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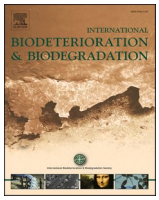

Antifungal activity of carvacrol-based solids and their effects on Whatman and Kraft paper

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ABSTRACT

The degradation of cellulose-based materials by fungi represents a menace to the cultural heritage conservation. Carvacrol-based β-cyclodextrins and cocrystals proved effective antifungal remedies *in vitro* but their effects on paper structure and properties were not studied. The aim of this study was to investigate possible structural modifications and alterations of the mechanical, optical and chemical properties of artificially aged and unaged Whatman and Kraft paper subjected to the treatment with carvacrol-based β-ciclodextrins and cocrystals. The pH of the samples did not significantly change after the treatment, as well as no colour-related alterations were detected ($1.00<\Delta E<2.00$). The tensile strength of both Whatman and Kraft paper was not affected by the vapours of carvacrol and spectroscopic analysis (FTIR and XRD) revealed no carvacrol-related damages of paper structure. The antifungal efficacy of the carvacrol-cocrystal was also proved on a book prototype made of Whatman and Kraft paper, kept under 98% of humidity for 28 days, and purposely inoculated with a mix of fungal species (*A. alternata*, *Aspergillus* sp. section Nigri, *C. cladosporioides*, and *T. orientale*). These results show the applicability of a carvacrol-releasing system, effective as antifungal remedy, and at the same time not harmful to Whatman and Kraft paper, as these materials did not show treatment-induced degradation.

1. Introduction

Paper represents the main support humankind has been using since its invention to collect written knowledge, giving reason for an extremely wide heritage of manuscripts, parchments, and many other cellulose-based documents requiring safeguarding. Paper is primarily composed of cellulose and is derived from plants through processes that have evolved over time involving various raw materials, additives, and manufacturing techniques. Cellulose is a naturally occurring polymer composed of repeated glucose units $(C_6H_{10}O_5)_n$, representing one of the most abundant organic materials on Earth (French, 2017; Seddiqi et al., 2021). The degree of polymerization (DP) of cellulose, which refers to the number of glucose units in a cellulose molecule, varies widely depending on the source and processing methods. Indeed, the DP is directly correlated with the mechanical properties of paper (Hallac and Ragauskas, 2011; Kim and Jang, 2013). Cellulose exhibits a complex multi-level structure, consisting of aggregates of fibrils. Each fibril comprises both large, well-ordered (crystalline) domains and smaller, less-ordered (amorphous) domains (Ioelovich, 2008). The ratio between crystalline and amorphous cellulose in paper significantly influences its properties and susceptibility to degradation. Typically, the crystalline regions provide structural integrity and resistance to enzymatic attack, whereas the amorphous regions are more accessible to enzymes and thus more prone to degradation (Brunšek et al., 2023).

Historically, cellulose for paper production was primarily sourced from cotton until the mid-19th century. With the advent of industrialization and increasing demand for paper, wood pulp became the predominant source of cellulose, thanks to its abundance and lower cost

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(Valente, 2012).

As all the materials of organic composition, paper undergoes over time decay due to a concerted action of weathering and biotic agents, these latters better known as "biodeteriogens" (Bloom, 2001; Sequeira et al., 2012). The macro-category of biodeteriogens comprehends any living organism able to induce mechanical, chemical or aesthetical alterations to an organic material to promote its growth and reproduction (Pinzari et al., 2006). Within this class, filamentous fungi are very critical in terms of paper degradation. Key fungal genera involved in paper degradation include *Aspergillus*, *Alternaria*, and *Cladosporium* (Menicucci et al., 2022). Together with bacteria such as *Micrococcus* and *Bacillus*, these microorganisms form biofilms that lead to materials' alterations on both surface and inner areas, causing long-term decay and loss of strength (Egil et al., 2022; Cirone et al., 2023).

Paper exhibits high bioreceptivity (i.e. susceptibility to colonization) to fungi due to its nature and composition rich in carbon sources (cellulose, hemicelluloses, lignin, adhesives, sizings). The physical and chemical forms of cellulose influence paper's bioreceptivity: processed cellulose with more amorphous sites is more susceptible to biodeterioration (Gallo et al., 1998; Allsopp et al., 2004). Lignin removal during papermaking enhances bioreceptivity, despite improving paper quality (Allsopp et al., 2004).

When affected by fungi, paper accumulates metabolic products that continue to exert their deleterious effects even after the fungus is no longer active (Florian, 2002). Excreted lipids can oxidize, forming free radicals and peroxides that contribute to brown discoloration. Pigments produced through metabolic processes can further impede the readability of the paper (Florian, 2002; Abdel-Kareem, 2010). Cellulolytic fungi decompose cellulose using extracellular enzymes called cellulases. Specifically, endogluconase and exogluconase break down the macromolecule into soluble sugars, and this process is finalized within the fungal cells by β-glucosidase. Additionally, initial stages of cellulose degradation can occur non-enzymatically. This combined enzymatic and acidic degradation leads to a gradual loss of mechanical strength in paper, eventually resulting in disintegration and loss of information (Valentín, 2007; Abdel-Kareem, 2010).

