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journal homepage: www.elsevier.com/locate/ecoenvGenome sequencing of *Porostereum spadiceum* to study the degradation of levofloxacin

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ABSTRACT

Despite various plans to rationalize antibiotic use, antibiotic resistance in environmental bacteria is increasing due to the accumulation of antibiotic residues in the environment. This study aimed to test the ability of basidiomycete fungal strains to biotransform the antibiotic levofloxacin, a widely-used third-generation broad-spectrum fluoroquinolone, and to propose enzyme targets potentially involved in this biotransformation. The biotransformation process was performed using fungal strains. Levofloxacin biotransformation reached 100% after 9 days of culture with *Porostereum spadiceum* BS34. Using genomics and proteomics analyses coupled with activity tests, we showed that *P. spadiceum* produces several heme-peroxidases together with H₂O₂-producing enzymes that could be involved in the antibiotic biotransformation process. Using UV and high-resolution mass spectrometry, we were able to detect five levofloxacin degradation products. Their putative identity based on their MS² fragmentation patterns led to the conclusion that the piperazine moiety was the main target of oxidative modification of levofloxacin by *P. spadiceum*, leading to a decrease in antibiotic activity.

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1. Introduction

Antibiotics are antimicrobial drugs that can kill bacteria (bactericidal) or inhibit bacterial growth (bacteriostatic) (Danner et al., 2019), and are widely used to treat or prevent bacterial infections in humans and animals (Liu et al., 2018). Antibiotics are classified based on their chemical structures and mechanisms of action (Oberoi et al., 2019). Antimicrobial agents have been used in biotechnological applications ranging from human medicine (Abubakar, 2020) to food-animal production (Van et al., 2020), agriculture (Manyi-Loh et al., 2018), aquaculture (Lulijwa et al., 2020), and veterinary medicine (Lekagul et al., 2019).

Fluoroquinolones are a class of synthetic antibiotics characterized by activity against a broad spectrum of Gram-positive and Gram-negative bacteria (Karampela and Dalamaga, 2020). Four generations of fluoroquinolone-class antibiotics have been produced over the years (Brar et al., 2020), and they have generated promising treatments for urinary tract infections, gastrointestinal infections, respiratory tract infections, sexually-transmitted diseases, and skin infections. However, fluoroquinolone antibiotics can have severe side effects, such as mood alteration, peripheral neuropathy, diarrhea, gastrointestinal irritation, and hepatic toxicity (Brar et al., 2020). Consequently, some fluoroquinolones (i.e. trovafloxacin) were rapidly withdrawn from the market following reports of serious long-term adverse effects and several deaths (Marchant, 2018). Other widely-used fluoroquinolones, such as levofloxacin, can have other side effects, such as hypoglycemic emergency (Liao et al., 2022), QT prolongation (Shojaei et al., 2020), tactile hallucinations, and acute anxiety reactions (Maharani et al., 2019).

Recent studies have highlighted the limitations of current wastewater treatment systems with respect to emerging pollutants such as antibiotics (Phoon et al., 2020; Langbehn et al., 2021). Even at low concentrations, antibiotics or their transformation products remain active, and so treated effluents that get reused for other purposes such as irrigation and soil amendments ultimately play a role in the acquisition of resistance by bacteria (Oberoi et al., 2019; Wang et al., 2020). To address the rapid development of bacterial resistance, powerful new antimicrobial agents have been generated, such as levonadifloxacin, zabofloxacin, and nemonoxacin, and are widely used in severe infections where no other treatments are required (Rusu et al., 2021; Herbert et al., 2022). However, there is an urgent need for efficient solutions to treat antibiotic waste (Kushwaha and Chatterjee, 2020).

As conventional wastewater treatment plants leave traces of antibiotics in effluents, several physicochemical techniques have been developed and applied to address these residual molecules, such as adsorption (Gao et al., 2022; Wu et al., 2022), oxidation (Kim et al., 2020), advanced oxidation processes (Anjali and Shanthakumar, 2019), and ozonation (González-Labrada et al., 2020). These treatments are efficiently active on antibiotics, but they generate a number of toxic and hazardous by-products (Crini and Lichtfouse, 2019; Bhatt and Chatterjee, 2022), and biological techniques have been proposed as a sustainable alternative solution. These techniques use biological organisms that produce extracellular ligninolytic enzyme arsenal composed of laccases and heme-peroxidases (Manasfi et al., 2020). The organisms used include fungal species such as *Trichoderma* sp. (*T. harzanium* and *T. asperellum*) (Manasfi et al., 2020), *Phanerochaete chrysosporium* and *Pycnoporus sanguineus* (Gao et al., 2018), and bacterial species such as *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Elizabethkingia miricola* (Shaker et al., 2022). Pan et al. (Pan et al., 2018) investigated the removal of several fluoroquinolones, including as ciprofloxacin, norfloxacin, and ofloxacin. Other researchers have reported enzymatic oxidation of fluoroquinolones by free or immobilized laccases (Shao et al., 2019; Mathur et al., 2021b), and heme-peroxidases [chloroperoxidase, MnP (Lueangjaroenkit et al., 2019)]. Biotransformation can follow numerous pathways depending on the type of microorganism involved and the antibiotic structure, but the most common pathways correspond to modification of the piperazine ring associated with

oxidation, hydroxylation, acetylation, and formylation of the parent compound (Mathur et al., 2021a; Shang et al., 2021; Sodhi and Singh, 2021).

This study aimed to evaluate the ability of two basidiomycete fungal strains to transform levofloxacin-spiked pharmaceutical wastewater. The candidate strains were selected for their proven ability to efficiently degrade several recalcitrant micropollutants, such as dyes, phenols, and other antibiotics (e.g. diclofenac) (Gao et al., 2020; Dhiman et al., 2022). The genome of the best strain was sequenced to conduct proteomic analysis and provide insight into the potential enzymes involved in fluoroquinolone biotransformation. In addition, the products of the biotransformed fluoroquinolones were annotated by high-resolution mass spectrometry (HR-MS), and the main products of these enzymes were proposed.

2. Materials and methods

2.1. Fungal strains and culture media

To test the ability of fungi to biotransform levofloxacin, we used two basidiomycete strains: *Porostereum spadiceum* strain BS34 [ON340794] and *Bjerkandera adusta* strain TM11 [ON340793] (found at <https://www.ncbi.nlm.nih.gov/>).

Solid cultures of the two strains were performed on potato dextrose agar (PDA) media, using 39 g of dehydrated media (Accumix®, Geel, Belgium) suspended in 1000 mL of distilled water and sterilized by autoclaving at 120 °C for 30 min. Liquid precultures were performed to inoculate in 500 mL Erlenmeyer flasks containing 100 mL of M7 medium at pH 5.5, as described in Ben Ayed et al. (Ben Ayed et al., 2022). Cultures were incubated at 30 °C with stirring at 160 rpm, and 300 µM CuSO₄ was added as a laccase inducer on day 3 of incubation, and levofloxacin was added on day 4 at a concentration of 50 mg L⁻¹.

