Antibacterial Effects of Wood Structural Components and Extractives from *Pinus sylvestris* and *Picea abies* on Methicillin-Resistant *Staphylococcus aureus* and *Escherichia coli* O157:H7

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Antibacterial properties of wood structural components and extractives were investigated against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* O157:H7 by placing bacterial inoculum on the model surfaces and incubating them for 2, 4, and 24 h. After incubation, the amount of viable bacteria on the surfaces was studied. The film coverage and thickness were evaluated with atomic-force microscopy (AFM) and X-ray photoelectron spectroscopy (XPS). The extracts were analyzed with gas chromatography–mass spectrometry (GC-MS). The results showed that films fully covered the glass surfaces. The XPS results confirmed the analysis of GC-MS, which revealed more similarities between the extractives of pine heartwood and spruce heartwood than between pine heartwood and pine sapwood. Only the pine heartwood extract showed an antibacterial effect against *E. coli* O157:H7. In contrast, MRSA was susceptible to all of the extracts and milled wood lignin (MWL).

INTRODUCTION

Solid Scots pine wood has been shown to have antibacterial properties against several bacterial strains (Koch *et al.* 2002; Milling *et al.* 2005a; Milling *et al.* 2005b; Vainio-Kaila *et al.* 2011, 2013). In addition, Norway spruce, larch (Kavian-Jahromi *et al.* 2015), and several hardwoods (Ak *et al.* 1994; Koch *et al.* 2002) have antibacterial properties. It has been suggested that the antibacterial properties of wood are a combination of the chemical composition of the wood and the drying effect of the surface of the wood, caused by its porosity (Milling *et al.* 2005a).

Wood is composed mainly of cellulose, hemicelluloses, lignin, and extractives. The extractives are a large group of different chemical substances. Their composition depends on the wood species and the location in the tree (Willför *et al.* 2003b,c). Knotwood extracts of Scots pine and several other wood species have both antibacterial and antifungal properties (Lindberg *et al.* 2004; Välimaa *et al.* 2007). A limited amount of literature is available on the antibacterial effects of wood extracts. Scots pine heartwood (Laireiter *et al.* 2013) and sapwood and spruce heartwood (Vainio-Kaila *et al.* 2015) extracts inhibit the

growth of several Gram-positive bacterial strains, while pine sapwood extract inhibits a Gram-negative strain. Extracts of Alaska cedar, western juniper, and several North American hardwoods have some antibacterial effects (Omar *et al*. 2000; Johnston *et al*. 2001). Several separate compounds such as terpenes (Himejima *et al*. 1992; Mourey and Canillac 2002), pinosylvins (Välimaa *et al*. 2007; Plumed-Ferrer *et al*. 2013), resin acids (Smith *et al*. 2005), and some fatty acids (Desbois and Smith 2010) have shown antibacterial effects, mostly against Gram-positive bacteria strains. Spruce resin has antimicrobial effects against several bacterial species, including methicillin-resistant *Staphylococcus aureus* (MRSA) (Rautio *et al*. 2007).

In addition to extractives, lignins have various positive health effects, including antibacterial properties. Dong *et al*. (2011) found commercial kraft lignin to have strong antibacterial properties, probably due to its high pH, and lignin extracted from residue of corn stover in ethanol production has also shown antibacterial properties. Lignin from bagasse and cotton stalk pulping has demonstrated antibacterial properties, depending on the cooking conditions (Nada *et al*. 1989). In addition, phenolic fragments from lignin (Zemek *et al*. 1979; Baurhoo *et al*. 2008) and nanolignin on linen fabric (Zimniewska *et al*. 2008) exhibit antibacterial properties. Milled wood lignin (MWL), which best resembles natural wood lignin, has not been investigated in regard to its antibacterial properties. Cellulose has been modified in various ways to create antibacterial material (Adamopoulos *et al*. 2007; Roy *et al*. 2007; Hou *et al*. 2009), where unmodified cellulose is used as a non-antibacterial control. Hemicelluloses stimulate probiotic bacteria and, hence, indirectly operate against harmful bacteria (Polari *et al*. 2012; Rajani *et al*. 2016).

