ENVIRONMENTAL MICROBIOLOGY

Plant-derived compounds as natural antimicrobials to control paper mill biofilms

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J Ind Microbiol BiotechnolJ Ind Microbiol BiotechnolJ Ind Microbiol BiotechnolAbstract   Biofilms can cause severe problems in industrial paper mills, particularly of economic and technological types (clogging of filters, sheet breaks or holes in the paper, machine breakdowns, etc.). We present here some promising results on the use of essential oil compounds to control these biofilms. Biofilms were grown on stainless-steel coupons with a microbial white water consortium sampled from an industrial paper mill. Five essential oil compounds were screened initially in the laboratory in terms of their antimicrobial activity against planktonic cells and biofilms. The three most active compounds were selected and then tested in different combinations. The combination finally selected was tested at the pilot scale to confirm its efficiency under realistic conditions. All the compounds tested were as active against biofilms as they were against planktonic cells. The most active compounds were thymol, carvacrol, and eugenol, and the most efficient combination was thymol–carvacrol. At a pilot scale, with six injections a day, 10 mM carvacrol alone prevented biocontamination for at least 10 days, and a 1 mM thymol–carvacrol combination enabled a 67 % reduction in biofilm dry matter after 11 days. The use of green antimicrobials could constitute a very promising alternative or supplement to the treatments currently applied to limit biofilm formation in the environment of paper mill machines.

Keywords   Biocide · Decontamination · Carvacrol · Thymol · Papermaking

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Introduction

The environment in paper mills provides highly conducive conditions for the formation of biofilms [12]. The process waters in paper mills contain high levels of biodegradable matter from wood; temperatures range from 30 to 50 °C; air humidity levels are high and various environments from aerobic to fully anaerobic may be encountered. In addition, the recycling of water for environmental purposes increases the microbial load, and when recycled paper is used for production, this raw material is highly contaminated and the water cycles are particularly rich in nutrients. It is therefore not surprising to find very thick and complex biofilms on paper-making machines. In these environments, biofilms can cause the clogging of filters and shower nozzles, an impairment of dewatering properties, and malodor problems [12]. In addition, when they grow on surfaces above paper sheet production, biofilms can be transformed into long, slimy "stalactites" that can suddenly fall on the sheets and cause serious damage, such as sheet breaks or holes in the paper. These problems generate costs related to cleaning and/or machine downtime. Furthermore, these biofilms can constitute health risks for operatives by: (1) producing aerosols if they contain pathogens, or (2) releasing highly toxic compounds into the atmosphere, such as hydrogen sulphide (H_2S), which is produced by some micro-organisms under anaerobic conditions.

Biofilms are structured communities of microbial cells embedded in a complex polymeric matrix [11], which are known to display specific properties including an increased resistance to biocide treatments. The antimicrobial concentrations required to achieve the same reduction in biofilm and planktonic populations may range from 1 to 1,000 times higher [6]. Chemical biocides such as chlorine, bromine, glutaraldehyde, or quaternary ammonium compounds, are continually applied at different points in the process to prevent the deleterious effects of microbiological growth in paper mills. However, although these antimicrobials can limit biofilm proliferation, they are not able to eradicate surface contamination because of biofilm resistance and microbial habituation. Besides, some disinfectants in use at present will probably be banned during the next few years because of regulatory changes (Biocide directive [3]; REACH regulation [4]).

In this context, there is an increasing need to develop novel and environmentally safe strategies to improve the control of biofilms, particularly in the setting of paper mill environments. Optimizing matrix breakdown through the use of specific enzymes could enable improvements in the efficiency of disinfection. However, this strategy necessitates the precise characterization of matrix composition so that the most appropriate enzymes can be employed. The matrix produced by paper mill biofilms has been shown to comprise species-specific polymers, with enormous variability depending on the consortium composition. Polysaccharides can be degraded by specific enzymes, like 1,4-β-fucoside hydrolase for colanic acid [33, 42]. When the matrix was constituted of proteins, it was demonstrated that several proteases, and notably Savinase®, were efficient in removing biofilms [23]. A second line of enquiry is to develop new strategies using antimicrobials that would exert high lethal activity against the microbial consortium found in paper mills, would be capable of penetrating the biofilm structure, and would be easily eliminated in wastewater treatments. The use of some natural compounds may thus provide a solution. For example, plants are a huge source of active volatile molecules with antimicrobial properties [21] and recent studies have shown that some of these compounds may display some efficiency against mono-species biofilms [7, 30]. Nevertheless, attention should be paid to that all natural compounds are not necessarily environmentally safe and non-toxic.

In this paper, we present results regarding a promising alternative for the control of multimicrobial biofilms in industrial paper mills. Five antimicrobials extracted from plants were selected regarding their recognized broad spectrum of action against Gram-negative and Gram-positive bacteria, their high volatility and their generally recognized as safe (GRAS) status. They were first tested for their efficiency against white water microbial consortium biofilms.

An appropriate combination of two compounds was then optimized at the laboratory scale and its efficiency was finally tested at a pilot scale under realistic conditions close to the industrial reality.

Materials and methods

### The paper mill microbial consortium, growth conditions, and preparation of suspensions

A sample of white water (WW) was collected from the outlet of a white water storage tank in a French paper mill that produces newsprint using combination of new and recovered fibers. Sent from the paper mill to the laboratory and aliquoted, this sample was used as an inoculum for biofilm production both at the laboratory and pilot scales. It contained 10^7–10^8 cultivable cells/ml (counted by plating on Plate Count Agar in aerobic conditions). At reception, the WW consortium was mixed immediately with glycerol 40 % (1:1) and stored at −80 °C.