2.2. Sample collection and isolation

The environmental samples *P. spadiceum* strain BS34 and *B. adusta* strain TM11 were collected from various habitats in northwest Tunisia (Bousalem) (Daâssi et al., 2016). Samples were collected in sterile tubes using a sterile spatula and stored at 4 °C until further use.

A small piece of wood sample was inoculated on 3.9% (w/v) PDA (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and 1.8% (w/v) malt extract (Sigma-Aldrich) with 0.1% (w/v) chloramphenicol to prevent bacterial growth, and incubated at 30 °C for three days until fungal growth was observed. An apparent monomorphic culture obtained after at least two transfers onto fresh agar plates was then further authenticated using molecular tools to check for strain purity and identity.

2.3. Identification of fungal isolates by sequencing the ITS region

P. spadiceum cultures were performed in 50 mL M7 medium added with 300 µM CuSO₄ at day 3 of culture and incubated at 30 °C with stirring at 160 rpm. On day 7 of incubation, the cultures were filtered through Miracloth (EMD Millipore Corp, Billerica, MA), and the resulting mycelia was carefully harvested with a spatula to separate it from the culture supernatant. DNA was extracted from 40 to 80 mg of mycelium powder using a GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA) as per the manufacturer's instructions, and its concentration was measured using a Nanodrop 2000 systems (Thermo Fisher Scientific, Wilmington, DE). The extracted DNA was used as the PCR template to amplify the partial sequences of two DNA loci: the internal transcribed spacer region (ITS), and the translation elongation factor 1 α region (TEF-1 α), as described in Ben Ayed et al. (Ben Ayed et al., 2022).

RNA was extracted from 100 mg of mycelia using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Madison, WI) as per the manufacturer's instructions. After purification, total RNA was quantified

using a NanoDrop spectrophotometer (Thermo Scientific) and further purified by LiCl (8 M) precipitation. All RNA samples were then collected, and their quality was evaluated using the High-Sens RNA Bioanalyzer (Agilent, Santa Clara, CA).

2.4. *Porostereum spadiceum* genome sequencing and gene annotation

Genomic DNA and RNA for cDNA libraries were extracted from mycelia cultured as described in Section 2.1. and homogenized in liquid nitrogen following the standard phenol/chloroform method (Green et al., 2012). The transcriptome used for genome annotation was sequenced using the Illumina sequencing platform (Illumina Inc., San Diego, CA). Plate-based RNA sample prep was performed on a PerkinElmer Sciclone NGS robotic liquid handling system (PerkinElmer, Waltham, MA) using Illumina's TruSeq Stranded mRNA HT sample prep kit with poly-A selection of mRNA following the protocol outlined by Illumina in their user guide (found at https://support.illumina.com/sequencing/sequencing_kits/truseq-stranded-mrna.html), under the following conditions: total RNA starting material was 1 µg per sample and 8 cycles of PCR was used for library amplification. The prepared library was quantified using KAPA Biosystem's next-generation sequencing library qPCR kit (Roche, Indianapolis, IN) and run on a Roche LightCycler model-480 real-time PCR instrument. The quantified library was then multiplexed with other libraries, and the pool of libraries was prepared for sequencing on an Illumina NovaSeq 6000 sequencing platform using NovaSeq XP v1.5 reagent kits and an S4 flow cell, following a 2 × 150 indexed run recipe.

Raw RNA-Seq reads were filtered and trimmed for artifact sequences by kmer matching (kmer=25) allowing one mismatch, and the detected artifact was trimmed from the 3' end of the reads. RNA spike-in reads, phiX reads, and reads containing any Ns were removed. Quality trimming was performed using the phred trimming method, set at Q6. Finally, following trimming, reads under the length threshold (minimum length of 25 bases or 1/3 of the original read length, whichever was longer) were removed. Filtered reads were assembled into consensus sequences using Trinity v2.11.0 (Grabherr et al., 2011).

The genome was sequenced on a PacBio sequencing platform (PacBio Inc., Menlo Park, CA). An input of 1.5 µg of genomic DNA was sheared to approximately 10 kb using Megaruptor 3 (Diagenode). The sheared DNA was treated with exonuclease to remove single-stranded ends, DNA damage repair enzyme mix, and end-repair/A-tailing mix, and ligated with barcoded overhang adapters using the SMRTbell Express Template Prep Kit 2.0 (PacBio). Up to 16 libraries were pooled in equimolar concentrations and purified using AMPure® PB Beads (PacBio). Pooled libraries were size-selected using 0.75% agarose gel cassettes with Marker S1 and the High-Pass protocol on BluePippin (Sage Science, Beverly, MA). The PacBio Sequencing primers were then annealed to the SMRTbell template library (PacBio), and sequencing polymerase was bound to them using the Sequel II Binding kit 2.0 (PacBio). The prepared SMRTbell template libraries were then sequenced on a Pacific Biosystems Sequel IIe sequencer using SMRT Link 10.2, 8 M v1 SMRT cells, and a sequencing chemistry with a 1 × 1800 sequencing movie.

The filtered subread data was processed to remove artifacts, and CCS reads were then assembled using Flye version 2.9-b1768 (found at <https://github.com/fenderglass/Flye>) [-t 32 -pacbio-hifi] and polished using two rounds of RACON version 1.4.13 [-u -t 36] (found at <https://github.com/lbcb-sci/racon>). The genome was annotated using the JGI Annotation pipeline (Grigoriev et al., 2014).

All putative proteins of *P. spadiceum* genome (Klosterman et al., 2011) were compared to the entries in the CAZy database (Drula et al., 2022). A homemade pipeline which combines BlastP (found at <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and HMMER3 (found at <http://hmmer.janelia.org/>) tools was used to compare protein models with the sequences of the CAZy modules. The proteins with E-values smaller than 0.1 were further screened by a combination of BlastP searches against libraries created from the sequences of the catalytic and non-catalytic

modules. HMMER3 was used to query against a collection of custom-made hidden Markov model (HMM) profiles constructed for each CAZy family. This was followed by a manual inspection by expert curators to resolve borderline cases.

2.5. Determination of residual levofloxacin on Mueller-Hint on agar plates

Fungi were cultivated in 50 mL of M7 medium (see Section 2.1.). From day 4 to day 10 of culture, 50µL aliquots were collected from supernatants and loaded on a Mueller-Hinton agar medium (Merck, Darmstadt, Germany), as described in Ben Ayed et al. (Ben Ayed et al., 2022). Two controls were used in parallel with the other tests: a culture medium in the presence of levofloxacin but without fungus, and a fungal culture without levofloxacin. Experiments were performed in triplicate, and the diameters of the zones of complete inhibition were measured using a ruler on the undersurface of the Petri dish for each fungal strain.

