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Antibacterial properties of Scots pine and Norway spruce

Tiina Vainio-Kaila

Aalto University

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Tiina Vainio-Kaila

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Abstract

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Abstract

Wooden surfaces in interior use hold potential for improving human health and wellbeing. The antibacterial properties of wood might reduce the possibility of cross-contamination from surfaces. In order to be able to control the hygienic quality of the wooden surface, the antibacterial effect should be better understood. The main aim of this thesis was to identify and evaluate the antibacterial properties of wood and its components.

Different methods were developed and used to study the antibacterial properties of Scots pine and Norway spruce, heartwood and sapwood. The solid wood surface showed clear antibacterial properties, even when the extractives had been removed with acetone. Studies with the extracts showed several human pathogens, including methicillin-resistant *Staphylococcus aureus*, to be susceptible to pine heartwood and sapwood in particular, and also, to some extent, spruce. Besides extractives, lignin was the only separate wood component showing antibacterial properties. Wood volatile organic compounds (VOCs), which were studied in gaseous form, showed an antibacterial effect against various human pathogens.

Several antibacterial compounds were found in all the extracts, however, they did not always explain the order of antibacterial activity between wood species. No single compound could alone explain the effect, hence the antibacterial effect derives either from different mechanisms in different species or from a synergistic effect. α-pinene and limonene could partly explain the antibacterial effect of the VOCs, but other components were also found to have an influence.

Wood was found to have various antibacterial parts and a diverse range of bacterial pathogens that were sensitive to it. These results offer a good ground for the exploitation of the hygienic properties of wood and a good starting point for enhancing them further. Additionally, the extracts showed promising qualities and they should be studied further in regard to resistant pathogens.

Keywords Antibacterial wood, Scot pine, Norway spruce

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Tiivistelmä

Puupinnat sisätiloissa vaikuttavat positiivisesti ihmisten terveyteen ja hyvinvointiin. Puun antibakteeriset ominaisuudet saattavat vähentää pintojen kautta tapahtuvan kontaminaation todennäköisyyttä. Antibakteeristen ominaisuuksien parempi ymmärrys mahdollistaa puupintojen hygieenisen laadun paremman hallinnan. Tämän väitöskirjan päätavoite oli selvittää puun ja sen komponenttien antibakteerisia ominaisuuksia.

Männyn ja kuusen sydän- ja pintapuun antibakteerisuuden tutkimiseen käytettiin osin tätä työtä varten kehitettyjä menetelmiä. Puupinnan todettiin olevan antibakteerinen myös silloin, kun puun uuteaineet oli poistettu asetonilla. Uutteiden tarkempi tutkimus osoitti erityisesti männyn sydän- ja pintapuun, mutta jonkin verran myös kuusen uutteiden ehkäisevän useiden tautia aiheuttavien bakteereiden mm. metisilliinille resistentin *Staphylococcus aureuksen* (MRSA) kasvua. Uutteiden lisäksi ligniini oli ainoa erillinen komponentti, jolla todettiin antibakteerisia ominaisuuksia. Puusta haihtuvilla orgaanisilla yhdisteillä (VOC) todettiin antibakteerisia ominaisuuksia useita tautia aiheuttavia bakteerikantoja kohtaan.

Kaikissa uutteissa todettiin useita antibakteerisia yhdisteitä, mutta niiden määrä ei aina selittänyt eri puulajien antibakteerisuutta suhteessa toisiin puulajeihin. Mikään yksittäinen yhdiste ei yksin selittänyt antibakteerista vaikutusta, joten ilmiö johtuu joko eri puulajeilla eri mekanismeista tai synergisistä vaikutuksista. α-pineeni ja limoneeni selittivät osin VOCien antibakteerisia ominaisuuksia, mutta myös muilla yhdisteillä todettiin olevan vaikutusta.

Puun antibakteerisuuden todettiin johtuvan useista eri aineista ja tehoavan useisiin eri bakteereihin. Nämä tulokset tarjoavat hyvän lähtökohdan puun hygieenisten ominaisuuksien hyödyntämiseen ja niiden kehittämiseen. Uutteet osoittautuivat myös tehokkaiksi ja niiden ominaisuuksia erityisesti suhteessa resistentteihin bakteereihin kannattaisi tutkia lisää

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PREFACE

The work reported in this thesis was carried out in the Department of Bioproducts and Biosystems at Aalto University during the years 2007-2017. A significant part of the experimental studies were made in THL, National Institute for Health and Welfare, in Helsinki. Some analyses were also made in Thünen Institut, in Hamburg, Germany and in Åbo Akademi, in Turku.

The work started as a Tekes-funded project, Multifunctional properties of wood, which I am grateful for. I would also like to thank Suomen Kulttuuri Rahasto, Professori Eero Kivimaan Stipendirahastosäätiö, Puumiesten Ammattikasvatussäätiö, Puuteollisuusinsinöörit, TKK:n tukisäätiö, Doctoral Programme of Chemical Tehnology and Tekniikan Edistämissäätiö for their financial support. COST FP 1407 is acknowledged for supporting the short-term scientific visit in Hamburg.

I was lucky to work with several inspiring supervising professors, Pertti Viitaniemi and Matti Kairi who helped me to get started and Lauri Rautkari, who helped and encouraged me already before becoming a professor. I am very grateful for the help and support of my thesis advisor, professor emerita Anja Siitonen, who gave me a chance to work in the bacterial laboratory in THL and, together with Aino Kyyhkynen, guided me to the wonderful world of microbiology. Working with real pathogens was so exciting! The last years I was privileged to have also Tuomas Hänninen as my thesis advisor. Without you, I would have aimed for lower rungs of the thesis ladder, thank you for encouraging me to reach higher!

I want to also thank all my co-authors and my friends and colleagues in both Puu 2 and Puu 1. It was fun in Puu 2, good conversations and always good company for lunch. Moving to Puu 1 opened my mind to wood chemistry and coffee with the pulp girls, which was also fun. It was beneficial to end up in an office next to Kyösti, the chemist, thank you for answering many questions! I got a lot of help from technical staff, which I am grateful for.

Special thanks to my sister-in-law, Halle, for proofreading and correcting my language and for the on-line support. Also my parents and all my brothers and their wives have been important in bringing joy in my life outside of the thesis and helping me to choose the color for the covers.

The greatest price for this thesis has been paid by my dear husband Touko, and our precious children Vilho, Martta and Miina. Thank you for your love and support!

"*The land produced vegetation: plants bearing seed according to their kinds and trees bearing fruit with seed in it according to their kinds. And God saw that it was good*."
-Genesis 1:12

I totally agree with Him!