The WW consortium was grown in VTT medium [43] containing: glucose 20 g/l, yeast extract 0.5 g/l, K_2HPO_4, $3H_2O$ 5.2 g/l, KH_2PO_4, $3H_2O$ 3.18 g/l, $(NH_4)_2SO_4$ 0.6 g/l, $MgSO_4$, $7H_2O$ 0.3 g/l, $FeSO_4$, $7H_2O$ 0.6 mg/l, $ZnSO_4$, $7H_2O$ 0.2 mg/l, $MnSO_4$, H_2O 0.2 mg/l, $CuSO_4$, $5H_2O$ 0.2 mg/l, and $CaCl_2$ 50 mg/l. Frozen consortium was used as an inoculum (1:20) in VTT medium and growth was performed at 37 °C under shaking for 18 h before the suspension was used. Aerobic conditions were chosen in order to mimic growth conditions on air exposed surfaces. The consortium was not sub-cultured in order to minimize any modifications to the equilibrium among cultivable microbial species. The microbial consortium was then harvested by centrifugation (20 °C, 7,000 × g during 10 min) and washed twice in 150 mM NaCl. The optical density (600 nm, 1 cm) of the microbial consortium was adjusted to 0.1 in 150 mM NaCl in order to obtain approximately 10^8 CFU/ml (verified by plating). This calibrated suspension was used either for the formation of biofilms or for the testing of disinfectant activity on planktonic cells.

Antimicrobial agents

Thymol (Th), carvacrol (Ca), eugenol (Eu), *trans*-cinnamaldehyde (t-Ci) and α-terpineol (α-Ter) were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). These compounds have a recognized high efficiency against Gram-negative and Gram-positive bacteria [8, 15]. Stock solutions (1 M) were prepared by dissolving them in absolute ethanol and stored at 4 °C. The final concentrations were prepared by diluting the stock solution in deionized water on the day of use.

Fig. 1 Diagram of the pilot chain: coupons were sprayed continuously with the VTT medium inoculated with the white water consortium in order to form a biofilm. The bioreactor enabled control of the environmental parameters influencing biofilm formation. The hydraulic retention time within the circuit was maintained at 6 h. The Ca + Th treatment was injected by shocks directly into the spray nozzle
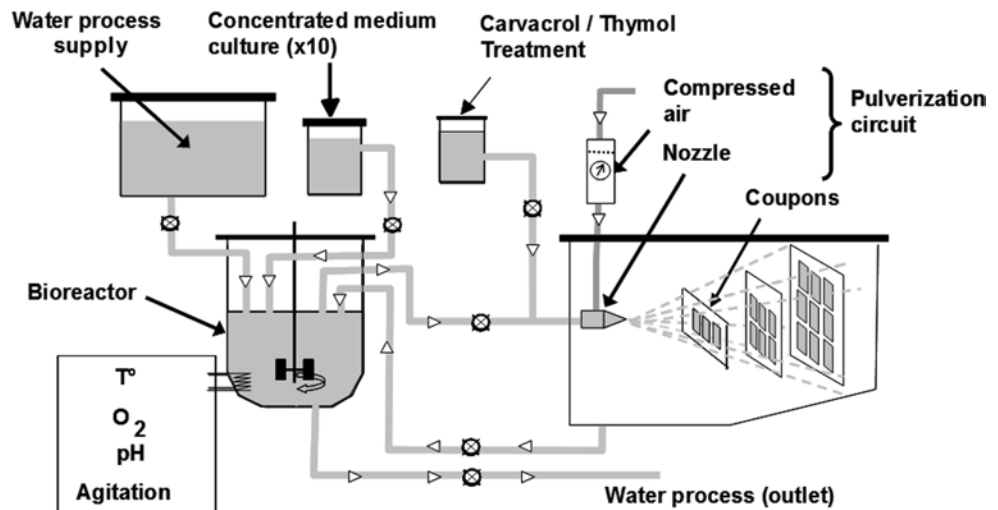
Biofilm formation

At the laboratory scale, biofilms were grown on 1-cm^2 stainless-steel AISI 316 2R coupons (Goodfellow, Cambridge Science Park, UK). Before use, the coupons were placed for 10 min under stirring at 50 °C in a 2 % v/v solution of surfactant RBS 35 (Société des traitements chimiques de surface, Lambersart, France), after which they were rinsed with sterile deionized water five times at 50 °C and five times at ambient temperature [25]. They were then stored in sterile deionized water for a maximum of 24 h before use. For biofilm formation, the coupons were settled in the wells of a polystyrene 24-well microtiter plate (Techno Plastic Products, Switzerland) and 1 ml of the microbial consortium as previously prepared was poured into the wells. Adhesion was ensured by sedimentation for 2 h at 37 °C. After the planktonic microbial consortium had been removed, the coupons were rinsed once again with VTT medium (1 ml) and incubated with a new batch of VTT medium (1 ml) at 37 °C for 24 h without shaking.

At the pilot scale, biofilms were grown in the system represented in Fig. 1. This pilot comprised three bioreactors connected to separate lines in order to test different treatment conditions (one line being used as a control and two for the tests). The bioreactor enabled control of the main physicochemical parameters that influence biofilm formation: dissolved oxygen, pH, agitation, and temperature. The pulverization circuit consisted of a pump feeding the nozzle spray with the fluid contained in the bioreactor. Spraying was achieved by mixing the air and fluid in circular, pneumatic atomizing spray nozzles. Fluid pressures and flow settings enabled the definition of spray characteristics in terms of the average fluid flow and average diameter of spray droplets. This system generated an aerosol with characteristics comparable to those of the paper mill aerosol. In the pilot plant, the flow rate was between 0.09 and

1.2 ml min^{-1} cm^{-2} (0.4 ml min^{-1} cm^{-2} for paper mill aerosols) and the drop size was between 20 and 500 µm (200–600 µm for paper mill aerosols). The biofilm was formed on AISI 316 stainless-steel coupons placed in the pulverization area. Three, six, and nine coupons were arranged at distances of 25, 40, and 50 cm from the spray nozzle, respectively (Fig. 1). Another pneumatic pump ensured recirculation of the fluid being sprayed, between the pulverization area and bioreactor. The pilot system was fed with VTT medium and inoculated with the WW consortium. During biofilm formation, the pH of the medium was maintained at 7.6, under gentle shaking, with a temperature of 48 °C and dissolved oxygen at a value higher than 30 % of saturation. The temperature of the spray aerosol reaching the coupons was around 34 °C. The pilot feed and pulverization were started up on the third day (the system having previously been operating under batch conditions). By generating an aerosol for several days, this pilot system enabled the formation of an air biofilm on coupons placed in the pulverization area. The hydraulic retention time (HTR) of the pilot was adjusted to 6 h during the experiment so that a biofilm would form in the control line with a growth kinetic comparable to that of industrial biofilms.