2.6. Follow-up of concentration time-course in the culture medium

The levofloxacin concentration in the wastewater from the Philadelphia PHARMA facility in Sfax (Tunisia) was determined by HPLC-UV analysis (Agilent 1260 Infinity HPLC system, Wilmington, DE) using commercial levofloxacin (Lovik, Philadelphia Pharma, Sfax, Tunisia) as a standard, as previously described in Ben Ayed et al. (Ben Ayed et al., 2022).

The time-course change in levofloxacin concentration was monitored by HPLC-MS in each fungal culture at two different timepoints (day 4 and day 10 of culture). Aliquots of the culture supernatants were filtered (0.45 µm, GHP Acrodisc, Pall Gelman, Port Washington, NY) and then injected into an ultra-HPLC system (UHPLC; Thermo Scientific) coupled with an electrospray ionization mass spectrometer (ESI-MS) and UV co-detection, as described in Ben Ayed et al. (Ben Ayed et al., 2022). The levofloxacin peak was assigned based on the retention time of the standard and the mass-to-charge ratio (m/z) of the protonated ions $[M + H]^+ = 362.1$ amu. The residual levofloxacin concentration was determined relative to that of a commercial standard. Levofloxacin was detected using a UV-Vis diode array detector at 254, 280, and 330 nm.

2.7. Enzymatic activity assays

Over 10 days of culture, aliquots from fungal supernatants under both conditions (with or without levofloxacin) were collected, centrifuged at 10.000 g for 5 min at 30 °C, and used to measure laccase-like and peroxidase-like activity, as described in Ben Ayed et al. (Ben Ayed et al., 2022). 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) and 2,6-dimethoxyphenol (DMP) were used for laccase and peroxidase-like activity assays, respectively.

2.8. Proteomic analysis of *Porostereum spadiceum* secretomes

Cultures of *P. spadiceum* were prepared under both conditions (i.e. with and without levofloxacin) in triplicate (see Section 2.1.) as described by Ben Ayed et al. (Ben Ayed et al., 2022) and corresponding proteomes were prepared in three replicates for each culture condition.

A total of 10 µg of proteins dissolved in 40 µL LDS buffer (26.5 mM Tris HCl, 35.25 mM TRIS base, 0.5% LDS, 2.5% glycerol, 0.13 mM EDTA, supplemented with 5% beta-mercaptoethanol) was incubated at 99 °C for 5 min. For both conditions (with or without levofloxacin), 20 µL equivalent to approximately 5 µg of protein was subjected to electrophoresis for 5 min under denaturing conditions on a NuPAGE 4–12% gradient gel using MES SDS as running buffer (50 mM MES ([2-(N-morpholino) ethane sulfonic acid), 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3). Each proteome sample was extracted as a unique polyacrylamide strip and treated as described by Rubiano-Labrador et al. (Rubiano-Labrador et al., 2014), before proteolysis with trypsin Gold

(Promega) in 50 mM NH_4HCO_3 in the presence of ProteaseMax detergent (Promega). About one-fifth of the resulting peptide mixture was pumped into a C18 PepMap100 nanosized capillary column (3 μm , 100 \AA , 75 μm ID \times 50 cm length, LC Packings, CA), then washed and then resolved with an acetonitrile gradient at a rate of 0.2 $\mu\text{L min}^{-1}$ for 90 min. Peptides were analyzed by MS/MS using a Q-Exactive HF instrument (Thermo Scientific) (Grenga et al., 2022) in data-dependent acquisition mode with a full scan of peptide ions acquired at 60,000 resolution. High-energy collisional dissociation and MS/MS scans were performed after each MS scan at 15,000 resolution on the 20 most abundant precursor ions with a 2⁺ or 3⁺ ion charge and dynamic exclusion of 10 s

2.9. UHPLC-UV and HR-MS analyses of *Porostereum spadicum* secretome extracts for the dereplication of major levofloxacin degradation products

UHPLC analyses coupled with high-resolution mass spectrometry (HR-MS) were performed on secretome extracts of *P. spadicum*, prepared as previously described (Ben Ayed et al., 2022), to putatively identify the products of levofloxacin degradation in the fungal culture. Comparative UHPLC-UV-MS analyses were performed on two (control samples) to four (assay) replicates of *P. spadicum* cultures supplemented with or without levofloxacin and grown for 4, 7, and 10 days. Data-dependent MS/MS acquisitions were performed on the three major features from 50 to 1200 amu at 4 Hz in positive mode with a collision energy of 40 eV. Each MS¹ and MS² spectrum was individually analyzed and annotated for further dereplication of the levofloxacin degradation products (Fig. S2-S8). The spectra were also analyzed with SIRIUS software to corroborate the annotation, notably with regard to structural information available in various databases using both fragmentation tree and CSI:FingerID. All MS raw data (mzXML and SIRIUS reports) are freely available on ZENODO (10.5281/zenodo.7602180).

3. Results

3.1. Phylogeny analysis

The two fungal strains studied in this work were isolated from various habitats in northwest Tunisia (Bou Salem forest) in winter, and were selected for their capacity to modify levofloxacin (see below) compared to previously tested strains *Thielavia* sp HJ22, *Thielavia* sp CH1, and *Corioloropsis gallica* (Ben Ayed et al., 2022) isolated from the same area. *Bjerkandera adusta* TM11 has already been fully characterized (Daassi et al., 2016). For strain BS34, molecular analysis was performed to identify related genera. A phylogenetic tree was constructed based on the ITS region amplified from BS34 genomic DNA. This phylogenetic tree was divided into two clades. Clade I contained five sequences from *Porostereum spadicum* (3) and *Ceriporiopsis carnegieae* (2) strains (Fig. S1). *Ceriporiopsis carnegieae* strains were phylogenetically close to *P. spadicum* but only shared 87.20% identity with *P. spadicum* BS34 at a bootstrap value of 100. However, strain BS34 shared 100% identity with *Porostereum spadicum* voucher CREA-DC TPR OL.117 and was named *P. spadicum* strain BS34 in the following experiments.

3.2. Fungal biotransformation of levofloxacin

From day 4 to day 10 of culture, aliquots from fungal culture media with or without levofloxacin were loaded onto a medium pre-spread with *E. coli* to test the ability of both *P. spadicum* and *B. adusta* to biotransform levofloxacin. The *E. coli* growth inhibition zones were measured in the presence of levofloxacin solution with or without (negative control) fungal pretreatment. In a previous study, we had tested the ability of three strains to biotransform the same antibiotic, and *C. gallica* was found to be the most efficient, with a 15% reduction of

the *E. coli* halo in 10 days (Ben Ayed et al., 2022). Here, the results showed a decrease in inhibition zone diameter from day 6 with *B. adusta* and from day 5 of culture with *P. spadicum*. The decrease in the inhibition zone reached 100% day 10 of culture with *B. adusta* culture media and at day 9 of culture with *P. spadicum* (Fig. 1), showing that *P. spadicum* was the best strain to biotransform levofloxacin.

