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Methylobacterium oryzae Influences Isoepoxydon Dehydrogenase Gene Expression and Patulin Production by *Penicillium expansum*

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Abstract: Biofilms can be considered the main source of microorganisms in drinking water distribution systems (DWDS). The ecology of a biofilm is dependent on a variety of factors, including the presence of microbial metabolites excreted by its inhabitants. This study reports the effect of the Gram-negative bacteria *Methylobacterium oryzae* on the *idh* gene expression levels and patulin production of *Penicillium expansum* mature biofilms. For this purpose, a RT-qPCR method to quantify *idh* mRNA levels was applied. In addition, the *idh* expression levels were compared with the patulin production. The results obtained revealed that the effect of the bacterium on pre-established *P. expansum* biofilms is dependent on the time of interaction. More mature *P. expansum* biofilms appear to be more resistant to the inhibitory effect that *M. oryzae* causes towards *idh* gene expression and patulin production. A positive trend was observed between the *idh* expression and patulin production values. The results indicate that *M. oryzae* affects patulin production by acting at the transcriptional level of the *idh* gene.

Keywords: bacteria; fungi; inter-kingdom biofilms; *idh* gene expression; patulin; quorum sensing inhibitor

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1. Introduction

Water biofilms can be considered the main source of microorganisms in drinking water distributions systems (DWDS) representing over 95% of biomass in these systems [1,2]. They can cause the deterioration of drinking water quality through a variety of factors: Changes in the organoleptic properties of the water, act as a reservoir for pathogenic microorganisms, increase corrosion and blockage of pipes, and be responsible for the production of toxins [3–7]. In DWDS, under natural conditions, biofilms are considered complex communities ruled by complex relationships involving inter- and intraspecies interactions [8,9]. Fungi and bacteria are known to coexist inside DWDS by forming and developing biofilms [8,10]. The inter-kingdom interaction is dependent on a complex mix of prevailing growth conditions, hydrodynamic forces, and the presence of microbial metabolites and molecules [11–13].

Quorum sensing (QS) is a mechanism employed by microbial species that allows the perception of population density by the production, release, and detection of small molecules (autoinducers), which in turn modify bacterial gene expression [14]. QS is used to control and regulate different bacterial population-density processes, including pathogenicity, stress resistance, biofilm formation, and production of secondary metabolites and toxins [15,16]. In contrast, fungi have the ability to produce metabolites that interfere with bacterial QS [17]. Some of these are mycotoxins and they can act as QS inhibitors (QSI) by mimicking the autoinducer structure or function, acting as antagonists to or hydrolysing QS molecules, and by interfering with the stability and function of the regulator protein or autoinducer synthase [18]. Filamentous fungi have also been associated with the production of mycotoxins in water [2,19,20]. The term mycotoxin refers to

harmful secondary metabolites produced by fungi. Initially thought to be waste products, fungal secondary metabolites are now being considered as important players in ecological settings allowing the fungus to secure its environmental niche by providing protection against other microbes [17]. Fungal secondary metabolites are known to be involved in the disruption of QS in bacteria [21]. For instance, fusaric acid acts as a quorum quencher against *Pseudomonas chlororaphis* [22]. Furthermore, two other mycotoxins produced by the genus *Fusarium*, zearalenone, and fumonisin have been shown to inhibit QS in the bacterium *Chromobacterium violaceum* [23]. Filamentous fungi from the genus *Penicillium*, have also been investigated for their ability to produce QSIs [24]. Patulin, a well-known mycotoxin with a low molecular weight (154.12 g mol^{-1}) and high polarity was shown to downregulate genes related with QS in *Pseudomonas aeruginosa* by 45% indicating specificity for QS-regulated gene expression (Rasmussen et al. 2005). In addition, this mycotoxin has been reported to reduce biofilm formation and the QS signaling system by *Halomonas pacifica*, as well as modulating biofilm formation by *P. aeruginosa* and *Achromobacter* sp. [25,26]. Furthermore, patulin has been shown to reduce biofilm formation by *Methylobacterium oryzae* [13].

Despite some known effects of patulin on biofilm formation and/or bacterial QS-regulated gene expression, little to no information is available on the effect and possible modulation that bacteria themselves cause on the expression of genes involved in the patulin biosynthesis and/or patulin production. The patulin biosynthetic pathway consists of approximately 10 steps [27,28]. The enzyme isoepoxydon dehydrogenase has been identified as a precursor in the late steps of the patulin biosynthetic pathway and the *idh* gene encoding this enzyme has been cloned and sequenced from *P. expansum* [29]. Real-time reverse transcription polymerase chain reaction (RT-qPCR) is the method of choice for sensitive, specific, and reproducible quantification of mRNA levels of a transcribed gene [30].

The goal of this study was to investigate the effect of the Gram-negative bacterium *M. oryzae* on the *idh* gene expression levels and patulin production of *P. expansum* mature biofilms.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

Penicillium expansum MUM 00.02, supplied by Micoteca da Universidade do Minho fungal culture collection (MUM, Braga, Portugal), was used in this work and chosen based on its occurrence in the tap water of the north of Portugal and since it is able to form single and inter-kingdom biofilms [12,13,31,32]. It is also a well-known producer of patulin. *P. expansum* was maintained on malt extract agar (MEA: Malt extract 20 g, peptone 5 g, agar 20 g, distilled water 1 L).