Antimicrobial efficiency of natural antimicrobial agents

At the laboratory scale, disinfection assays were performed on both biofilms and planktonic cells in order to evaluate biofilm resistance by comparison with intrinsic WW consortium resistance. Planktonic cells were challenged with antimicrobials using the EN 1040 standard protocol [2]. Briefly, planktonic cells (~10^7 CFU/ml in 150 mM NaCl) were exposed to the antimicrobial solution for a selected duration. The action of the antimicrobial was halted by transfer (1:9) to a quenching solution (3 g/l L-α-phosphatidyl choline, 30 g/l Tween, 80.5 g/l sodium

thiosulphate, 1 g/l L-histidine, 30 g/l saponine). After 10 min at 20 °C, serial dilutions were made in 150 mM NaCl, and the survivors were enumerated using the 6 × 6 drop count method [10] on Plate Count Agar (BD Difco™, Sparks, USA). The control was performed in the same way with sterile deionized water instead of the disinfectant. The logarithm reduction achieved was the difference between the $\log_{10}$ survivors after the test with deionized water and the $\log_{10}$ survivors after the test with the antimicrobial agent.

An adaptation of this method was developed to test biofilm resistance to antimicrobials under similar conditions. After biofilm formation, the coupons were rinsed with 150 mM NaCl in order to eliminate planktonic and weakly adherent cells. The coupons were then challenged with antimicrobial solutions. After the appropriate period, the coupons were removed from the antimicrobial solution and placed for the same period in the quenching solution. They were then placed in 45-mm Petri dishes with 5 ml of deionized water, and the adherent cells were detached by scratching. The survivors were enumerated and log reductions were determined as previously described. The results are the mean of at least four experiments performed on independently grown cultures.

At the pilot scale, an antimicrobial solution was injected into the system at the input of the pump feeding the spray nozzle with culture medium. This antimicrobial solution was a 200 mM carvacrol solution with or without thymol, prepared in deionized water from the 1 M stock solution. When carvacrol was used with thymol, they were mixed at equimolar concentrations. The flow rate was adjusted to obtain a carvacrol concentration in the medium sprayed on the coupons of 10 mM during the first test, and a carvacrol/thymol mixture concentration of 1 mM during the second test. The antimicrobial treatment begins 2 h after the pulverization of the coupons has started. It was injected in shocks lasting $t = 30'$. One of the test lines was treated once a day while the other line was treated six times a day. During this experiment, the amount of dry matter (corresponding to the biofilm that had formed on the coupons) was measured. For this purpose, coupons from the pilot line (with and without treatment) were removed, kept at 80 °C for 48 h, and then weighed. The dry matter thus obtained was expressed in mg/surface unit.

Confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM)

Directly after biofilm formation, or after subsequent treatment with 10 mM Ca for 15 min, the stainless-steel coupons were rinsed twice with 150 mM NaCl in order to remove planktonic cells and/or traces of quenching solution. For CLSM observations, the biofilms were stained fluorescently with the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen/Molecular Probes, Eugene, USA) for 15 min at room temperature in the dark. This kit contains the nucleic acid stains SYTO 9, which enables the labeling of total cells and propidium iodide (PI) that stains cells with damaged membranes. The biofilms were scanned using a 40×/water immersion objective lens on an inverted confocal microscope (Leica SP2 AOBS, Leica Microsystems, France) at the INRA MIMA2 platform (http://www.jouy.inra.fr/mima2). The excitation of fluorescent markers was performed at 488 nm using an argon laser. The green and red fluorescences emitted were recorded respectively within 500–600 nm (SYTO 9) and 650–750 nm (PI). Three-dimensional acquisitions were made at 400 Hz with a z-step of 1 μm between each xy image for the z-stack. Three-dimensional projections of biofilm structure were then reconstructed using the Easy 3D function of Imaris 7.1 software (Bitplane, Switzerland).

For SEM observations, the biofilms were fixed in a solution containing 2.5 % glutaraldehyde and 0.1 M sodium cacodylate (pH 7.4). The samples were then washed three times for 10 min with a solution containing 0.1 M sodium cacodylate before being transferred into 50 % ethanol. They were progressively dehydrated by passage through a series of ethanol solutions graded from 50 to 100 %. Finally, they were critical point dehydrated (Quorum Technologies K850, Elexience, France) using carbon dioxide as the transition fluid, and coated with platinum (272 Å thickness) in an automatic sputter (Polaron SC7640, Elexience, France). High-magnification imaging of the biofilms was performed at an operating voltage of 2 kV under a S-4500 Hitachi SEM (Hitachi, Japan) at the INRA MIMA2 platform.

Statistical analysis

Statistical analyses of the data (one-way analysis of variance) were performed using Statgraphics v6.0 software (Manugistics, Rockville, MD, USA). The p values tested the statistical significance of each factor by means of F tests. At $p < 0.05$, these factors had a statistically significant effect at the 95 % confidence level.