3.3. HPLC determination of residual levofloxacin after fungal biotransformation

The residual concentration of levofloxacin was determined by HPLC-UV at 280 nm. Under our conditions, no degradation of the antibiotic alone was detected after 10 d of culture (Fig. 2). In a previous study, we found 25% biotransformation of levofloxacin with *C. gallica* strain CLBE55 (Ben Ayed et al., 2022) but no recorded biotransformation for either of the two *Thielavia* strains. On day 10 of culture, only 50% of the levofloxacin had been biotransformed by *B. adusta* whereas levofloxacin had completely disappeared in the presence of *P. spadicum*. *P. spadicum* was thus selected as the best strain for biodegrading levofloxacin and was used for further experiments.

3.4. Laccase-like and peroxidase-like activities of *Porostereum spadicum* secretomes

As oxidative enzymes can potentially participate in antibiotic degradation, laccase-like activity and peroxidase-like activity were assayed over 10 days of culture under both tested conditions (with or without levofloxacin antibiotic). There was no detectable laccase-like activity. Peroxidase-like activity showed a peak at day 8 of culture but with no significant difference between conditions with or without antibiotic (0.74 nkatal mL⁻¹ and 1.01 nkatal mL⁻¹, respectively; Fig. 3). In conclusion, heme-peroxidases seems to be the main enzymes produced by *P. spadicum* BS34 and their production were not modified in the presence levofloxacin.

3.5. Genome sequencing and annotation of auxiliary activities of *Porostereum spadicum*

To further identify the enzymes potentially involved in the degradation of levofloxacin, the genome of *P. spadicum* was sequenced and its main CAZymes were annotated (<http://www.cazy.org/>) to characterize the global CAZome of *P. spadicum* (Drula et al., 2022). Compared to other CAZomes from related fungi, the *P. spadicum* CAZome is equipped with the classical families involved in cellulose and hemicellulose degradation, as well as a fully versatile enzymatic arsenal for lignin breakdown (Table S1 and Table S2). Note that *P. spadicum* has a slightly higher global number of CAZymes (534) than its homologs, mainly because of its higher number of enzymes encoding auxiliary activities (AA) (i.e. n = 127 versus 70 and 108 for *Phlebiopsis gigantea* and *Bjerkandera adusta*, respectively), which is an indication in favor of this strain.

Among the predicted lignin-degrading activities that could be involved in the biotransformation of levofloxacin, *P. spadicum* contains six representatives of the AA1 (multicopper oxidase, MCO) and AA3-2 (ferroxidase) families, but no AA1_1 (laccase) model was identified in its genome (Table 1). Regarding the second group of enzymes known to be central to lignin degradation that could be potential biocatalysts for the modification of antibiotic, we identified 29 representatives of class-II peroxidases together with enzymes potentially involved in hydrogen peroxide production, which are necessary for the peroxidase activities. A few enzymes have been proposed to supply H₂O₂, including the best-established candidate, which is glyoxal oxidase of family AA5_1 (9 representatives), as well as GMC oxidoreductases from family AA3_2, which includes aryl-alcohol oxidases and glucose oxidases (31 representatives).

We then compared the numbers of AA2 (putative class-II

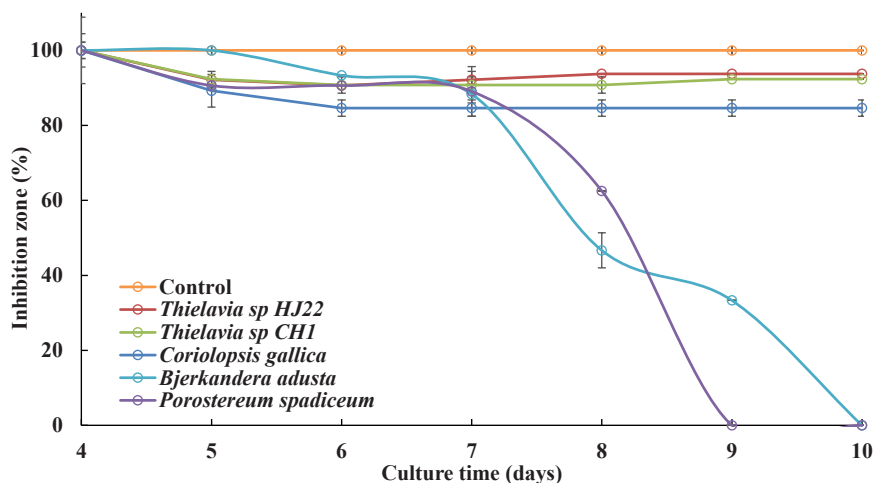


Fig. 1. The diameter of the inhibition zone decreased from day 4 to day 10 of culture in media containing 50 mg L⁻¹ of levofloxacin: antibiotic control in M7 medium (orange), *Thielavia sp. HJ22* (red), *Thielavia sp. CH1* (green), *Corioliopsis gallica* (blue) (Ben Ayed et al., 2022a), *Bjerkandera adusta* (light blue), and *Porostereum spadicum* (purple) (here). The maximum inhibition zone (100%) was attributed to the antibiotic effect of levofloxacin in the culture medium without the fungus. Data are mean ± standard deviation of triplicate experiments.

