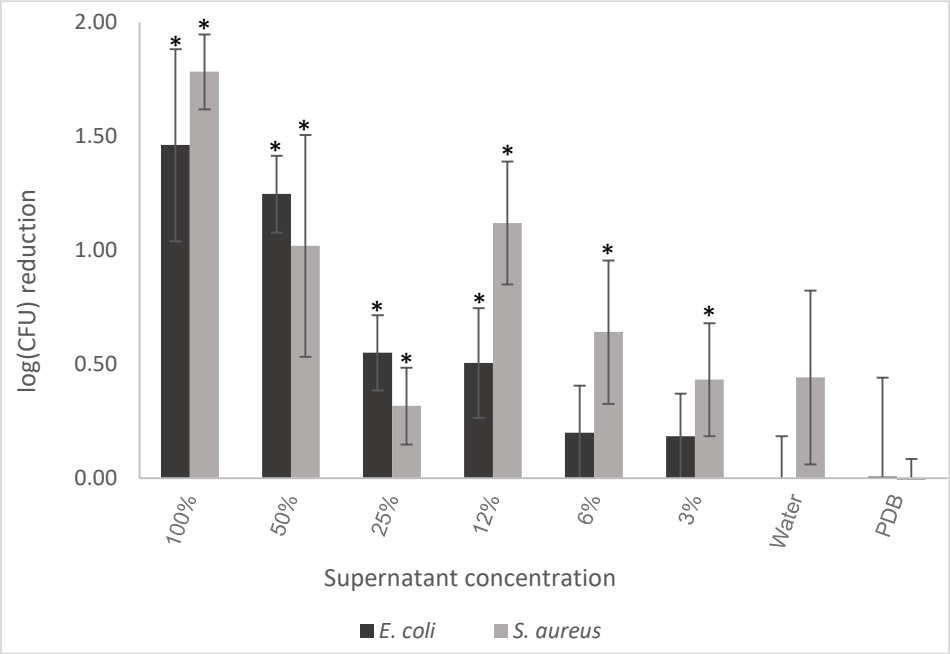


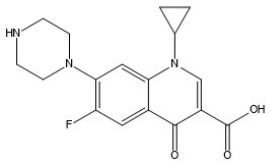
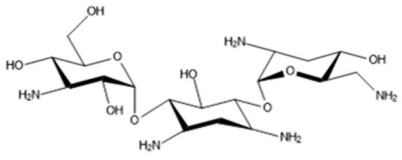
Figure 12. Effect of the supernatant concentration on log (CFU) reduction, for *E. coli* and *S. aureus*. Mean ± standard deviations are illustrated. Controls include distilled water and unfermented PDB. An * denotes results significantly different from the control (MHB).

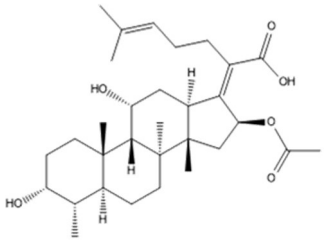
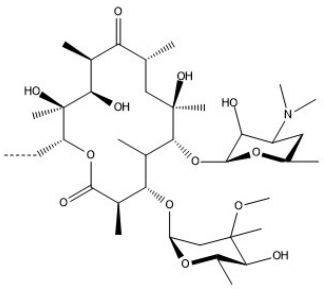
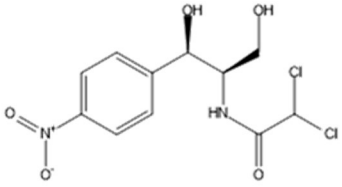
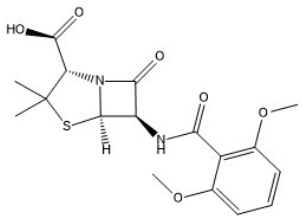
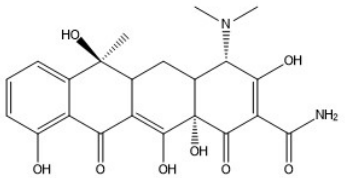
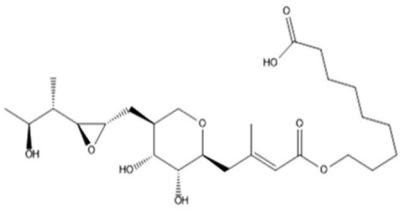
4.8. Combinatorial effect of active supernatants and antibiotics

Evaluation of combinatory effects in planktonic state

The inhibition zones of the combination of antibiotics and the supernatant of *C. spilospora* grown for 7 days on PDB, using an agitation of 120 rpm are presented in Table 7.

