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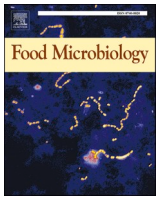

Antibiofilm activity of LAE (ethyl lauroyl arginate) against food-borne fungi and its application in polystyrene surface coating

R. Becerril [a], M. Precone [b], C. Nerin [a,*]

[a] I3A–Aragón Institute of Engineering Research, University of Zaragoza, María de Luna 3, 50018, Zaragoza, Spain
[b] University of Bologna, Via Zamboni, 33, 40126, Bologna, BO, Italy

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ABSTRACT

Several filamentous fungi species as *Fusarium oxysporum* or *Cladosporium* sp. can form biofilms by themselves or by participating in polymicrobial biofilms with bacteria. However, despite the high impact of biofilm on the food industry and the high efforts done to control biofilm produced by bacteria in the food area, there has been little study of strategies to control fungal biofilm in this area. In this study, the antibiofilm activity of the safe antimicrobial compound ethyl lauroyl arginate (LAE) was investigated against food spoilage fungi (*Cladosporium cladosporioides*, *Aspergillus ochraceus*, *Penicillium italicum*, *Botrytis cynerea* and *Fusarium oxyspoum)*. Finally, the efficacy of a varnish-based coating incorporating LAE and coated onto polystyrene microtiter plates has been evaluated as a strategy to reduce fungal biofilm formation. The results of the 2,3-bis-(2-metoxi-4-nitro-5-sulfofenil)-2H-tetrazoilo-5-carboxanilida (XTT) assay, which measure the biofilm metabolic activity of moulds, demonstrated that LAE reduced significantly the formation of fungal biofilm at concentrations from 6 to 25 mg/L. This reduction was confirmed by the micrographs obtained by scanning electronic microscopy (SEM). In addition, LAE also showed antifungal activity against established biofilms. Particularly, it reduced their metabolic activity and viability at concentrations from 6 to 25 mg/L according to results obtained in the XTT assay and observations made by confocal laser scanning microscopy (CLSM). Finally, active coating incorporating from 2% of LAE proved to reduce significantly the biofilm formation in *C. cladosporioides*, *B. cynerea* and *F. oxyspoum* according to the results obtained in the XTT assay. However, the released studies indicated that the retention of LAE in the coating should be improved to prolong their activity.

1. Introduction

Food spoilage caused by filamentous fungi is a worldwide problem that triggers important health problems and economic losses (da Cruz Cabral et al., 2013). Fungi can grow on diverse food products, including cereals, meat, milk, fruit, vegetables, nuts, and fats (da Cruz Cabral et al., 2013; Ribes et al., 2017) causing their decay by the development of off-flavours, acidification, discolouring, and disintegrating (Filtenborg et al., 1996; Pitt and Hocking, 2009). Indeed, it is estimated that between 5% and 10% of the world's food production is lost due to fungal spoilage (Pitt and Hocking, 2009). In addition, some common fungal strains present in food products produce mycotoxins, like patulin, alternariols, and citrinin, which pose a risk to the health of consumers (Samson et al., 2004; Pitt and Hocking, 2009). Contamination by fungi in agricultural food products is produced generally in pre-harvest steps and also during storage and manufacturing production processes (Salas et al., 2017). However, in other products such as dairy products, some types of meat or baked goods, contamination is produced mainly due to the food processing environment (Asefa et al., 2010; Garcia et al., 2019; Garnier et al., 2017). The application of efficient and safe strategies to prevent fungal contamination in the food industry are essential to reduce the negative impact of fungal growth in the food industry.

Microorganisms are able to attach to inert or living surfaces forming complex communities known as biofilms (Agarwalla et al., 2019; Peiqian et al., 2014; Siqueira and Lima, 2013). These self-organized structures increase the protection of cells from the environment and improve nutrient availability, promoting the survival and propagation of cells (Jabra-Rizk et al., 2004; van Acker et al., 2014). Although bacterial biofilms are by far the most studied, it has been widely demonstrated that several fungal species can form biofilms, by themselves or in combination with bacteria (Afonso et al., 2020; Rosero-Hernández and Echeverri, 2020). The formation and presence of biofilms in food

* Corresponding author.
E-mail address: cnerin@unizar.es (C. Nerin).

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industrial environments is a serious problem that compromised the safety and quality of food (Coughlan et al., 2016). Biofilms attached to the surface act as a source of microorganisms, contaminating food products by direct contact or in an indirect way, for example, by contaminating the water used during processing or by spreading detached organisms to other areas of processing plants (Afonso et al., 2020; Coughlan et al., 2016). Besides, biofilms formed in food products reduce the effectiveness of sanitization procedures and the activity of added preservatives reducing their shelf life and safety (Cui et al., 2020; Zhang et al., 2020).

The control of biofilm produced by bacteria in the food area has been widely studied and numerous strategies have been developed to prevent their formation (Coughlan et al., 2016). These include the cleaning and disinfection with new antimicrobials compounds, the use of antibiofouling surfaces, or the use of packaging materials with antimicrobials coatings (DeFlorio et al., 2021; Lu et al., 2022; Prabhawathi et al., 2014; Gherardi et al., 2016). However, despite the problems that fungi caused in the food industry, biofilms produced by fungi have attracted less attention and very little study there is about their formation and control in this area. Fungal strains commonly present in food have been detected in biofilms attached to several types of surfaces used in the food industry like water supplied systems, dishwashers, plastic debris, or even in some vegetables such as onions or tomato stems (Abdel-Aziz et al., 2019; Babič et al., 2017; Motaung et al., 2020; Zupančič et al., 2018). Therefore, the use of novel technologies that also help to reduce the biofilms produced by filamentous fungi should be a matter of concern in the food industry.

Ethyl lauroyl arginate (LAE) is a cationic surfactant derived from lauric acid and arginine that possesses notable antimicrobial properties due to its chemical structure and surfactant properties (Higueras et al., 2013). This compound is rapidly metabolized and hydrolyzed by the human body to natural components such as lauric acid, L-arginine and ethanol (Haghighi et al., 2019). As a result, it has been approved as a safe antimicrobial food preservative by the Food and Drug Administration (FDA) for several foods such as fresh fish, cheese or processed fruits and by the European Food Safety Authority (EFSA) for heat-treated meat products. The bactericidal activity of LAE has been demonstrated against a wide range of bacteria such as a Listeria innocua, *Escherichia coli* or *Pseudomonas aeruginosa* with MIC values ranging from 8 to 100 mg/L (Becerril et al., 2013; Nerin et al., 2016; Higueras et al., 2013). Besides, LAE has shown also activity against food spoilage fungi such as *Aspergillus niger* or *Penicillium chrysogenum*. However, this activity was lower than that found to bacteria with MIC values ranging from 24 to 400 mg/L and a general bacteriostatic behaviour (Higueras et al., 2013; Nerin et al., 2016; Xu et al., 2018). LAE has been applied as an antimicrobial agent in active packaging materials proving to reduce or inhibit microbial growth *in vitro* and in food applications (Higueras et al., 2013; Haghighi et al., 2019, 2020; Li et al., 2021; Muriel-Galet et al., 2015). Regarding their antibiofilm activity, it has been assessed against several bacterial strains such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp. or *Pseudomonas aeruginosa* (Kim et al., 2017a, 2017b; Sadekuzzaman et al., 2017). However, to our knowledge, there are no studies about their activity against fungal biofilms.

The aim of this work is to evaluate the activity of LAE on the formation and eradication of biofilm produced by common food-related filamentous fungi, in order to assess the potential use and effectiveness of this compound in food industry applications. For this purpose, the activity of LAE was assessed against *Cladosporium cladosporioides, Aspergillus ochraceus, Fusarium oxysporum, Botrytis cinerea and Penicilium italicum*. All these strains are commonly isolated from food and can produce the decay of numerous food products such as strawberries (*Botrytis cinerea*), citrus (*Penicilium italicum*) or onion bulbs (*Fusarium oxysporum)* (Samson et al., 2004). Moreover, *Aspergillus ochraceus*, which is often isolated from coffee beans or spices, produces mycotoxins that may be found contaminating these products (Samson et al., 2004).

Finally, this work also aims to investigate the ability of an active coating incorporating LAE to prevent the formation of fungal biofilm on abiotic surfaces.