Results

3D architecture of WW consortium biofilms

The global architecture of the WW consortium biofilms grown in VTT medium for 24 h was analyzed using CSLM and SEM. Figure 2a–c groups one representative three-dimensional view of a biofilm (including a virtual shadow projection on the right-hand side, which represents the biofilm section) obtained from a CLSM image series, and
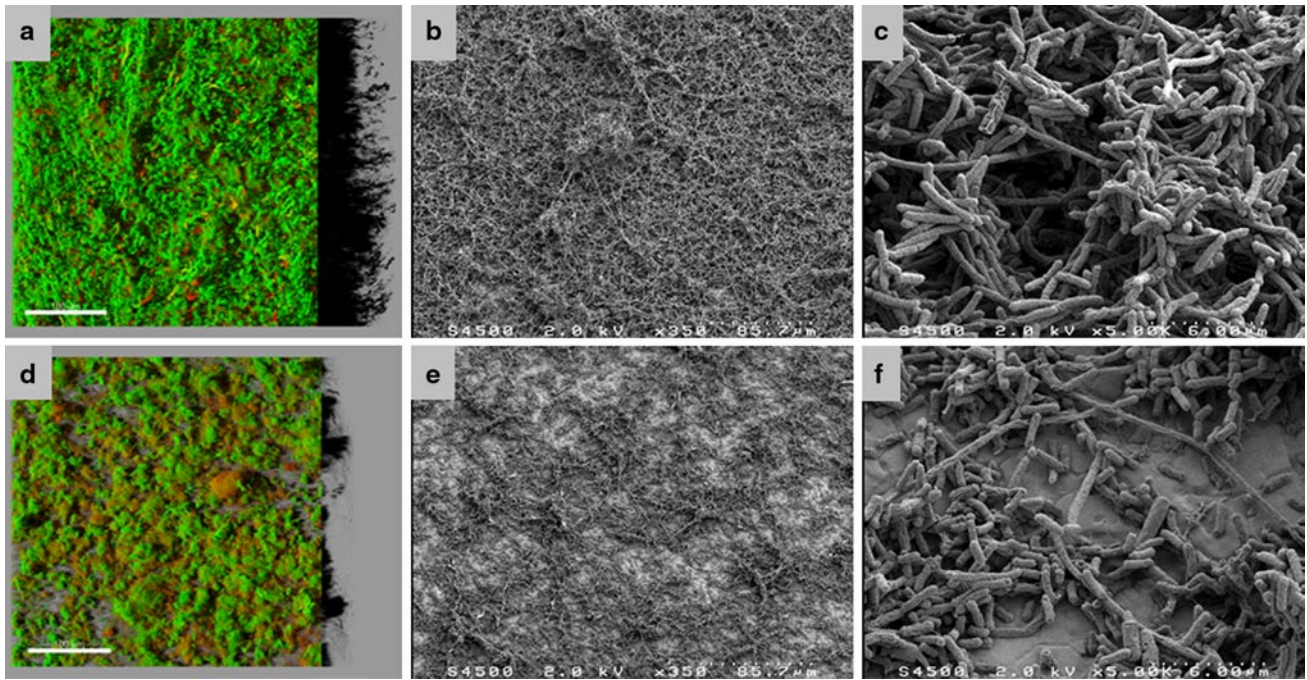
Fig. 2 CSLM (**a**, **d**) and SEM (**b**, **c**, **e**, **f**) observations of WW consortium biofilms before (**a–c**) and after (**d–f**) treatment with carvacrol (10 mM, 15 min). The biofilms were stained using a live/dead Bac- Light viability kit before CSLM observations. Two different magnifications for both cases are shown: 350 (**b**, **e**) and 5,000 (**c**, **f**)

two electron micrographs obtained using SEM (at two different magnifications). The three-dimensional reconstruction of the biofilm obtained using CSLM (Fig. 2a) revealed the globally loose and heterogeneous architecture of the biofilm with some structures that could reach 50 μm, particularly in the highest portion of the biofilm. Interestingly, SEM observations at a similar magnification (Fig. 2b) also displayed this type of pattern, thus indicating that these structures were partially resistant to the fixation and dehydration steps required for SEM preparation. At a higher magnification (Fig. 2c), note was made of microbial diversity in the WW consortium, which contained long filamentous micro-organisms and bacilli with different shapes and sizes.

Moreover, according to the plate counts, the biofilms obtained on stainless steel in VTT medium from a WW inoculum gave reproducible results in terms of their total populations ($7.7 \log_{10} \pm 0.2$).

Antimicrobial activities of five plant-derived compounds on WW consortium planktonic cells and biofilms

WW consortium planktonic cells or biofilms were challenged with five compounds, used separately (Th, Ca, Eu, t-Ci, α-Ter) at a dose of 10 mM for 1 h. The decimal logarithmic reductions in cultivable cells are presented in Fig. 3. Surprisingly, no significant differences were noted between
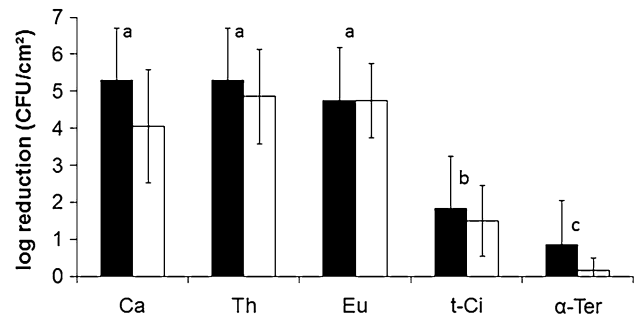
Fig. 3 Log reductions (CFU/cm^{-2}) of the WW consortium in planktonic state (*black bars*) and biofilms (*white bars*) after 1 h treatment with a 10 mM antimicrobial solution (Ca, Th, Eu, t-Ci, α-terpineol). *Error bars* represent standard deviations from the mean. Different *lowercase letters* between antimicrobials in the same state indicate a significant difference ($p < 0.05$)

the planktonic cells and biofilms, whatever the antimicrobial agent. Ca, Th, and Eu achieved the greatest reductions in both planktonic cells (log reductions of 5.3 ± 1.4, 5.3 ± 1.4, and 4.7 ± 1.4, respectively) and biofilms (log reductions of 4.0 ± 1.5, 4.8 ± 1.3, and 4.7 ± 1.0, respectively) when compared with t-Ci and α-Ter. No significant differences could be seen between the efficiencies of Ca, Th, and Eu. Thus, all three compounds were selected for the subsequent testing of combinations. Before the combinations were tested, optimizations in terms of the duration

of challenge and the concentration used were achieved with Ca as a model. First of all, the biofilms were challenged with a 10 mM Ca solution for 5, 10, 15, 30, and 60 min, and the resulting log reductions were determined. The log reduction did not rise significantly after periods longer than 15 min (data not shown). Secondly, the biofilms were challenged with various Ca concentrations (2, 4, 6, 8, and 10 mM) for 15 min. The logarithmic reductions increased regularly in line with the concentrations, and the greatest reduction was achieved with 10 mM (data not shown). For subsequent experiments, we therefore chose to challenge the biofilms for 15 min with 10 mM as the total concentration of antimicrobial agents.