Table 7. IZDs (mm) of commercial antibiotics in regular plates and plates enriched with the lyophilized supernatant of *C. spilospora* grown on PDB, for 7 days, at 120 rpm. The values on a white background correspond to *S. aureus* and the ones highlighted to *E. coli.*

Antibiotic	Class	Structure	IZD_a	IZD_{a+S}
Ciprofloxacin	Fluoroquinolone		28.6 ± 0.4	33 ± 2 * (++)
			30.3 ± 0.8	32 ± 2 (+)
Tobramycin	Aminoglycoside		21.1 ± 0.9	22.5 ± 1.2 (+)
			18 ± 2	19 ± 2 (+)

Fusidic acid			36 ± 4	38 ± 3 (+)
			6.25 ± 0	6.7 ± 0.9 (+)
Erythromycin	Macrolide		23 ± 2	23.5 ± 0.6 (+)
			6.25 ± 0	6.25 ± 0 (+)
Chloramphenicol	Amphenicol		26.3 ± 0.2	27.2 ± 0.6 * (+)
			27.4 ± 0.5	28.2 ± 0.9 (+)
Methicillin	Penicillin		28 ± 2	30 ± 5 (+)
			7.4 ± 1.4	6.25 ± 0 (+)
Tetracycline	Tetracycline		24 ± 2	26 ± 2 (+)
			19.8 ± 1.3	19 ± 2 (+)
Mupirocin			45.5 ± 0.7	48 ± 2 (+)
			19 ± 5	21 ± 2 (+)
Supernatant	-	-	8.1 ± 1.3	
			7.8 ± 1.1	

* The IZD in combination is significantly different from the antibiotic ($p < 0.05$); (+) indifferent interaction; (++) additive interaction; Structures generated by ChemDraw Ultra 12.0 software.

The combination that promoted the biggest statistically significant difference between inhibition halos was the supernatant paired with ciprofloxacin, but only in *S. aureus*. That combination was further tested in a biofilm scenario.

Biofilm mass removal quantification

The combination of the supernatant with ciprofloxacin did not produce any more effect on the biofilm's mass than the isolated antibiotic. The isolated supernatants also reduced the mass, but the effect of their concentration was unexpected. It, however, may be due to an effect similar to what was observed in 0, in Figure 8. At a particular concentration, that in this case could have been 1 mg/L of lyophilized supernatant, the biofilm is not as much removed. The biofilm mass removals of the lower supernatant concentrations are both significant and thus harder to explain, as, unlike previously, those are not being diluted in water and compared to fresh MHB medium. As such, it was expected that these results would be very similar to the control group, considering that higher concentrations also showed little effect (Figure 13). The difficult reproducibility of this test, coupled with the lack of time to produce more replicates may be the limiting factor in drawing conclusions.

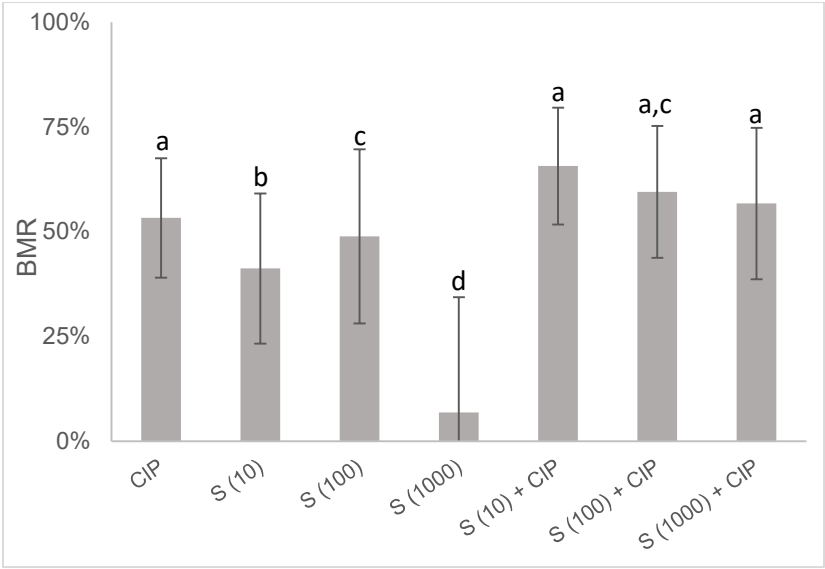


Figure 13. BMR of the combination of ciprofloxacin at the MIC and the lyophilized supernatant (S), at various concentrations (in mg/L, in parenthesis). Mean ± standard deviation are illustrated. Controls include the antibiotic and the supernatant isolated at the same concentrations. Each value is compared to both of their controls and columns with the same letter are not statistically different.

Biofilm cells metabolic activity reduction quantification

The metabolic activity measured was more in line with what was expected, as can be seen in Figure 14. The only supernatant that decreased metabolic activity was the least

concentrated, but although this value is significant, it is small and not very meaningful. Despite being a small difference, the combination of the supernatant at the highest concentration and the antibiotic had a significantly higher decrease in metabolic activity than just the antibiotic. This interaction can thus be classified as synergistic [160].