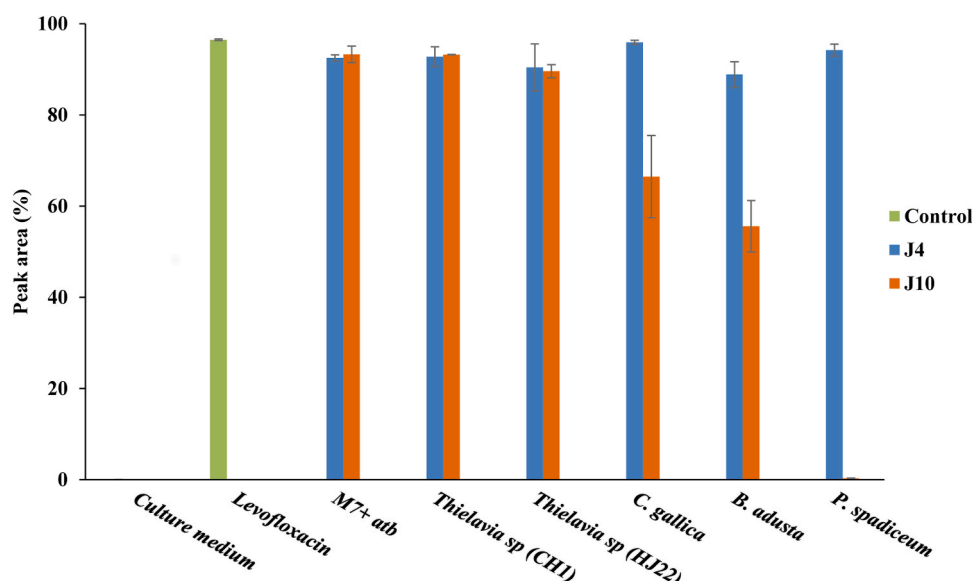


Fig. 2. Time-course data on levofloxacin degradation by *Thielavia sp. CH1*, *Thielavia sp. HJ22*, *Corioliopsis gallica* (Ben Ayed et al., 2022a), *P. spadicum*, and *B. adusta* at day 4 (here, in blue) and day 10 (orange) of culture. Culture medium alone (M7), levofloxacin at 50 mg L⁻¹ in water (green), and culture medium (M7) with 50 mg L⁻¹ levofloxacin (on day 4 and day 10) were used as controls. Each datapoint is the mean ± standard deviation of triplicate experiments.

peroxidases) and potential hydrogen providers, such as representatives of AA3_2 (aryl-alcohol oxidases and glucose oxidase) and AA5_1 (glyoxal oxidase) between fungi phylogenetically related to *P. spadicum* (29, 31 and 9 candidates, respectively). *Phanerochaete chrysosporium* (17, 35 and 7 candidates, respectively), which has long been used as a reference model for lignocellulose degradation, and its phylogenetically related strain *Phanerochaete carnosus* (12, 40 and 6 candidates, respectively) contained approximately the same number of representatives of these families (Suzuki et al., 2012) (Table 2). *B. adusta* (21, 30 and 7 candidates, respectively), which is also a wood decayer and is known as a secondary colonizer in the succession of fungal communities, had the closest enzyme profile to *P. chrysosporium* and contained more representatives of AA2 and fewer H₂O₂ enzyme providers than *P. carnosus* (Hiscox et al., 2017). The white-rot fungus *P. gigantea* had the lowest number of representatives of these three families (10 AA2, 19 AA3_2 and 6 AA5_1), with a total of just 35, which is roughly half the number of the three other fungi (Hori et al., 2014). *P. spadicum* counted the highest

number with a total of 69 representatives, for which the number of H₂O₂ providers are approximately the same whereas its number of AA2 representatives was the highest of all five fungi, i.e. 29 versus 10 and 21 for *P. gigantea* and *B. adusta*, respectively. From the genomic analysis, we concluded that *P. spadicum* has a highly versatile enzymatic arsenal for lignin breakdown, mainly represented by heme-peroxidases, and no laccase, in agreement with the enzymatic assays conducted in the previous section. In conclusion, we can confirm from this genomic analysis that *P. spadicum* has the enzymatic potential to efficiently degrade antibiotics via a large panel of oxidative enzymes.

3.6. Proteomic analysis of the fungal cultures in the presence or absence of levofloxacin

To identify the enzymes potentially involved in levofloxacin biotransformation, we performed proteomics analysis under both growth conditions with and without levofloxacin. As expected, no

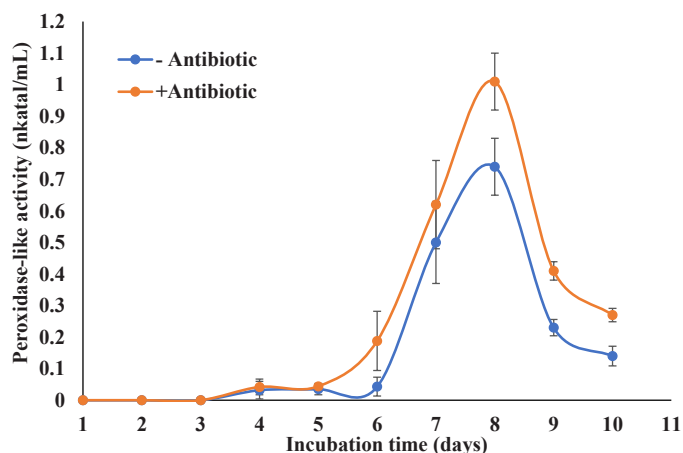


Fig. 3. Peroxidase-like activity tested with and without levofloxacin antibiotic. Each datapoint point is the mean \pm standard deviation of triplicate experiments.

Table 1

Global composition of AA-encoding genes found in *P. spadicum*.

Family	Known activities	Total number
AA1	Multicopper oxidase	5
AA1_2	Ferroxidase	1
AA2	Class-II peroxidase	29
AA3_1	Cellobiose dehydrogenase	1
AA3_2	Aryl-alcohol/Glucose oxidase	31
AA3_3	Alcohol oxidase	5
AA3_4	Pyranose oxidase	1
AA5_1	Glyoxal oxidase	9
AA6	1,4-benzoquinone reductase	7
AA8	Iron reductase domain	2
AA9	Lytic polysaccharide monooxygenase	30
AA12	Pyrrroquinoline quinone-dependent oxidoreductase	1

Table 2

Comparison of the repertoires of class-II peroxidases and hydrogen peroxide-providing enzymes among fungi related to *P. spadicum*.

AA	AA2	AA3_2	AA5_1	Total
<i>Bjerkandera adusta</i>	21	30	7	58
<i>Phanerochaete carnososa</i>	12	40	6	58
<i>Phanerochaete chrysosporium</i>	17	35	7	59
<i>Phlebiopsis gigantea</i>	10	19	6	35
<i>Porostereum spadicum</i>	29	31	9	69

laccase was identified in *P. spadicum* secretomes, whereas 21 proteins out of the 29 class-II peroxidase AA2 representatives were produced at different levels in the culture medium of *P. spadicum* (Table 3). No protein was found to be induced in the presence of levofloxacin, as none of the proteins met the fold criterion ($\text{fold} \geq 1.5$) with a low p -value ($p \leq 0.05$) (Table 3 and S3), which was confirmed by the above results (Section 3.4., Fig. 3).

Of these 21 putative proteins, Contig_2031650 was the strongest candidate protein potentially involved in the levofloxacin modification process, even though it was not overproduced when levofloxacin was added to the culture medium. Six other proteins (from Contig-2080941, 1981152, 1981122, 2156615, 1423290, and 972133) were strongly produced, and proteins from Contig_2080941 and 2156615 (“green” statistical class) with a high fold-change of 7.92 and 4.68 were selected as potential biocatalysts for levofloxacin modification, even though they did not meet both the statistical criteria for first selection. In this case, heterologous production of these two proteins should be carried out, and the proteins should be tested for their ability to modify levofloxacin. Six

other proteins were moderately produced (Contig_1997301, 832455, 2219202, 2081092, 831168 and 2089642) with fold-change of 2.83 to 4.45 and could be considered as a second set of biocatalysts for the levofloxacin modification process. The eight other proteins were poorly represented in the *P. spadicum* secretomes. Proteins from Contig_2081093 and 20881098 with a fold-change of 28.3 and 10.5, were added to the set of target proteins tested for their ability to modify levofloxacin.