The global architecture of the WW consortium biofilm challenged for 15 min with 10 mM carvacrol was analyzed using CSLM and SEM. Figure 2d–f shows the CLSM and SEM images of the biofilm after disinfection and neutralization. The three-dimensional reconstruction of the biofilm obtained using CSLM (Fig. 2d) showed a drastic reduction of biofilm thickness compared to control (Fig. 2a). Some parts of the stainless-steel surface were no longer covered by the biofilm. Nevertheless, small clusters remained on the stainless-steel surface forming a thin heterogeneous biofilm. SEM observation at a similar magnification (Fig. 2e) revealed the same biofilm pattern on the stainless-steel surface. At a higher magnification (Fig. 2f), it was seen again that the WW consortium was made up of several microbial types but the amount of the principal bacterial type was markedly reduced.

Antimicrobial combinations of two compounds among thymol, carvacrol, and eugenol

WW consortium biofilms were challenged for 15 min with combinations of two compounds at various ratios, the total concentration remaining constant at 10 mM (Fig. 4). The ratios applied were 1:1 (5, 5 mM), 1:3 (2.5, 7.5 mM), and 3:1 (7.5, 2.5 mM). The same compounds were tested alone

in parallel to act as controls. Thymol–eugenol and carvacrol–eugenol combinations did not produce results that differed significantly from those achieved using the compounds separately. By contrast, thymol–carvacrol combinations are significantly more efficient than each compound used alone. As all the antimicrobial solutions (combinations or compounds alone) have the same total concentration, synergy between antibacterial activity of thymol and carvacrol is demonstrated. The impact of the ratios between compounds did not appear to be marked. The Th–Ca combination at a ratio of 1:1 was therefore chosen for tests at the pilot scale for the sake of convenience regarding its preparation.

Antifouling activity of carvacrol alone or thymol–carvacrol combination at a pilot scale

A treatment based on one or six daily applications of 10 mM Ca solution was used during the formation of air biofilms in the pilot plant. In both cases, no visual biocontamination of the coupons was noted during the 10 days of the test (dry matter <0.05 mg cm^{-2}), while the control line allowed the formation of thick biofilms (0.62 mg cm^{-2} dry matter) (data not shown). Moreover, total flora counts showed that bacterial concentrations remained between 10^4 and 10^5 CFU/ml in the treated waters while the values determined in the waters of the control bioreactor (without treatment) ranged from 10^7 to 10^8 CFU/ml. It is important to note that the residual carvacrol concentration in the bioreactor was relatively high between two injections and was estimated at being theoretically around 1 mM, 3.5 h after the 30′ injection.

The next experiment was designed to test at a pilot scale the most efficient combination selected during laboratory tests. An equimolar carvacrol/thymol combination was prepared and sprayed onto the coupons one or six times a day. Given the high efficiency of carvacrol at 10 mM, the total concentration was reduced to 1 mM. The results

Fig. 4 Log reductions (CFU/cm^{-2}) in the WW consortium biofilms after 15 min treatment with individual compounds or combinations (total concentration is always set at 10 mM). *Error bars* represent standard deviations from the mean. Different *lowercase letters* between antimicrobials in the same state indicate a significant difference ($p < 0.05$)
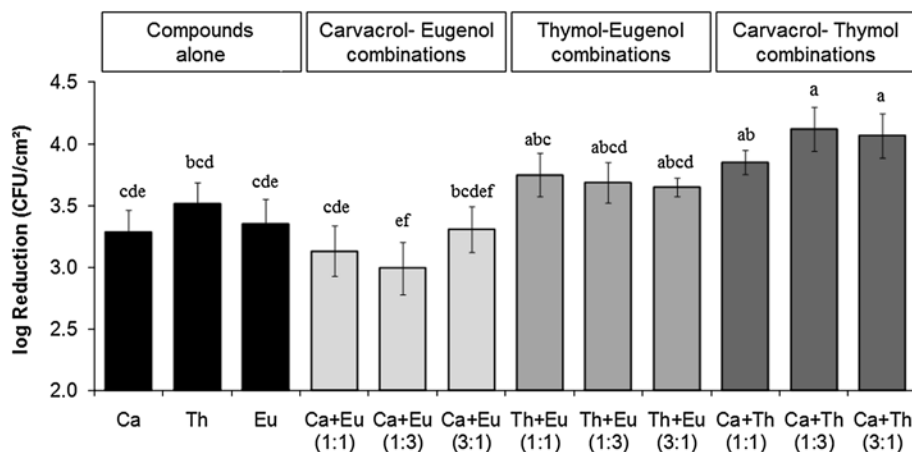
Fig. 5 Kinetics of biofilm formation on stainless-steel coupons in the pilot system. The coupons were not treated (*black squares*) or treated with thymol–carvacrol mixture (1 mM) once (*white squares*) or six times a day (*white triangles*). Different *lowercase letters* between conditions at the same time point indicate a significant difference ($p < 0.05$)
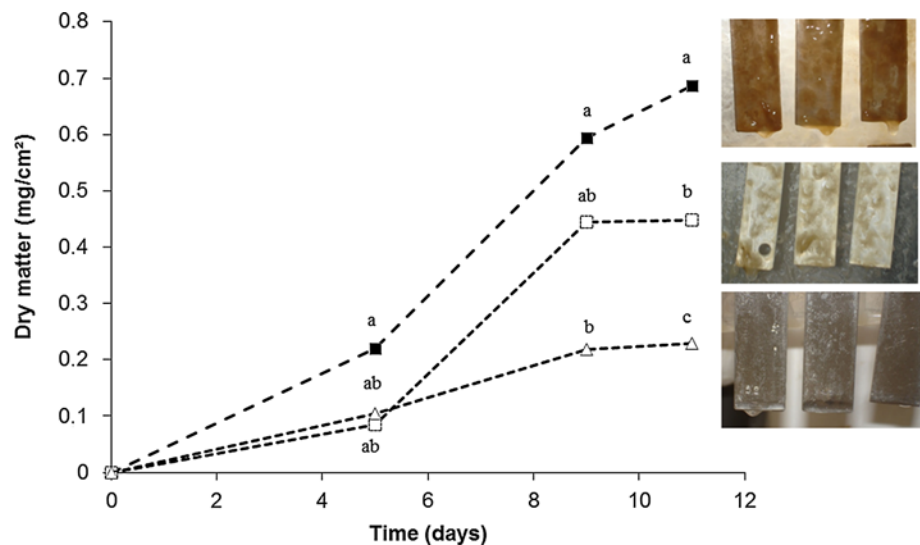
showed that the two treatment conditions (one and six applications/day) similarly delayed biofilm formation during the first 5 days (Fig. 5). After that period, the growth rate of the biofilm treated once a day became identical to that of the control and after 11 days of spraying, the biofilm that had formed on the coupons was overall reduced by a factor of 1.5 (35 %). The treatment based on six applications a day significantly and constantly reduced the biofilm growth rate. Using this latter treatment, the biofilm formation kinetic was 2.2×10^{-2} mg cm^{-2} day^{-1}, while it was 6.4×10^{-2} mg cm^{-2} day^{-1} in the pilot line without treatment (Fig. 5). After 11 days of spraying, the biofilm formed on coupons had been reduced by a factor of 3 (67 %).