Methylobacterium oryzae was previously isolated from a model laboratory DWDS by Simões et al. [33]. This bacterium was chosen for being representative of drinking water bacteria and due to its ability to form complex single species biofilms, as well as inter-kingdom biofilms with this fungus [12,13,34]. *M. oryzae* was grown for 72 h before the start of the assay. It was grown in batch cultures using 100 mL of R2A broth (yeast extract 0.5 g, proteose peptone 0.5 g, casein hydrolysate 0.5 g, glucose 0.5 g, soluble starch 0.5 g, dipotassium phosphate 0.3 g, magnesium sulphate 0.024 g, sodium pyruvate 0.3 g, distilled water 1 L) at room temperature (25 ± 2 °C) and under agitation (150 rpm). Afterwards, the bacteria were harvested by centrifugation (10 min at 13,000× *g*, room temperature), washed twice in 0.1 *M* saline phosphate buffer, and resuspended in a volume of 50% R2A broth and 50% apple juice (APJ) to obtain a cellular density of 10^8 cells mL^{-1}. This was the bacterial concentration used for biofilm formation assays.

2.2. Stock Solution of Fungal Spores

The stock solution of fungal spores was prepared according to Simões et al. [32]. Briefly, spores of *P. expansum* were harvested from 7-day old pure cultures in MEA at 25 °C

by flooding the surface of the agar plates with 2 mL of TWS solution (0.85% NaCl plus 0.05% Tween 80) and rocking gently. The suspension was then homogenized by vortexing and used for large scale production of spores. The final spore suspension was homogenized by vortexing before quantification using a Neubauer count chamber. Aliquots of spore suspension with 10% of glycerol were cryopreserved at −80 °C in order to allow the use of the same spore suspension in all the biofilm assays. Stock spore suspensions were resuspended in the volume of R2A broth necessary to achieve a density of 10^5 spores mL^{-1}. This was the spore concentration used for biofilm formation assays.

2.3. Apple Puree Agar Medium (APAM) and Apple Juice (APJ) Preparation

APAM was prepared as described by Baert et al. [35]. Briefly, apples (cultivar Golden Delicious) were cut into small pieces (with the peel, but without the core) and mixed until a fine puree was obtained. This puree was stored at −20 °C. To prepare APAM, 100 g of apple puree was weighed in a sterile Schott bottle and heated during 45 min in a hot water bath at 99 °C. This was done to kill vegetative cells. In addition, 50 mL sterile agar solution was then added to the puree to make the APAM. Under aseptic conditions, Petri dishes were filled with 20 g APAM.

To prepare APJ, 50 g of apple puree was weighed in a sterile Schott bottle and 300 mL of sterile H_2O was added to the puree. The Schott was heated at 99 °C for 10 min to help mix the puree with the water. After this step, the solution was filtered using a first layer of gauze followed by a second filtration using a 12–15 µm qualitative filter paper (VWR, Radnor, PA, USA) into a new sterile Schott bottle. After the filtering process, the Schott bottle containing the final APJ, similarly to APAM, was heated during 45 min in a hot water bath at 99 °C.

2.4. Biofilm Formation

Biofilms were developed according to the modified microtiter plate test used by Stepanović et al. [36] for bacteria and Simões et al. [32] for fungi. Briefly, wells of sterile polystyrene 96-well flat bottom culture plates (Greiner Bio-One Cellstar®, Kremsmünter, Austria) were filled under aseptic conditions with 200 µL of spore suspension (10^5 spores mL^{-1} in 50% R2A broth and 50% APJ) or 200 µL of a cell suspension (10^8 cell mL^{-1} in 50% R2A broth and 50% APJ) for the single-species biofilms, which were also used as positive controls. To promote biofilm formation, all plates were incubated aerobically at room temperature (25 ± 2 °C) and under agitation (150 rpm) for 120 h. *P. expansum* biofilms were developed for 96 h. Each 24 h, the depleted medium was removed, and a fresh, clean medium was added to each well. In parallel, at 48 and 72 h in different batches of *P. expansum* biofilms, the depleted medium was also removed, but the fresh medium inoculated with 10^8 cells mL^{-1} of *M. oryzae* was added. This co-growth was analyzed for 24 h, meaning that the interaction between these two microorganisms was evaluated from 48 to 72 h and from 72 to 96 h. *M. oryzae* single species biofilms at 24 h were also used as positive controls. At each sampling time (72 and 96 h) the content of each well was removed and washed two times with 200 µL of sterile distilled water to remove non-adherent and weakly adherent cells. The plates were air dried for 30 min to remove excess water by evaporation. The remaining attached cells were analyzed in terms of biomass adhered on the inner walls of the wells, in terms of colony forming units (CFU) for bacteria. The rinsing and drying procedures were as previously used with bacterial and fungal biofilms [32–34]. Negative controls were obtained by incubating the wells with only 50% R2A broth and 50% APJ without adding any fungal spores or bacterial cells. Experiments were performed in triplicate with at least 3 repeats.