Seventeen other proteins, other than class-II peroxidase AA2 representatives (Table S3), met both the criteria (fold-change and p -value) and were overexpressed in the presence of levofloxacin in the medium. Among these enzymes, two proteins of the AA3_2 family encoded by Contig_2086199 (fold-change of 7.25) and 2018111 (fold-change of 2.13) were of interest. These two AA3_2 representatives were aryl alcohol oxidase and glucose 1-oxidase, which are enzymes that provide H_2O_2 and are required by other AA enzymes, including heme peroxidases, for the oxidation of aromatics and lignin (Sützl et al., 2018). These two biocatalysts could be candidates for testing as enzyme partners for class-II peroxidases.

3.7. UHPLC-UV-MS analyses of *Porostereum spadicum* secretome extracts for the dereplication of levofloxacin degradation products

Porostereum spadicum secretomes from culture media with or without levofloxacin at day 4, 7 and 10 were studied by UHPLC-UV-MS to tentatively dereplicate the biotransformation products of levofloxacin. Five major degradation products were detected in the UV chromatograms obtained at 325 nm (Table 4). Levofloxacin m/z 362.1539 $[\text{C}_{18}\text{H}_{20}\text{FN}_3\text{O}_4 + \text{H}]^+$ eluted at 7.3 min and was still detected on UV chromatograms at days 4 and 7, but not at day 10. No degradation products were detected on day 4. High-resolution MS^1 and MS^2 spectra were further analyzed to identify putative structures for these five compounds. The MS^2 spectra were individually annotated (Fig. S2-S8) and the results were corroborated using SIRIUS software with a fragmentation tree and CSI:FingerID (Dührkop et al., 2015, 2019; Böcker and Dührkop, 2016).

Two major degradation products, 3 and 4, were characterized as m/z 392.1259, corresponding to $[\text{C}_{18}\text{H}_{18}\text{FN}_3\text{O}_6 + \text{H}]^+$ and m/z 279.0776 for $[\text{C}_{13}\text{H}_{11}\text{FN}_2\text{O}_4 + \text{H}]^+$, eluted successively at 8.0 and 8.2 min. Analysis of the MS^2 fragmentation spectrum of compound 3 indicated an oxydation and cleavage of the piperazine cycle, suggesting a structure closely related to the compound with CAS Number 2489671-26-1. Three fragments at m/z 58.0650 $[\text{C}_3\text{H}_8\text{N}]^+$, m/z 86.0598 $[\text{C}_4\text{H}_8\text{NO}]^+$, and m/z 114.0551 $[\text{C}_5\text{H}_8\text{NO}_2]^+$, consistent with the proposed structure, were formed by successive loss of the carbonyl ($\text{C}=\text{O}$) function. The structure of compound 4, the major degradation product detected, was annotated as levofloxacin with a primary amine in place of the piperazine cycle, as previously described for ofloxacin (Hori et al., 2014; Tønnesen et al., 2007; Hubicka et al., 2013; Jimenez-Villarín et al., 2016). In these studies, both the MS spectra and retention times were in accordance with the proposed structure for degradation product 4.

A minor degradation product 1 (m/z 298.0719 $[\text{C}_{13}\text{H}_{12}\text{FNO}_6 + \text{H}]^+$) was detected only on day 10. This suggests the possible formation of 1 from the most abundant product remaining, i.e. compound 4, by replacing the primary amine with a hydroxyl group. Degradation product 2 (m/z 307.0729 $[\text{C}_{14}\text{H}_{11}\text{FN}_2\text{O}_5 + \text{H}]^+$) may be produced from 3 through the loss of the aliphatic chain in the alpha position of the amide function. Compound 5 (m/z 364.1301 $[\text{C}_{17}\text{H}_{18}\text{FN}_3\text{O}_5 + \text{H}]^+$), which was detected only at trace levels on days 7 and 10, likely appeared due to the loss of the carbonyl ($\text{C}=\text{O}$) function from the tertiary amine of 3.

In conclusion, all major biotransformation processes seemed to occur mainly on the piperazine ring (oxidation reactions and ring cleavage). Further confirmation of the structural identity of these degradation products would require a spectral comparison with purified reference standards, which are currently not commercially available.

Table 3

Putative heme peroxidases potentially identified in *Porostereum spadiceum* grown in the presence (+L) or absence (-L) of levofloxacin modification, together with their percent protein identity shared with *Bjerkandera adusta* homologs and corresponding annotation (https://mycocosm.jgi.doe.gov/Bjead1_1/Bjead1_1.home.html), and their abundances based on spectral counts.

	<i>B. adusta</i> protein ID	Identity (%)	Putative annotation	Molecular mass	Fold	P-value	Statistical classes	-L1	-L2	-L3	+L1	+L2	+L3
Contig_2031650	43095	84.5	VP1	28.708	1.24	8.16E-02	Red	502	535	656	663	737	705
Contig_2080941	172246	85	LiP6	39.855	7.92	8.88E-02	Green	26	12	138	186	608	573
Contig_1981152	43329	86.4	MnP1	37.71	-1.11	1.22E-01	Red	292	331	326	372	282	303
Contig_1981122	43329	80.5	MnP1	37.512	-1.25	1.45E-02	Orange	371	402	408	321	309	317
Contig_2156615	172246	86.7	LiP6	39.832	4.68	5.77E-02	Green	31	19	146	193	443	292
Contig_1423290	306404	90.4	MnP3	37.717	1.44	9.24E-02	Red	175	175	277	264	315	328
Contig_972133	306404	85.7	MnP3	37.671	1.33	1.13E-01	Red	154	169	235	219	257	269
Contig_1997301	116816	92.2	LiP3	34.306	4.45	8.44E-02	Green	18	13	68	72	207	172
Contig_832455	116816	90	LiP3	39.533	2.83	1.06E-01	Green	17	13	60	53	127	80
Contig_2219202	120002	86.6	LiP4	40.037	3.27	9.74E-02	Green	13	13	42	43	112	74
Contig_2081092	269481	89.3	LiP9	39.45	3.76	3.48E-01	Green	7	0	48	30	159	26
Contig_831168	116816	91.2	LiP3	39.372	3.68	6.98E-02	Green	9	7	38	40	97	70
Contig_2089642	306863	84	MnP4	38.012	-1.05	6.48E-01	Red	62	64	77	63	72	59
Contig_1941337	41982	91.8	LiP2	40.715	12.22	1.49E-01	Green	0	0	6	6	49	52
Contig_2081093	172152	86.7	MnP5	38.076	28.33	1.99E-01	Green	0	0	0	10	16	56
Contig_2081098	41982	91.5	LiP2	40.35	10.5	1.57E-01	Green	0	0	3	3	26	31
Contig_1313540	121664	88.7	LiP5	39.66	2.07	3.16E-01	Green	4	3	19	6	31	20
Contig_2132150	121664	88.8	LiP5	38.699	1.93	3.63E-01	Green	4	3	19	5	29	19
Contig_445179	173495	70.6	GP1	38.344	1.43	1.50E-01	Red	3	3	5	7	4	6
Contig_2072951	172152	75.2	MnP5	38.207	3.80	3.49E-01	Green	0	1	1	2	1	13
Contig_1361988	175536	83.9	MnP6	42.229	5.33	3.77E-01	Green	0	0	0	1	0	12

*Statistical classes. Orange class: did not meet the fold criterion but warrant a second look as they have low p-values; Green class: met the fold-change criteria but this was most likely by chance, so they should be ruled out; Red class: did not meet the fold criteria and p-value criteria, so they should be ruled out.