2. Materials and methods

2.1. Materials

Ethyl Lauroyl Arginate (CAS 60372-77-2) was supplied by VEDEQSA S.A. (Barcelona, Spain). The fungal strains *Cladosporium cladosporioides*, CECT 2111, *Aspergillus ochraceus* CECT 2093, *Penicillium italicum* CECT 2294, *Botrytis cinerea* CECT 2100 and *Fusarium oxysporum* CECT 20201 used to evaluate the antibiofilm activity of LAE were obtained from the Colección Española de Cultivos Tipo, Burjasot, Valencia, Spain. The moulds were cultured in Potato Dextrose Agar (PDA) plates at 25 °C for 7 days in the case of *C. Cladosporioides*, *P. italicum* and *A. ochraceus* and 14 days in the case of *B. cinerea* and *F. oxysporium* to ensure adequate sporulation.

2.2. Minimal inhibitory concentration

Minimum inhibitory concentration (MIC) was determined by using a broth microdilution method. Briefly, serial two-fold dilutions of LAE in Yeast Malt broth (YM), ranging from 800 to 6 mg/L, were prepared in a 96-well plate (100 μl/well). Then, 100 μl of a spore suspension in YM broth (10^5 CFU/mL) was added to each well. Spores were collected by washing the surface of the cultured PDA plates with approximately 3 mL of sterile water containing 0.1% of Tween 20. The turbidity of the stock spore suspension was measured at 620 nm and subsequently appropriately diluted in NaCl (0.9%) to obtain an inoculum of 10^5 CFU/mL. Controls were prepared by replacing the spore suspension volume with the YM media. The plates were incubated at 25 °C for 24 h in the case of *C. cladosporioides*, *A. ochraceus* and *P. italicum* and 48 h in the case of *B. cynerea* and *F. oxyspoum*. After incubation, MIC was defined as the lower concentration of antimicrobial where tubidity was not observed. At least three independent assays were performed, and the modal MIC values were selected.

2.3. Effect of LAE on biofilm formation

To evaluate the antibiofilm activity of LAE, 96-well microtiter plates were inoculated with 100 μl of a suspension of the tested mould (10^5 CFU/mL) in NaCl 0.9%. Subsequently, LAE at different concentrations (ranging from the MIC value to 1/16 of MIC value) was added to the test wells and incubated at 25 °C h for 24 h in the case of *C. cladosporioides*, *A. ochraceus* and *P. italicum* and 48 h in the case of *B. cynerea* and *F. oxyspoum*. As the latter two strains require longer germination and growth times and therefore longer times to form the biofilm, they were incubated for a longer time. Positive controls without LAE and negative controls without moulds were also prepared. After incubation, planktonic cells were removed from each well by washing twice with 0.9% NaCl and an XTT reduction assay was carried out to determine the metabolic activity of the formed biofilm. Briefly, 100 μl of culture medium (YM) and 25 μl of XTT solution (1 mg/mL of XTT containing 20 μg/mL of menadione sodium bisulfite) were added at each well and incubated for 2 h at 25 °C in the dark. Then, 75 μl of supernatant were transferred to a new microplate and the absorbance of each plate was measured at 450 nm using a Multiskan microtiter plate reader. Values were expressed as mean absorbance (450 nm) ratios between inoculated samples and negative controls.

2.4. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to observe fungal biofilms grown in the presence or absence of LAE. Treated fungal biofilms were prepared by growing the tested mould in YM media in

presence of the minimum concentration of LAE that reduced the biofilm formation according to the data obtained in the previous antibiofilm formation assay (25 mg/L for *F. oxysporum* and *B. cinerea*, 12.5 mg/L for *C. cladosporioides* and *A. ochraceus*, and 6 mg/L for *P. italicum*) or 48 h (*B. cinerea* and). Moulds suspension were incubated at 25 °C for 24 h (*C. cladosporioides*, *A. ochraceus* and *P. italicum*) or 48 h (*B. cinerea* and *F. oxysporum*). Samples without LAE as control were prepared.

The biofilms of *C. cladosporioides* and *A. ochraceus* were prepared in silanized coverslip. However, because *P. italicum*, *B. cinerea*, and *F. oxysporum* did not attach well to this surface, they were prepared in μ-Slide 8 Well with a thin ibidi Polymer Coverslip Bottom (Ibidi, Munich, Germany). After incubation, planktonic hyphae were removed by washing with NaCl 0.9%. Then, biofilms were fixed with glutaraldehyde (2.5% in phosphate buffer 0.1 M pH 7.4), dyed with 2% of OsO_4 and dehydrated in graded alcohols. Subsequently, samples were critical-point-dried and coated with gold. The observation was carried out using a scanning electronic microscope JEOL JSM 6360-LV at an accelerating voltage of 15 kV.

2.5. Activity of LAE against established biofilms

The activity of LAE against formed fungal biofilm was studied in polystyrene 96-well microtiter plates. 200 μl of inoculated YM (10^5 CFU/mL) were added to each well and incubated at 25 °C to form the biofilm. After the incubation (24 h for *C. cladosporioides*, *A. ochraceus* and *P. italicum* and 48 h for *B. cinerea* and *F.oxyspoum*), the supernatant was removed carefully, and the attached cells were washed twice with NaCl 0.9%. Subsequently, 300 μl of YM containing different concentrations of LAE, ranging from the MIC to 8xMIC, were added to each well and incubated for 24 h at 25 °C. Positive control without LAE and negative controls without mould inoculum were also prepared. After incubation, each well was washed twice with 0.9% NaCl and the biofilm metabolic activity was determined using the XTT reduction assay described in section 2.3.

2.6. Confocal laser scanning microscopy CSLM observations

The activity of LAE against established biofilms was also observed by CSLM. For this purpose, 100 μl of inoculated YM (10^5 CFU/mL) were added to each well of a μ-Slide 8 Well with a thin ibidi Polymer Coverslip Bottom (Ibidi, Munich, Germany) and incubated at 25 °C for 24 or 48 h. Subsequently, samples were washed twice with NaCl 0.9% to remove planktonic hyphae and 300 μl of YM containing concentrations of LAE of 4xMIC were added. After 24 h at 25 °C, each well was washed twice with 0.9% NaCl and biofilms were stained using a LIVE/DEAD® BacLight™ Bacterial Viability Kit (L-13152; Molecular Probes Inc. Eugene, OR) to ascertain the viability of the biofilm fungal cells. Stained cells were observed by CLSM (Zeiss, LSM880).

2.7. Active coating incorporating LAE

The active coating solution was prepared by adding LAE to an organic solvent-based varnish (Artibal, Sabiñánigo, Spain) at concentrations of 0.5, 1, 2, 4 and 8% (w/w of LAE with respect the varnish solids content). 50 μl of this solution were added to each well of a 96-well microplate, then dried at 50 °C overnight and sterilized by exposing to UV light for 30 min in a safety cabinet (MSC-Advantage, Thermo Fisher Scientific, Inc., MA, USA). As a UV source, the lamp from the laminar hood was used (36 W, 254 nm). The grammage of varnish added to each well was 24.7 g/m^2. This value was calculated taking into account the amount of solids present in the varnish and the amount of varnish added to each well. Controls were prepared by adding 50 μl of the varnish without LAE in the wells of the 96-well microplate.

2.8. Antibiofilm activity of the LAE active coating

The antibiofilm activity of the active coating incorporating LAE was evaluated against *C. cladosporioides*, *B. cinerea* and *F. oxysporum* since *P. italicum* and *A. ochraceus* did not form biofilm on the control coating (coating without LAE). For this purpose, 200 μl of inoculated YM (10^5 spores/mL) were added to each well of the coated plates and incubated at 25 °C for 48 h. Positive controls were prepared in wells coated with the control coating (coating without LAE). Negative controls without mould inoculum were also prepared in coated and non-coated wells in order to detect possible microbial contaminations. After 48-h the incubation, biofilm quantification was carried out using the XTT assay previously described.

2.9. Release of LAE from the LAE active coating

To study the release of LAE from the active coating, plates 55 mm in diameter were coated with the active varnish. For that 3.08 mL of the varnish solution containing 8, 2 or 0.5% of LAE (w/w respect to the varnish solids content) was added to each plate and dried at 50 °C overnight. The coating was dried at 50 °C overnight and sterilized with UV. For the release test, 12.50 mL of water was added to each plate and incubated at 25 °C at different times. Subsequently, an aliquot of the added water was analysed to measure the amount of released LAE. Using this method, two different assays were carried out. In the first assay, plates coated with 8, 2 and 0.5% of LAE (w/w respect to the varnish solids content) were analysed at 24 h and 48 h. In a second assay, plates coated with 2% of LAE were analysed at 2, 4, 8, 14, 24, 48 and 96 h.