2.5. Biofilm Mass Quantification by Crystal Violet

The biomass adhered on the inner walls of the wells was quantified by the crystal violet (CV) method according to the procedure described by Stepanović et al. [36]. The biofilms in the 96-well plates were fixed with 200 µL $well^{-1}$ of 98% methanol (VWR,

Carnaxide, Portugal), for 15 min. Afterwards, the methanol was discarded, the plates left to dry and then the fixed biofilm was stained with 200 µL well^{-1} of CV (Merck KGaA, Darmstadt, Germany) for 5 min. The excess stain was rinsed out by placing the plate under a slow running tap water. After this, the plates were air dried and the dye bound to the adherent cells was resolubilized by adding 200 µL well^{-1} of 33% ($v\ v^{-1}$) glacial acetic acid (Panreac, Cascais, Portugal). The optical density of the obtained solution was measured at 570 nm using a microtiter plate reader (BioTek, Winooski, VT, USA) and the biofilm mass was presented as $OD_{570\,nm}$ values.

2.6. Number of Bacteria in Single and Inter-Kingdom Biofilms

The number of bacterial cells present in *M. oryzae* single-species biofilms and in inter-kingdom biofilm after 24 h of co-growth with *P. expansum* was determined in terms of CFU using a plate count assay upon biofilm release. Briefly, *M. oryzae* single-species biofilms and inter-kingdom biofilms were grown in a 96-well plate as described above. After 24 h of incubation period, the supernatant was removed, and the plate was washed three times with sterile water. A volume of 200 µL of sterile phosphate buffer saline (pH 7.4) was added into each well. Afterwards, biofilms were scrapped with a pipette tip and, in addition, the 96-well plate was covered with the lid and placed into an ultrasonic bath (Bandelin electronic GmbH & Co. KG, Berlin, Germany). To release bacterial cells from the biofilm, the plate was sonicated for 1 min (5 s sonicate, 10 s interval) at 35 kHz. Three replicates were used for each condition and bacterial cells were plated onto R2A agar plates for CFU determination.

2.7. RT-qPCR Method to Measure Idh Gene Expression of P. expansum

2.7.1. RNA Extraction and DNase Treatment

Biofilms were prepared as described in Section 2.4. At each sampling time (72 and 96 h) the content of each well was removed and washed two times with 200 µL of sterile distilled water to remove non-adherent and weakly adherent cells. After this washing step, the contents of at least 3 wells were scrapped off the microtiter plate and transferred to a 2 mL microcentrifuge tube (Frilabo, Maia, Portugal) with 0.6 g of 710–1180 µm glass beads, acid-washed (Sigma-Aldrich, St. Louis, MO, USA). The microcentrifuge tube was immediately placed on ice until all the samples to be tested were collected. When all the samples were collected (time taken < 5 min), RNA was extracted by means of the PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). Briefly, 600 µL of Lysis Buffer was added to each microtube containing fungal biomass and glass beads, which were then homogenized in a FastPrep-24™5G Instrument (MP Biomedicals, Irvin, CA, USA). The homogenization procedure was as follows: One homogenization cycle for 45 s at 6 m s^{-1}, followed by 30 s on ice, followed by a second homogenization cycle for 45 at 6 m s^{-1}. The microtubes were again placed on ice and the rest of the RNA extraction procedure followed the manufacturer's instructions. Total RNA was eluted in a final volume of 50 µL nuclease-free H_2O (Invitrogen, Carlsbad, CA, USA).

The DNase I, RNase-free (Thermo Scientific, Waltham, MA, USA) was used to remove any residual DNA. Two microliters of DNase I buffer and 1 U DNase I were added to a 200 µL centrifuge tube containing 20 µL total RNA. After gentle inversion, the mixture was incubated for 30 min at 37 °C. After the incubation period, 2 µL EDTA was added to the RNA mixture and thoroughly resuspended by smooth pipetting, and incubated for 5 min at 65 °C to inactivate DNase I. The tube was then stored at −20 °C until the next day when further analysis was conducted.

RNA samples were visually checked after staining a 1.5% (*w/v*) agarose gel (NZYTech, Lisbon, Portugal) with GreenSafe Premium (NZYTech). Non-denaturing electrophoresis was carried out at 80 V for 45 min. The Nanodrop® ND-1000 spectrophotometer (Thermo Scientific) was used to measure the RNA concentration and nucleic acid purity based on the A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios for protein contamination and polysaccharide and/or chaotropic salt contamination, respectively.

2.7.2. The cDNA Synthesis

The cDNA synthesis was done with the Xpert cDNA synthesis kit (GRiSP, Porto, Portugal) according to the manufacturer's instructions. All DNase treated RNA extracts were 1/10th diluted before converting to cDNA to prevent the possible presence of sample inhibition [37]. Prior to the cDNA synthesis by means of reverse transcription, a heat shock treatment was applied to the mixture of template RNA, dNTPs, and primers for sequence denaturation. In this way, the absence of secondary structures of RNA and/or primers is assured, guaranteeing a more efficient cDNA synthesis. Hence, a mixture of 50 ng of total RNA, 1 µL of random hexamer primers (10 µ*M*), 1 µL of dNTPs (10 m*M* each), and RNase-free water up to 14.5 µL (GRiSP) were subjected to heat shock for 5 min at 65 °C followed by 2 min cooling on ice. After the heat shock, 4 µL of 5× reaction buffer, 0.5 µL of RNase inhibitor (40 U μL^{-1}), and 1 µL of Xpert reverse transcriptase (200 U μL^{-1}) were added to the reaction mixture obtaining a final volume of 20 µL. Reverse transcription was carried out with a C1000^{TM} Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the following conditions: 10 min at 25 °C, 15 min at 50 °C, and 5 min at 85 °C. To check for residual genomic DNA (gDNA), each RNA sample was also subjected to a cDNA synthesis reaction without the addition of reverse transcriptase enzyme (noRT) [38]. cDNAs were then stored at −20 °C until the qualitative real-time PCR assay was performed.