In this study, thin films of MWL, cellulose nanofibrils (CNF), galactoglucomannan (GGM), and extractives of Scots pine (*Pinus sylvestris*) heartwood and sapwood and Norway spruce (*Picea abies*) heartwood were prepared, and their antibacterial properties were studied in order to gain further understanding of the roles of the different structural compounds and extractives in the antibacterial properties of wood. MRSA and *Escherichia coli* O157:H7 were chosen as relevant pathogenic bacterial strains representing Gram-positive and Gram-negative strains, respectively. MRSA is one of the strains that hospitals struggle to contain (Hierholzer *et al*. 1995; Dancer 2008). In day care environments, *E. coli* O157:H7 has caused outbreaks of severe diarrhea (Reida *et al*. 1994; Rimhanen-Finne *et al*. 2014).

EXPERIMENTAL

Materials

Wood material for the extracts was obtained from Koskisen Oy sawmill in Järvelä, Finland, within two weeks of kiln drying. The sawn wood was taken from mature logs (about 60 to 100 years old). The Scots pine (*Pinus sylvestris* L.) sapwood and heartwood samples were selected based on the visible color difference between sapwood and heartwood. Norway spruce (*Picea abies* (L.) H. Karst.) heartwood samples were taken approximately 5 cm from the pith. Wood material was milled with a Wiley mill to a particle size of < 1 mm. After milling, the wood dust was stored at -20 °C until use. Particles were extracted with acetone using a Soxhlet apparatus for 6 h. The concentration of the remaining solution was determined by weighing and drying a small amount, and the solution was heated to 40 °C to evaporate excess acetone until a concentration of 5.4 g/L was reached. Gas chromatography-mass spectrometry (GC-MS) was performed as

described by Willför *et al*. (2003c) to determine the total amount (mg/g dry weight) of each extractive component.

The chemical composition of lignin is highly dependent on the lignin source and extraction method. Milled wood lignin is the closest to native lignin (Nunn *et al*. 1985) and was thus used as the lignin model substrate. The MWL was prepared from spruce sawdust according to Björkman (1956).

Cellulose nanofibrils were obtained from never-dried kraft birch pulp from a Finnish pulp mill by mechanical fibrillation using a Microfluidics M-110Y high-pressure fluidizer (Microfluidics Int. Corp., Westwood, MA). No chemical or enzymatic treatments were applied to the pulp prior to fluidizing it using 12 passes.

Galactoglucomannan was extracted from spruce thermomechanical pulp by hot water extraction followed by a purification process (Willför *et al*. 2003a).

Polystyrene (PS, average molecular weight about 280,000 g/mol), toluene, and polyethyleneimine (PEI) of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO), and 1,4-dioxane (analytical standard) and acetone (ACS reagent) were purchased from VWR International (Radnor, PA).

Bacterial Strains

The bacterial strains used were MRSA (ATCC 43300) and *E. coli* O157:H7, without the *stx* genes (RHE5402). Prior to use, both strains were subcultured at least twice on sheep blood (MRSA) or Drigalski-Conradi (*E. coli*) agar plates. The plates were incubated at 37 °C overnight in ambient atmosphere. Bacterial colonies were moved directly from the agar to physiological NaCl solution, and their concentration was adjusted to 0.5 McFarland using a photometer (Gene-Trak Systems, Hopkinton, MA). This was equivalent to a concentration of 1.5×10^8 (colony forming units per milliliter (CFU/mL) and was further diluted in physiological NaCl solution to correspond to a concentration of 1.5×10^7 CFU/mL.

Preparation of Single Wood Component Substrates

The CNF films were prepared by spin-coating a CNF dispersion onto a glass surface previously coated with PEI to enhance the CNF adsorption, as previously described (Valle-Delgado *et al*. 2016). First, 40 µL 2.5 mg/mL PEI solution was dropped on the glass cylinder. After 10 min adsorption, the glass cylinder was rinsed with water and dried with nitrogen gas. The CNF dispersion was then spin-coated atop the PEI film by sonicating a 1.35 g/L CNF dispersion at 25% amplitude for 1 min, without heating, with a Branson sonifier S-450 D (Branson Corp., Danbury, CT) and then centrifuged to remove the large fibril bundles at 8000 g (where g is the relative centrifugal force) for 30 min at 20 °C with an Eppendorf centrifuge 5804R (Eppendorf AG, Hamburg, Germany).