Espoo, September 14, 2017

Tiina Vainio-Kaila

LIST OF PUBLICATIONS

This thesis consists of an overview of the following five publications, which from this point forward are referred to in the text by their Roman numerals. Additionally, some unpublished results are presented.

I **Vainio-Kaila, T.**, Kyyhkynen, A., Viitaniemi, P., Siitonen, A. (2011) Pine heartwood and glass surfaces: easy method to test the fate of bacterial contamination. *European Journal of Wood and Wood Products*, 69:391-395.

II **Vainio-Kaila, T.**, Rautkari, L., Nordström, K., Närhi, M., Natri, O., Kairi, M. (2013) Effect of extractives and thermal modification on antibacterial properties of Scots pine and Norway spruce. *International Wood Products Journal* 4:248-252.

III **Vainio-Kaila, T.**, Kyyhkynen, A., Rautkari, L., Siitonen, A. (2015) Antibacterial effects of extracts of *Pinus sylvestris* and *Picea abies* against *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli*, and *Streptococcus pneumoniae*. *BioResources*, 10:7763-7771.

IV **Vainio-Kaila, T.**, Hänninen, T., Kyyhkynen, A., Ohlmeyer, M., Siitonen, A., Rautkari, L., (2017) Effect of volatile organic compounds from *Pinus sylvestris* and *Picea abies* on *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae* and *Salmonella enterica* serovar Typhimurium. *Holzforschung* DOI: 10.1515/hf-2017-0007

V **Vainio-Kaila, T.,** Zhang, X., Hänninen, T., Kyyhkynen, A., Johansson, L-S., Willför, S., Österberg, M., Siitonen, A., Rautkari L. Antibacterial effect of wood structural components and extractives from *Pinus sylvestris* and *Picea abies* on methicillin resistant *Staphylococcus aureus* and *Escherichia coli* O157:H7. *BioResources,* 12:7601-7614.

AUTHOR'S CONTRIBUTION

I The author took primary responsibility for the research plan under the supervision of Prof. Anja Siitonen and Prof. Pertti Viitaniemi, prepared the samples and carried out the experimental work with help from M.Sc. Aino Kyyhkynen, and wrote the first draft of the manuscript.

II The author took primary responsibility for the research plan with help from the co-authors and carried out the experimental work in the microbiology laboratory under the supervision of Prof. Katrina Nordström and Prof. Matti Kairi with support from M.Sc. (Tech) Olli Natri and M.Sc. (Tech) Marko Närhi. Tiina Vainio-Kaila also performed the CA measurements with Prof. Lauri Rautkari. Prof. Rautkari performed the thermal modification and extraction of the wood samples and wrote those respective parts of the manuscript. The author wrote the other parts of the manuscript.

III The author took primary responsibility for the research plan with help from Prof. Anja Siitonen and Prof. Lauri Rautkari, prepared the samples and carried out the experimental work with help from M.Sc. Aino Kyyhkynen, and wrote the first draft of the manuscript.

IV The author took primary responsibility for planning the work, for the interpretation of the results and for writing the publication under the supervision of Prof. Anja Siitonen and Prof. Lauri Rautkari. The author prepared the samples and carried out the experimental work in the microbiology laboratory with help from M.Sc. Aino Kyyhkynen. D.Sc. (Tech) Tuomas Hänninen was instructing and supporting both the experimental work and the writing process. The analysis of the VOCs was made in collaboration with Dr. Martin Ohlmeyer and his group.

V The author took primary responsibility for planning the work, for the interpretation of the results and for writing the publication under the supervision of Prof. Lauri Rautkari and Prof. Anja Siitonen. The author carried out the microbiological trials with help from M.Sc. Aino Kyyhkynen. D.Sc. (Tech) Tuomas Hänninen was instructing and supporting both the experimental work and the writing process. The model surfaces were prepared by M.Sc. (Tech.) Xue Zhang under supervision of Prof. Monika Österberg. M.Sc. (Tech.) Xue Zhang was also responsible for the AFM imaging and writing the respective parts of the manuscript. The XPS was done by PhD. Leena-Sisko Johansson. Prof. Stefan Willför carried the GC-MS analysis of the extracts.

NOMENCLATURE

Abbreviations

AFM	Atomic Force Microscopy
CFU	colony forming unit
CNF	cellulose nanofibril
DCA	dynamic contact angle
FAB	fastidious anaerobe broth
FFA	free fatty acid
GC-MS	gas chromatography–mass spectrometry
GGM	galactoglucomannan
HW	heartwood
MIC	minimal inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
MWL	milled wood lignin
PEI	polyethyleneimine
PS	polystyrene
RH	relative humidity
SEM	scanning electron microscopy
SW	sapwood
VOC	volatile organic compound
VRE	vancomycin resistant *Enterococcus faecalis*

Table of Contents

1 INTRODUCTION AND THE OBJECTIVES OF THE THESIS

Wood is used extensively in interior surfaces. In certain environments, such as hospitals, day care centers, schools or elderly care homes, the hygienic properties of the surfaces are of special importance. Contaminated surfaces are one of the ways that infections are spread (Hierholzer et al., 1995, Dancer, 2008) and with constant pressure to save money, proper surface cleaning in healthcare and daycare environments may be jeopardized. In order to exert some level of control over the hygienic properties of interior surfaces, an understanding of the microbiological properties of the material used is critical. This enables effective choice of the correct material for each end-use. Also, it encourages the development of desired properties of and new modifications to interior surfaces.

Wood has been widely researched from many perspectives and the structural and chemical properties are well known. The microbiological properties of wood surfaces in regard to bacteria, are, however, still quite unknown. These properties are affected by both chemical and physical characteristics of the wood material. Different wood species and parts of wood differ from each other in these aspects. The focus of this work is on pine and spruce heartwood and sapwood, as they are the most commonly used wood species in Northern Europe. Their antibacterial properties were known better from earlier literature than for example birch, which is also widely used in interior surfaces. Also the higher amount of extractives in pine and spruce makes them more interesting regarding bacterial inhibition.

The main objective of this thesis was to study the phenomena behind the antibacterial properties of wood. Antibacterial properties of pine and spruce were studied using both solid surfaces and individual components of the wood, such as extractive and structural components. The test method for studying the antibacterial properties of wooden surfaces was developed and studied in Paper I. Also in this paper, the effect of bacterial adherence on the results was investigated. The physical properties were further studied in Paper II, where wettability of the different surfaces was measured and the results were compared to the antibacterial results. The antibacterial effect of extracts of Scots pine and Norway spruce was studied in Paper II by removing them from wood samples and measuring the antibacterial properties of wood in their absence, while Papers III and V further investigated the extracts by taking the acetone-extracted components and studying their antibacterial properties. Volatile organic compounds were studied in Paper IV, where only the volatile matter was in contact with bacterial suspension. Cellulose, hemicellulose, lignin and extractives were studied separately in Paper V as surfaces.