Most techniques to preserve paper objects from biodeterioration involve the use of biocides, with a critical fall-out in terms of health risks on those people working in/attending libraries and archives (Sequeira et al., 2012; Karbowska-Berent et al., 2018). The standard conservation approach should therefore be revised by introducing non-invasive methodologies based on no/low-toxicity substances. It is also relevant to note that if paper is kept dry, decay cannot proceed, underscoring the importance of environmental control as a non-biocidal preservation strategy. However, this kind of strategy can be challenging in environments with high humidity.

Essential oils (EOs) are complex organic mixtures produced by plants. They have been widely used in traditional medicine and for embalming rituals since most ancient times. Their range of properties (i.e., antimicrobial, antioxidant, antiviral, insecticidal, etc.) justifies a multitude of applications encompassing agriculture, medicine, food industry, cosmetics and so on (Bakkali et al., 2008; Sharifi-Rad et al., 2017; Ferreira et al., 2021; Derbassi et al., 2022). Major components of EOs are terpenes and phenols, volatile organic compounds (VOCs) playing a key role in plant defense and signaling (Sharifi-Rad et al., 2017; Menicucci et al., 2023). One of the main challenges implied by the use of such substances is preventing their immediate volatilization, in order to extend their action through time. With this in mind, different types of EO/VOC-based antimicrobial technologies and formulations (e.g. nanoparticles, beads, cyclodextrins) have been experimented also in the field of cultural heritage conservation, but only few publications were specifically addressed to paper-based items (Benkovïcová et al., 2019; Campanella et al., 2021; Menicucci et al., 2022).

Among innovative technologies, β-cyclodextrins and cocrystals proved to be effective tools for the controlled release of VOCs. The former are cyclic hydrosoluble sugars able to interact with a guest molecule according to a dynamic equilibrium. β-cyclodextrins may host a VOC or an EO in their hydrophobic cavity and find large use in medicine as drug delivery systems, thanks to low levels of toxicity (Marques, 2010; Kfoury et al., 2019). Cocrystals are composed of a coformer molecule and an active ingredient (e.g. a VOC), which is stabilized within a solid network through weak bonds (Mazzeo et al., 2019, 2020). Similarly to β-cyclodextrins, cocrystals are widely used in the pharmaceutical industry (Schultheiss and Newman, 2009).

Recently, VOC-based β-cyclodextrins and cocrystals have been experimented in solid form against a set of biodeteriogens affecting paper, and those ones capsulating carvacrol showed good levels of antimicrobial activity and insect repellency (Menicucci et al., 2022). However, possible side effects due to paper exposure to the treatment were not under investigation at this stage of research. Aiming to alternative non-invasive conservation approaches, the experimentation of innovative technologies must go hand-in-hand with a careful evaluation hinged on three aspects: effectiveness, safety and harmlessness. The main object of this work was therefore the assessment of the effects of carvacrol-based β-cyclodextrins and cocrystals on two types of paper (Kraft and Whatman), to investigate possible treatment-related side effects. In particular, paper structure and properties were analyzed before and after carvacrol treatment. For this purpose, spectroscopic techniques (Fourier-transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD)) were used, and mechanical, pH and colour measurements were carried out on the material. Additional objective was that of evaluating the antifungal activity of the carvacrol-based cocrystal on a book prototype kept at controlled conditions.

2. Materials and methods

2.1. Synthesis and characterization of carvacrol-based β-cyclodextrins and cocrystals

Carvacrol (CARV) (Sigma-Aldrich) was included into β-cyclodextrin (CD) (Sigma-Aldrich) or complexed with phenazine (PHE) (Sigma-Aldrich) following the protocols detailed in our previous publications (respectively, Menicucci et al., 2022; Mazzeo et al., 2019), at a 1:1 (β-cyclodextrin/carvacrol) and 1:2 (phenazine/carvacrol) stoichiometric ratio. The obtained powders (CD-CARV and PHE:CARV) were subjected to XRD analysis for purity checking and the amount of carvacrol included into β-cyclodextrin was determined via GC-MS analysis, data not shown (Menicucci et al., 2022).

2.2. Tests on Whatman and Kraft paper before and after exposure to carvacrol-based treatments

All the material analysis (subparagraphs 2.2.1.-2.2.5.) were performed on Whatman filter paper (No. 1) and Kraft paper samples (90 g/m^2) of aged and unaged material, before and after the treatment with carvacrol (i.e. exposure to the vapours of carvacrol used as pure (liquid state), or as solid (as a powder of CD-CARV and PHE:CARV)). These types of paper were selected as standard reference materials, characterized by high cellulose (Whatman) and lignin content (Kraft), respectively. The Kraft paper was provided by the Forte Belvedere archive (Florence, IT), where it is used as protective covering of the volumes. Artificial aging was realized exposing the samples at 80 °C, 65% RH, for 21 days, as previously reported by (Rakotonirainy and Lavedrine 2005), according to the standardized procedure (ISO 5630-3). The indirect treatment of both aged and unaged paper was carried out by exposing the specimens to the vapours of carvacrol, both in its pure form (CARV) and as solid in the form of β-cyclodextrin complex or cocrystal (i.e. CD-CARV or PHE:CARV), at 27 °C, 98% RH, for 21 days. Fourty-eight μL of carvacrol, 507 mg of CD-CARV and 76 mg of PHE:CARV were placed within Ø 150 mm Petri dishes (Sigma-Aldrich). The control (Ctrl RH) of these samples was kept at 27 °C, 98% RH. For all experiments, the untreated paper, not exposed to 98% humidity, was

kept as reference control (Ctrl NO RH). In all graphs the following acronyms were attributed to the treatments: CARV, PHE, PHE:CARV, CD, CD-CARV (for simplicity, RH was omitted in the treatment acronyms).