The hemicellulose substrates were prepared by dropping 80 µL of a 2.5% wt. aqueous GGM solution atop CNF films prepared as described above. The substrate was immediately dried in an oven at 50 °C for 30 min.

Polystyrene-coated glass surfaces were used as substrates for the preparation of lignin and extractive films, with 0.5 wt.% PS solution in toluene spin coated onto the glass surfaces in three steps: firstly, at 300 rpm for 3 s with an acceleration of 500 rpm/s; secondly, at 1000 rpm for 5 s with an acceleration of 800 rpm/s; and, finally, at 2000 rpm for 30 s with an acceleration of 800 rpm/s.

Milled wood lignin films were obtained by spin coating 0.5 wt.% MWL solution in 1,4-dioxane onto PS-coated glass surfaces in three steps (Tammelin *et al*. 2006): firstly, at

400 rpm for 3 s with an acceleration of 2400 rpm/s; secondly, at 500 rpm for 5 s with an acceleration of 6000 rpm/s; and, finally, at 1000 rpm for 2 min with acceleration of 4000 rpm/s. The procedure was repeated four times to get full MWL coverage.

Extractive films were prepared by spreading 40 µL of 5.4 wt.% extractive solution onto PS-coated glass surfaces and drying at 50 °C for 5 min. This process was repeated four times for each extractive in order to achieve full coverage.

Atomic-Force Microscopy (AFM)

A MultiMode 8 atomic force microscope equipped with a NanoScope V controller (Bruker Corporation, Billerica, MA) was used to obtain high resolution images of the films. Images in at least three different spots of each film were obtained in tapping mode in air using NCHV-A probes (Bruker) with a tip radius around 10 nm. Research NanoScope 8.15 software (Bruker) was used for image analysis. The only image correction applied was flattening. The films were scratched and scanned with a scan size of 30 µm × 30 µm at 6 different positions in order to estimate each film's thickness.

X-ray Photoelectron Spectroscopy (XPS)

A Kratos Analytical AXIS Ultra electron spectrometer (Manchester, UK) was used to analyze the chemical compositions and evaluate the coverages of the extractive films on PS substrate. A monochromated A1 Kα X-ray source at 100 W was applied. A spin-coated neat PS film was measured as a reference. Furthermore, a pure cellulose specimen was measured with each sample batch, as an *in situ* reference for the experimental conditions in ultra-high vacuum. Low resolution survey spectra and high resolution spectra of O1s and C1s regions were collected at several positions across the sample. For the C1s region, CasaXPS software (ver 2.3.17.PR1.1) and a four-Gaussian component fit tailored for lignocelluloses were used (Johansson and Campbell 2004).

Cultivations

Bacterial testing was made by cultivating the bacterial strains on glass cylinders coated with the test materials. The cylinders were placed in empty Petri dishes, and 20 µL of bacterial solution was pipetted on top. Clean glass surfaces were used as an inert control. After incubation at room temperature for 2, 4, and 24 hours, the cylinders were placed in test tubes with 7.5 mL of physiologic NaCl solution and shaken vigorously, and 200 µL from the solution and a 1:10 dilution was spread on sheep blood or Drigalski-Conradi agar plates. After overnight incubation at 37 °C, the CFUs on the plates were counted. The bacterial cultivations were performed with three parallel samples, and the averages and standard variations were calculated.

RESULTS AND DISCUSSION

Extractive Composition

The most abundant compound groups detected in GC-MS analysis were resin acids in the spruce and pine heartwood and triglycerides in the pine sapwood (Table 1). In general, the results agree well with earlier reports on pine and spruce extracts (Martínez-Iñigo *et al*. 1999; Hovelstad *et al*. 2006). Triglycerides were the largest group in the extract of pine sapwood, but Martínez-Iñigo *et al*. (1999) found resin acids to be the largest group.

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Some differences can be expected as the amount of different substances varies even within the heartwood of a single tree (Ekeberg *et al*. 2006).