Visual inspection (photos in Fig. 5) showed that biofilms from the pilot line treated one or six times a day were light-brown, odorless, and poorly cohesive when compared with those from the control line. Optical microscopic observations highlighted the presence of bacterial assemblage, string bacteria, and filaments in smaller quantities than those seen in the control biofilm without treatment.

Discussion

The issue of biofilms in industrial paper mills is a major concern because they can cause industrial and health problems due to microbial slimes. At present, high levels of biocides are added to paper pulp in order to control these biofilms, but this method is poorly successful. Moreover, in recent years, an additional problem has become more and more important: the environmental impact of the release of large quantities of chemical antimicrobials, which are very difficult to eliminate by treating waste water. In this context, we are convinced that alternative processes could be

developed, particularly by using natural compounds such as volatile antimicrobials extracted from aromatic plants.

First of all, biofilms were obtained with WW consortium at both the laboratory and pilot scales. The reproducibility of their 3D architectures and biovolumes was verified, despite the broad diversity of micro-organisms: filamentous micro-organisms, which probably contribute to biomass cohesion, and bacilli of different shapes and sizes where observed, contrarily to cocci. Many teams in the past have attempted to identify the composition of microbial biofilm populations associated with paper mills. It appears that regardless of the identification method used (a conventional or molecular biological method), a very broad spectrum of micro-organisms was shown to colonize paper mill environments, although this diversity largely depends on local conditions. The diversity results from the origin of micro-organisms introduced via local water, raw materials, the air, and equipment, and from the growth environmental factors such as temperature, nutrients, etc. [12]. The most common micro-organisms isolated in paper mills are mesophilic and thermophilic bacilli [37, 38], Desulfovibrionacae and Desulfobacteriacae [24], Enterobacteriacae and many other bacteria, which include *Deinococcus geothermalis* [31], *Bacillus* spp. [37], or *Burkholderia* spp. [22]. When low concentrations of peracetic acid have been used for several years in the machines, *Deinococcus* is generally not detected, probably because this species is more sensitive to this biocide [32]. Nevertheless, a study of microbial diversity was carried out in our test paper mill and the microbial community was found to be relatively stable at each sampling point in the paper machine circuit [26].

The purpose of this study was to find a mixture of phytochemicals that would be highly efficient against WW consortium biofilms. Plants are well known for their antimicrobial activities. Terpenes and phenolic compounds in

particular are recognized for their efficiency against bacteria and fungi [5, 8, 28, 39, 40]. They are able to damage the cell membrane leading to lysis, the leakage of cell contents and the inhibition of proton motive force [8]. Among these, and in line with our results, thymol and carvacrol are generally recognized as being the most efficient in terms of growth inhibition or inactivation of various planktonic bacteria [8, 13, 14, 27]. However, eugenol, which is generally thought to be less efficient, demonstrated here the same efficiency as the two other compounds against the WW consortium. In contrast, t-Ci and α-Ter were here less efficient, although it had previously been reported that t-Ci might possibly be highly efficient against *Salmonella* Enteritidis for example [16].

Increasing interest has recently focused on the use of essential oils or their components to control bacterial biofilms in different environments. After an initial report on the treatment of bacterial biofilms with carvacrol in 2001 [18], numerous studies were able to demonstrate that essential oils, and especially thymol, carvacrol, eugenol and *trans*-cinnamaldehyde could be highly effective against single-species biofilms at the laboratory scale [1, 7, 17, 30, 35, 41]. Our work revealed that these compounds could also be efficient against biofilms formed with a natural consortium isolated in an industrial paper mill. Moreover, it appears that they are equally efficient against biofilms and planktonic cells. This result is quite unusual because it is well known that cells embedded in a biofilm matrix can express phenotypes that differ from those of their planktonic counterparts and can display specific properties, including increased resistance to biocidal treatments [6]. However, this similar resistance had previously been reported with thymol or carvacrol. For example, their concentration ratio necessary to achieve the same log reduction in biofilms and planktonic cells was 0.125, 2, or 4 for *Staphylococcus*, depending on the strain [17, 30], and 2 for *Salmonella* [36]. Essential oil compounds such as thymol or carvacrol could be able to penetrate easily into biofilm matrices because they benefit from both hydrophobic and hydrophilic properties [29]. They thus can cause detachment of the biofilm mass, as well as inactivating cells. Other hypotheses can also be advanced to explain the similar resistance levels of planktonic cells and biofilms obtained in our experiments, such as: (1) the diversity of micro-organisms in the WW consortium, and (2) the 3D architecture of the biofilm. Indeed, the WW consortium is made up of numerous micro-organisms (not only bacteria), each type having its own resistance profile. In that context, the global measurement of resistance may be due to the better survival of certain species in both biofilm and planktonic states. This may be supported by the fact that no further inactivation of the population was achieved when the duration of Ca applications increased (data not shown). Moreover, the complexity of the consortium, made up of

micro-organisms with varying (notably filamentous) shapes, induced the formation of an aerial biofilm with holes in its structure, as it could be seen from SEM and CSLM observations. This aerial edifice enabled the good penetration of antimicrobial into the structure, so that it could no longer act as an efficient barrier.