2.2.1. pH measurements

The pH measurement was performed following the procedure for the cold extract of paper reported in ISO 6588. Briefly, 0.5 g of finely chopped paper underwent cold extraction with 50 mL of deionized water for 1 h. A Mettler Toledo SevenEasy pH Meter was used for pH measurements and four replicates per each thesis were prepared.

2.2.2. Colour alteration

To provide information on possible colour alteration of the paper, measurements of untreated/treated samples were performed using a spectrophotometer Konica Minolta mod. Chroma Meter CM-700d. According to the CIELAB 1976 method, with standard illuminant D65 and observer 10° (EN 15886:2010), the colour coordinates L*, a* and b* were registered in each selected area (Ø 8 mm) before and after the treatments. The calibration against a SPECTRALON® was performed before any measurement. Each colorimetric value (L*, a*, b*) was the autoaveraging of three subsequent measurements. Four different spots were randomly scanned on the surface of each sample, and the registered values were expressed following this formula (Karbowska-Berent et al., 2018):

$$\Delta E^* = [(L_1^* - L_c^*)]^2 + (a_1^* - a_c^*)^2 + (b_1^* - b_c^*)^2]^{1/2}$$

where:

L_1^*, a_1^*, b_1^* results of the treated samples, L_c^*, a_c^*, b_c results of the untreated control samples.

2.2.3. Tensile properties

The tensile breaking properties were investigated adopting the methodology described in TAPPI 494 with few modifications. Samples (2.5 cm × 12 cm) were subjected to a constant rate of elongation (4.5 mm/min until breaking, within a time frame of 50 s. A Universal Testing Machine, Instron mod. 5567, equipped with a 30 kN load cell (precision 0.1%) was used. Ten specimens per thesis were processed and those breaking within or close to the clamping jaws were discarded.

2.2.4. Fourier-transform infrared spectroscopy

Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy was performed on the specimens with an ALPHA FT-IR spectrometer (Bruker Optics) equipped with a SiC Globar source and a DTGS detector. The instrument mounted an ATR module with Platinum single reflection diamond. Spectra were recorded in the 4000–400 cm^{-1} range, with a resolution of 4 cm^{-1} over 24 scans. All the spectra were processed using OPUS 7.2 software.

2.3. Antifungal activity of the carvacrol-based cocrystal (PHE:CARV): assessment on a book prototype

The antifungal effect of PHE:CARV was evaluated on a book prototype kept under controlled environmental conditions. The book prototype was composed of a stack of Whatman paper (54 pages; 13 cm × 6.5 cm) and two identical prototypes were assembled. Eight rectangular areas of 2 cm^2 were outlined on the first (pag n. 1), the central (pag n. 27), and the last page (pag n. 54) of each book, before sterilizing the material. A fungal suspension of about $1x10^5$ conidia/mL was prepared as detailed in our previous work (Menicucci et al., 2022), for each following species: *A. alternata*, *Aspergillus* sp. section Nigri, *C. cladosporioides*, and *T. orientale*. A mixed suspension of the same concentration was obtained mixing equal volumes of the four fungal suspensions. 20 μL of this suspension were pipetted in correspondence of each rectangular square on books' pages. After inoculation, each book was wrapped in a sterile protective casing made of Kraft paper and placed at the bottom of a 5.5 L dryer. 2088 mg of PHE:CARV were placed in the first dryer, as treatment, whereas no powder was added to the second dryer, as control. The prototypes were kept at 25 °C, 98% RH for 28 days, as to reproduce critical conservation conditions over a long period. The ATP bioluminescence assay (3M Clean-Trace Surface ATP) kit was used to assess the fungal proliferation on paper, by swabbing four inoculated areas outlined on the books' pages. This test quantifies the ATP bioluminescence, reflecting the number of living and metabolically active cells present on a surface (Rakotonirainy and Dubar, 2013). Values were expressed as RLU (Relative Light Units)/cm^2.

2.4. Statistical analysis

Statistical analysis was conducted using GraphPad Prism 5 software. For all tests, at least four replicates were considered for each treatment, and the results were presented as mean ± standard error (SEM). Statistical comparisons among the treatments were performed using the non-parametric Kruskal-Wallis test, followed by the Mann-Whitney *U* test for pairwise comparison, with a significant threshold set at 5 % ($p < 0.05$). An analysis of variance (ANOVA) was performed with tensile strength as dependent variable, using R® software. Shapiro–Wilk normality test and then Levene's test for the homogeneity of variance were first carried out. A post-hoc analysis, i.e. Tukey's honest significant difference (HSD) test, was calculated for significant differences obtained by ANOVA.