The antibacterial properties of a surface are naturally strongly affected by the surface treatment, if such is applied. However, this thesis is only concerned with non-treated wooden surfaces and the components of wood material from the xylem. It is important to have a better understanding of the material properties of wood in order to be able to choose the best possible surface treatment, or to leave the surface untreated, depending on the desired qualities. Also, a better understanding of the material enables the development of new products.

2 BACKGROUND

2.1 Properties of wood as an interior material

Wood is widely used as an interior material. It is common in wall paneling, flooring, in saunas and in furniture. Wooden interiors are experienced as warm and natural (Masuda, 2004, Rice et al., 2006) and have been shown to decrease the stress level of humans (Zhang et al., 2017). The construction of massive wood-structured houses is increasing as technology in fire protection has improved and innovations in timber structure design have been developed (Muller, 2010). The hygienic properties of wood are, consequently, important. Hygienic or microbiological properties vary within and between different wood species and wood components. In this chapter, the hygienic properties of wood are briefly discussed.

2.1.1 Heartwood and sapwood

Most wood species have separate sapwood (SW) and heartwood (HW). HW is formed in the inner layers of the trunk as the parenchyma cells in SW die and cease to contribute to the transfer and storage of water and nutrients (Dinwoodie, 2000). Since the heartwood cells are no longer alive, they need no nutrition or water and are more economical for the tree (Bamber, 1976). In some species, such as pine, the distinction can be made based on visible color difference, where HW is darker than SW (Fig.1). Better durability of HW in moist conditions depends on lower permeability, a lesser amount of nutrition and a higher amount of extractives that protect it from fungi (Viitanen, 1994).

Figure 1. Heartwood and sapwood are visibly different in Scots pine.

2.1.2 Wood components

Wood consists mainly of cellulose, hemicellulose, lignin and extractives. Wood tissue is formed from wood cells, which in pine and spruce are mainly tracheids. A schematic picture of wood cells and their composition is shown in Fig. 2. Middle lamella fills the space between wood fibers and is composed mostly of lignin. The tracheids have a hollow cavity called lumen. Cellulose molecules form cellulose microfibrils (CMFs) which are aligned in bundles and surrounded by hemicelluloses and lignin. Together with the hemicelluloses and lignin, these bundles form the secondary wall, which is the thickest layer of the wood fiber.

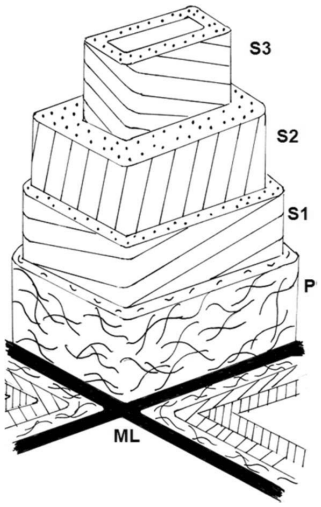

Figure 2. Structure of wood cell wall, ML= middle lamella, between the fibers, P= primary wall and S1, S2 and S3 form the secondary wall. (Hänninen, 2011) Reprinted with permission from the artist.

Cellulose is one of the main wall-building constituents of timber (Dinwoodie, 2000). It is a large, linear homopolysaccharide. The cellulose molecules form bundles with crystalline and amorphous regions. (Stenius, 2000) Cellulose, which is not antibacterial in itself, has been modified in various ways to create antibacterial materials (Roy et al., 2007, Adamopoulos et al., 2007, Hou et al., 2009). In these studies, unmodified cellulose is used as a non-antibacterial control. Cellulose produced by bacteria has been recently investigated for use in bio-medical end uses as implants or scaffolds (Petersen and Gatenholm, 2011, Tang et al., 2015, Sulaeva et al., 2015). Cellulose nanofibrils are also used in hydrogels to grow three-dimensional cell cultures (Bhattacharya et al., 2012, Lou et al., 2013).

Hemicelluloses form structures that crosslink the CMF bundles and together with lignin they form modules surrounding the CMF bundles (Terashima, 2009). Hemicelluloses in softwoods consist of galactoglucomannan and the rest of arabinoglucoronoxylan (Dinwoodie, 2000). Hemicellulosis are heteropolysaccharides and less well-defined than cellulose (Stenius, 2000). Hemicelluloses have been found to stimulate probiotic bacteria and, hence, to play an indirect role in preventing the growth of harmful bacterial species (Polari et al., 2012, Rajani et al., 2016). Direct antibacterial properties have not been reported.

Lignin differs from the other structural compounds of wood. It is a complex three-dimensional aromatic molecule composed of phenyl groups. Woods stiffness is mainly due to lignin. (Dinwoodie, 2000) As a non-hygroscopic material, it regulates water absorption in the cell wall. Lignin from Nordic softwoods has not been studied regarding bacterial inhibition, but several other lignins have shown antibacterial properties. Dong et al. (2011) found commercial Kraft lignin to have strong antibacterial properties, probably due to its high pH, but also lignin extracted from residues of corn stover in ethanol production showed antibacterial properties. Lignin from bagasse and cotton stalk pulping showed antibacterial properties depending on cooking conditions (Nada et al., 1989). Phenolic fragments from lignin (Zemek et al., 1979, Baurhoo et al., 2008) and nanolignin on linen fabric (Zimniewska et al., 2008) also demonstrated antibacterial properties.

Extractives are a wide and varying group consisting of e.g., resin acids, di- and triglycerides, terpenoids, lignans and stilbenes, depending on the wood species (Pohjamo et al., 2003, Willför et al., 2003b, Willför et al., 2003c, Hovelstad et al., 2006). They are separated from wood by extracting with different solvents, such as water, methanol or acetone. Composition varies depending on the solvent used. Research into the location of extractives within wood cells is scant and the results are varied and partly contradictory (Kuo and Arganbright, 1980, Kuroda et al., 2014). Within Scots pine trunk, extractives are more plentiful in HW than in SW, 5.1-8.4% and 3.1-3.7%, respectively (Martínez-Iñigo et al., 1999, Ekeberg et al., 2006, Rautkari et al., 2012, Arshadi et al., 2013). Resin acids, β-sitosterols and stilbenes are more plentiful in HW, whereas SW contains more triglycerides (Martínez-Iñigo et al., 1999, Willför et al., 2003c, Ekeberg et al., 2006). The amount of extractives in spruce is low, below 1 % in both SW and HW (Bertaud and Holmbom, 2004). In spruce, free fatty acids are more abundant in HW than in SW and in SW triglycerides are more abundant than in HW (Bertaud and Holmbom, 2004). Extractives are believed to be the main contributors to pine HW's superior durability against fungi (Martínez-Iñigo et al., 1999, Harju et al., 2002, Blom and Bergström, 2005, Blom et al., 2013, Kirker et al., 2013).