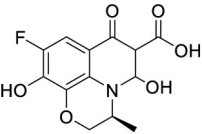
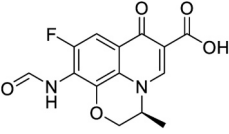
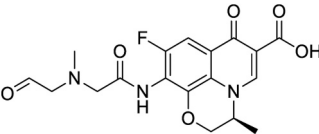
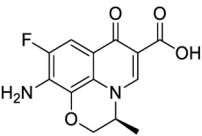
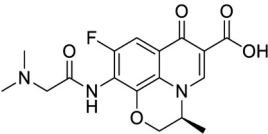
4. Discussion

This study aimed to evaluate the capacity of two basidiomycete fungal strains (*P. spadiceum* and *B. adusta*) to biotransform the fluoroquinolone levofloxacin and to identify the potential enzymes involved in the process. In this study, *P. spadiceum* and *B. adusta* showed complete levofloxacin biotransformation in both solid and liquid media on days 9 and 10 of culture, respectively. Other previously tested fungal strains including *Thielavia* sp. HJ22, CH11 and *C. gallica* did not exhibit such levels of levofloxacin biotransformation under the same experimental conditions (Ben Ayed et al., 2022). *C. gallica* was shown to biotransform only 25% of levofloxacin in 10 days of culture (Ben Ayed et al., 2022). In comparison with other fluoroquinolone drugs, the ascomycete fungus *Trichoderma asperellum* was able to degrade 81% and 44% of ciprofloxacin and norfloxacin, respectively, after 13 days of incubation (Manasfi et al., 2020), whereas Chakraborty and Abraham (Chakraborty and Abraham, 2017) showed that the basidiomycete fungus *Ganoderma lucidum* JAPC was able to degrade 100% of norfloxacin after 4 days of incubation but was unable to degrade ciprofloxacin. Comparing these studies and the variations in biotransformation rate, it could be hypothesized that the differences in removal efficiencies could be due to

difference in the molecular structure of the antibiotics and the selected fungal species (Chakraborty and Abraham, 2017).

Antibiotic treatment can be mediated by several reactions, such as bioadsorption, biomineralization (bio-precipitation), and biotransformation and biodegradation mediated by enzymatic systems (Olicón-Hernández et al., 2017). Here we focused on the biotransformation of levofloxacin by fungal enzymatic systems, and in particular the action of extracellular enzymes involved in lignin degradation, i.e. class-II peroxidases and laccases. From our knowledge, only a few works were performed to identify the enzymes potentially involved in the antibiotic degradation. In a recent proteomic approach, *C. gallica* was demonstrated to secrete a diverse panel of laccases with dye-decolorizing peroxidases (DyP), but no class-II peroxidase AA2 representatives, that are potential enzymes involved in this process (Ben Ayed et al., 2022). Information pertaining to the metabolic activities of *P. spadiceum* is scarce. *P. spadiceum* is known to be halotolerant (up to 50 mM) and is involved in the production of plant growth-promoting gibberellins (Gas) as well as volatile compounds that act as inhibitors of plant-pathogenic bacteria and fungi (Hamayun et al., 2017; Hamamoto et al., 2021). Given the paucity of available information, we assessed the enzymatic activities of the *P. spadiceum* secretomes together with genome

Table 4
Proposed biotransformation products of levofloxacin in the presence of *P. spadicum*.

#	Rt (min)	[M + H] ⁺ m/z	Molecular formula	Relative occurrence		Putative structure	SMILES (CAS Number)	Confidence Level ^a
				d7	d10			
1	7.5	298.0719	C ₁₃ H ₁₂ FN ₂ O ₆	Tr.	+		OC1=C(C=C2C(C(O)=O)C(O)N3C2=C1OC[C@@H](C)C(=O)F)(-)	2b
2	7.6	307.0729	C ₁₄ H ₁₁ FN ₂ O ₅	+	++		N(C=O)C=1C2=C3C(C=O)C(C(O)=O)=CN3[C@@H](C)CO2)=CC1F(2305668-58-8)	2b
3	8.0	392.1259	C ₁₈ H ₁₈ FN ₃ O ₆	+++	++		C[C@H]1COc2c(NC(=O)CN(C)CC=O)c(F)cc3c(=O)c(C(=O)O)cn1c23(-)	2b
4	8.2	279.0776	C ₁₃ H ₁₁ FN ₂ O ₄	++++	++++		O=C1C=2C=3N(C=C1C(O)=O)[C@@H](C)COC3C(N)=C(F)C2(151250-74-7)	2b
5	8.4	364.1301	C ₁₇ H ₁₈ FN ₃ O ₅	Tr.	Tr.		C[C@H]1COc2c(NC(=O)CN(C)C)c(F)cc3c(=O)c(C(=O)O)cn1c23(123975-27-9)	2b

Compound number; Rt: Retention time; ^a Identification Confidence Level according to Shymanski et al., 2014 (Schymanski et al., 2014).

sequencing and proteomic analysis to determine the enzyme machinery involved in the levofloxacin biotransformation process.

Our enzymatic assays, performed with or without levofloxacin, revealed that only peroxidase activity was found in the extracellular medium of *P. spadicum*. Spina et al. (Spina et al., 2018) indicated that *P. spadicum* MUT 1585 mainly produces peroxidases rather than laccases. In contrast, Gul et al. (Gul et al., 2022) found that *P. spadicum* strain AGH786 showed high polyphenol oxidase activity. This result indicates that different enzymatic activities could be deployed as a function of strain identity. As a frame for comparison, we previously assayed both laccase-like activity and peroxidase-like activity in *C. gallica* culture medium during the biotransformation of levofloxacin, and found that laccase activity was stronger (Ben Ayed et al., 2022). Here, we found no significant difference in the peroxidase-like activity of *P. spadicum* in the presence vs. absence of levofloxacin, indicating that the enzyme machinery was neither induced or repressed in the presence of levofloxacin.