2.7.3. Quantitative Real-Time PCR (qPCR) Assay

Optimization of the qPCR is essential for an accurate quantification [38]. The *idh* gene expression levels in each sample were quantified relative to the expression of the reference gene *β-tubulin*. The set of primers used in this study were selected from De Clerc et al. [37] (Table 1).

Table 1. Primers and primer efficiency.

Gene	Primer Set	F Primer Sequence (5′ to 3′)	R Primer Sequence (5′ to 3′)	Product Length (bp)	qPCR Efficiency (%)
idh	EK-RT-2F/EK-RT-2R	GCAGTTTCGCGATCGATGT	GTAGGGAGTAGCCGCCTTGA	59	92.1
β-tubulin	BTub-2F/BTub-2R	GGTCCCTTCGGCAAGCTT	TGTTACCAGCACCGGACTGA	64	93.0

For the qPCR itself, a reaction mixture containing 5 µL Xpert Fast SYBR Mastermix (GRiSP), 300 n*M* (10 m*M* μL^{-1}) of each of the appropriate gene-specific primers, 2 µL of template cDNA (1/10th diluted to prevent the presence of inhibitors), and ultra-pure water up to 10 µL was prepared for each sample. Reaction mixtures were added to a clear, low profile 96-well PCR plate (Thermo Scientific) and sealed using an adhesive sealing foil (Frilabo). For each sample (duplicates), a relative quantification of the mRNA levels of the gene of interest against the reference gene was done using a CFX96^{TM} real-time system (Bio-Rad) coupled to a C1000^{TM} Thermal Cycler (Bio-Rad). Thermal cycling conditions were as follows: 2 min at 95 °C to activate the polymerase enzyme, followed by 40 cycles of denaturation at 95 °C for 5 s, and a one-step annealing and elongation during 20 s at 60 °C. The data were collected during each elongation step. A melting-curve analysis between 65 and 95 °C was performed after each PCR to check the specificity of the amplification product. Samples were tested for residual gDNA by comparing the Cq values of RT cDNA and noRT cDNA reactions. When $\Delta Cq > 7$ was obtained, gDNA contamination was considered negligible [37]. In each run, a negative control (without cDNA) was included. Both genes were quantified with biological duplicates for each independent assay. The expression of *idh* was normalized to the expression of the reference housekeeping gene *β-tubulin* using the Pfaffl method and considering *P. expansum* single-species biofilms at 72 and 96 h as controls [39].

Preliminary results of qPCR amplification in the conditions selected for this work, revealed high Ct values (>28 cycles) for the *idh* gene, which would not allow for enough 10× serial dilutions of cDNA to recalculate primer efficiency. For this reason, efficiency was recalculated in house for the *P. expansum* strain used in this study in APAM. Briefly, *P. expansum* MUM 00.02 was middle-point inoculated onto APAM Petri plates and incubated at 25 °C for 7 days. Sporulating mycelium was then scrapped into a 2 mL microcentrifuge tube and RNA was extracted by the method described in Section 2.7.1. After cDNA synthesis and qPCR analysis, efficiency was determined with the CFX Manager™ Software version 3.1 (Bio-Rad) using the dilution curve method. Then, cDNA was 10× serial diluted, and the amplification curves were used for efficiency calculation (Table 1).

2.8. Patulin Extraction and Quantification by UHPLC

2.8.1. Reagents and Chemicals

Pure patulin standard (≥98%) was purchased from Cayman Chemical (Ann Arbor, MI, USA). A stock solution of 5 mg mL^{-1} was prepared in dimethyl sulfoxide (DMSO) (Merck KGaA) and stored at −20 °C until use. Ethyl acetate (AcOEt) and acetonitrile (ACN) (HPLC gradient grades) were supplied by Fischer Chemical (Hampton, NH, USA). HPLC-grade water was generated by a Milli-Q-grade purification system (Millipore, Darmstadt, Germany).