A number of extractives have been found to exhibit medicinal properties. Extracts from Scots pine were found to exhibit antibacterial action against *Staphylococcus aureus*, *Enterococcus faecium* and *Bacillus subtilis* (Laireiter et al., 2013), bark extract against *S. aureus* (Rauha et al., 2000) and several *Pinus* species against environmental bacterial strains found in paper mills (Lindberg et al., 2004). Essential oil of *Picea excelsa* has been found to have antibacterial properties, though only against Gram-positive strains (Canillac and Mourey, 2001). The resin of Norway spruce (*Picea abies*) has been found to produce antibacterial effects against several Gram - positive bacteria (Sipponen et al., 2007).

Extracts from several other wood species besides Scots pine and Norway spruce have been studied in regard to their health effects. Juniper species have received wider attention in the literature than other coniferous species regarding effects on health. Besides antibacterial (Clark et al., 1990, Johnston et al., 2001, Filipowicz et al., 2003, Meng et al., 2016) and antifungal (Clark et al., 1990, Filipowicz et al., 2003) properties, the essential oils and extracts of Juniper have been shown to have diuretic, antidiabetic and anti-inflammatory effects (Seca and Silva, 2006). Extracts from the wood and bark of *Maclura tinctoria* have been shown to have antibacterial effects against several bacterial strains such as *Streptococcus mitis, Streptococcus sanguinis* and *Streptococcus mutans* (Lamounier et al., 2012). Extracts from the leaves of *Laurus nobilis* demonstrated an antibacterial effect against MRSA and VRE (Otsuka et al., 2008). Several hardwood extracts have also shown antibacterial effects against *S. aureus*, *Bacillus subtilis* and *Mycobacterium phlei* (Omar et al., 2000).

Several extractive components have been found to possess antibacterial properties. Table 1 presents some of the compounds, which can be found in Scots pine and Norway spruce, and susceptible bacterial strains. Additionally, pinosylvins, which are found in the HW and knotwood of pine, have demonstrated antioxidant (Koskela et al., 2014), anti-inflammatory (Laavola et al., 2015) and anticarcinogenic (Park et al., 2013, Yatkin et al., 2014) effects. Similarly, hydroxymatairesinol, which is found in spruce, especially in the knotwood, has been shown to have anti-inflammatory (Cosentino et al., 2010) antioxidant and antitumor properties (Kangas et al., 2002). Piperidine alkaloids found in the needles and bark of spruce have also been found to have an antibacterial effect against several pathogenic strains (Fyhrquist et al., 2017).

Table 1. Antibacterial extractive compounds and the susceptible bacterial strains.

Compound	Bacterial species	Ref.
α-pinene	*Listeria monocytogenes*	1
	Staphylococcus aureus	2,8
	Enterococcus faecalis	2
	Escherichia coli	2
	Erwinia carotovora	2
	Klebsiella pneumoniae	2
Limonene	*Clostridium sporogenes*	2
	Escherichia coli	2
	Flavobacterium sauveolens	2
	Salmonella serovar Pullorum	2
Pinosylvin and pinosylvin monomethyl ether	*Escherichia coli,*	3
	Salmonella serovar Infantis	3,4
	Pseudomonas fluorecens	3
	Bacillus cereus	3
	Staphyloccus aureus	3,4
	Listeria monocytogenes	3
	Salmonella serovar Typhimurium	4
Abietic acid	*Bacillus subtilis*	5
	Brevibacterium ammoniagenes	5
	Staphylococcus aureus	5,6,7
Dehydroabietic acid	*Staphylococcus aureus*	6
Isopimaric acid	*Staphylococcus aureus*	7

[1] Mourey and Canillac, 2002
[2] Dorman and Deans, 2000
[3] Välimaa et al., 2007
[4] Plumed-Ferrer et al., 2013
[5] Himejima et al. 1992
[6] Söderberg et al. 1990
[7] Smith et al. 2005
[8] Raman et al. 1995

2.1.3 Volatile organic compounds

Most materials used in built environments emit substances, called volatile organic compounds (VOCs), into the indoor air. The most frequent VOCs from pine and spruce are terpenes, such as α-pinene, 3-carene, β-pinene and limonene, and aldehydes, such as hexanal and pentanal (Risholm-Sundman et al., 1998, Englund, 1999). Besides these, there are also small amounts of acids, alcohols, hydrocarbons and ketones (Hyttinen et al., 2010). In pine, HW usually emits more VOCs than SW (Roffael, 2006). The amount of VOCs from green wood is remarkably higher than dried wood (Englund, 1999) and the amount continues to decrease over the first couple of years in newly constructed buildings (Park and Ikeda, 2006).

Studies on the beneficial properties of VOCs from wood in relation to human health are rare. However, volatile components in essential oils and their properties have been studied widely (Bakkali et al., 2008) and are known to have various positive effects on health (Holley and Patel, 2005). Several phenolic components of essential oils have been found to exhibit antimicrobial activity (Burt and Reinders, 2003, Holley and Patel, 2005, Puupponen-Pimiä et al., 2005) and even the essential oil from wood consisting mainly of terpenes, such as α-pinene, β-pinene and limonene, has been found to be antibacterial (Canillac and Mourey, 2004). Also, volatiles from wood have been reported to have a suppressing effect on the activity of house dust mites (Hiramatsu and Miyazaki, 2001) and α-pinene to decrease tumor growth in mice (Kusuhara et al., 2012).

Wood VOCs in gaseous form have been studied in regard to fungi (Suolahti, 1951) and were found to induce the growth of fungi, the effect being stronger for volatiles from dried wood compared to green wood. On the other hand, terpenes have not only been shown to inhibit the growth of fungi (Hintikka, 1970, De Groot, 1972, Bridges, 1987), but also to induce the growth of fungi connected to wood-attacking insects (Bridges, 1987). Nonanal, an aldehyde emitted by conifers, has been found to strongly induce the growth of wood-rotting fungi (Fries, 1960). Some other aldehydes, including hexanal, pentanal and butanal, in gaseous form, have been shown effective against some insects (Hammond et al., 2000).

2.2 Wood species and antibacterial activity

Antibacterial properties vary greatly among different wood species. Scots pine has been found to exhibit antibacterial activity (Schönwälder et al., 2002, Milling et al., 2005a, Milling et al., 2005b) as well as oak (Koch et al., 2002, Milling et al., 2005a). Norway spruce has been studied less and the results are more varied. Milling et al. (2005a) compared spruce chips with plastic chips and found spruce to have greater antibacterial effect when tested with *Enterococcus faecium*, but no clear difference between the two existed when *Escherichia coli* was used as the test bacteria. Sipponen (2013) has studied Norway spruce resin and found it to be antibacterial against several bacterial strains. In addition to those mentioned above, other wood species that have been found to exhibit antibacterial properties include ash, basswood, beech, birch, butternut, cherry, hard maple, oak and black walnut (Ak et al., 1994a, Ak et al., 1994b). In the literature, various wood species have been shown to inhibit many bacterial pathogens (Table 2).