The concentration of LAE released into the water was determined using the method described by Silva et al. (2019). Briefly, 3.5 mL of a Co $(SCN)_4^{2-}$ stock reagent solution (35.0 g of NH_4SCN and 15.0 g of Co $(NO_3)_2$ in 35 mL of H_2O) was mixed with 25 mL of LAE aqueous solutions and 2.5 mL of 1,2-dichloroethane. Samples were vigorously vortexed for 1 min, and the samples were centrifuged at 4000 rpm for 10 min. After centrifugation, the bottom organic phase was recovered and the absorbance of the blue dichloroethane extracts was recorded at 623 nm. Calibration graphs were constructed with LAE standards with concentrations ranging from 1.1 to 25 ppm in water.

2.10. Statistics

Differences in the values of absorbance ratios obtained in the XTT assays between the control samples and the samples treated with LAE were analysed using PSPP software. For this purpose, t-Student test with equal variances and t-Student with unequal variances was used. The verification of the equal variances hypothesis was performed using Levene's test. The differences were considered significant at a 0.05 significance level.

All the experiments were performed at least in triplicate.

3. Results

3.1. Minimal inhibitory concentration

LAE was tested for its antifungal activity before assessing its

Table 1
Minimal inhibitory concentration (MIC) of LAE (modal values).

Fungal strain	MIC (mg/L)
A. ochraceus	50
P. italicum	50
C. cladospoiroides	25
F. oxysporium	100
B. cynerea	50

antibiofilm efficacy. MIC values obtained ranged from 25 to 100 mg/L (Table 1) confirming that LAE possessed high fungi-static activity against the selected moulds. *F. oxysporum* was the most resistant mould with a MIC of 100 mg/L while *C. cladosporioides* was the most sensitive strain with a MIC of 25 mg/L. *B. cinera*, *P. italicum* and *A. ochraceus* showed a MIC of 50 mg/L.

The result obtained for *C. cladosporioides* agrees with Higueras et al. who found a MIC of 24 mg/L for this mould. In the case of *F. oxysporum*, *B. cinera*, *P. italicum* and *A. ochraceus*, this is the first time that the MIC of LAE has been determined, and the obtained values are within the range found for other fungi (24–400 mg/L) (Higueras et al., 2013; Nerin et al., 2016; Xu et al., 2018). Besides, the sensitivity of the tested moulds is similar to slightly higher than that found for some bacteria such as *Pseudomona aeruginosa* (100 mg/L), *Listeria innocua* (25 mg/L) or *Escherichia coli* (25, 11.8 mg/L) (Nerin et al., 2016).

3.2. Effect of LAE in biofilm formation

The effect of LAE in the fungal biofilm formation was studied at different sub-MICs using an XTT reduction assay. The reduction of XTT by metabolically active cells to a coloured water-soluble formazan derivative correlates with the biofilm metabolic activity of moulds and can be used to evaluate the efficacy of antifungal compounds to reduce the formation of biofilms (Kischkel et al., 2020; van Dijck et al., 2018).

According to Fig. 1, controls whithout LAE showed high metabolic activity (measured as a ratio of absorbance at 450 nm) that indicates the formation of fungal biofilms on the 96-well plate surface. The treatment with certain concentrations of LAE, decreased notably the biofilm metabolic activity, which indicates a reduction of the biofilm formation. Particularly, LAE showed the highest antibiofilm activity against *P. italicum*, inducing biofilm reduction from a concentration of 6 mg/L (1/8 MIC), with a significant reduction of the ratio of absorbance (450 nm) comparing with the control. Conversely, the lowest activity of LAE was observed against *B. cinerea* and *F. oxysporum*, requiring 25 mg/L of LAE (1/2 and ¼ of MIC respectively) to produce some effect in biofilm formation. In the case of *A. ochraceus* and *C. cladosporioides*, biofilm reduction was observed from 12.5 mg/L (1/2 and ¼ of MIC respectively) of LAE.

The *in vitro* activity of LAE against biofilm formation have assessed against bacteria in previous works (Gracia-Vallés et al., 2018, 2022; Kim and Park, 2016). Biofilms of *S. aureus*, *E. faecalis*. *P. aeruginosa* and *E. coli* were reduced using concentrations of LAE higher than 0.5–3.9 mg/L, which are values lower than that obtained by the more susceptible mould (6 mg/L) in the current work. It should be noted that the mechanism of biofilm formation by bacteria and filamentous fungi have some dissimilarities that could influence the different susceptibility to antimicrobials. In their initial stages of adsorption and adhesion, for example, bacteria adhere to the surface and begin to undergo a series of changes that adapt them to life on a surface while in the case of filamentous moulds are the germinating spores that adhere to form the biofilm (Shay et al., 2022). Moreover, the composition of the extracellular matrix generated by biofilms that differs from bacteria and moulds can also play an important role in antimicrobial susceptibility (Breitenbach et al., 2022).

The effects of sub-MIC concentrations of LAE on biofilm structure and biomass were studied by SEM. For this purpose, moulds were grown in the presence or absence of LAE and the obtained micrographs were compared (Figs. 1–6). The concentration of LAE used for each fungal strain was the minimum that reduced the biofilm formation according to the results of the previous assay (25 mg/L for *F. oxysporum* and *B. cinerea*, 12.5 mg/L for *C. cladosporioides* and *A. ochraceus*, and 6 mg/L for *P. italicum*).

Figs. 1–6 confirmed that LAE reduced the antibiofilm formation of the fungal strains, however, some minor differences in the results were observed with respect to the previous assay. In the case of *C. cladosporioides* (Fig. 2) and *B. cinerea* (Fig. 3), a higher reduction of biofilm formation was observed in the SEM images. Probably these differences could be due to the different attachment of the fungal strains to the supports in which the moulds were grown in the different assays. In the previous assay, a 96-well plate was used while in SEM silanized coverslip and treated μ-Slide 8 wells were used.

As can be seen in the micrographs, *C. cladosporoides* treated with ½ MIC (12.5 mg/L) (Fig. 1 c and d) and *B. cinera* treated with concentrations of LAE of ½ MIC (25 mg/L) (Fig. 3 c and d) clearly reduced the biofilm formation with respect to the control samples. The reduction is especially important in the case of *B. cinerea* where only some occasional growth was observed (Fig. 3c). In both fungal strains, the treatment with LAE reduced the length of the hyphae and the smoothness. Besides, a reduction of EPS was observed, especially in *C. cladosporioides* samples. The formation of unusual bulges on the surface of *B. cinerea* has been also observed when treated *B. cinerea* with other antimicrobial compounds like zinc oxide nanoparticles (He et al., 2011) or phenazine-1-carboxylic acid (Simionato et al., 2017). It is important to point out that *C. cladosporioides* and *B. cinerea* showed effects in biofilm formation only when treated with ½ of MIC while the rest of the moulds showed some effect when treated with ¼ or even 1/8 of MIC. This fact could be related to the presence of EPS observed in the untreated *C. cladosporioides* and *B. cinerea,* since the presence of EPS improve the adhesion of the cell to the surface, lend stability to the biofilm by mediating cellular aggregation and protect against antimicrobial substances (Breitenbach et al., 2022).

The micrographs of *A. ochraceus* (Fig. 4) and *P. italicum* (Fig. 5) showed that treatment with ¼ MIC (12.5 mg/L) and 1/8 MIC (6 mg/L) of LAE respectively produced a decrease of biofilm formation, especially in the *P. italicum* strain. Treated samples showed a reduction of conidial growth and shorter and more branched hyphae. However, no differences

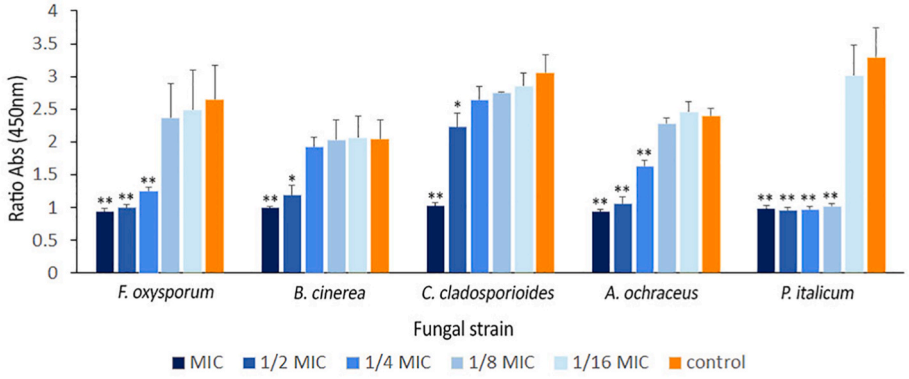

Fig. 1. Fungal biofilm formation in the presence or absence of LAE (control). Results are given as mean absorbance (at 450 nm) ratios between inoculated samples and negative controls. Error bars correspond to SD values. Bars with asterisk are statistically significantly different from them control. *p < 0.05, **p < 0.01.