Table 1. Wood Extract Components (mg/g dry weight)

	Spruce Heartwood	Pine Sapwood	Pine Heartwood
Terpenoids	17	2	18
Fatty acids	109	19	232
Stilbenes	nd	nd	174
Resin acids	141	133	290
Hydroxy resin acids	6	12	9
Lignans	129	nd	nd
Sterols	28	5	5
Monosaccharides	27	4	35
Steryl esters	97	40	34
Diglycerides	39	36	26
Triglycerides	22	414	3

nd = not detected

Wood Component Substrates

A typical fibrillary network of CNF is clearly visible both in the 25 µm^2 and the 1 µm^2 AFM height images (Fig. 1a and 1b).
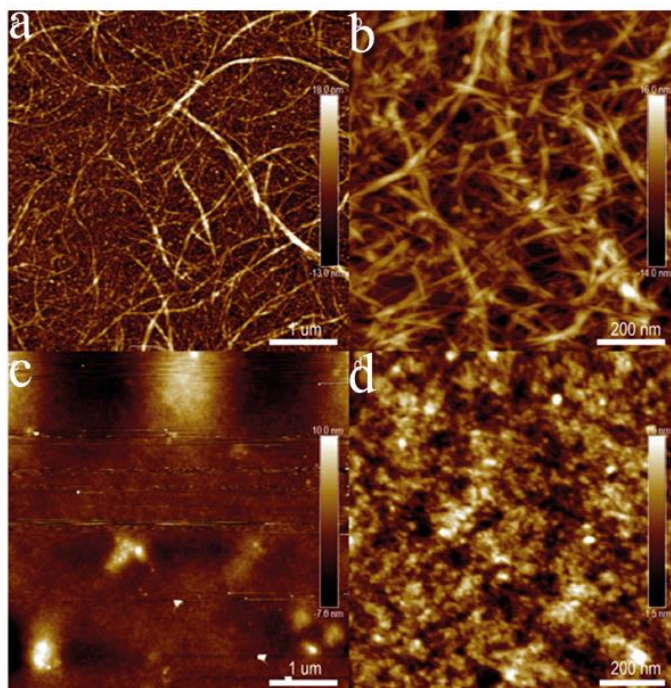

Fig. 1. Atomic-force microscopy height images of spin-coated CNF film with scan sizes of (a) 25 µm^2 and (b) 1 µm^2 and deposited GGM film with scan sizes of (c) 25 µm^2 and (d) 1 µm^2

The fibrils evenly covered the substrate, and no open areas were observed. The AFM height images of GGM deposited on top of CNF substrates (Fig. 1c and 1d) showed that the CNF fibril network could no longer be seen, which indicates that the GGM had fully covered the CNF films with an even film. The average thickness of the film, including GGM, CNF, and PEI, was 36 ±1.7 nm. Because the PEI film was 1 to 2 nm thick and the CNF film about 5 to 8 nm (Eronen *et al*. 2011), the GGM film was approximately 26 nm.

The AFM images show that the glass surface (Fig. 2a) was fully covered by PS (Fig. 2b). The spin-coated MWL film (Fig. 2c) appeared to fully cover the PS, and the images were very similar to previously published AFM images of spin-coated MWL films (Tammelin *et al*. 2006; Salas *et al*. 2012). The thickness of the MWL layer was 10 ±0.46 nm.
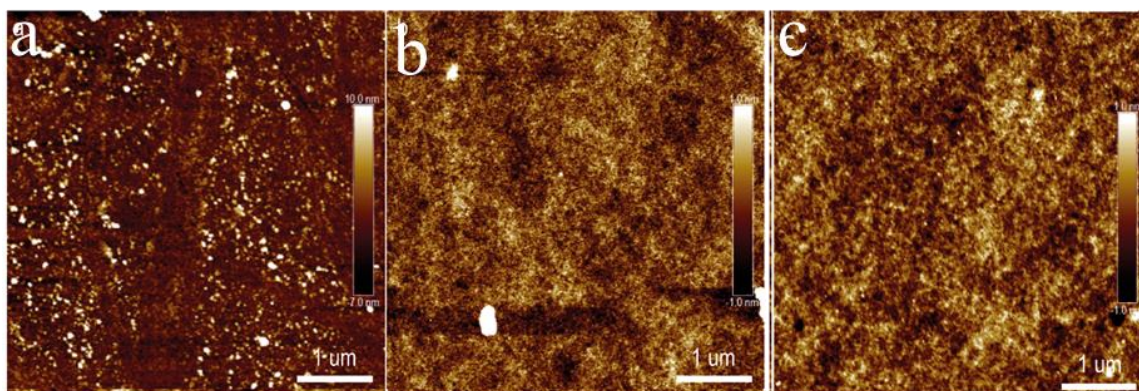