Bacterial strains can be roughly divided into two groups, Gram-positive and Gram-negative, based on the differences in their cell walls. The differentiation is based on the structure of the cell wall. Gram-positive bacteria have a thicker cell wall, while Gram-negative bacteria have a thinner cell wall with an additional outer membrane, which

prevents large water-soluble molecules from entering the cell (Vaara et al., 2003). Gram-positive bacteria tend to be more sensitive to natural antibacterial agents than Gram-negative (Jouvenaz et al., 1972, Takahashi et al., 2004, Tyagi and Malik, 2011). This has also been true in the case of bacteria exposed to wood and its components (Söderberg et al., 1990, Välimaa et al., 2007, Sipponen, 2013, Laireiter et al., 2013). There are, however, exceptions, for example wood and its components have been found to exert a greater effect against certain strains of Gram-negative bacteria such as *Klebsiella pneumoniae* (Kavian-Jahromi et al., 2015) and *E. coli* pIE639 (Schönwälder et al., 2002) compared to the Gram-positive strains of *S. aureus* and *E. faecium*.

Table 2. Wood species that have been shown to inhibit certain bacterial strains. Heartwood (HW)/sapwood (SW) is mentioned when it has been reported.

Wood species	Bacterial strain	Gram +/-	Reference
Basswood, maple	*Listeria monocytogenes*	+	Ak & al., 1994a
Beech, birch, maple	*Escherichia coli* O157:H7	-	Ak & al., 1994a
Birch, maple	*Salmonella* Typhimurium	-	Ak & al., 1994a
Larch, both HW and SW	*Klebsiella pneumoniae*	-	Kavian-Jahromi & al., 2015
Larch, both HW and SW	*Staphylococcus aureus* (Methicillin-resistant)	+	Kavian-Jahromi & al., 2015
Oak, beech, ash, pine, spruce	*Bacillus subtilis*	+	Koch & al., 2002
Oak, beech, ash, pine, spruce	*Pseudomonas fluorescens*	-	Koch & al., 2002
Scots Pine, Norway spruce and oak, mixed HW and SW	*Enterococcus faecium*	+	Milling & al., 2005a
Scots Pine and oak, mixed HW and SW	*Escherichia coli*	-	Milling & al., 2005a
Scots Pine HW	*Enterococcus faecium*	+	Schönwälder & al., 2002
Scots Pine HW , larch	*Escherichia coli*	-	Schönwälder & al., 2002
Scots Pine, HW	*Bacillus subtilis*	+	Laireiter & al., 2013
Scots Pine, HW	*Enterococcus faecium*	+	Laireiter & al., 2013
Scots Pine, HW	*Staphylococcus aureus*	+	Laireiter & al., 2013
Poplar	*Bacillus cereus*	+	Revol-Junelles et al., 2005
Poplar	*Escherichia coli*	-	Revol-Junelles & al., 2005

Different bacteria can have very different optimal conditions for living and multiplying. In this thesis, only a few bacterial strains are used, all of them human pathogens. They grow best at 35-37°C, in neutral or slightly alkali environments (Vaara et al., 2003). Besides water, microbial cells need nutrition, e.g. hydrogen, oxygen, carbon, nitrogen, sulfur, phosphorus and potassium. The nutrient requirements are very diverse depending on the microorganism. (Stanier et al., 1987)

2.3 Methods used for studying the antibacterial or hygienic properties of wood

2.3.1 Methods used in studies on solid surfaces

In earlier research on the hygienic properties of the surfaces of solid wood, the most commonly used methods are the agar contact plate method or its variations (Ak et al., 1994a, Ak et al., 1994b, Gough and Dodd, 1998, Schönwälder et al., 2002, Zangerl et al., 2010, Kavian-Jahromi et al., 2015). In the agar contact plate method, the inoculated surface is pressed directly onto the agar plate, which is then cultivated and the colony forming units (CFUs) are counted. Another method involves swabbing the inoculated surface with a sterile swab and shaking it in a broth, which is consequently cultivated with subsequent counting of CFUs (Gilbert and Watson, 1971, Welker et al., 1997, Koch et al., 2002). Other methods involve destruction of the samples by, e.g., planing the inoculated surface off and investigating the bacteria from the shavings (Zangerl et al., 2010). There are two ISO standards describing testing methods for antibacterial surfaces. ISO 22196 (2011) is for plastic, non-porous surfaces and ISO 20743 (2013) is for textile products. Neither of them is directly suitable for studying wooden surfaces as wood is porous and hard, and also these standards are meant for antibacterial-treated surfaces, which is not the case in this thesis.

2.3.2 Methods used in studies on wood extracts

In studies of the antibacterial properties of extracts, the most commonly used methods are the disk diffusion test or an investigation into the minimal inhibitory concentration (MIC) in various ways. In the disk diffusion method, the substance studied is added to sterile paper disks and consequently placed on agar plates previously seeded with bacteria (Rauha et al., 2000, Omar et al., 2000). The results after incubation are shown as the inhibition zone around the paper disks where no visible growth of bacteria can be seen. MICs have been studied by mixing the sample substance at different concentrations in a medium and investigating the concentration at which there is no more bacterial growth in the broth (Canillac and Mourey, 2004, Otsuka et al., 2008, Lamounier et al., 2012). The MIC test is made in 96-well microtiter trays (Otsuka et al., 2008, Lamounier et al., 2012), where the bacterial growth can be followed based on the turbidity, or on another medium, such as an agar plate (Söderberg et al., 1990, Rautio et al., 2007), where bacterial growth is followed

manually. Rautio et al. (2007) also studied spruce resin as such, without diluting it into any solvent, in fastidious anaerobe broth (FAB), where the turbidity could be seen after incubation overnight.

2.3.3 Methods used in studies on wood VOCs and microbes

The effect of wood VOCs on fungi growing on the bottom of an Erlenmeyer flask was studied by hanging a piece of wood from the lid above the culture and comparing the fungal growth to a similar culture without the wood piece (Suolahti, 1951). Most studies on the effects of separate volatile compounds on fungi have been made in desiccators or similar containers, with the compound under investigation at the bottom of the container and the cultivated organism above it (Hintikka, 1970, De Groot, 1972, Väisälä, 1974, Bridges, 1987).