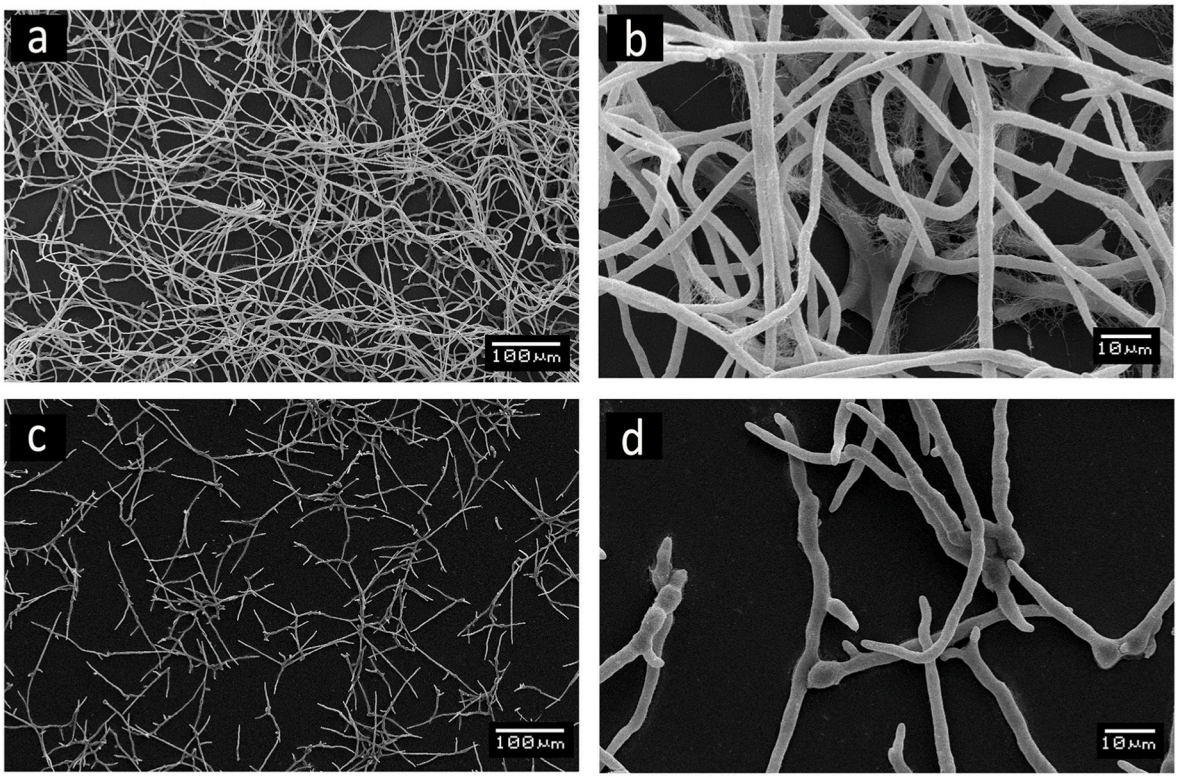

Fig. 2. SEM micrographs of *C. cladosporioides* biofilm growth in (c, d) presence or (a,b) in absence of LAE.

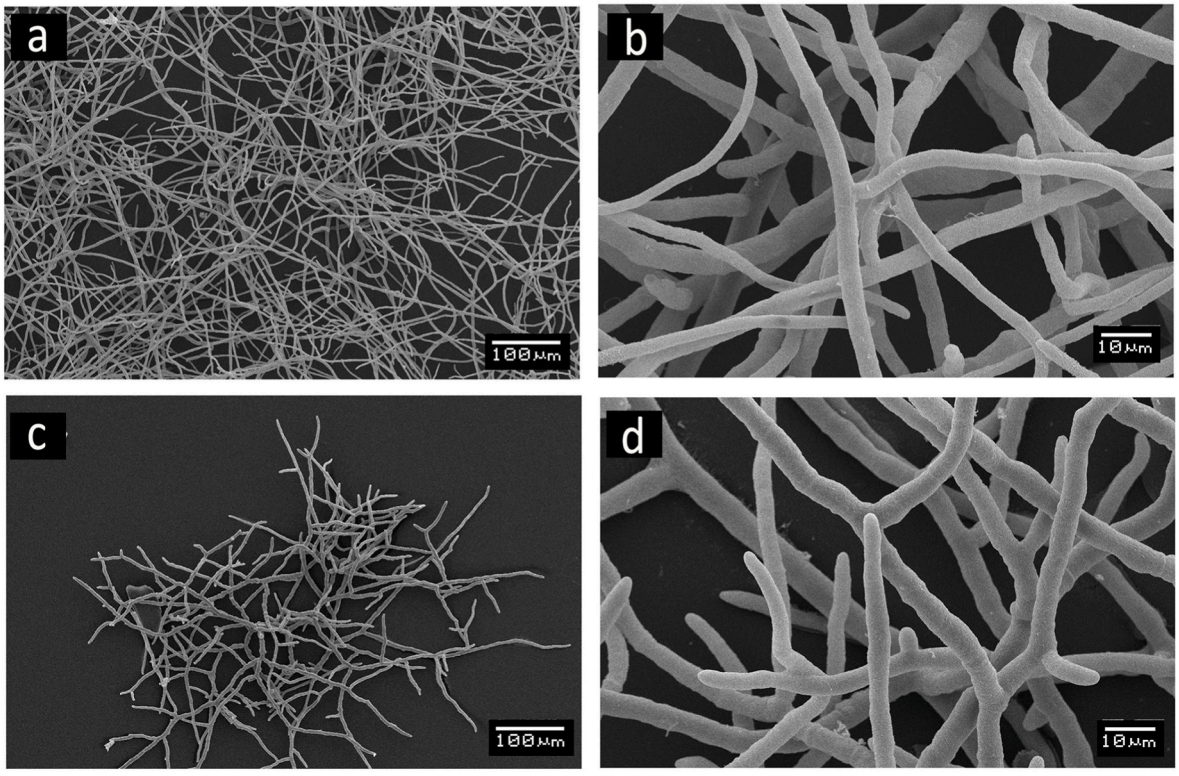

Fig. 3. Fig. 2. SEM micrographs of *B. cinerea* biofilm growth in (c, d) presence or (a,b) in absence of LAE.

between treated and control samples were observed in the hypha surface.

In the case of *F. oxysporum*, the minimum concentration of LAE that produced biofilm reduction (1/4 of MIC, 25 mg/L) resulted in total inhibition of fungal attachment, therefore a lower concentration (1/8 of MIC, 12.5 mg/L) was tested (Fig. 6). However, this concentration of LAE