Fig. 2. Atomic-force microscopy height images of (a) glass cylinder surface, (b) PS substrate, and (c) MWL film spin-coated atop the PS substrate

Extractive films were also coated on PS substrates (Fig. 3a and 3b). Due to the presence of a relatively large amount of resin acids, the extractive films were sticky, which made it hard to obtain high resolution topographical images. The pine sapwood was so sticky that no representative image could be obtained.
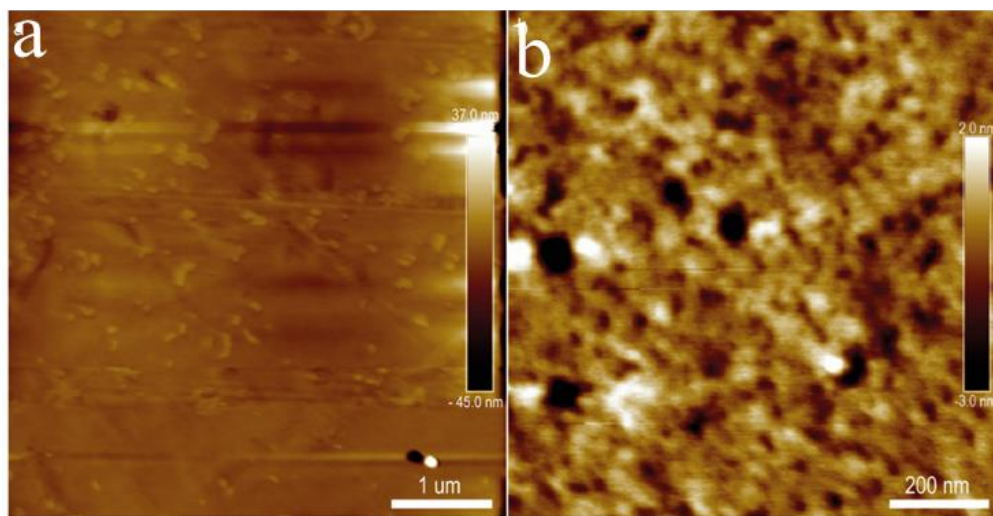

Fig. 3. Atomic-force microscopy height images of extractives deposited on PS-coated glass: (a) pine heartwood, (b) spruce heartwood

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Variation in the compositions of the extractives created very uneven films. Average thicknesses of 229 ± 40 nm, 197 ± 5 nm, and 175 ± 59 nm were obtained for the pine heartwood, pine sapwood, and spruce heartwood, respectively.