2.4 Mode of action of antibacterial components from natural sources

There are different ways antibacterial compounds can affect bacterial cells. Bactericidal action means that the bacteria are directly killed while bacteriostatic action is when growth is inhibited and cell division is prevented. It can be assumed that after enough time, the inhibition of bacterial growth will cause a bacterium to die. (Desbois and Smith, 2010) Therefore, no further distinction is made between these two actions in this thesis.

Despois and Smith (2010) described various mechanisms for the antibacterial action of free fatty acids (FFAs). The FFAs might disrupt the electron transport chain, cause leakage of cell metabolites or cause inhibition of enzymes. FFAs might also cause nutrient uptake to be impaired. Different mechanisms for the antibacterial action of essential oils have also been widely studied. For example, essential oil from fennel seeds increased permeability of the *Shigella dysenteriae* membrane leading to leakage of electrolytes and hence, to the death of the cell (Diao et al., 2014). In like manner, cinnamon essential oil led to an increase in permeability of *E. coli* and *S. aureus* membranes (Zhang et al., 2016). Essential oil from Juniper caused damage of the cytoplasmic membranes of *K. pneumoniae*, which led to the loss of large molecules (DNA and RNA) (Meng et al., 2016). Scanning electron microscopy (SEM) images of cell damage caused by essential oil of Juniper is shown in Fig. 3.

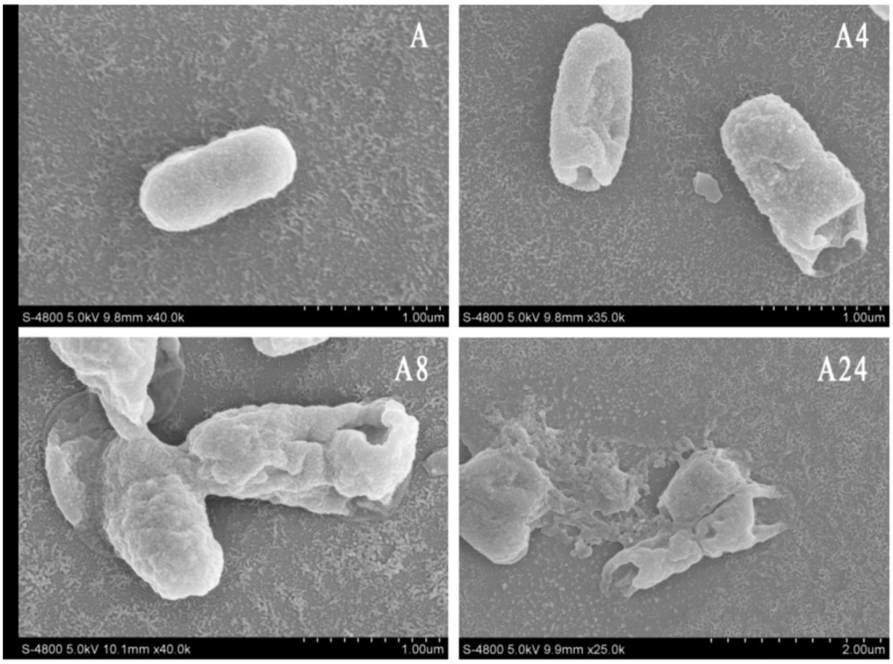

Figure 3. The SEM micrograph of *K. pneumoniae* for A, untreated bacteria; A4, A8 and A24, bacteria treated with the essential oil from the leaves of *J. rigida* at 1×MIC for 4, 8 and 24 h respectively (Meng et al., 2016). Reprinted with permission from Elsevier.

3 MATERIALS AND METHODS

3.1 Sample materials

All wood materials were collected from sawmills in southern Finland. The wood species used are Scots pine (*Pinus sylvestris* L.) (Papers I-V), from now on referred to as pine, and Norway spruce (*Picea abies* [L.] H. Karst.) (Papers II-V), from now on referred to as spruce. These wood species were chosen as they are the most common species processed in Nordic countries. All wood was kiln-dried prior to use. More detailed information can be found in Papers I-V.

3.1.1 Wood and glass cylinders

In Papers I and II, solid wood surfaces were investigated. The wood material used was the SW and HW of pine and the HW of spruce, having average densities (RH 65%, 20°C) of 500, 500 and 460 kg cm^{-3}, respectively. The samples were produced by drilling cylinders with a diameter of 15 mm and a surface area of circa 176.7 mm^2 (Fig. 4 A). The thickness was 10 mm. The proportion of earlywood and latewood was not controlled and could cause some variation, as the chemical composition differs between them, e.g. the amount of lignin is higher in earlywood than latewood (Lamlom and Savidge, 2003).

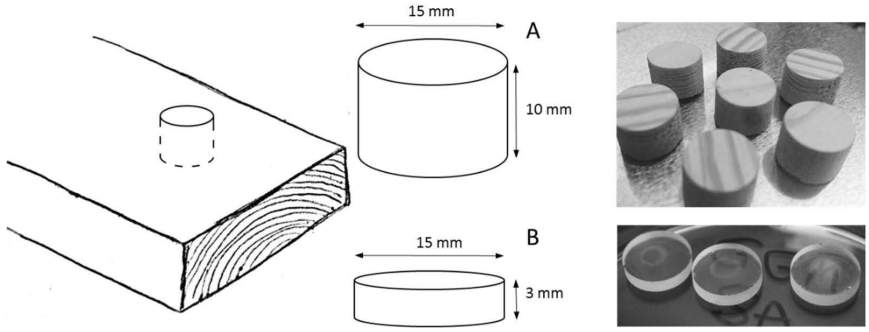

Figure 4. Wooden samples (A) were drilled from the tangential-longitudinal surface. Glass samples (B) were ordered from Lasinpuhaltamo Siljander Oy.

Glass surfaces (Fig. 4 B) were used as control, since glass is considered to be an inert and homogenous material, and therefore more suitable as a reference than, e.g., plastic. Glass cylinders of the same diameter and surface area were ordered from Lasinpuhaltamo Siljander Oy, Finland (www.laborexin.fi), a company specializing in laboratory equipment. The thickness of the glass samples was 3 mm. The same glass cylinders were used in all experiments in Papers I-V. After each use, the cylinders were washed with detergent and hot water and sterilized.

3.1.2 Wood particles for the extracts and the VOCs

Wood particles were used for preparing the extracts (Papers III and V) and as a source for the VOCs (Paper IV). Wood particles were prepared by milling with a Wiley-mill to a particle size of < 1mm. The extracts were prepared from pine HW and SW and spruce HW. The wood particles for the VOCs were prepared from HW and SW of both pine and spruce. In Paper IV, sterile water (cleaned by reverse osmosis, deionized, filtered and active carbon- and pyrogen- sterilized), α-pinene (98%, Sigma-Aldrich, USA), and limonene (97%, Sigma-Aldrich, USA) were used as controls.