2.8.2. Patulin Extraction

The patulin extraction method was based and adapted from Sargenti and Almeida [40]. The contents of 8 wells of each condition (1.6 mL), including medium and biomass, were scrapped and transferred to 15 mL centrifuge tubes (Orange Scientific, Braine-I'Alleud, Belgium). The double volume of AcOEt was then added to the Falcon tube. The extraction procedure was as follows: 5 min vortexing, followed by 10 min in an ultrasonic bath (Bandelin electronic GmbH & Co. KG, Berlin, Germany) at 35 kHz, followed by vortexing for another 5 min. The AcOEt phase was separated and transferred to a 4 mL vial and placed under at 40 °C under a flow of gentle nitrogen stream to evaporate the solvent. When the residue was completely dry, it was kept at −20 °C until all the samples were extracted. The residues were then resuspended in 1 mL of HPLC-grade water adjusted to pH 4.0 with acetic acid and vortexed for 1 min. Finally, the extracts were filtered into microvials using a 2 mL Omnifix syringe (B. Braun, Melsungen, Germany) coupled with a 0.22-µm nylon syringe filter (Nantong FilterBio Membrane, Nantong City, China) and analyzed by liquid chromatography. Quantification was performed by means of internal calibration curves of increasing concentrations: One in a range from 0.1 to 2.5 µg mL^{-1} and another one from 10 to 100 µg mL^{-1} of pure patulin standards. The patulin recovery percentage using the present method was calculated by spiking the medium with two different concentrations of patulin, in this case, 1 and 10 µg mL^{-1}, in triplicates and the chromatographic analysis was performed in duplicates. The recovery percentage varied from 68.74 to 84.23% with a mean of 79.78 ± 5.65%.

2.8.3. UHPLC Apparatus and Conditions

Liquid chromatography was performed using a Nexera X2 UHPLC System equipped with a DGU-20A5R degassing unit, LC-30AD pump, SIL-30AC autosampler, CTO-20AC column oven, and an SPD-M20A photodiode array detector set at 276 nm (Shimadzu Corp., Kyoto, Japan). Chromatographic separation was carried out with a Chromolith® Performance RP-18 endcapped 100–4.6 HPLC column (Merck KGaA). The oven temperature was maintained at 25 °C. The injection volume was set at 5 µL and the sample flow rate at 0.75 mL min^{-1}. The run time was 10 min with a mobile phase of H_2O:ACN (95:5, *v/v*). The patulin average retention time was obtained at around 4.4 min.

2.9. Statistical Analysis

The data were analyzed using the software GraphPad Prism version 8.0.2 (GraphPad Software, La Jolla, CA, USA). The mean and standard error (SE) were calculated for all the cases. The statistical significance of results was determined by the unpaired *t*-test. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Biofilm Mass Quantification

In order to assess the effect of *M. oryzae* on pre-established biofilms of *P. expansum*, the standard 96-well microtiter plates with CV was used. Results for biofilm mass quantification are presented in Figure 1. *P. expansum* single-species biofilms were used as controls. The results revealed that *P. expansum* single-species biofilms increased in mass until 96 h. The introduction of *M. oryzae* into pre-established *P. expansum* biofilms at 48 and 72 h, and consequent 24 h interaction until 72 and 96 h, respectively, caused a significant decrease of *P. expansum* biofilm mass at both times ($p < 0.001$).

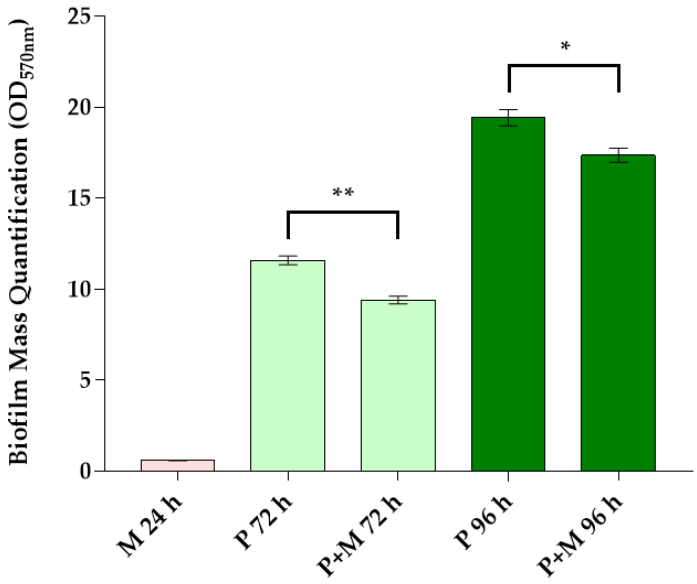

Figure 1. Biofilm mass quantification for single-species and inter-kingdom biofilm formation. Legend is as follows: 'M'—*M. oryzae*; 'P'—*P. expansum*. The means ± SE for three independent experiments are illustrated. Significant differences are depicted with: * $p < 0.001$; ** $p < 0.0001$.

3.2. Bacterial Cell Density in Single and Inter-Kingdom Biofilms

The number of *M. oryzae* cells present in either single-species biofilms and in inter-kingdom biofilms after 24 h of interaction with 48 or 72 h pre-established *P. expansum* biofilms was determined as CFU and the results obtained are presented in Figure 2. CFU from *M. oryzae* single-species biofilms at 24 h was used as control. Analyzing the results, the interaction of *M. oryzae* with pre-established *P. expansum* biofilms from 48 to 72 h did not cause a statistically significant change ($p > 0.05$) in this bacterium CFU. However, a significant decrease ($p < 0.0001$) in CFU was observed from the interaction with pre-established *P. expansum* biofilms from 72 to 96 h indicating a higher inhibition of *P. expansum* against *M. oryzae* at this time. This inhibition was also observed when comparing the interaction from 72 to 96 h with 48 to 72 h ($p < 0.001$).