3. Results and discussion

3.1. pH measurements

For the unaged Kraft paper, the comparison between the controls, namely Ctrl NO RH and Ctrl RH, showed that the exposure to humidity (RH) caused a significant although limited pH increase (Fig. 1). All the treatments (e.g. CARV, PHE, CD, and CD-CARV) except PHE:CARV, showed pH values comparable to that of the RH control, as no significant differences were detected by pairwise comparison. Kraft paper exposure to PHE:CARV treatment induced a significant pH decrease with respect to the RH control, but the value did not significantly differ from that of the NO RH control. The presence of carvacrol as cocrystal seems therefore to be related to a slight acidification of paper, that in any case was fully comparable to that of the untreated paper (Ctrl NO RH).

For the unaged Whatman paper, no significant differences were registered among the controls (Ctrl NO RH and Ctrl RH) and all the carvacrol-based treatments (CARV, PHE:CARV, and CD-CARV), no matter as pure nor as formulation (Fig. 1). The pH value registered after paper exposure to CD was higher but not significantly different from that of the control (Ctrl RH), whereas a significant increase of the pH was observed after exposure to PHE. Despite this difference, it should be noted that in all cases, the mean pH was between a minimum of 7.35 and a maximum of 7.64, a range of values whose accuracy of measurement is negatively affected by its closeness to neutrality.

No significant differences were found among the treatments and the controls (Ctrl RH and Ctrl NO RH) of aged Kraft and Whatman paper (Fig. 2).

The comparison between aged and unaged Kraft paper revealed no significant differences (Fig. S1). This may be due to Kraft composition, notoriously poorer in cellulose content. Normally, aged paper is characterized by more acid pH values than unaged one, as the aging process involves cellulose degradation and a consequent acidification of the substrate itself (Zervos 2010). The non-acidification of the Kraft paper after aging is therefore probably due to its low cellulose - high lignin content, which seems to bestow more resistance on this material. On the contrary, a significant difference was detected between aged and unaged Whatman paper, the latter showing significantly higher pH values, with the only exception of PHE:CARV treatment (Fig. S1). Such values are in agreement with Whatman paper composition, which is basically given

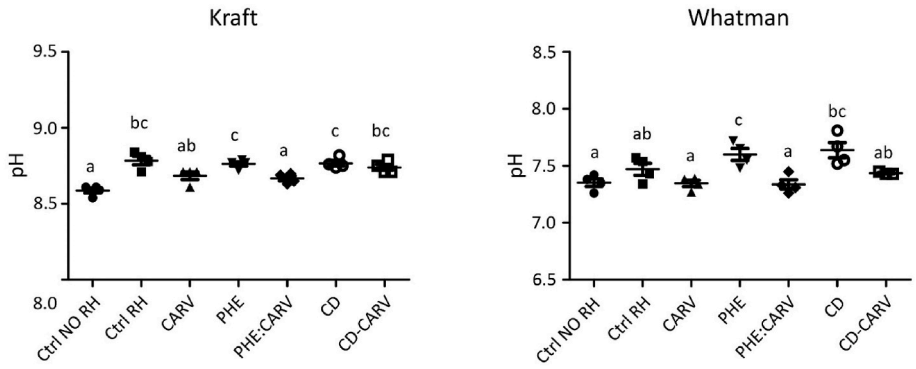

Fig. 1. Mean pH for unaged Kraft and Whatman paper after exposure to indirect contact with carvacrol-based treatments. Different letters indicate significant differences among the seven treatments (Ctrl NO RH: untreated paper; Ctrl RH: untreated paper exposed to 98% of relative humidity; CARV: paper exposed to carvacrol (RH 98%); PHE: paper exposed to phenazine (RH 98%); PHE:CARV: paper exposed to phenazine-carvacrol cocrystal (RH 98%); CD: paper exposed to β-cyclodextrin (RH 98%); CD-CARV: paper exposed to β-cyclodextrin-carvacrol (RH 98%)).

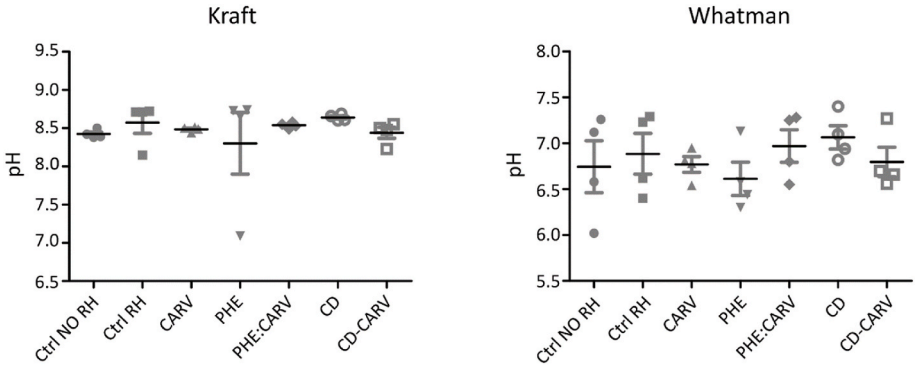

Fig. 2. Mean pH for aged Kraft and Whatman paper after exposure to indirect contact with carvacrol-based treatments. Ctrl NO RH: untreated paper; Ctrl RH: untreated paper exposed to 98% of relative humidity; CARV: paper exposed to carvacrol (RH 98%); PHE: paper exposed to phenazine (RH 98%); PHE:CARV: paper exposed to phenazine-carvacrol cocrystal (RH 98%); CD: paper exposed to β-cyclodextrin (RH 98%); CD-CARV: paper exposed to β-cyclodextrin-carvacrol (RH 98%).

by pure cellulose.