X-ray photoelectron spectroscopy measurements were performed on the spin-coated extractive films, with the spin-coated, polystyrene-only sample as a reference (Fig. 4). The data for the neat reference was typical of thin, air-exposed polystyrene film. In the survey, only a strong carbon signal with a negligible oxygen trace (due to exposure to ambient air) was observed. Furthermore, the carbon high resolution region showed only aromatic carbon features, namely, a sharp component at 285 eV and a clear plasmonic feature at around 292 eV. In contrast, all extractive films showed carbon and oxygen as main components, and both with strong tailing backgrounds, indicating the presence of films thicker than XPS analysis depth (Tougaard 1998). In the high resolution region, the main component was located at 285 eV, but it was much broader than in polystyrene and without the plasmonic feature. The results indicated that the substrate polystyrene was well covered by the resin films. Furthermore, XPS high resolution data on carbon C 1s showed subtle differences in the three resins studied. The two heartwood samples had fairly similar carbon regions, while the sapwood sample was clearly richer in the acidic functionalities, seen as the 290 eV component of the normalized C 1s spectra in Fig. 4 (insert). This result is in agreement with the GC-MS chemical analysis, where more similarities were seen between the two heartwood samples than between the pine heartwood and sapwood samples. The heartwood formation process in the transition zone might explain the similarities between pine and spruce heartwood.
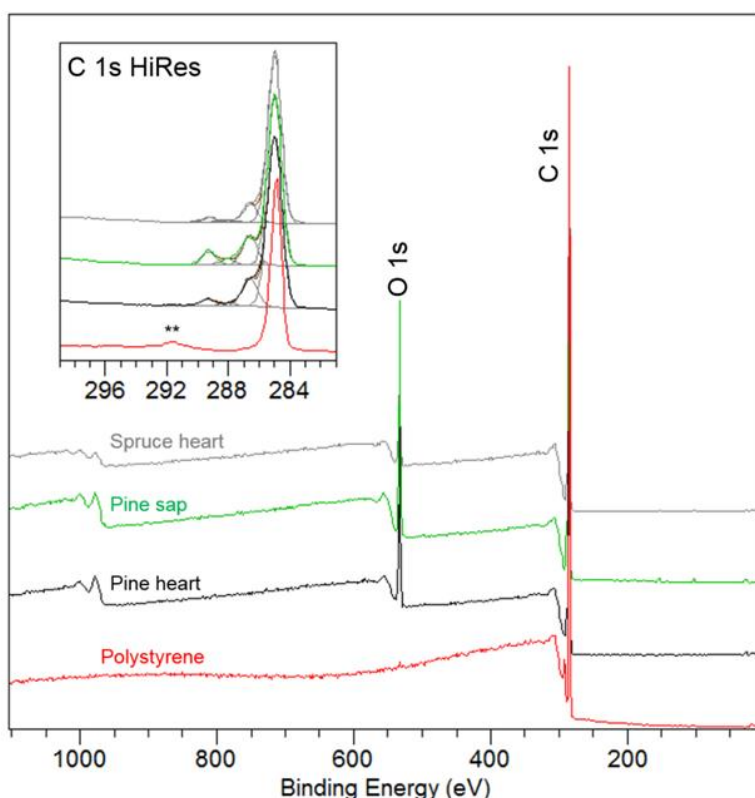

Fig. 4. X-ray photoelectron spectroscopy spectra (survey scans and high resolution C 1s regions) of the spin-coated resin films on polystyrene substrate. Two asterisks (**) in the insert indicate the plasmonic feature typical of aromatic C 1s species. This feature was observed on the neat polystyrene surface only.