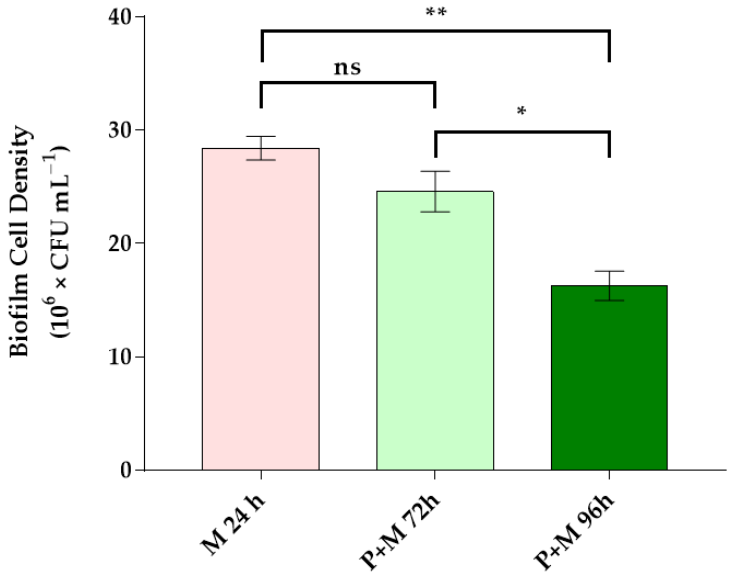

Figure 2. Biofilm cell density for *M. oryzae* while forming single-species or inter-kingdom biofilms with pre-formed *P. expansum* biofilms. Legend is as follows: 'M'—*M. oryzae*; 'P'—*P. expansum*. The means ± SE for three independent experiments are illustrated. Significant differences are depicted with: * $p < 0.001$; ** $p < 0.0001$; 'ns'—not significant.

3.3. Idh Gene Expression in Single and Inter-Kingdom Biofilms

The effect of the introduction of *M. oryzae* on the *idh* gene expression of pre-established *P. expansum* biofilms was investigated in vitro on 50% R2A + 50% APJ. Figure 3 shows the results of the normalized expression levels (fold change) of the *idh* gene in relation to the reference gene *β-tubulin*. *P. expansum* single-species biofilms at 72 and 96 h were set as controls, while the treatment was the presence of *M. oryzae* at each respective time (Figure 3a,b). Through the results it is possible to observe a decrease in the expression of the *idh* gene when *M. oryzae* cells were introduced to *P. expansum* biofilms from 48 to 72 h ($p < 0.0001$). The normalized *idh* expression levels at this time, in the different independent assays, varied from a minimum of 0.12 to a maximum of 0.45 which is equivalent to a fold decrease of 8.3 and 2.2, respectively. At 96 h, there was a slight increase in *idh* gene expression in the presence of *M. oryzae*, however, it was not considered statistically significant ($p = 0.099$). The *Idh* normalized expression levels at 96 h varied from a minimum of 1.3 to a maximum of 3.1-fold increase.

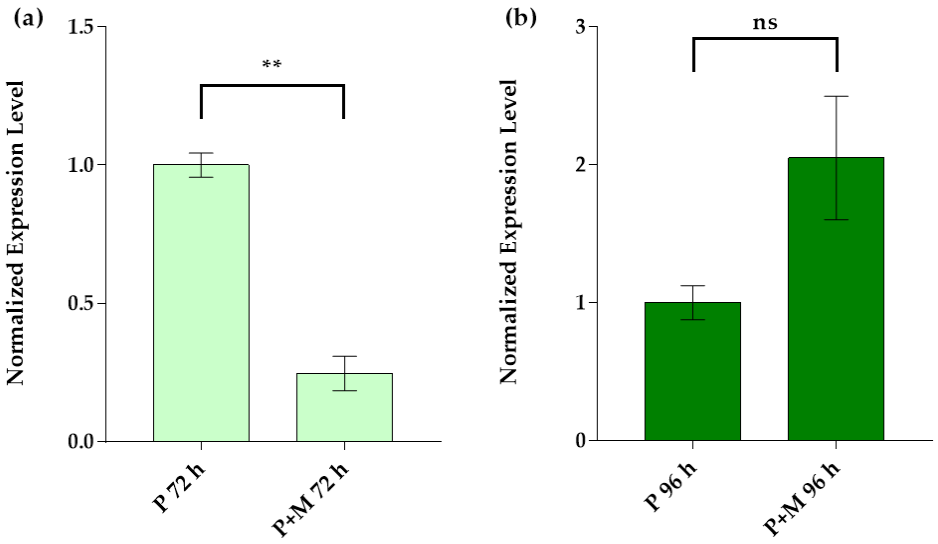

Figure 3. *Idh* gene normalized expression levels relative to the reference gene *β-tubulin* in *P. expansum* biofilms at 72 (**a**) and 96 h (**b**) in the presence of *M. oryzae*. Legend is as follows: 'M'—*M. oryzae*; 'P'—*P. expansum*. The means ± SE for four independent experiments are illustrated. Significant differences are depicted with: ** $p < 0.0001$; 'ns'—not significant.