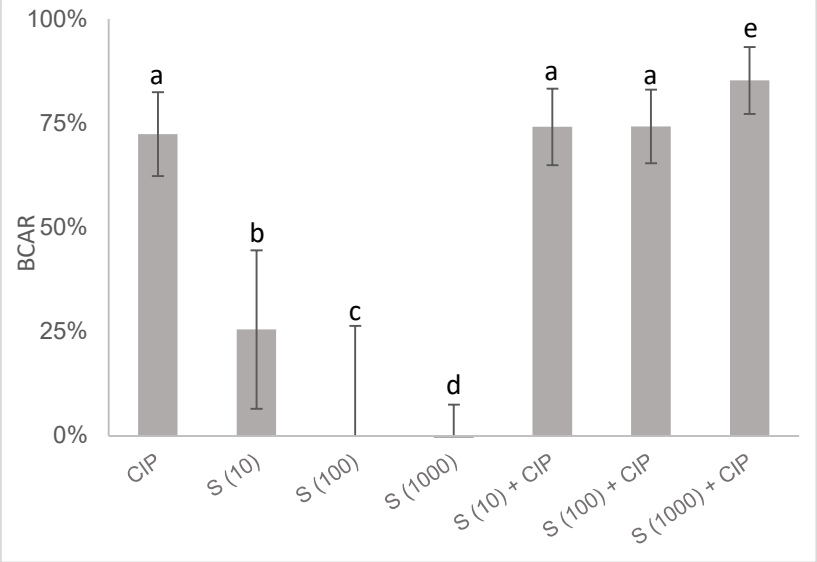


Figure 14. BCAR of the combination of ciprofloxacin at the MIC and the lyophilized supernatant (S), at various concentrations (in mg/L, in parenthesis). Mean ± standard deviation are illustrated. Controls include the antibiotic and the supernatant isolated at the same concentrations. Each value is compared to both of their controls and columns with the same letter are not statistically different.

Biofilm culturable cells' quantification

Once again, as represented in Figure 15, the supernatants did not produce a high effect. The one at the lowest concentration significantly reduced culturable cells count, but only to a small degree. The reduction of the cell count by ciprofloxacin was 1.02 ± 0.12 log (CFU). That was significantly smaller than when combined with 1000 mg/mL of the supernatant, which reached a reduction of 1.4 ± 0.2 log (CFU), making that combination synergistic.

These results are particularly interesting. Even if the observed effect is modest, it can be improved if the active molecule was isolated and tested individually, at higher concentrations. When illudins S and M were first discovered, their reported yield on an optimized culture medium was 0.33 g/L and 0.04 g/L, which would be only a small part of the supernatant mass [149]. Fluoroquinolones' activity is due to its binding to the DNA gyrase-DNA complex and inhibition of topoisomerase IV. It is plausible that the damage provoked to the DNA by illudins increases the efficacy of that kind of action, for example by increasing the antibiotics affinity to the target [144,161]. It is also possible that it interferes with biofilm structures and thus decreases ciprofloxacin resistance. It may, for example increase the biofilm permeability

to this antibiotic, exposing its cells to higher concentrations thereof, through an unknown mechanism. If the illudins are, in fact, the reason for the increase in ciprofloxacin activity, it would be a very promising discovery, as no examples of synergistic effects involving any illudin or their derivatives were found in the literature, except related to cancer research [144,162].[144,162].

The intriguing part of this explanation is that dihydroilludin M and other illudins have literature reported antibiotic activity, as was found in 0 [149]. However, that was not verified with the use of solutions of lyophilized supernatant, as the most concentrated one did not show significant reduction in biofilm mass, cells' metabolic activity, or number of cells. Illudins are sensitive to light and temperature, and although they were handled carefully, the activity might have been partly lost during storage or the lyophilization process. [149]. Their concentration, thus, might have been too low to have antibiofilm properties, but enough to synergize with a more effective antibiotic. It is also possible that the synergy is due to an entirely different molecule, not detected in the supernatant because of their low concentration, or for being an isomer of a detected metabolite.