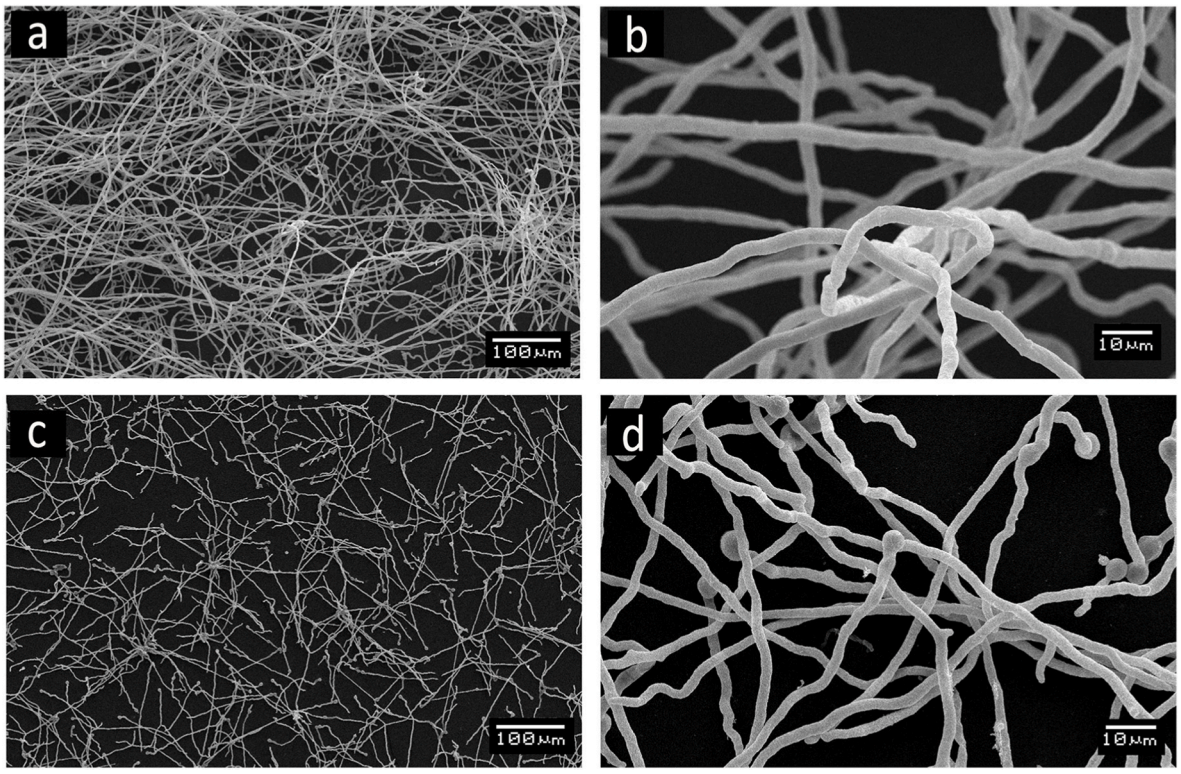

Fig. 4. SEM micrographs of *A. ochraceus* biofilm growth in (c, d) presence or (a,b) in absence of LAE.

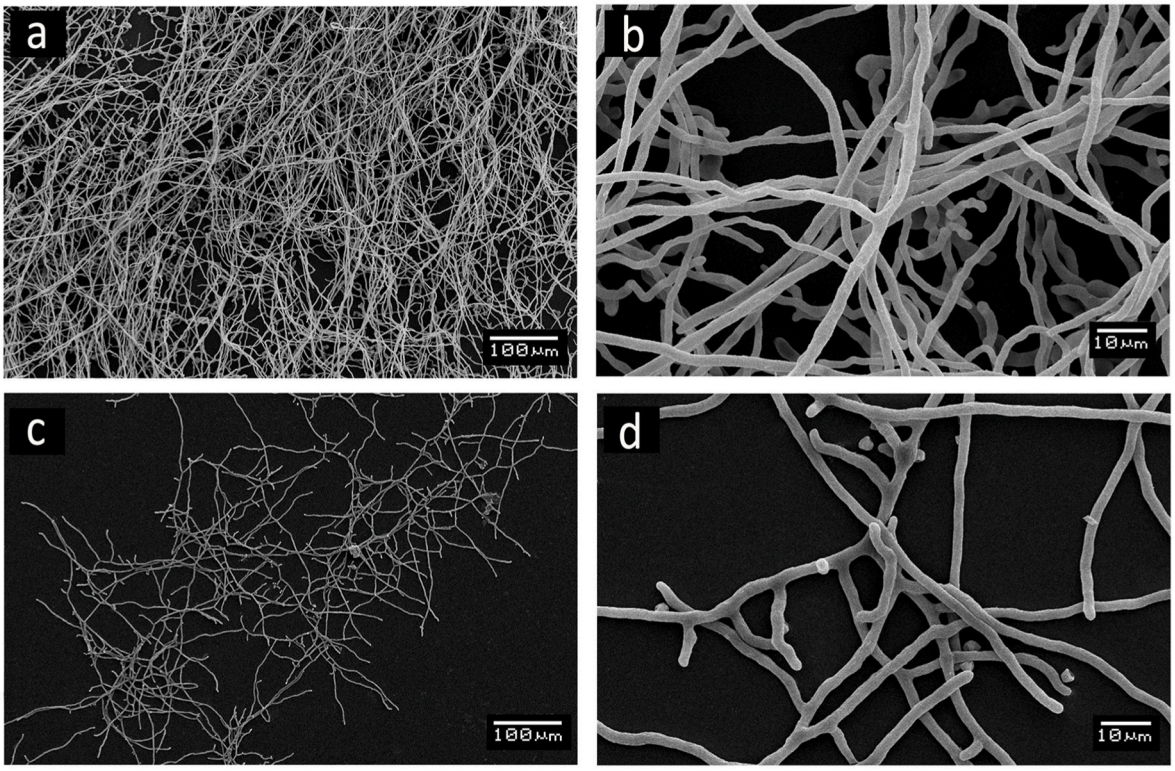

Fig. 5. SEM micrographs of *P. italicum* biofilm growth in (c, d) presence or (a,b) in absence of LAE.

did not produce any change in either the amount of biofilm or the structure of *F. oxysporum* with respect to the control samples (without LAE treatment).

3.3. Activity of LAE against established biofilms

The activity of different concentrations of LAE (higher than the MIC) against established fungal biofilms was evaluated using the XXT

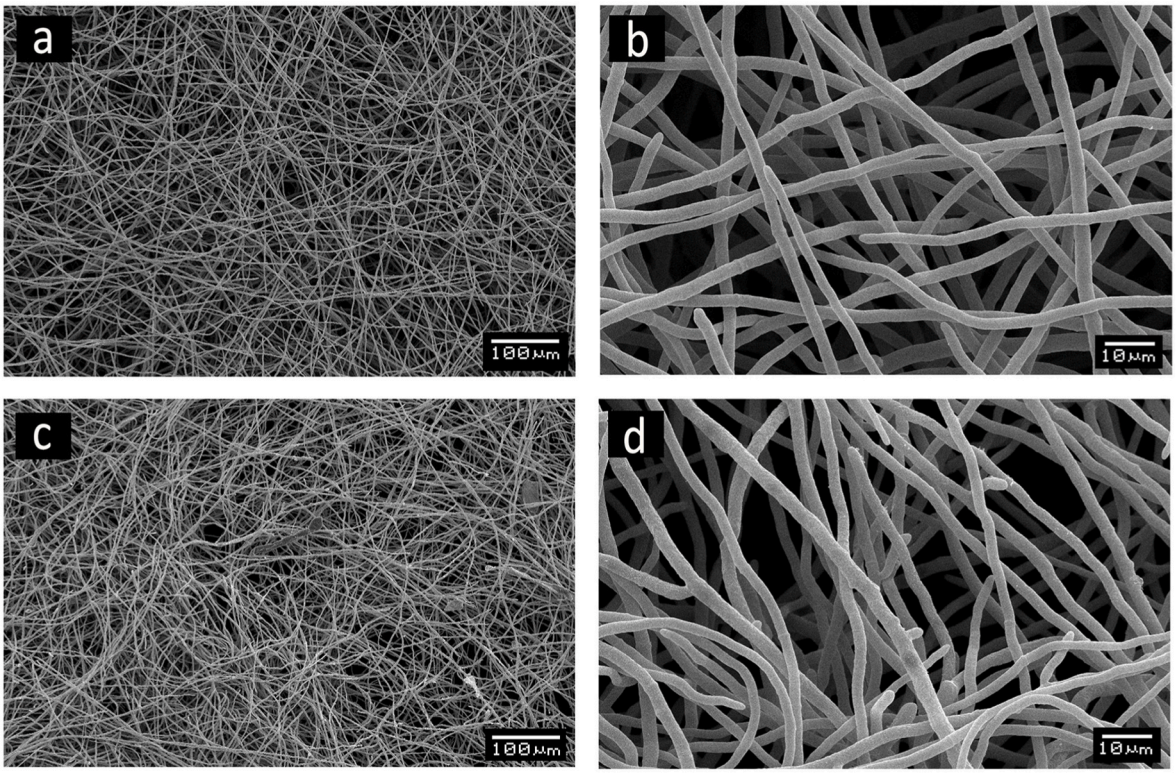

Fig. 6. SEM micrographs of *F. oxysporum* biofilm growth in (c, d) presence or (a,b) in absence of LAE.

reduction assay (Fig. 7). According to the results, the metabolic activity of all the fungal strains tested decreased in a dose-dependent manner compared to control after 24-h treatment with LAE. Indeed, metabolic activity in all fungal biofilms was practically inhibited when treated with four times the MIC, indicating a high effect of LAE in biofilm matrices.