3.4. Patulin Production in Single and Inter-Kingdom Biofilms

The effect of the introduction of *M. oryzae* on patulin production of 48 and 72 h pre-established *P. expansum* biofilms was investigated in vitro on 50% R2A + 50% APJ. Table 2 shows the results of the relative fold change of patulin in the presence of *M. oryzae* in relation to *P. expansum* single-species biofilm controls at 72 and 96 h. The individual results of patulin production from the three independent assays revealed a high variation of patulin concentration between them. For instance, among three independent assays, concentrations ranged from 1.14–7.09 μg mL^{-1} in *P. expansum* 72 h biofilms, 0.19–2.61 μg mL^{-1} in *P. expansum* + *M. oryzae* 72 h biofilms, 0.59–1.05 μg mL^{-1} in *P. expansum* 96 h biofilms, and 1.43–5.14 μg mL^{-1} in *P. expansum* + *M. oryzae* 96 h biofilms. For this reason, the relative fold change difference in relation to each individual control was calculated in order to normalize the results. Through the normalization we can observe that *P. expansum* biofilms growing in the presence of *M. oryzae* from 48 to 72 h results in a decrease of patulin production. However, the inverse is observed when *M. oryzae* is introduced to 72 h *P. expansum* biofilms, where an increase in patulin is observed at 96 h.

Table 2. Patulin relative fold change production by *P. expansum* biofilms at 72 (a) and 96 h (b) in the presence of *M. oryzae*.

Conditions	Patulin Relative Fold Change to Control
P 72 h − P + M 72 h	0.322 ± 0.048 ($p < 0.0001$)
P 96 h − P + M 96 h	3.349 ± 1.627 ($p = 0.067$)

A positive trend between *idh* gene expression and patulin production values is observed. The results obtained from both patulin production and *idh* gene expression, reveal a tendency of decrease in both parameters when *M. oryzae* is introduced to *P. expansum* from 48 to 72 h biofilms, while the opposite is detected when this bacterium is introduced to *P. expansum* biofilms at 72 h.

4. Discussion

The present study aimed at analyzing the changes that occur when *M. oryzae*, a Gram-negative bacterium found in DWDS is introduced to mature *P. expansum* biofilms. For this purpose, *M. oryzae* was introduced to pre-established fungal biofilms at 48 and 72 h, the

interaction was allowed to occur for 24 h, and different parameters were evaluated at 72 and 96 h, respectively. The parameters analyzed to understand this interaction were biofilm mass, bacterial CFU, *idh* gene expression, and patulin production from the developed biofilms. These microorganisms were chosen based on different characteristics: Their ability to form mature single-species biofilms between 48 and 72 h growth and since they have been assessed while forming inter-kingdom biofilms [12,13,32,34]. *P. expansum* was also selected due to its well-known ability to produce patulin as a secondary metabolite [37,41,42]. Preliminary assays leading to this work revealed that *P. expansum* biofilms developed in R2A did not produce detectable amounts of patulin for as long as 120 h of biofilm formation. For this reason, apple juice made from apple extract was added to induce patulin production. In the conditions tested in the present work (50% R2A–50% APJ), patulin was detected from 72 h onwards, thus, the time-points selected to evaluate the effect of *M. oryzae* on *P. expansum* biofilms patulin production and *idh* gene expression were from 48 to 72 h and 72 to 96 h.

Regarding biofilm mass quantification, we can observe that at 72 and 96 h, after 24 h of co-growth with *M. oryzae*, a decrease in *P. expansum* biomass was detected at both times, indicating an inhibitory effect of *M. oryzae* towards *P. expansum*. This inhibition has been observed in previous studies where *M. oryzae* was inoculated at the same time as *P. expansum* [12,13]. This could mean that, despite the beginning of interaction with *M. oryzae*, albeit inoculated at the same time or added to pre-established *P. expansum* biofilms, this bacterium has the ability to interfere with fungal growth. This could be due to the direct competition for available nutrients, the production of metabolites that might inhibit fungal growth or a combination of both factors [43,44].

To analyze the effect that pre-established *P. expansum* biofilms had on *M. oryzae* itself, bacterial CFU were determined with *M. oryzae* single-species biofilms at 24 h being used as control. A reduction in this bacterium CFU was observed when it was added to 48 and 72 h *P. expansum* biofilms, although it was only statistically significant when it was added to the latter. A significant reduction was also observed when comparing bacterial CFU at 96 with 72 h, meaning a stronger inhibitory effect of the fungus towards *M. oryzae* in more mature biofilms. This inhibitory effect had also been observed in previous studies where these two microorganisms were inoculated at the same time [12,13]. The presence of exogenous concentrations of patulin highlighted this inhibitory effect [13]. In the present study, the inhibitory effect accentuated at 96 h might be related to the increased concentrations of patulin detected at this time (0.19–2.61 μg mL^{-1} compared to 1.43–5.14 μg mL^{-1}).

RT-qPCR is a molecular technique that allows the quantification of fungi in environmental samples and the study of host-pathogen interactions and changes in gene expression in response to certain conditions [45]. Its application has become the method of choice for quantitatively assessing steady state mRNA levels [37]. In the present study, the primers used for qPCR were selected from a study by De Clercq et al. [37]. An optimization process for the conditions required for this study was performed, as well as the recalculation of primer efficiency for both sets of primers used presently. The presence of PCR inhibitors is a major drawback of the PCR, as it decreases sensitivity and can give false-negative results [46]. For this reason, a 1/10th dilution of both RNA prior to the cDNA synthesis as well as cDNA prior to the qPCR assay were done to remove possible inhibitors [37,47].