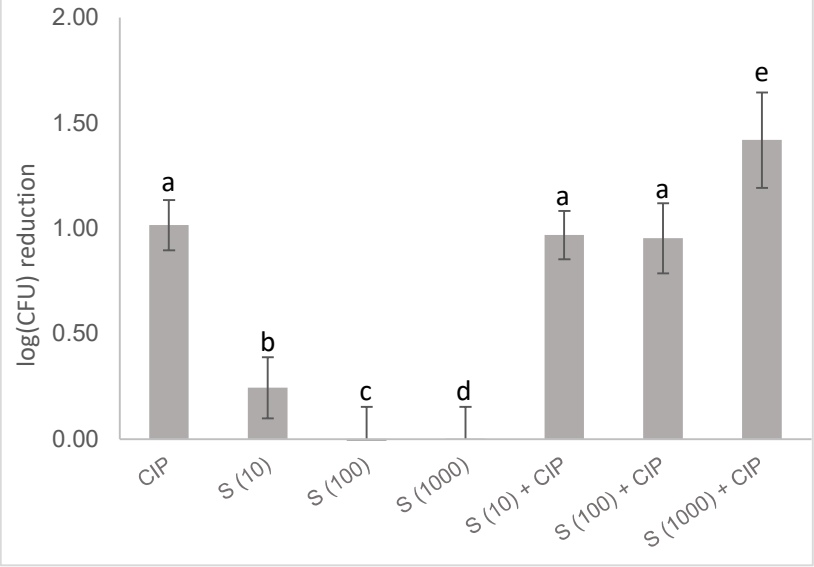


Figure 15. Logarithmic reduction of culturable cells of the combination of ciprofloxacin at the MIC and the lyophilized supernatant (S), at various concentrations (in mg/L, in parenthesis). Mean ± standard deviation are illustrated. Controls include the antibiotic and the supernatant isolated at the same concentrations. Each value is compared to both of their controls and columns with the same letter are not statistically different.

5. Conclusions

In this study, the antibiotic and antibiofilm properties of eight under-explored fungal species were evaluated. It was found that *C. spilospora*, when grown on PDB, has remarkable antibacterial properties, probably due to the production of compounds of the illudin family. The agitation speed and duration of the fermentation were proven to be deciding factors in the activity of the supernatant. The most effective supernatant reduced *S. aureus* biofilm mass by 78 ± 20%, culturable cells count by 1.8 ± 0.2 log, and totally inhibited the cells' metabolic activity. It also showed a significant, although more modest effect against *E. coli*, remarkably reducing viable cells count by 1.5 ± 0.4 log. Other species like *D. phillipsii* gave promising results, although still requiring some optimization.

It was verified that the supernatant of *C. spilospora* has a synergistic effect with ciprofloxacin against *S. aureus* biofilms, being the first time such effect is reported, assuming that dihydroilludin M is the main responsible of the supernatant's activity.

This study highlights the importance of bioprospecting new sources of active molecules, namely for antibiotic discovery. It also shows how culture conditions may affect the outcome of a fermentation and cannot be overlooked for the production of secondary metabolites.

6. Future Prospects

This study produced several promising results worth pursuing. The genome of *C. spilospora* could be sequenced and compared with other dihydroilludin producing species like *C. illudens* to identify the associated BCG and possibly further improve dihydroilludin M yield [163,164].

The combinatory effect of the *C. spilospora* supernatant with ciprofloxacin is also worth exploring. A checkerboard assay may be useful in determining optimal concentrations of supernatants and ciprofloxacin [165]. The mode of action of the supernatant could be evaluated by verifying whether it has a bactericidal or bacteriostatic effect through flow cytometry and scanning electron microscopy (SEM), combined with a viability test [166]. Optical coherence tomography (OCT) can be useful to determine if the effect of the supernatant is related to the biofilm structure, for example by increasing its permeability to the antibiotic [167]. On the other hand, molecular docking studies could verify if the damage done to DNA by illudins enhances ciprofloxacin affinity to its target [168]. As it is still a complex combination of metabolites, the fractionation of the supernatant through an extraction with an organic solvent, or, preferably, by liquid chromatography, may be fruitful in identifying the compound that most contributed to its effects [169]. Alternatively, standard solutions of the detected metabolites, namely dihydroilludin M, could be tested to validate these results in a more controlled environment, or to exclude this metabolite as the cause of the synergy, and look for other possible causes. The use of the supernatant produced using a higher agitation could further improve the effect found. Lastly, testing this combination against a biofilm of an MRSA strain could make the results more relevant to a potential clinical context.

The antibiofilm properties of *D. phillipsii*, especially against *E. coli* biofilms were proven, but left largely unexplored. It would be interesting to test the supernatants produced in different culture media, especially the ones produced at higher agitation, and to identify bioactive molecules present, similar to what was done for *C. spilospora*. The number of species being discovered and deposited at MUM and other culture collections is enormous, so, in the future, other species may be bioprospected for antibiotics and biofilm inhibitors similarly to the species in the present study [45].

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Appendices

Appendix A – Additional information about the selected fungal species

The selected species are generally very poorly studied Table A.1 contains the number of results of the specified terms in Google Scholar, in English, excluding citations and patents.

Table A.1. Number of results obtained for each search term in Google Scholar, on 17th March 2023.