F. oxysporum biofilms were the most resistant to LAE action, requiring 200 mg/L (2xMIC) to reduce the metabolic activity of established biofilms. Contrarily, *P. italicum* and *C. cladosporioides* strains were the most sensible, requiring 50 mg/L of LAE (MIC and 2xMIC respectively).

The effect of LAE in formed biofilms has been studied previously against several bacteria (Gracia-Vallés et al., 2022; Kim and Park, 2016; Sadekuzzaman et al., 2017; Turovskiy et al., 2012). Biofilms of *P. aeruginosa* and *G. vaginalis* were reduced significantly when treated with 31.3 mg/L for 24 h (dynamic flow conditions) and 100 mg/L of LAE for 25 h (static conditions) respectively (Kim and Park, 2016; Turovskiy et al., 2012). Besides, a high reduction was observed in biofilms of *E. coli*, *L. monocytogenes* and *S. typhimurium* when treated for 2 h with 50 mg/L of LAE (Sadekuzzaman et al., 2017). Microbial cells embedded in biofilms may exhibit higher resistance to antimicrobial substances than planktonic cells. The extracellular polymeric substances among other resistance factors allow biofilms to defend against antimicrobial compounds penetrating, which protects attached cells (Bazargani and Rohloff, 2016). The results obtained for bacteria in those works together with the results obtained for fungal strains in the current work indicated that LAE can be used as a safe effective compound to reduce food contamination caused by biofilms.

It is important to note that LAE reduced the metabolic activity of established biofilms, and therefore could kill fungal cells but did not remove detached biofilm formed in most of the strains tested. Optical microscopy images (supplementary material S1) showed that only the

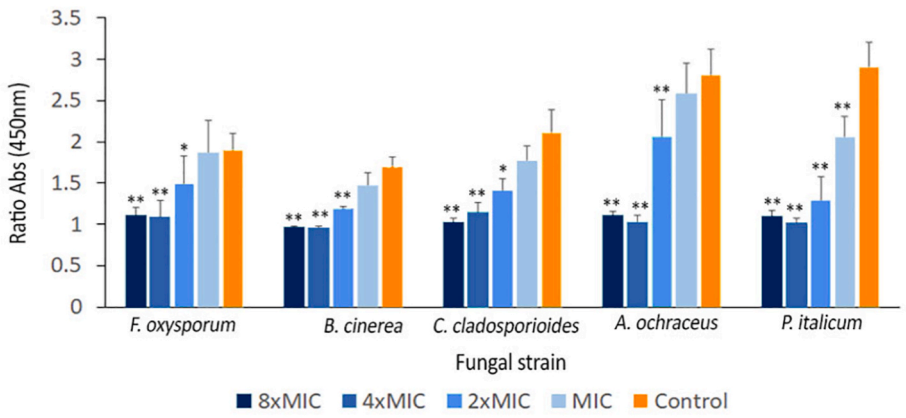

Fig. 7. Activity of different concentrations of LAE against established fungal biofilms. Results are given as mean absorbance (at 450 nm) ratios between inoculated samples and negative controls. Error bars correspond to SD values. Bars with asterisk are statistically significantly different from the control. *$p < 0.05$, **$p < 0.01$.

biofilm of *F. oxysporum* was detached when treated with concentrations from 4xMIC (400 mg/L).

The activity of LAE (4xMIC) against established biofilms was also studied by CLSM after fluorescence staining. The staining was carried out by a combination of two dyes (SYTO 9 and propidium iodide) that differ in their ability to cross membranes. When treated with a mixture of these dyes, living moulds with intact membranes stain green and cells with damaged membranes (dead cells) red or yellow. Fluorescence images of biofilms of *P. italicum*, *A. ochraceus*, *F. oxyporum* and *C. cladosporioides* are shown in Figs. 8–11. *B. cinera* was not possible to observe because cannot be stained correctly.

As can be seen in the images, the treatment of LAE produced a high increase of red fluorescence in all tested moulds, indicating a significant reduction of viable cells in the biofilm. It is important to point out that control samples of *A. ochraeus* and *P. italicum* showed some red fluorescence in cell envelopes that were not related to dead cells (Figs. 8 b and 11 b). A cell is considered dead when the red fluorescence dye can penetrate the damaged membrane and stain the inner of the cell (Figs. 8 d and 11 d).

In concordance with results observed in optical microscopy (Fig. S1), CLSM images confirmed that LAE did not remove the biofilm from the surface of the plates when used at 4xMIC. In this assay, *F. oxysporum* was not also removed probably due to the stronger adherence of biofilms to the surface of the slides used (coated with a thin ibidi Polymer Coverslip Bottom).

3.4. LAE active coating

After demonstrating the efficacy of LAE to reduce fungal biofilms, a varnish-based coating incorporating LAE was tested as a strategy to reduce the formation of biofilms in abiotic surfaces. For that purpose, a polystyrene 96-well microplate was coated with the active varnish and biofilm formation was measured using a XTT assay This hydrophobic coating was previously used to prepare active films incorporating active compounds (Becerril et al., 2007; Gutiérrez et al., 2009; Wrona et al., 2015) showing good performance *in vitro* and in food applications.

Because the capacity of a microorganism to form biofilms depends directly on the properties of the attachment surface (e.g. roughness, charge density, wettability, stiffness) (Krsmanovic et al., 2021; Zheng et al., 2021), preliminary tests were carried out to evaluate the capacity of moulds to form biofilms on the varnish free of LAE. Results indicated that *P. italicum* and *A. ochraceus* were not able to form biofilm in the varnish-coated surface, therefore, the activity of the coating incorporating LAE was only tested against *C. cladosporioides*, *F. oxysporum* and *B. cinerea*.

The antibiofilm properties of the active coating were evaluated using the XTT reduction assay in a coated 96-well plate. Controls coated without LAE were also tested. As can be seen in Fig. 12, the metabolic activity of biofilms formed in the wells incorporating 2% or more of LAE (w/w with respect to varnish weight) was significantly reduced in all tested moulds compared with the wells coated with the solution without LAE. Besides, some reduction was observed in the formation of *C. cladosporioides* biofilm in the well coated with 1% of LAE.

In the last years, numerous antimicrobial polymeric materials (including biopolymers) incorporating LAE have been developed, especially for food packaging applications. These materials have demonstrated antibacterial but also antifungal effects (Cui et al., 2020; Kashiri et al., 2019; Li et al., 2021; Manso et al., 2021; Motta et al., 2020; Pattanayaiying et al., 2019). The results obtained in the current assay suggest that active coating incorporating LAE not only can possess antimicrobial activity but also antibiofilm activity, protecting the materials from fungal biofilm attachment.

The release of LAE from active coating incorporating different concentrations of LAE was evaluated over time. For that, two assays were carried out.

In a first assay, the release from coatings incorporating different concentrations of LAE (0.5, 2 or 8%) was studied and compared. The data obtained (Fig. 13 a), shows a release of LAE between 51.9 and 63.7% after 48 h immersed in water. This means that after 48, in the coating containing 0.5% of LAE the concentration decreased to 0.24%, in the 2%–0.81% and in the 8%–2.9%.