Several studies regarding the expression of fungal genes involved in mycotoxin biosynthesis have been quantified relative to one reference gene [48–51]. In our study, we used *β-tubulin* as the reference housekeeping gene as it maintained very stable Ct values throughout the different conditions tested.

Overall, the results of our in vitro study show that the presence of *M. oryzae* affects the patulin production of *P. expansum* mature biofilms on 50% R2A-50% APJ (Table 2), by influencing the transcriptional level of the *idh* gene (Figure 3). This influence is observed by a significant decrease of the *idh* expression level and consequent patulin production from a 24 h interaction with 48 h *P. expansum* biofilms. In contrast and besides the variability observed, with 72 h *P. expansum* biofilms until 96 h the *idh* expression level remained

significantly unchangeable. To the best of our knowledge, this is the first study evaluating the effect of a bacterial presence on the *idh* gene expression and patulin production of *P. expansum* biofilms. Nevertheless, compared with studies in different matrixes/conditions, a positive trend was also found between *idh* expression and patulin production in a study performed by De Clercq et al. [37]. The use of RT-qPCR to monitor gene expression related to the biosynthesis of mycotoxins has also been described for other fungal species. For instance, the mRNA levels of two genes involved in the biosynthetic pathway of aflatoxin in *Aspergillus parasiticus* revealed an absence in the transcription of both genes when this fungus was grown in a medium that did not support aflatoxin production [48]. Jiao et al. [50] investigated the effect of different carbon sources on the trichothecene production and induction mechanisms of *Fusarium graminearum* and suggested that this fungus recognizes the sucrose molecules, activates the expression of a specific gene, and induces trichothecene production.

As previously stated, in the present study, *M. oryzae* caused a decrease in *idh* gene expression and patulin production after 24 h of interaction with a 48 h pre-established *P. expansum* biofilm. Different studies have reported the use of bacteria to inhibit the growth and mycotoxin production of different fungal species. Taheur et al. [52] demonstrated that using different lactic acid bacteria (LAB) in agar medium led to a growth inhibition of *Aspergillus flavus* and *Aspergillus carbonarius*, as well as the reduction of different mycotoxins in almonds. In this study, aflatoxin B1, B2, and ochratoxin A were reduced by 97.22, 95.27, and 75.26%, respectively. Another study using LAB, revealed a growth inhibition of *F. graminearum* and *A. parasiticus* by *Lactobacillus rhamnosus* and *L. plantarum* [53]. In addition, a decrease in zearalenone production was observed as a result of the interaction of both *Lactobacillus* strains with *F. graminearum*, while the interaction between *A. parasiticus* and *L. plantarum* resulted in an increased aflatoxin B1 production [53]. The use of viable and nonviable bacteria has also been studied regarding the reduction of patulin from different matrixes, including apple juice. For instance, Hatab et al. [54] demonstrated that LAB have the ability to remove patulin from an aqueous solution. In this study, the maximum patulin uptake was achieved by *Bifidobacterium bifidum* and *L. rhamnosus* (52.9 and 51.1% for viable and 54.1 and 52.0% for nonviable cells) after 24 h of incubation. The same authors reported in another study that incubation for 24 h with *L. rhamnosus* and *Enterococcus faecium* caused a decrease of patulin in apple juice by 80.4 and 64.5%, respectively [55]. Yuan et al. [56] also reported the use of inactivated *Alicyclobacillus* spp. cells to reduce patulin concentration in apple juice. These authors reported patulin reduction rates of 88.8 and 81.6% by two strains of *A. acidoterrestris* achieved after a 24 h incubation period [56].

The increase in patulin production from the 24 h interaction of *M. oryzae* with the 72 h pre-established *P. expansum* biofilm, might be related to the overall metabolic state of the fungus being more active or heterogeneous, allowing it to develop potential diverse strategies, including the subcellular compartmentalization of biosynthetic pathway [28] or molecular trafficking machinery [57], to contest the presence of *M. oryzae*, by increasing the production of secondary metabolites (i.e., patulin).

Results for patulin production revealed high variablility in the concentration of mycotoxin obtained among the independent experiments despite the good recovery percentage of the extraction method [40]. This variability in the patulin concentration values, despite all efforts to control the conditions, could also be due to differences in the composition of APJ medium or the low sample volume used in each extraction. However, when the results were normalized to each independent experiment control, a clear tendency was observed.

In conclusion, our results show that the effect of *M. oryzae* on pre-established *P. expansum* biofilms is dependent on the time of interaction as demonstrated by the different resulting tendencies when the bacterium is introduced to 48 or 72 h *P. expansum* pre-established biofilms. More mature *P. expansum* biofilms appear to be more resistant to the inhibitory effect that *M. oryzae* causes towards *idh* gene expression and patulin production. This is a result of the increased concentration of patulin at 96 h and reciprocates an inhibitory effect of *P. expansum* towards *M. oryzae*. A trend was observed between the *idh* expression

and patulin production values. The results indicate that *M. oryzae* affects patulin production by acting at the transcriptional level of the *idh* gene. These findings indicate that patulin plays a vital role in inter-kingdom communication and/or microbial assembly processes that can influence biofilm formation between fungi and bacteria.

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