In order to enhance the efficacy of antimicrobial agents, synergistic properties have often been considered between several compatible compounds. This so-called "hurdle technology" is gradually attracting attention from industry because it could increase global efficiency as well as enabling a reduction in doses that complies with organoleptic and regulatory requirements [20, 34]. During the present study, we highlighted synergy between antibacterial activity of thymol and carvacrol against biofilms. It had previously been shown that some terpenes display synergies in inhibiting bacterial growth [13, 27] or in displaying bactericidal activity against planktonic cells [14]. However, as far as we know, such synergistic effects have never been optimized to ensure biofilm control.

In order to confirm the efficiency of our selected combination under realistic conditions, we tested it on a WW consortium biofilm grown in a pilot system that mimicked conditions existing in industrial paper mills. The 1 mM equimolar Th–Ca combination (selected at the laboratory scale) was then challenged once or six times a day. Despite applying this non-biocidal concentration, a marked limitation of the biofilm biomass was demonstrated after 11 days of applications. The efficacy of this treatment was comparable to that of a usual oxidizing biocide treatment in paper mills. For example, tests carried out using sodium hypochlorite enabled a 40 % reduction in biofilm formation (data not shown). Several hypotheses have been advanced to explain the considerable efficiency of carvacrol at a non-biocidal concentration on biofilm formation: essential oils interact with bacterial cell surfaces and impair bacterial adhesion [30]; these oils provoke growth inhibition [19]; and they can limit motility and flagella production [9]. Some essential oils (e.g., *trans*-cinnamaldehyde) can also act as quorum-sensing inhibitors, interfering with cell–cell communication and thus compromising the ability to form robust biofilms [35].

Overall, the data presented here show for the first time that carvacrol and its combination with thymol display considerable efficiency in controlling WW consortium biofilms grown on stainless-steel surfaces. Use of these green antimicrobials could therefore constitute a very promising alternative or complement to the treatments currently used to restrict biofilm formation in industrial paper-making environments. It could be particularly interesting to spray the antimicrobial solutions at some focused points of the paper machine circuits where biofilms are highly deleterious. Further investigations are nevertheless required to

assess the potentiality of phytochemical combinations with specific enzymes, in order to enhance the global efficacy through the combination of several activities: prevention of adhesion and growth, hydrolysis of the biofilm matrix, and destruction of the cells.

References

1. Amalaradjou MAR, Venkitanarayanan K (2011) Effect of *trans*-cinnamaldehyde on inhibition and inactivation of *Cronobacter sakazakii* biofilm on abiotic surfaces. J Food Prot 74:200–208

2. Anonymous (1997) Chemical disinfectants and antiseptics—basic bactericidal activity: test method and requirements (phase 1). Ed AFNOR: NF EN 1040

3. Anonymous (1998) Directive 98/8/CE of the European parliament and of the council of 16 February 1998 concerning the placing of biocidal products on the market. Official Journal of the European Communities

4. Anonymous (2006) Regulation (EC) N° 1907/2006 of the European parliament and of the council of 18 December 2006 concerning the registration, evolution, authorisation and restriction of chemicals (REACH). Official Journal of the European Union

5. Ben Arfa A, Combes S, Preziosi-Belloy L, Gontard N, Chalier P (2006) Antimicrobial activity of carvacrol related to its chemical structure. Lett Appl Microbiol 43:149–154

6. Bridier A, Briandet R, Thomas V, Dubois-Brissonnet F (2011) Resistance of bacterial biofilms to disinfectants: a review. Biofouling 27:1017–1032

7. Budzynska A, Wieckowska-Szakiel M, Sadowska B, Kalemba D, Rozalska B (2011) Antibiofilm activity of selected plant essential oils and their major components. Pol J Microbiol 60:35–41

8. Burt S (2004) Essential oils: their antibacterial properties and potential applications in foods—a review. Int J Food Microbiol 94:223–253

9. Burt SA, van der Zee R, Koets AP, de Graaff AM, van Knapen F, Gaastra W, Haagsman HP, Veldhuizen EJA (2007) Carvacrol induces heat shock protein 60 and inhibits synthesis of flagellin in *Escherichia coli* O157: H7. Appl Environ Microbiol 73:4484–4490

10. Chen CY, Nace GW, Irwin PL (2003) A 6 × 6 drop plate method for simultaneous colony counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli*. J Microbiol Methods 55:475–479

11. Costerton J, Lewandowski Z, Caldwell D, Korber D, Lappin-Scott H (1995) Microbial biofilms. Annu Rev Microbiol 49:711–745

12. Flemming HC, Meier M, Schild T (2013) Mini-review: microbial problems in paper production. Biofouling 29:683–696

13. Gallucci MN, Oliva M, Casero C, Dambolena J, Luna A, Zygadlo J, Demo M (2009) Antimicrobial combined action of terpenes against the food-borne microorganisms *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*. Flavour Frag J 24:348–354

14. Garcia-Garcia R, Lopez-Malo A, Palou E (2011) Bactericidal action of binary and ternary mixtures of carvacrol, thymol, and eugenol against *Listeria innocua*. J Food Sci 76:M95–M100

15. Guillier L, Nazer A, Dubois-Brissonnet F (2007) Growth response of *Salmonella* Typhimurium in presence of natural and synthetic antimicrobials: estimation of MICs from three different models. J Food Prot 70:2243–2250

16. Johny AK, Darre MJ, Donoghue AM, Donoghue DJ, Venkitanarayanan K (2010) Antibacterial effect of *trans*-cinnamaldehyde, eugenol, carvacrol, and thymol on *Salmonella* Enteritidis and *Campylobacter jejuni* in chicken cecal contents in vitro. J Appl Poult Res 19:237–244

17. Karpanen TJ, Worthington T, Hendry ER, Conway BR, Lambert PA (2008) Antimicrobial efficacy of chlorhexidine digluconate alone and in combination with eucalyptus oil, tea tree oil and thymol against planktonic and biofilm cultures of *Staphylococcus epidermidis*. J Antimicrob Chemother 62:1031–1036