3.2. Colour alterations

After 21 days of treatment, there was no significant colour-related alteration detectable on both aged and unaged Whatman paper exposed to the carvacrol-based treatments, with all ΔE values below 1.00 (Table 1). According to the scale reported by Drzewinska (2002), such values correspond to unnoticeable alterations. Regarding Kraft paper, the exposure to humidity (RH) caused increased ΔE values for both the unaged and aged material (respectively +0.83 and + 0.88 compared to Ctrl NO RH). However, even in this case, all values were still in the range of the very small difference (1.00<ΔE<2.00) for all the considered treatments. These data point out that, at the tested concentration, neither the pure carvacrol nor its formulates significantly alter the colour of the substrate, suggesting the non-invasiveness of the experimented treatments as regards possible optical changes of Whatman and Kraft paper.

3.3. Tensile properties

For the unaged Kraft paper, the exposure to 98% of relative humidity caused a significant decrease of the tensile strength, as lower values were registered for all treatments with respect to the control (Ctrl NO

Table 1
ΔE values for Whatman and Kraft paper of aged and unaged material, exposed to indirect contact with carvacrol-based treatments.
Ctrl NO RH: untreated paper; Ctrl RH: untreated paper exposed to 98% of relative humidity; CARV: paper exposed to carvacrol (RH 98%); PHE: paper exposed to phenazine (RH 98%); PHE:CARV: paper exposed to phenazine-carvacrol cocrystal (RH 98%); CD: paper exposed to β-cyclodextrin (RH 98%); CD-CARV: paper exposed to β-cyclodextrin-carvacrol (RH 98%).

unaged material	ΔE		aged material	ΔE	
Treatment	***WHATMAN***	***KRAFT***	***Treatment***	***WHATMAN***	***KRAFT***
Ctrl NO RH	0,217	0,575	Ctrl NO RH	0,586	0,705
Ctrl RH	0,373	**1401**	Ctrl RH	0,436	**1582**
CARV	0,294	**1283**	CARV	0,461	**1565**
CD	0,245	**1597**	CD	0,380	**1685**
CD-CARV	0,315	**1148**	CD-CARV	0,486	**1408**
PHE:CARV	0,300	0,599	PHE:CARV	0,505	**1353**
PHE	0,331	0,500	PHE	0,433	**1977**

ΔE<1,00 - unnoticeable difference; 1,00<ΔE<2,00 - very small difference, noticeable to an experienced observer only; 2,00<ΔE<3,50 - medium difference, noticeable to an experienced observer only; 3,50<ΔE<5,00 - significant difference (Drzewinska 2002; Karbowska-Berent et al., 2018).

RH). The strength of paper is related to a number of factors that act together to determine the ultimate resistance: the strength of the individual cellulose fibers; the surface contact area of these cellulose fibers with each other (which is in turn affected by their size and length); the shear strength that develops between them due to hydrogen bonding related to the presence of surface hydroxyls in the cellulose fibers (Vaidyanathan and Basu, 1979; Hon, 1989; Bandyopadhyay et al., 2002; Rhim, 2010). The hydroxyls are hydrophilic and therefore interact with water molecules from the environment in the presence of moisture. This determines the partial replacement of the fiber-fiber interaction, the reduction of hydrogen bonds and the resulting global decrease in strength, which was in fact observed in the tests. In contrast, the hydroxyls of carvacrol are hindered by its monocyclic terpene chemical structure and therefore carvacrol is less able to affect the strength of the paper, at least at high humidity conditions, as with the RH series. In fact, its presence had very little effect on tensile strength, the values obtained being very close to those of the control RH (Fig. 3; Table S1).

Similarly to the Kraft paper, for Whatman unaged paper the presence of carvacrol had very little effect on tensile strength compared to the Ctrl RH (Fig. 3; Table S1). It should be noted that Whatman strength was appreciably lower than that of the Kraft paper: for example, the average values were 31 MPa and 7 MPa for the controls RH Kraft and Whatman paper, respectively. This difference is related to the different origins of the two papers. In fact, Whatman paper is obtained from cotton linters that are subjected to an acid treatment to maximize α-cellulose and purity. This treatment tends to oxidize and partially depolymerize the cellulose. In contrast, Kraft paper is made from wood pulp using the sulfate process, an alkaline process that only removes most of the lignin originally present in the wood. However, this process is less impacting than the acid one, and actually some hemicelluloses are still present in the material (Soares et al., 1995; Tarasov et al., 2018), which also contribute to hydrogen bonding in the fibers (Adamo et al., 2001; Moise et al., 2012). Thus, the combination of a less degraded cellulose and a higher number of hydroxyl groups makes the Kraft stronger than the Whatman paper.

For both Kraft and Whatman aged papers, exposure to humidity caused a significant decrease in tensile strength compared to the unexposed paper (Ctrl NO RH) (Fig. 4; Table S2). This is due to the fact that in aged paper exposed to humidity, the two strength-reducing mechanisms mentioned above, namely partial depolymerization of cellulose (hence fiber weakening) and reduction of fiber-fiber interactions (replacement of cellulose OH–OH hydrogen bonds by water molecules), combine to determine the observed decrease. When comparing the RH-exposed treatments, no significant differences were observed in either Kraft or Whatman paper (Table S2).