To annotate the sequenced genome of *P. spadicum* and to identify the exact enzymes involved for these activities, we focused on the Auxiliary Activities enzymes involved in the aromatic and lignin degradation processes. No gene encoding laccase was found in the *P. spadicum* genome, but a diverse set of class-II peroxidases (n = 29 representatives) and putative enzymes providing hydrogen peroxide (n = 40 representatives) were found, thus corroborating the activity assays. *B. adusta* and *P. chrysosporium*, which are relatively well equipped for both categories of enzymes totalized, a lower number of 58 and

59 representatives, respectively, for class II peroxidase and hydrogen providers belonging to the AA3_2 and AA5_1 families. Considering this specificity and the results obtained with levofloxacin biotransformation, we suggest that *P. spadicum* warrants further attention for investigation in the levofloxacin degradation process.

To identify the potential enzyme machinery involved in levofloxacin biotransformation, we used a comparative proteomics approach to analyze the secretomes of *P. spadicum* cultivated in the presence and absence of the antibiotic. No protein of the class II peroxidase group was found to be differentially produced in the two culture conditions, as already been shown in the differential proteomic analysis of *C. gallica* (Ben Ayed et al., 2022). However, a total of 21 class-II peroxidase out of the 29 representatives identified in *P. spadicum* was found in the secretomes. One protein was strongly produced (Contig_2031650) and merits further study for levofloxacin modification. Six other proteins were also found to be relatively strongly produced, and the proteins encoded by Contig_2080941 and 216615 were proposed as alternative proteins of interest. Other proteins, such as glyoxal oxidases, glucose oxidases, and aryl alcohol oxidases, are involved in the production of hydrogen peroxide and are synergistically coupled with class-II peroxidases. In fact, class-II peroxidases use hydrogen peroxide as an electron acceptor to catalyze a number of oxidative reactions and, as such, are crucial enzyme partners for their activities. Two proteins encoded by Contig_2086199 and Contig_2018111 could be enzyme partners of peroxidases that play a role in the biotransformation of levofloxacin. An accurate functional annotation of these genes should be conducted,

together with cloning, heterologous production, and detailed biochemical characterization of each protein in parallel to evaluate their ability to biotransform levofloxacin and give some insights about the exact mechanism of the levofloxacin degradation. While laccases have already been demonstrated to be useful for the biotransformation of some antibiotics, few studies have focused on heme-peroxidase-degrading antibiotics. Lueangjaroenkit et al. (Lueangjaroenkit et al., 2019) reported that purified MnP from *T. polyzona* was able to degrade 100% and 73% of tetracycline and ciprofloxacin, respectively (Lueangjaroenkit et al., 2019).

Overall, the levofloxacin degradation process driven by *P. spadicum* remained relatively long, at 9 days to fully biotransform levofloxacin. It is possible that antibiotic degradation may only start once the fungi have consumed most of the more readily-accessible nutrients from the culture medium. Moreover, the five major levofloxacin degradation products conserved their quinolone core, and may thus retain some of their antibiotic properties, notably towards gram-positive bacteria (Pham et al., 2019).

5. Conclusion

Further experiments are warranted in an attempt to accelerate levofloxacin degradation by *P. spadicum* and possibly achieve biotransformation of the quinolone antibiotic core. For this purpose, two options can be considered: (1) using culture media with optimized compositions in response surface methodology (RMS) to induce specific enzymes involved in the degradation of antibiotics, and/or (2) co-culturing different basidiomycete strains known for their complementary metabolic properties. Another challenge is to elucidate the enzymatic mechanism involved in the degradation of levofloxacin. To solve it, we would need to produce the main heme-peroxidases and test them in a separate or a synergistic way to identify the main enzyme actors of the process. Nevertheless, the results reported in this manuscript increase our knowledge of the metabolic potential of *P. spadicum* and offer further perspectives for its application to fluoroquinolone biodegradation.

Environmental implications

Levofloxacin is a fluoroquinolone drug that is used to treat bacterial infections. Although it has benefits for human medicine, it has also been detected in the environment at a non-metabolization rate of 70%, which creates an environmental hazard and contributes to the spread of resistant bacteria. Antibiotic resistance is constantly increasing, which poses an increasing threat to the environment and while decreasing the effectiveness of the antibiotics we need. There is therefore an urgent need to develop efficient treatments to address these hazardous phenomena. This work is a proof of concept for applying the bioprocessing of a fluoroquinolone antibiotic as a sustainable and environmentally-friendly method that can use filamentous fungi and their enzymes to replace the current physical-chemical methods that are not only costly but also generate toxic byproducts.

CRediT authorship contribution statement

Maalej Amina: Methodology, Writing – review & editing. **Chamkha Mohamed:** Methodology, Writing – review & editing. **Faulds Craig B.:** Writing – original draft, Writing – review & editing. **Chaduli Delphine:** Data curation, Methodology, Writing – review & editing. **Lomascolo Anne:** Writing – original draft, Writing – review & editing. **Bertrand Emmanuel:** Writing – original draft, Writing – review & editing. **Turbé - Doan Annick:** Methodology, Writing – review & editing. **Drula Elodie:** Methodology. **Navarro David:** Data curation, Methodology, Writing – review & editing. **Kielbasa Mélodie:** Data curation, Methodology, Validation. **Akrout Imen:** Investigation, Methodology, Writing – original draft, Writing – review & editing. **Ben Ayed Amal:**

Conceptualization, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Armengaud Jean:** Conceptualization, Data curation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Savage Emily:** Methodology. **He Guifen:** Methodology. **Lipzen Anna:** Data curation, Methodology, Validation, Writing – original draft, Writing – review & editing. **LaButti Kurt:** Methodology. **RECORD Eric:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **Ahrendt Steven:** Data curation, Methodology, Software, Validation, Writing – review & editing. **Mechichi Tahar:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing. **Simmler Charlotte:** Conceptualization, Data curation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. **Sciara Giuliano:** Conceptualization, Methodology, Writing – review & editing. **Greff Stéphane:** Conceptualization, Data curation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. **Zouari-Mechichi Héla:** Methodology, Validation, Writing – original draft, Writing – review & editing. **Albert Quentin:** Conceptualization, Writing – original draft, Writing – review & editing. **Martin Francis:** Resources, Writing – original draft, Writing – review & editing. **Staita Karima:** Investigation, Methodology, Writing – original draft. **Grigoriev Igor V:** Resources, Writing – original draft, Writing – review & editing. **Barry Kerrie:** Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ben Ayed Amal reports financial support was provided by Programme Hubert Curien - Utique. Grigoriev Igor V reports financial support was provided by US Department of Energy Office of Science.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2023.115808](https://doi.org/10.1016/j.ecoenv.2023.115808).

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