Search terms	Number of results
"trichoderma aestuarinum"	3
"colletotrichum coccodes" -potato -tomato -velvetleaf -pepper -soybean	444 *
"coprinopsis spilospora"	9
"coprinus spilosporus" -"coprinopsis spilospora"	4 **
"penicillium tunisiense"	14
"talaromyces saxoxalicus"	1
“cladosporium rubrum”	14
“diaporthe phillipsii”	5
"neopestalotiopsis scalabiensis"	1

**C. coccodes* is much more widely studied than the other species. It is a common plant parasite [170], so some plants it commonly infects were removed from the results. The production of secondary metabolites is reported but it is beyond the scope of most of these studies.

** *C. spilospora* was formerly known as *Coprinus spilosporus* [140].

Although little information is available regarding those species, there is more literature related to the genus as a whole.

Trichoderma aestuarinum

Trichoderma species are parasitic or saprophytic fungi highly adaptable to several ecological conditions and lifestyles. They are widely used in agriculture as biopesticides, especially against phytopathogenic fungi, that they parasitize. Many strains colonize plant roots and produce elicitors that promote their growth and increase tolerance to abiotic stresses like drought. Their secondary metabolites have a proven antagonistic activity against bacteria and fungi. Over 1000 compounds are estimated to be produced by this genus [171,172]. They

reproduce asexually but evidence suggests that hybridization may lead to strain improvement [172].

T. aestuarinum was isolated from the estuary of Ria de Aveiro (Portugal) in 2019 and its closest species is *T. paraviridescens* [173].

Penicillium tunisiense

Penicillium species are well known for their antibiotic production [45]. They are some of the most common fungi in many habitats, have a worldwide distribution, and a great economic significance because of food spoilage, biotechnology, plant pathology, and medicine. Their secondary include mycotoxins, but also antibacterial and antifungal compounds, immunosuppressants, and cholesterol-lowering agents [174].

P. tunisiense was isolated in 2018 from orchard apples purchased in a local market in Tunis (Tunisia). It appears to not be able to rot the apples or produce the mycotoxin patulin. It is a psychrotolerant species, with an optimal growth temperature below 25 °C [175].

Coprinopsis spilospora

Coprinopsis are macrofungi that decompose grass and have the ability to form mushrooms [176]. These basidiomycetes were previously in the same genus, *Coprinus*, together with *Coprinus sensu stricto*, *Coprinellus*, and *Parasola* [140].

C. spilospora is very rare but has been recorded in a few localities in Europe, and more recently, in Western Australia. It grows on bare, gravelly-calcareous soil, in moss beds in deciduous forests, and, sometimes, on burnt soil [177,178]. This species was first described in 1951 but there is not much research made on it [179].

Talaromyces saxoxalicus

Talaromyces are typically endophytes (live between plant cells) previously in the genus *Penicillium* that can be isolated from soil, plant, sponges, and foods [180]. This genus contains species with useful enzymes in the synthesis of saccharides, preparation of chiral building blocks or biotransformation, and others used in pest biocontrol. Some of their most common secondary metabolites are alkaloids, peptides, lactones, and polyketides, among others [181]. Some species produce pigments, more commonly red, but also yellow and purple, that may be produced by submerged fermentation and are industrially valuable [138,139,182].

T. saxoxalicus was isolated in 2021 in the old cathedral of Coimbra (Portugal) as potentially degrading this monument [183].

Colletotrichum coccodes

Colletotrichum species may be endophytes, saprobes, or, rarely, they may parasite insects [184]. They are among the most important genera of fungal plant pathogens causing anthracnose so their identification is routinely needed by plant pathologists, regulatory officials, and fungal biologists [185]. Anthracnose is a plant disease where masses of sunken necrotic tissue are formed, and orange conidia are produced. Some *Colletotrichum* parasitize only one species while others parasite hundreds [186]. The secondary metabolome of this genus contains many metabolites that cause the same symptoms to plants as the fungi itself, but also some that provide defense and have antimicrobial activity [186,187].

C. coccodes is widely reported as the cause of the black dot disease in potatoes but also is known to produce antimicrobial and antibiofilm metabolites [170,188,189].

Neopestalotiopsis scalabiensis

Neopestalotiopsis species commonly occur on plants as endophytes, pathogens, or saprobes, and are economically important plant pathogens along with other close genera, for example, in blueberries, as they cause canker, twig blight, and dieback symptoms [190-192]. Species of this genus can produce antibacterial polymers [193], small molecules [109], and even produce silver nanoparticles [194]

N. scalabiensis was discovered recently in blueberries, and it has been verified that it is a pathogen to this plant [191].