It is also interesting to compare aged and unaged papers. For the Kraft paper, higher values were observed for the unaged untreated paper (Ctrl NO RH) (Fig. S2). The statistical significance of this difference is indicated by the fact that the lower and upper extremes of the 95% confidence intervals do not overlap (Fig. S2). It is known that aging in an aggressive environment (higher than ambient temperature, humid conditions) induces a decrease in the degree of polymerization in cellulose due to hydrolysis (Zou et al., 1994). The resulting reduction in the average cellulose chain length leads to strength reduction, as explained above. Similar results have been obtained previously (Shahani et al., 1989; Isca et al., 2015). However, in this case the decrease was less pronounced, probably due to the slightly milder conditions used here (80 °C/65% RH here, 90 °C/60% RH in Shahani et al. (1989)). In fact, this difference was not observed for Whatman paper (Fig. S2), probably because cellulose chains in this paper are already partially depolymerized.

3.4. Spectroscopic analysis: FTIR and XRD

Fig. 5 shows FTIR-ATR spectra collected on unaged and aged Kraft and Whatman paper, before and after exposure to carvacrol treatments. Typical vibrations of cellulose are identified at 3331–3275 cm^{-1} (ν (OH) vibration of hydrogen bonded OH-groups from polysaccharides structures) (Maréchal and Chanzy, 2000), 2896 cm^{-1} (ν(CH)) (Xiao et al., 2015), 1427 cm^{-1} ($\delta(CH_2)$) (Ciolacu et al., 2010), 1318 cm^{-1} ($\delta(CH_2)$ related to the content of crystallized cellulose I) (Delmotte et al., 2008), 1158 cm^{-1} (v(C–O–C) stretching of the β-(1–4)-glycosidic linkage) (Nikonenko et al., 2000), 1106 cm^{-1} (asymmetric stretching of the glycosidic ring), 1029 cm^{-1} (ν(C–O) vibrations of primary alcohol) (Castro et al., 2011; Garside and Wyeth, 2003). No significant variations were visible for both Kraft and Whatman paper, with exception of sharp rotational absorption bands of water vapour molecules in the spectral regions 4000-3600 and 1800-1400 cm^{-1} (ROI1 and ROI2), which suggest moisture absorption due to exposure to RH. Details of the fingerprint spectral region are provided in Fig. S3.

None of the main bands of PHE (ν(CH) at 3058 cm^{-1} and γ(C–H) at 750 cm^{-1}) and of CARV (ν(CH) at 2970, 2896 cm^{-1}) (Fig. 5, spectra a and b) (Kellenberger et al., 2011) was detected on paper one month after treatments, suggesting the absence of residues on the exposed surfaces.

The powder diffraction spectra of Kraft and Whatman paper are shown in Supplementary Fig. S4. Only the Cellulose β phase is present in the Whatman paper sample, while in the Kraft one also the calcite and polyethylene polymer phases are found (Costa et al., 2014). The superimposing of the X-ray spectra of all recorded spectra for Whatman and Kraft samples did not show any significant change (Fig. S5). The degree of crystallinity reported in Table S3 highlighted that the treatments with carvacrol and carvacrol-based solids did not induce any significant change with respect to the Ctrl RH samples, for both Whatman and Kraft paper. For unaged material, the untreated sample (Ctrl NO RH) shows a slightly higher degree of crystallinity for the Whatman paper and a slightly lower one for the Kraft paper. For the aged material,

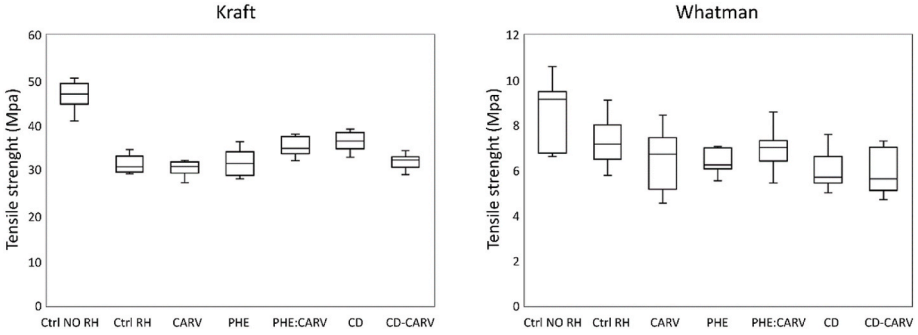

Fig. 3. Mean tensile strength for unaged Kraft and Whatman paper after exposure to indirect contact with carvacrol-based treatments. Ctrl-NO RH: untreated paper; Ctrl-RH: untreated paper exposed to 98% of relative humidity; CARV: paper exposed to carvacrol (RH 98%); PHE: paper exposed to phenazine (RH 98%); PHE:CARV: paper exposed to phenazine-carvacrol cocrystal (RH 98%); CD: paper exposed to β-cyclodextrin (RH 98%); CD-CARV: paper exposed to β-cyclodextrin-carvacrol (RH 98%).