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Antibacterial Effects

The only wood component surface shown to suppress the growth of *E. coli* O157:H7 was the extract of pine heartwood (Fig. 5). On the GGM and CNF surfaces, the amount of bacteria decreased at a slower rate than that of the control, which is probably due to the available nutrients in those surfaces. Galactoglucomannan consists of shorter chains and is more easily accessible than CNF, which is paracrystalline. However, hemicelluloses are also available on the CNF. The accessibility leads to GGM being a more readily available source of nutrition for the bacterial cells. Nanofibrillar cellulose is used in hydrogels for growing three-dimensional cell cultures (Bhattacharya *et al*. 2012; Lou *et al*. 2013; Liu *et al*. 2016). Thus, it could be expected not to be very antibacterial. Polystyrene, which was used as an anchoring substrate for both the extractives and the MWL, had a weak antibacterial effect. However, this clearly did not affect the bacterial viability on the films of the extractives and MWL, as MWL and the pine sapwood and spruce heartwood extracts produced no difference relative to the control. As discussed earlier, the films covered the whole surface area.
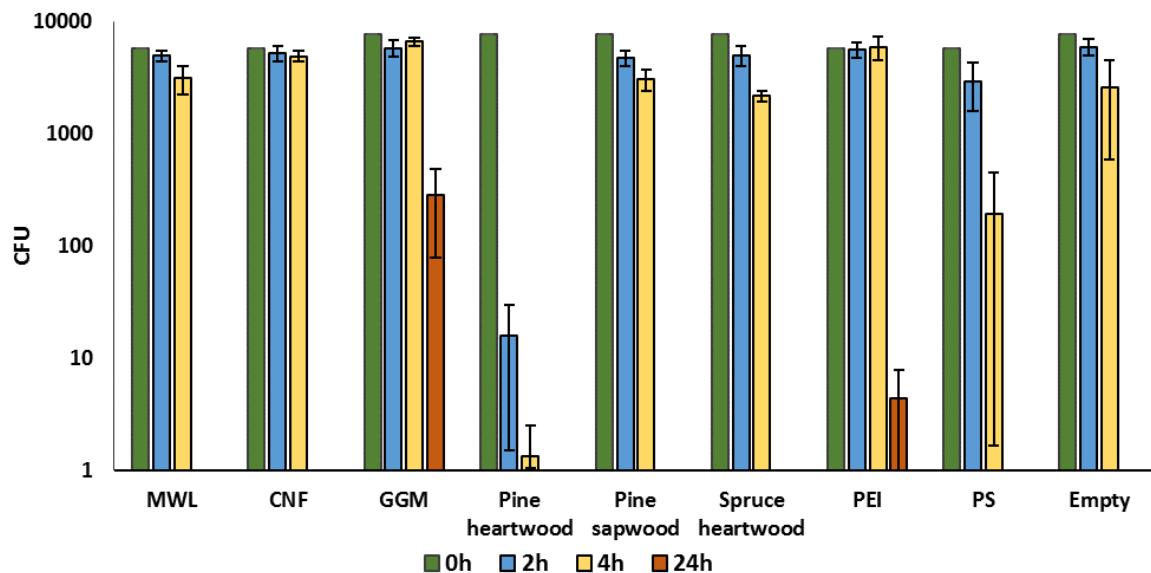

Fig. 5. The amount of colony forming units (CFU) after 0, 2, 4, and 24 h incubation of *E. coli* H157:O7 on different model surfaces of the structural components of wood, the extractives, and the glues used for preparing the surfaces. Clean glass surface was used as a control.

MRSA was more sensitive to all the extracts studied, pine heartwood being the most effective, followed by spruce heartwood and pine sapwood (Fig. 6); MWL also had an antibacterial effect on MRSA. On the GGM and CNF surfaces, the number of bacteria decreased at a slower rate than that of the control, which is probably due to the available nutrients, as discussed earlier. Neither PEI nor PS had any antibacterial effect on MRSA.

Gram-positive *S. aureus* was more susceptible to wood components than Gram-negative *E. coli*. This result was similar to several studies on wood components and bacteria (Himejima *et al*. 1992; Mourey and Canillac 2002; Rautio *et al*. 2007; Välimaa *et al*. 2007; Plumed-Ferrer *et al*. 2013). There are, however, contradictory findings, suggesting that wood or its components would be more effective against Gram-negative strains *Klebsiella pneumoniae* (Kavian-Jahromi *et al*. 2015) and *E. coli* (Schönwälder *et al*. 2002). When the wood extracts were studied in a broth against these same strains, the