Appendix B – Parameters of culture media

The following considerations were made to assess the culture media composition:

- Yeast extract has 390 mg/kg of heavy metals [195]. It can have carbohydrates, but in small amounts and so were not considered [196].
- Potato infusion was considered to have 70% carbohydrates in its composition and 8.24 mg/kg of heavy metals. Its salt content was considered negligible [197].
- Tryptone has no appreciable carbohydrates and LB as a whole has 1801 mg/kg of heavy metals [198].
- CSL is very complex and variable but was considered to have 50% water, 107 mg/kg heavy metals, 1.5% carbohydrates, and little salt content [199].
- Peptone has 20% carbohydrates [200]. No information on its metal nor salt content was found so it was not considered, although it probably is not negligible.
- Malt has approximately 2.5% salts and was considered to have 29 mg/kg of heavy metals, although this might be an underestimation [201,202].

Results are summarized in Table B.1 and can be visualized in Figure B.1.

Table B.1. Approximate composition of culture media (g/L).

Medium	Complex infusion	Carbohydrates	Total organics	Inorganic Salts	Heavy metals	pH
YES	20	150	170	0.25	1.1×10^{-2}	7.2
PDB	1.2	23	24	-	3.3×10^{-5}	5.1
LB	15	0	15	10	1.8×10^{-3}	7.0
PDC	11	23	34	-	2.2×10^{-3}	5.1
MME	4.4	28	32	0.76	4.3×10^{-3}	4.7
CYAS	5	30	35	54	2.0×10^{-3}	7.3
WATM	6.8	33	39	3.2	8.9×10^{-3}	6.5

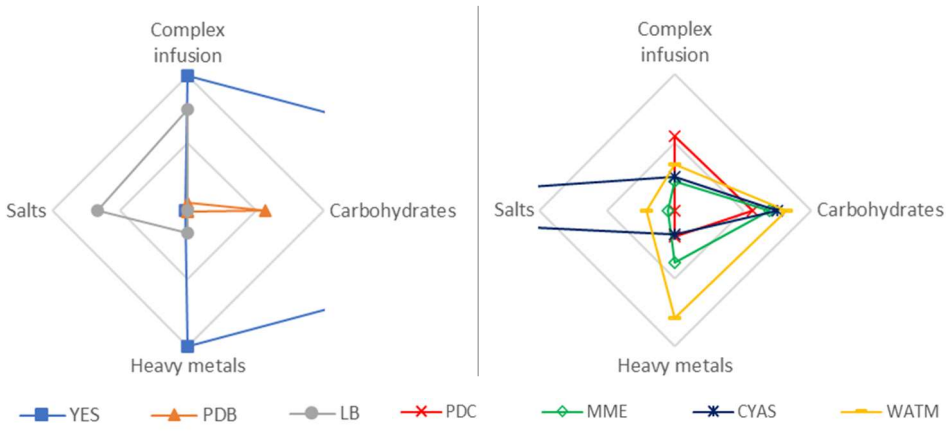
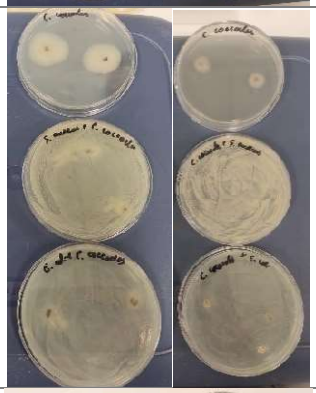


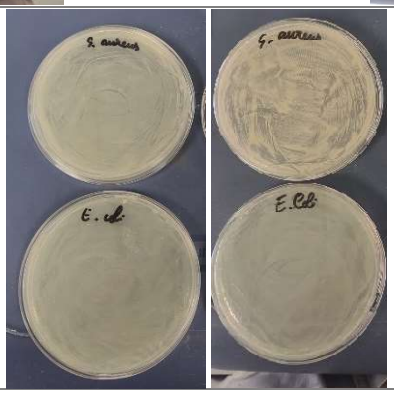
Figure B.1. Composition of culture media (arbitrary units).

Appendix C – Appearance of the fungal–bacterial co-cultures

Photographs of the 3 days co-culture test are presented in Table C1. Some time after these results were collected, the vicinities of the inoculation plugs of *D. phillipsii* co-cultured with *S. aureus* and *N. scalabiensis* co-cultured with *E. coli* developed a slight halo. Macro photographs are presented in

Table C1. Photographs of the co-cultures of the eight fungal species and two bacterial species. Bacteria are identified in the petri dish.