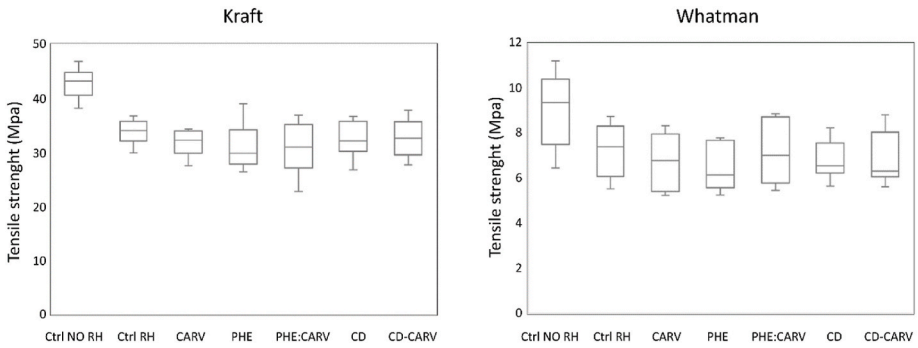

Fig. 4. Mean tensile strength for aged Kraft and Whatman paper after exposure to indirect contact with carvacrol-based treatments. Ctrl-NO RH: untreated paper; Ctrl-RH: untreated paper exposed to 98% of relative humidity; CARV: paper exposed to carvacrol (RH 98%); PHE: paper exposed to phenazine (RH 98%); PHE:CARV: paper exposed to phenazine-carvacrol cocrystal (RH 98%); CD: paper exposed to β-cyclodextrin (RH 98%); CD-CARV: paper exposed to β-cyclodextrin-carvacrol (RH 98%)).

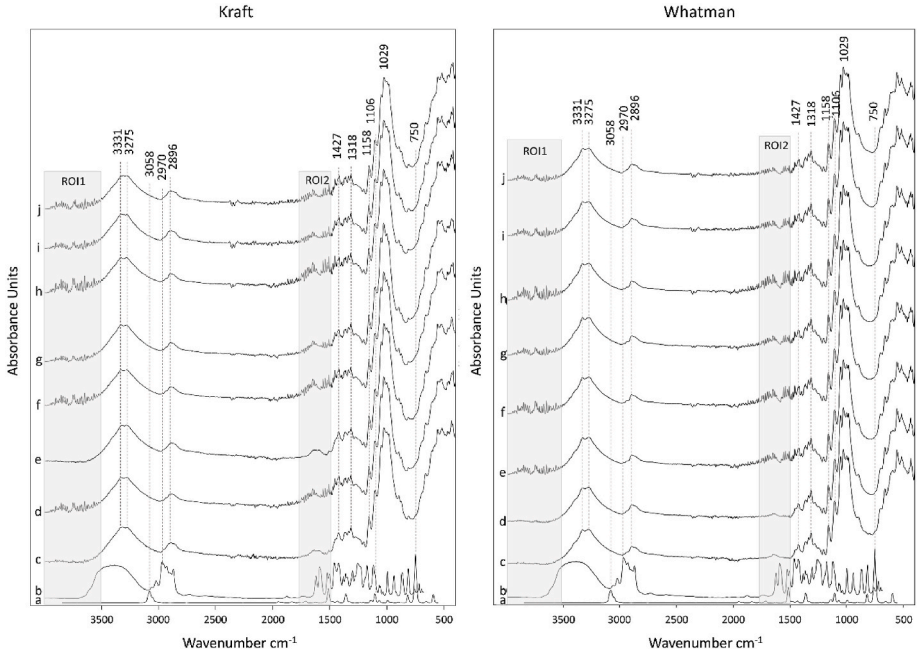

Fig. 5. FTIR-ATR spectra: (a) pure phenazine; (b) pure carvacrol; Kraft or Whatman paper (c) unaged (Ctrl NO RH); after artificial aging (d) with no exposure to RH (aged Ctrl NO RH); (e) with exposure to RH (CTRL RH); Kraft or Whatmanpaper after artificial aging, exposure to RH and to (f) CD; (g) CD-CARV; (h) CARV; (i) PHE:CARV; (j) PHE. Grey rectangles represent region of interest (ROI) where stretching (ROI1) and bending (ROI2) vibration modes of water are detected.

the differences are even smaller. As already shown by the other analysis, exposing the material to high humidity levels is the main factor that could influence the degree of crystallinity, whereas the effect of carvacrol is negligible.

3.5. *Antifungal activity of the carvacrol-based cocrystal: assessment on a book prototype*