As expected, coating with higher concentrations of LAE showed a faster release than those with lower concentrations, especially after 24 h. It is known that higher concentrations of active substances in

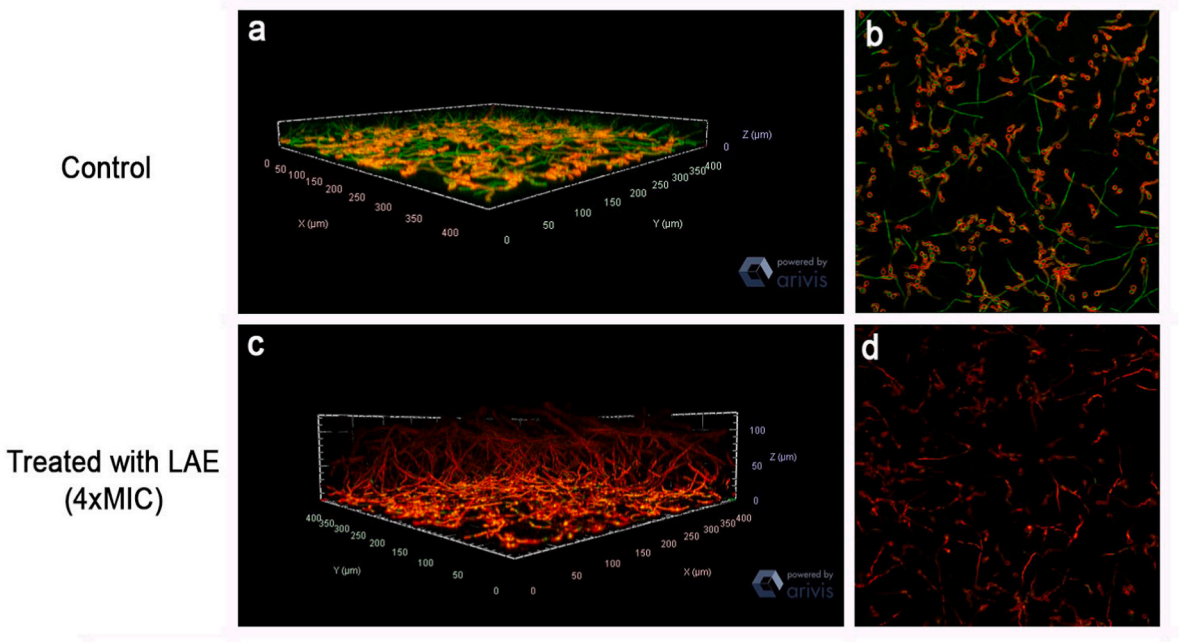

Fig. 8. CLSM images of Aspergillus ochraceus biofilms after treatment with LAE. Samples were stained with LIVE/DEAD staining. Green cells represent undamaged cells whereas red cells represent either damaged or dead cells. Figures a and c shows the three-dimensional construction of CLSMscans and b and d a representative one-plane image. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

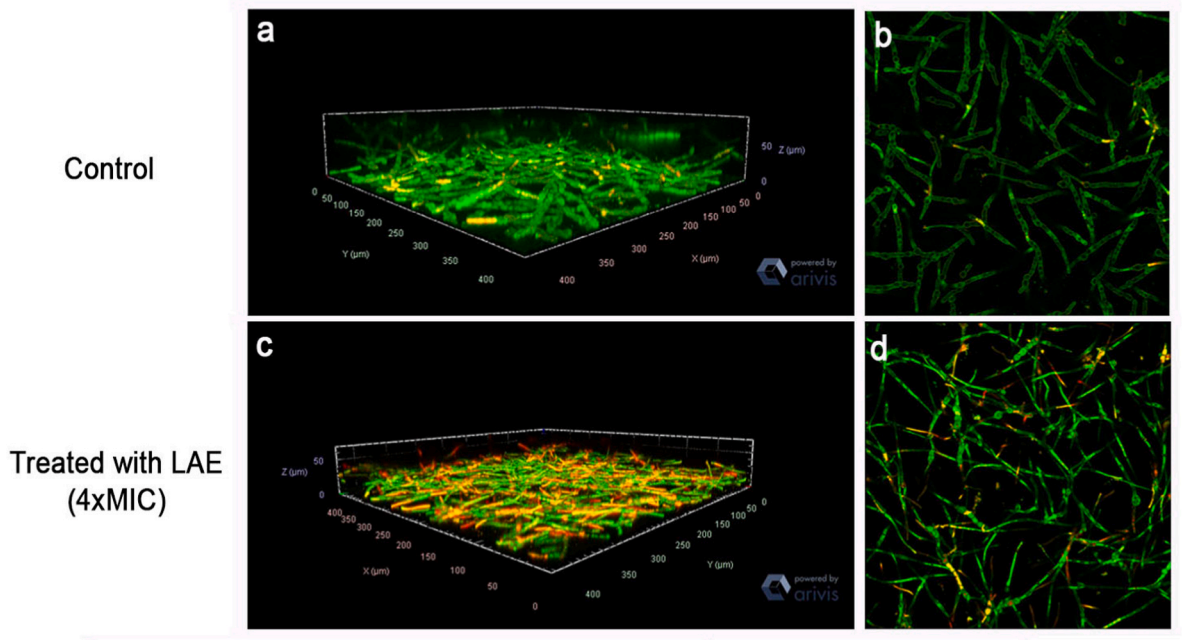

Fig. 9. CLSM images of *C. cladospoiroides* biofilms after treatment with LAE. Samples were stained with LIVE/DEAD staining. Green cells represent undamaged cells whereas red cells represent either damaged or dead cells. Figures a and c shows the three-dimensional construction of CLSMscans and b and d a representative one plane image. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

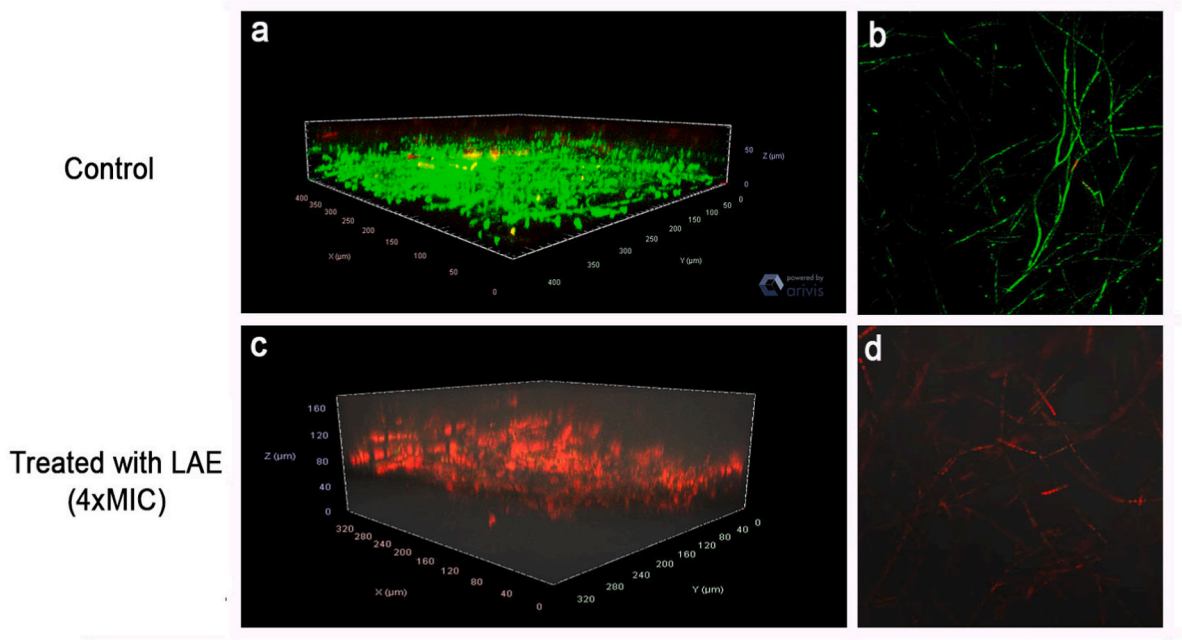

Fig. 10. CLSM images of *Fusarium oxysporum* biofilms after treatment with LAE. Samples were stained with LIVE/DEAD staining. Green cells represent undamaged cells whereas red cells represent either damaged or dead cells. Figures a and c shows the three-dimensional construction of CLSMscans and b and d a representative one plane image. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

materials increase the differences in concentration change and thus increase the diffusion force (Cheng et al., 2019). Besides, a higher proportion of LAE respect varnish reduces the interactions between the varnish and LAE and diminishes the retention capacity of the coating.

Because coating with 2% was the minimum concentration of LAE that produced a considerable decrease of biofilm of the three moulds tested, a second release assay was carried out to study the profile of LAE release from that coating over time (Fig. 13b). Data obtained indicate that there was an initial burst release of LAE until 8 h, after which the concentration of LAE remaining in the film was 1.37%, followed by a slower release until 96 h. At this time, most of the LAE contained in the coating (82.1%) had been released and a concentration of 0.35% remained in the film.