Fungal species	Co-culture	Fungal species	Co-culture
Trichoderma aestuarinum		***Talaromyces saxoxalicus***	
Colletotrichum coccodes		***Neopestalotiopsis scalabiensis***	
Coprinopsis spilospora		***Cladosporium rubrum***	

Penicillium tunisiense		***Diaporthe phillipsii***	
Control (bacteria)			

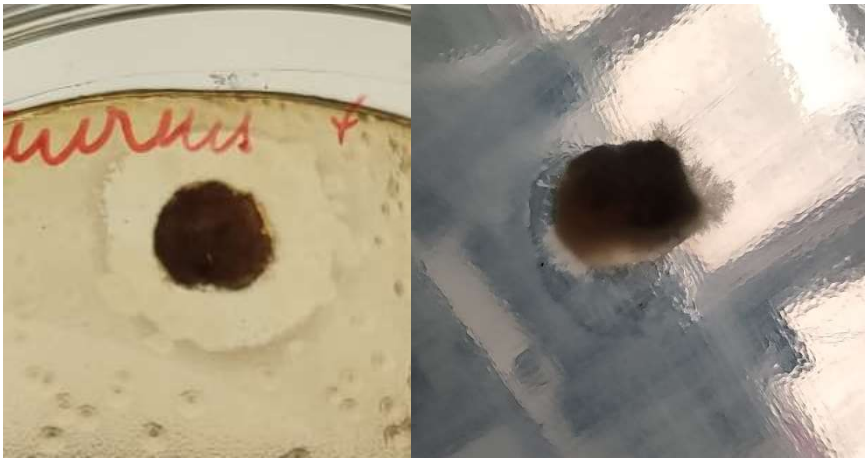
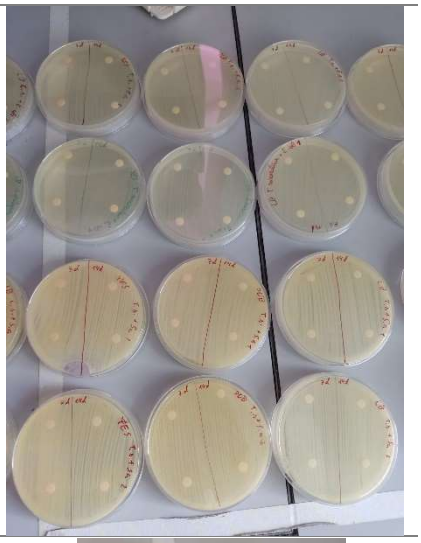
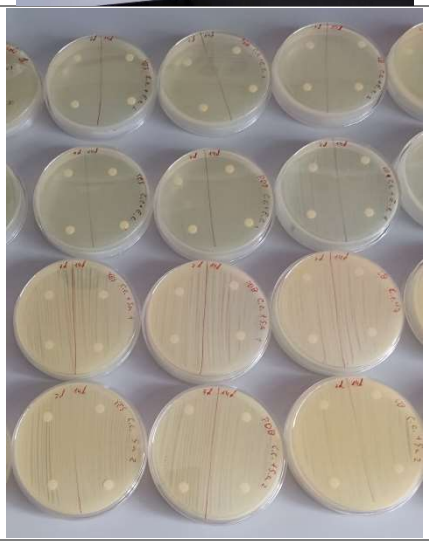
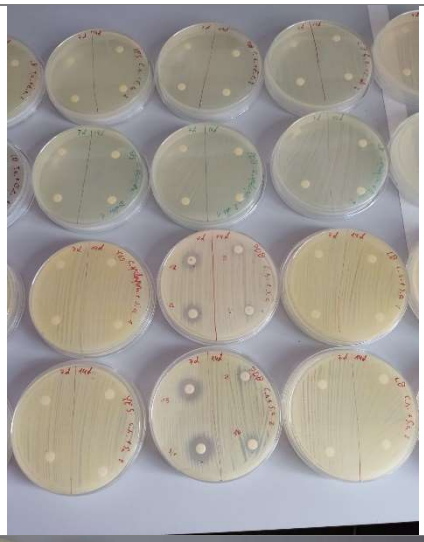
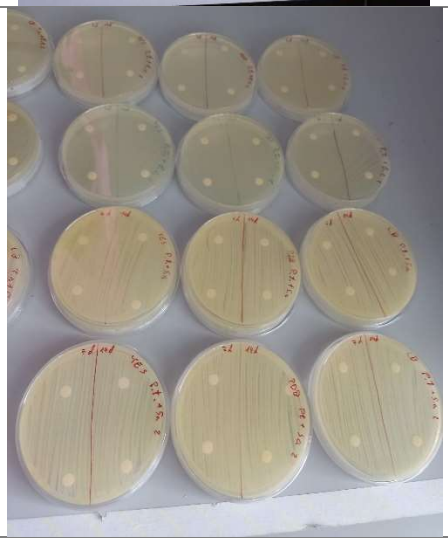
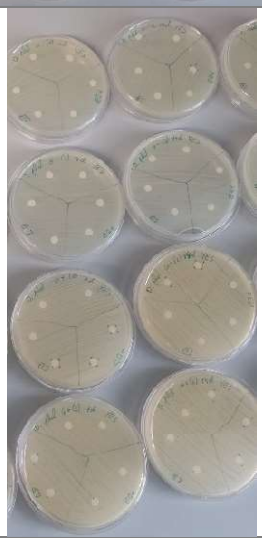
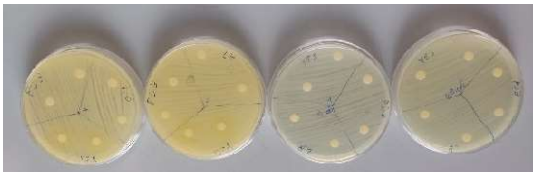