3.1.3 Wood structural components

Paper V examined the structural components of wood, including cellulose nanofibrils (CNF), hemicellulose galactoglucomannan (GGM) and milled wood lignin (MWL). Polyethyleneimine (PEI) was used as an anchoring substrate for CNF and GGM and polystyrene (PS) for MWL and extract surfaces.

CNF was prepared from never-dried kraft birch pulp from Finnish pulp mills 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 through 12 passes. GGM was extracted from spruce thermal mechanical pulp by hot water extraction followed by purification (Willför et al., 2003a). MWL, prepared according to Björkman (1956), was obtained from University of Helsinki. PS and PEI of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO).

3.2 Bacterial strains

Bacterial strains were chosen in order to simulate real-world hygienic challenges on various surfaces and to represent Gram-positive and Gram-negative strains (Table 3). *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium (briefly *S.* Typhimurium) are a challenge in food processing premises, while methicillin-resistant *Staphylococcus aureus* and vancomycin resistant *Enterococcus faecalis* are a growing concern in hospital environments (Hierholzer et al., 1995, Dancer, 2008). *Streptococcus pneumoniae*, one of the leading causes of ear infections and pneumonia (Bluestone et al., 1992, Van Beneden et al., 2000, Kilpi et al., 2001) and *Escherichia coli* serotype O157:H7, which causes severe diarrhea (Reida et al., 1994, Rimhanen-Finne et al., 2014), are both troublesome pathogens in, e.g., day care centers. The colonies of the bacterial strains used are shown in Fig.5.

Table 3. Bacterial strains that have been used in this thesis and the respective agars and the papers where the strains were used.

Bacterial strain	Specific information	Agars used	Papers
Gram-positive strains			
Listeria monocytogenes[1] 1/2a IH 83616	Isolated from a patient	Sheep blood	I
Staphylococcus aureus[2] ATCC 43300	Methicillin-resistant	Sheep blood / Müller-Hinton II	III,IV,V
Streptococcus pneumoniae[1] ATCC 49619		Sheep blood / Müller-Hinton II with 5% horse blood	III,IV
Enterococcus faecalis[2] ATCC 51299	Vancomycin resistant	Sheep Blood / Müller-Hinton II	III
Gram-negative strains			
Escherichia coli[3] MG 1655;		Drigalski-Conradi / Nutrient broth	I,II
Escherichia coli[1] O157:H7 RHE 5402	Without *stx*-genes	Drigalski-Conradi / Sheep blood / Müller-Hinton II	III,IV,V
Salmonella enterica serovar Typhimurium[1] RHS 1882	Isolated from a patient	Drigalski-Conradi	IV

Sources: [1] The strain collection of the National Institute for Health and Welfare (THL)
[2] Labema Oy
[3] Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH

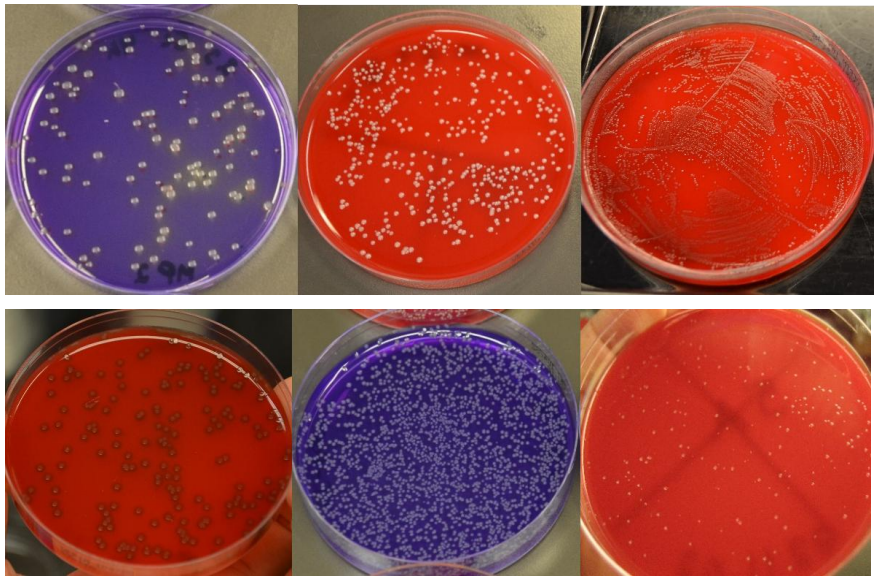

Figure 5. From the top left: *E. coli, S. aureus, E. faecalis, S. pneumoniae, S.* Typhimurium and *L. monocytogenes* colonies growing on agar plates after 24h incubation.

3.3 Extraction

3.3.1 Extracted wood

Wood cylinders were extracted with acetone (Paper II) using a Soxhlet apparatus for 6 h. Acetone-soluble extractive content was measured from approximately 10 g of drilled wood cylinders. Acetone was evaporated from the samples using a rotary evaporator (Büchi, Switzerland). Extractive content was calculated based on dry wood weight.

3.3.2 Preparation of the extracts

In order to prepare extracts for Papers III and V, wood particles were extracted as described above. Most of the acetone was evaporated from the extract using a rotary evaporator (Büchi, Switzerland) in a 40°C water bath and approximately 400 mbar pressure. Extracts in the remaining acetone were poured into a small container and the rest of the acetone was left to evaporate in ambient air. The extracts were stirred and weighed regularly until there was no observable change in weight. Extractive content was calculated based on dry wood weight.

3.4 Preparation of model surfaces

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). 40 µl 2.5 mg/ml PEI solution was first dropped on the glass cylinder. After 10 min adsorption, the glass cylinder was rinsed with water and dried with nitrogen gas. CNF dispersion was then spin-coated on top of 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., CT) and further centrifuged to remove the large fibril bundles at 8000 g for 30 min at 20°C with an Eppendorf centrifuge 5804R (Eppendorf AG, Germany).

GGM film was prepared by dropping 80 µl of a 2.5 % wt. aqueous GGM solution on top of a CNF film prepared as described above. After this, the substrate was immediately dried in the oven at 50°C for 30 min.

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

MWL 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 3s with an acceleration speed of 2400 rpm/s; secondly, at 500 rpm for 5s with an acceleration

speed of 6000 rpm/s; and finally, at 1000 rpm for 2 min with an acceleration speed of 4000 rpm/s. The procedure was repeated four times to obtain full MWL coverage. To further confirm MWL coating, lignin was also spin-coated directly onto the glass cylinder.