results were very similar to the present study, as would be expected (Vainio-Kaila *et al*. 2015). However, the effect of pine sapwood on *E. coli* was stronger than that of pine heartwood in the broth with the extracts, whereas the results in this study were the opposite. This could depend on the differences in water solubility of the extracts.
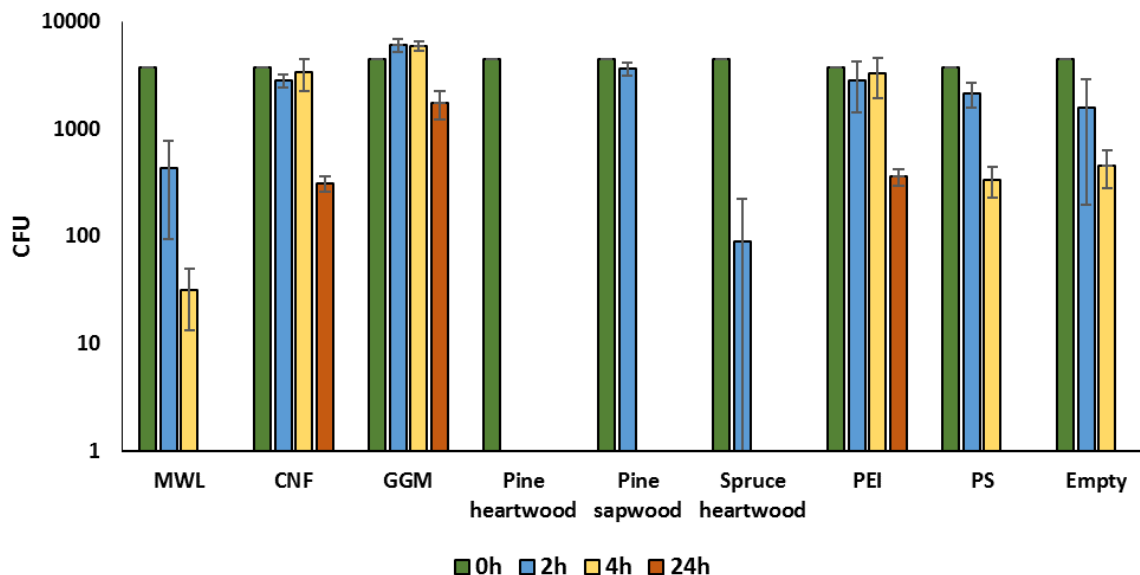

Fig. 6. The amount of colony forming units (CFU) after 0, 2, 4, and 24 h incubation of MRSA on different model surfaces of the structural compounds of wood, the extractives, and the glues used for preparing the surfaces. Clean glass surface was used as a control.

The antibacterial properties of the extracts could originate from various compounds found to have an antibacterial effect, which have been discussed earlier, or from a synergistic effect of several compounds. Some of the most common fatty acids found in all samples (Table 1), such as palmitoleic (C16:1), linoleic (C18:2), and linolenic (C18:3) acids, have been reported to have antibacterial properties (Kabara *et al*. 1972; Desbois and Smith 2010), though some have been shown to be effective only against Gram-positive strains. Most of the resin acids found in the samples, such as abietic, isopimaric, neoabietic, pimaric, and palustric acid, have been reported to have antibacterial effects (Söderberg *et al*. 1990; Smith *et al*. 2005) only against Gram-positive strains. Pine heartwood had the greatest amount of these acids, followed by spruce heartwood, although in spruce heartwood there was more isopimaric acid than in pine heartwood. Stilbenes, pinosylvin, and pinosylvin monomethyl ether exhibiting antibacterial activity (Plumed-Ferrer *et al*. 2013) were found exclusively in pine heartwood. The chemical composition of the extracts explains the results observed in this study well, as pine heartwood had stronger antibacterial effect than pine sapwood or spruce and it was also found rich in components with demonstrated antibacterial effects.

The antibacterial properties of the wood surface (Milling *et al*. 2005a,b; Vainio-Kaila *et al*. 2011, 2013) seem to be affected by several factors. Extractives, as has been shown earlier (Laireiter *et al*. 2013; Vainio-Kaila *et al*. 2015), are one of the main factors, but even lignin contributed to these properties. Lignin is more stable on the surface of wood; it does not dissolve as easily as extractives. Its antibacterial effect could therefore be considered more permanent than the effects of the extractives.

Comparing MWL and the extracts, however, is somewhat complicated. The differences in the thicknesses of the films are notable, and the forms of the materials are different. The extract surfaces were around 200 nm thick, whereas the MWL surfaces were only 10 nm thick. Most of the acetone extracts are not water-soluble, but they could affect bacteria in a water-based broth, which means that some active components of the extracts were dissolved in the broth (Vainio-Kaila *et al.* 2015). However, the MWL covered the whole surface, and its thickness should not affect the results if the effect is purely contact-based and not due to any migration of compounds. These two materials also differ in form, the extracts being softer and stickier and MWL being harder.

CONCLUSIONS

1. The antibacterial properties of wood are affected by both the extractives and lignin.

2. Pine heartwood had the strongest antibacterial effect of the extracts.

3. Cellulose and hemicellulose surfaces act as nutrition for bacteria.

4. The chemical composition of the surfaces was studied with XPS, which correlated well with the GC-MS results.

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