Figure C1. Macro photographs of the co-culture test of *D. phillipsii* and *S. aureus* (left), and *N. scalabiensis* and *E. coli* (right), taken circa 14 days after the inoculation.

Appendix D – Appearance of the disk diffusion test of the cell-free supernatants

Figure D1. Photographs of the disk diffusion tests of the cell-free supernatants of the eight fungal species after 7 and 14 days, grown on YES PDB and LB, against two bacterial species. Culture media, fermentation time, and bacterial species are identified in the petri dish.

Fungal species	Co-culture	Fungal species	Co-culture
Trichoderma aestuarinum		*Talaromyces saxoxalicus*	
Colletotrichum coccodes		*Neopestalotiopsis scalabiensis*	

Coprinopsis spilospora		***Cladosporium rubrum***	
Penicillium tunisiense		***Diaporthe phillipsii***	
Control (unfermented media)			
Control (ciprofloxacin g/L)			

Appendix E – Characterization of the growth of *C. spilospora* according to culture medium parameters

At the time that it became clear that the supernatants of *C. spilospora* had interesting properties, it was no longer feasible to measure the dry biomass, so the culture media used were qualitatively classified on a scale from 1 to 5 where 1 means that the culture did not develop outside the inoculation plug, and 4 means abundant biomass production. 5 was reserved for the cases where any kind of antimicrobial activity was detected. The classification is presented in Table E.1. Ideally, the scale would all be based on a quantitative scale of antimicrobial activity, but that would not have produced enough data points.

Table E.1. Classification of culture media for *C. spilospora growth* and antimicrobial production

Medium	Growth/ Antimicrobial production (y)
YES	4
PDB	5
LB	2
PDC	1
MME	4
CYAS	1
WATM	3

Two parameters – carbohydrates to complex infusion ratio (C/CI, as to approximate the C/N ratio), and salt concentration – were deemed the most impactful in the cultivation of *C. spilospora*. Ascribing a weight to each, summing the values, and plotting that against the growth classification, as done in Figure E.1, allows the determination of a general tendency that may be useful for the formulation of a culture medium in a scaled-up culture. It is important to note that the media have far more differences than what is being considered, so this approach does not intend to be accurate.

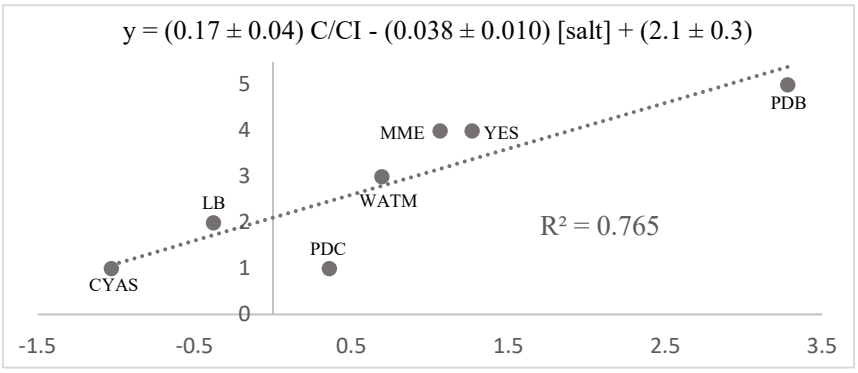


Figure E.1. Fungus growth plotted against aggregated carbohydrate to complex infusion concentrations ratio and salt concentration.

The main takeaway is that the fungus' growth and antibiotic production is heavily favored by a high proportion of carbohydrates to other substances. Lower salt concentrations are also beneficial. This is in line with the common medium in which *Coprinopsis* and *Coprinus* species are often cultivated: They have high concentrations of glucose or maltose, some salts, but only to buffer the pH or to supply micronutrients, and small amounts of complex infusions including combinations of yeast, malt, and horse dung extracts or tryptone [203]. The regression obtained has a small value of R^2, as expected, due to the number of parameters unaccounted for and for the qualitative classification of each culture media. It is, nonetheless, statistically significant ($p < 0.05$).