If comparing the release rate of LAE with that found in other studies we can observe that in this case, the release is slower (63.7% of release was observed after 48 h in the 8% coating). Kashiri et al., in 2019 found a release of almost 100% of LAE from a zein coating containing 5 or 10% of LAE at 37 °C in 4 h. In adittion, Muriel-Galet et al., in 2014 obtained

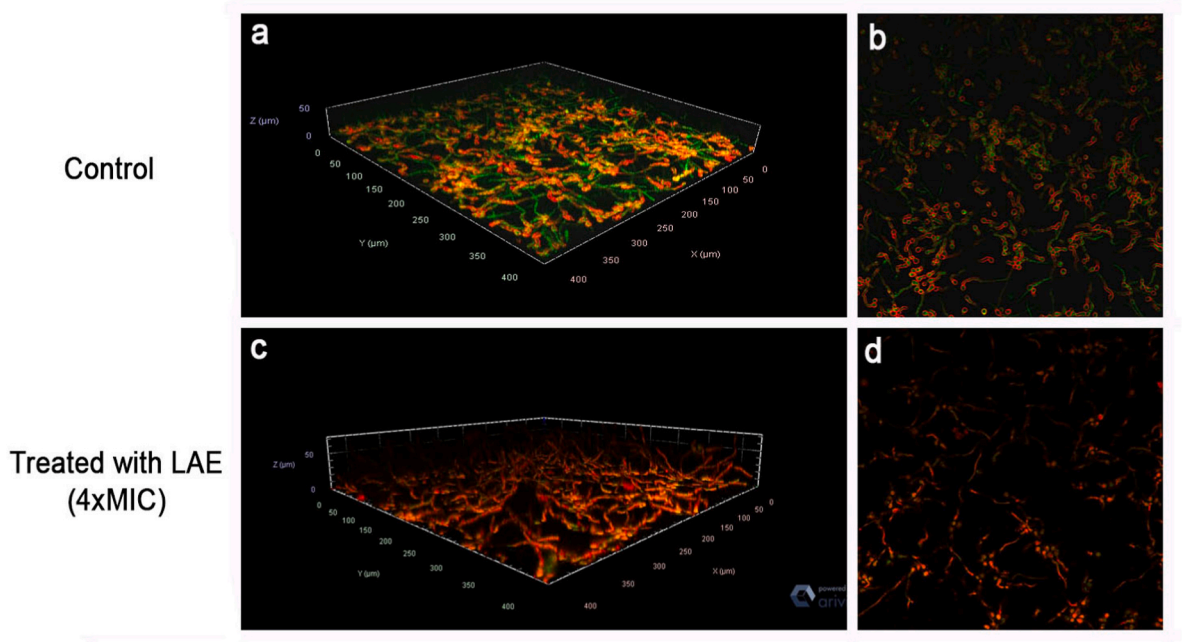

Fig. 11. CLSM images of *Penicillium italicum* biofilms after treatment with LAE. Samples were stained with LIVE/DEAD staining. Green cells represent undamaged cells whereas red cells represent either damaged or dead cells. Figures a and c shows the three-dimensional construction of CLSM scans and b and d a representative one plane image. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

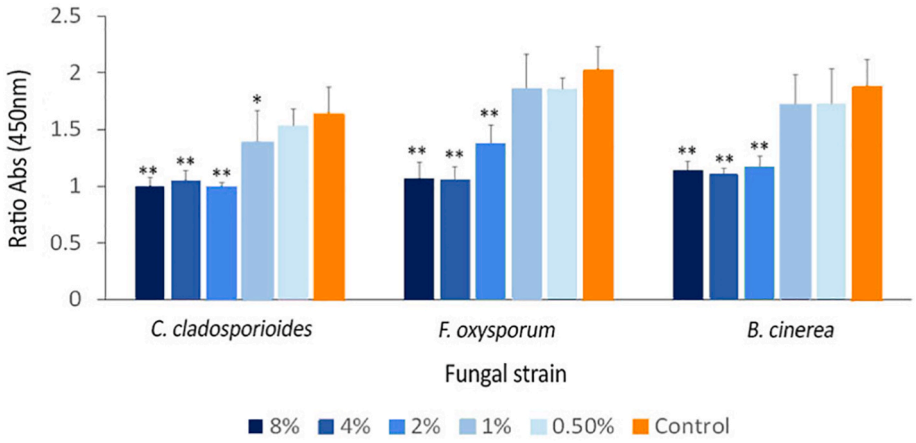

Fig. 12. Biofilm formation in coated wells. Results are given as mean absorbance (at 450 nm) ratios between inoculated samples and negative controls. Error bars correspond to SD values. Bars with asterisk are statistically significantly different from the control. *$p < 0.05$, **$p < 0.01$.

80% of release after 20 min at 23 °C from an ethylene-vynilalcohol film containing 5 or 10% of LAE. his could be due to the hydrophobic matrix-varnish used in this active coating as it has been demonstrated that less hydrophilic polymers retain better LAE because results in less matrix swelling and plasticization, and consequently slower LAE diffusion (Kashiri et al., 2016).

However, despite the higher retention capacity demonstrated in comparison to other hydrophilic matrices, the active coating should retain LAE better to obtain an active material with prolonged activity (Almasi et al., 2021; Becerril et al., 2020). For that, stronger interactions between the polymer matrix and the antimicrobial compound should be promoted. Silva et al., in 2019 demonstrated that biocomposite films containing cellulose nanofibrils strongly retain LAE due to electrostatic interactions between carboxylic groups of nanocellulose and LAE. Therefore, the inclusion of substances that increase interaction with LAE could improve the retention capacity of the coating.

4. Conclusions

In this work, the strong antimicrobial agent LAE has demonstrated high activity against biofilms formed by food spoilage fungal strains, particularly against, *P. italicum*, *B. cinerea*, *F. oxysporum*, *C. cladosporioides* and *A. ochraceus*. To our knowledge, this is the first study regarding the efficacy of LAE against fungal biofilms. As has been reported, the treatment with LAE reduced the formation of biofilms and acted against biofilms previously established. These results indicate that the use of LAE could be an effective strategy to prevent and reduce biofilms in the food industry. In this regard, this study evaluated a varnish-based coating incorporating LAE to reduce biofilm formation in abiotic surfaces. Results demonstrated high efficacy against *B. cinera*, *F. oxysporum* and *C. cladosporioides* but an elevated release of LAE from the coating. Future research should be carried out to improve the retention of LAE in the coating in order to extend their activity and to study the performance of the new coating in real conditions.

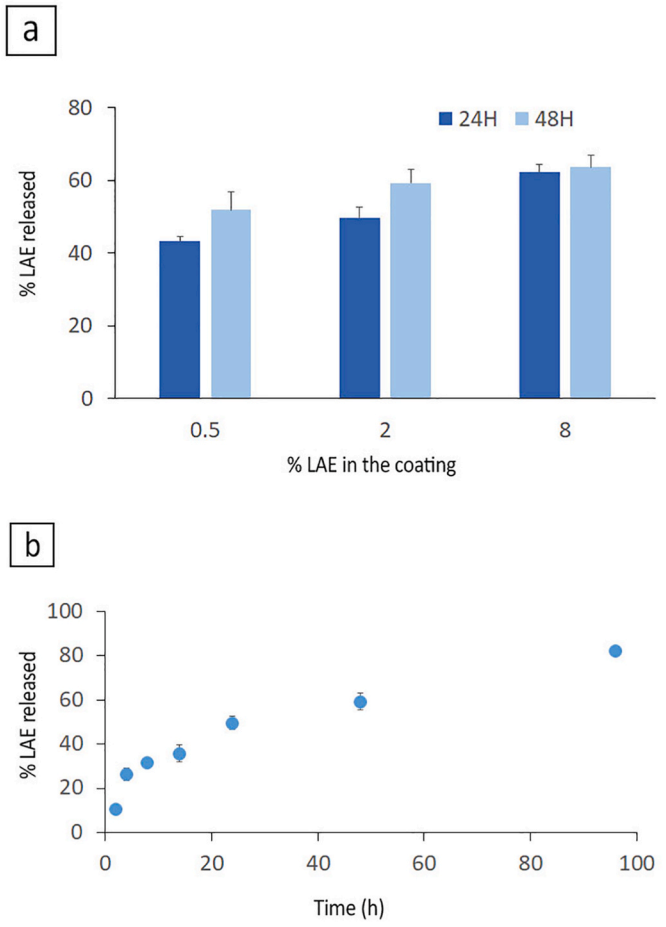

Fig. 13. Relative release of LAE from the active coating (a) with different concentrations of LAE (% w/w with respect to varnish weight) and (b) with a concentration of 2% of LAE over time. Values are given as % of LAE (LAE released/LAE contained in the coating *100).

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Conflict of interest and authorship conformation form

Please check the following as appropriate:

- o All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.
- o This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.
- o The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript
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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2023.104284.

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