18. Knowles J, Roller S (2001) Efficacy of chitosan, carvacrol, and a hydrogen peroxide-based biocide against foodborne microorganisms in suspension and adhered to stainless steel. J Food Prot 64:2001

19. Knowles JR, Roller S, Murray DB, Naidu AS (2005) Antimicrobial action of carvacrol at different stages of dual-species biofilm development by *Staphylococcus aureus* and *Salmonella enterica* serovar Typhimurium. Appl Environ Microbiol 71:797–803

20. Leistner L (1995) Food preservation by hurdle technology. Trends Food Sci Technol 6:41–46

21. Lewis K, Ausubel FM (2006) Prospects for plant-derived antibacterials. Nat Biotechnol 24:1504–1507

22. Lindberg LE, Holmbom BR, Vaisanen OM, Weber AML, Salkinoja-Salonen MS (2001) Sugar composition of biofilms produced by paper mill bacteria. Appl Microbiol Biotechnol 55:638–643

23. Marcato-Romain CE, Pechaud Y, Paul E, Girbal-Neuhauser E, Dossat-Letisse V (2012) Removal of microbial multi-species biofilms from the paper industry by enzymatic treatments. Biofouling 28:305–314

24. Maukonen J, Saarela M, Raaska L (2006) Desulfovibrionales-related bacteria in a paper mill environment as detected with molecular techniques and culture. J Ind Microbiol Biotechnol 33:45–54

25. Meylheuc T, van Oss CJ, Bellon-Fontaine MN (2001) Adsorption of biosurfactant on solid surfaces and consequences regarding the bioadhesion of *Listeria monocytogenes* LO28. J Appl Microbiol 91:822–832

26. Milferstedt K, Godon JJ, Escudie R, Prasse S, Neyret C, Bernet N (2012) Heterogeneity and spatial distribution of bacterial background contamination in pulp and process water of a paper mill. J Ind Microbiol Biotechnol 39:1751–1759

27. Nazer A, Kobilinsky A, Tholozan JL, Dubois-Brissonnet F (2005) Combinations of food antimicrobials at low levels to inhibit the growth of *Salmonella* sv. Typhimurium: a synergistic effect? Food Microbiol 22:391–398

28. Nostro A, Blanco AR, Cannatelli MA, Enea V, Flamini G, Morelli I, Sudano Roccaro A, Alonzo V (2004) Susceptibility of methicillin-resistant staphylococci to oregano essential oil, carvacrol and thymol. FEMS Microbiol Lett 230:191–195

29. Nostro A, Marino A, Blanco AR, Cellini L, Di Giulio M, Pizzimenti F, Roccaro AS, Bisignano G (2009) In vitro activity of carvacrol against staphylococcal preformed biofilm by liquid and vapour contact. J Med Microbiol 58:791–797

30. Nostro A, Roccaro AS, Bisignano G, Marino A, Cannatelli MA, Pizzimenti FC, Cioni PL, Procopio F, Blanco AR (2007) Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. J Med Microbiol 56:519–523

31. Peltola M, Oqvist CK, Ekman J, Kosonen M, Jokela S, Kolari M, Korhonen P, Salkinoja-Salonen M (2008) Quantitative contributions of bacteria and of *Deinococcus geothermalis* to deposits and slimes in paper industry. J Ind Microbiol Biotechnol 35:1651–1657

32. Rasimus S, Kolari M, Rita H, Hoornstra D, Salkinoja-Salonen M (2011) Biofilm-forming bacteria with varying tolerance to peracetic acid from a paper machine. J Ind Microbiol Biotechnol 38:1379–1390

33. Ratto M, Verhoef R, Suihko ML, Blanco A, Schols HA, Voragen AGJ, Wilting R, Siika-aho R, Buchert J (2006) Colanic acid is an exopolysaccharide common to many enterobacteria isolated from paper-machine slimes. J Ind Microbiol Biotechnol 33:359–367

34. Roller S (2003) Natural antimicrobials for minimal processing of foods. Woodhead Publishing Limited, Cambridge

35. Simoes M, Bennett RN, Rosa EAS (2009) Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. Nat Prod Rep 26:746–757

36. Soni KA, Oladunjoye A, Nannapaneni R, Schilling MW, Silva JL, Mikel B, Bailey RH (2013) Inhibition and inactivation of *Salmonella* Typhimurium biofilms from polystyrene and stainless steel surfaces by essential oils and phenolic constituent carvacrol. J Food Prot 76:205–212

37. Suihko ML, Sinkko H, Partanen L, Mattila-Sandholm T, Salkinoja-Salonen M, Raaska L (2004) Description of heterotrophic bacteria occurring in paper mills and paper products. J Appl Microbiol 97:1228–1235

38. Suihko ML, Skytta E (2009) Characterisation of aerobically grown non-spore-forming bacteria from paper mill pulps containing recycled fibres. J Ind Microbiol Biotechnol 36:53–64

39. Tunc S, Chollet E, Chalier P, Preziosi-Belloy L, Gontard N (2007) Combined effect of volatile antimicrobial agents on the growth of *Penicillium notatum*. Int J Food Microbiol 113:263–270

40. Ultee A, Gorris LGM, Smid ED (1998) Bactericidal activity of carvacrol towards the food-borne pathogen *Bacillus cereus*. J Appl Microbiol 85:211–218

41. Upadhyay A, Upadhyaya I, Kollanoor-Johny A, Venkitanarayanan K (2013) Antibiofilm effect of plant derived antimicrobials on *Listeria monocytogenes*. Food Microbiol 36:79–89

42. Verhoef R, Beldman G, Schols HA, Siika-Aho M, Ratto M, Buchert J, Voragen AGJ (2005) Characterisation of a 1,4-beta-fucoside hydrolase degrading colanic acid. Carbohydr Res 340:1780–1788

43. Verhoef R, Schols HA, Blanco A, Siika-aho M, Ratto M, Buchert J, Lenon G, Voragen AGJ (2005) Sugar composition and FT-IR analysis of exopolysaccharides produced by microbial isolates from paper mill slime deposits. Biotechnol Bioeng 91:91–105