To assess the antifungal activity of the carvacrol-based cocrystal (PHE:CARV) in a close-to-reality scenario, a long-term experiment was performed under controlled time, temperature and humidity conditions. The set-up involved two book prototypes which were infected with a mix of filamentous fungal species (Fig. 6, up right), previously isolated from a book collection stored at the Forte Belvedere archive (Florence, IT) and showing cellulolytic activity (Menicucci et al., 2022). After 28 days, the fungal proliferation was visible to the naked eye on all the inoculated pages of the untreated book prototype, on the adjacent pages, and on the inner side of its protective casing made of Kraft paper (Fig. 6, bottom). The contamination of the PHE:CARV-treated prototype was not detectable by eye. Significantly, lower ATP bioluminescence values were registered for all the treated book pages (n. 1, 27, and 54) than their respective controls (Fig. 6, up left). These values confirmed what observed by naked eye and pointed out the efficacy of the carvacrol-based treatment (PHE:CARV) over time, acting as an antifungal remedy. It is worth noting that the treatment was effective on the whole prototype: this indicates that the vapours released from the cocrystal in the surrounding atmosphere do not act just on the external part of a paper object exposed to the treatment, but do pass across its pages, exerting their antifungal activity. The antifungal activity of PHE:CARV had been previously investigated on the same fungal species with positive results, but only *in vitro* (Menicucci et al., 2022). The book prototype experiment also shows the broad spectrum efficacy of carvacrol, which proved effective not only on the singular fungal species, as found *in vitro*, but also against a mix of fungi which often colonize badly preserved paper objects and archives more in general. Similar results were obtained by Rakotonirainy and Lavédrine (2005) using linalool in its pure form on a book kept within a showcase, inoculated with a mix of fungi. In our work, the use of a carvacrol-based cocrystal was preferred

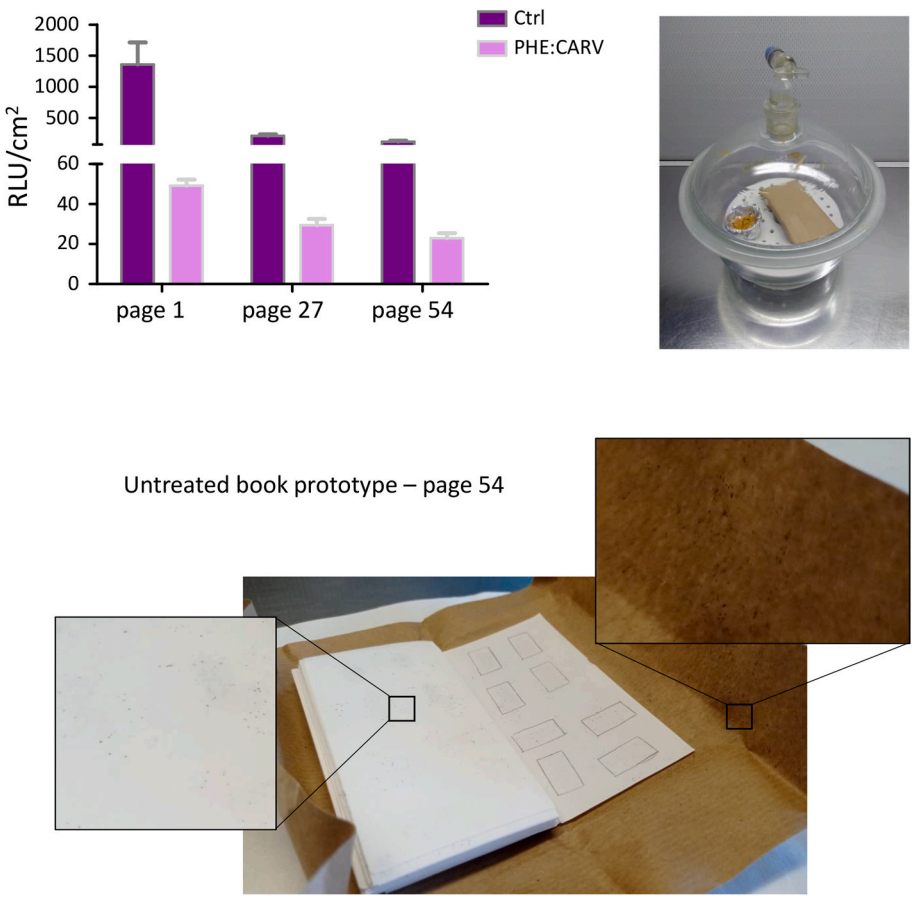

Fig. 6. Bioluminescence values (RLU/cm^2) registered on the treated and untreated book pages (up left). Ctrl: untreated book pages (i.e. prototype not exposed to PHE:CARV); PHE:CARV: treated book pages (i.e. prototype exposed to PHE:CARV). Experimental set-up for the treated book prototype (up right). Fungal proliferation on the untreated book prototype (bottom) after 28 days at 25 °C, 98% RH.

to the pure substance, as it ensures over time release and therefore improved long-term efficacy. As far as we know (Menicucci et al., 2023), there are no investigations of the use of technologies based on carvacrol and other essential oil components as antimicrobials for the preservation of paper items.

4. Conclusions

The exposure to carvacrol and its derived solids did not induce any significant acidification or colour alteration of the substrate, was it Kraft or Whatman and aged or unaged. Mechanical tests, as well as the FTIR and XRD analysis, showed that the alterations observed in all paper samples were mainly due to humidity and artificial aging process, and not to the carvacrol vapours exposure. The use of the carvacrol-releasing cocrystal significantly reduced the fungal proliferation on a book prototype infected with a mix of fungi. This remedy proved effective over long time (approximately one month) under critical conditions (98% of RH) and did not cause structural, optical or chemical alteration of this paper object.

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CRediT authorship contribution statement

Felicia Menicucci: Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Benedetto Pizzo:** Writing – original draft, Data curation. **Barbara Salvadori:** Writing – original draft, Data curation. **Laura Chelazzi:** Formal analysis. **Andrea Ienco:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis. **Eleonora Palagano:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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