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

3.5 Cultivations

3.5.1 Solid wood surface

In Papers I and II, 100µl of bacterial solution was placed with a pipette on the top surface of the wooden samples and glass samples as controls, which is equivalent to 1.5 x 10^5 CFU per sample. Five parallel samples were used for each incubation time. The samples were placed in petri dishes, five parallel samples in each. After incubation at room temperature for the respective times specified in Papers I and II, the samples were tested for recoverable bacteria. To remove bacterial cells that had possibly adhered to the surface of the samples, the samples were dropped in broth and vortexed in 15 mL brain heart infusion (BHI) or physiological salt solution, in Papers I and II respectively, for 5 s. Immediately after, 400 ml of the solution with bacteria was spread onto relevant agar plates (Table 3). After overnight incubation at 37°C, the CFUs on the plates were counted. In cases where the bacterial colonies formed a lawn on the agar plates, and thus were impossible to count separately, the CFUs were estimated to correspond to the original number of cells at 0 h (i.e. 1.5 x 10^5). The average and the standard deviation of the five parallel results were calculated and the results are shown as CFU/plate in a logarithmic scale. The cultivation procedure is shown in Fig. 6.

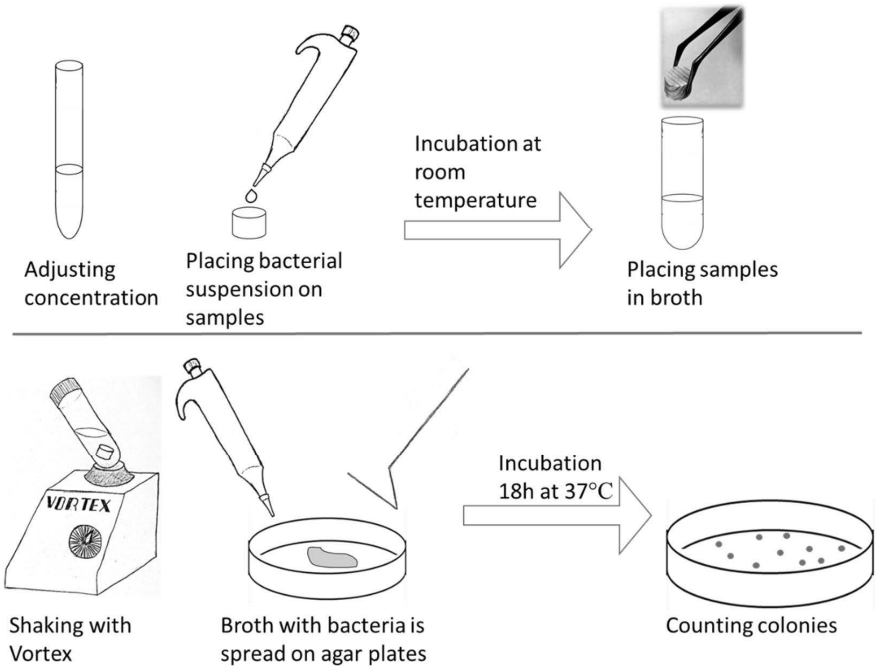

Figure 6. Schematic diagram of the cultivation procedure

3.5.2 Extracts in FAB-broth

The cultivations were carried out in three parallel samples in test tubes with 900 µl FAB (fastidious anaerobe broth), as described in Paper III. For each bacterial strain there was a control, which was cultivated without extract, only a bacterial dilution in the FAB. Of the extracts, 100 µl were placed in 900 µl of FAB (Fig. 7), shaken and left in ambient temperature for a minimum of one hour. To the broth, 100 µl of the bacterial dilutions (1.5×10^7 CFU ml^{-1} for *S. pneumoniae* and 1.5×10^5 CFU ml^{-1} for *S. aureus, E. faecalis* and *E. coli*) was then added making the final concentration in the tubes 1.5×10^6 CFU ml^{-1} for *S. pneumoniae* and 1.5×10^4 CFU ml^{-1} for other bacteria studied. *S. pneumoniae* was incubated in an atmosphere of 5% CO_2, whereas other bacterial strains were incubated in ambient atmosphere. After incubations of 24 and 48 h at 37°C, 50 µl of the broth was spread on sheep blood agar that was incubated at 37°C until the next day. Also, 10^{-2} dilution of the solution was cultivated. If the number of CFUs in the original broth could not be counted, the dilution results were used to calculate it. The mean values and standard deviations of the three parallel samples were calculated. Control samples were estimated to have CFU > 10^8 as they grew a full mat on the agar.

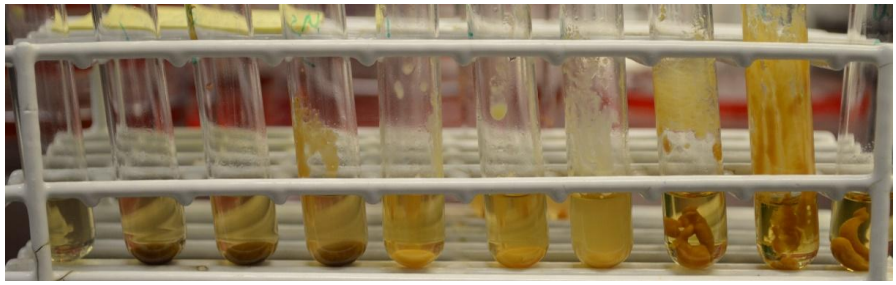

Figure 7. Wood extracts in FAB + *S. pneumoniae* before incubation. From the left: control, 3x pine HW, 3x pine SW and 3x spruce.

3.5.3 Sensitivity test with extracts

The antibacterial effect of the extracts was also studied using Müller-Hinton II sensitivity agar plates, supplemented for *S. pneumoniae* with 5% horse blood. With a pipette, 100 µl of each extract was dropped on the agar seeded with bacteria according to the EUCAST disc diffusion method (Matuschek et al., 2014). After incubation overnight at 37°C in ambient atmosphere, or 5% CO_2 for *S. pneumoniae*, the area around the extract drops without visibly growing bacteria was measured with a ruler.

3.5.4 Bacterial testing of model surfaces

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, glass cylinders were placed in test tubes with 7.5 ml physiologic NaCl solution, shaken vigorously, and 200 µl from the solution was spread on sheep blood or Drigalski-Conradi agar plates. Also, 10^{-1} dilution of the solution was cultivated. After overnight incubation at 37 °C, the CFUs on the plates were counted. If the number of CFUs in the original broth could not be counted, the dilution results were used to calculate it. The tests were made with three parallel samples and the average and standard variation were calculated.

3.5.5 Testing the effect of VOCs

Cultivations were carried out in closed glass containers with a volume of 1.9 l (Paper IV). On the bottom of the container, 70 g of wood particles was placed and 20 µl of bacterial solution was placed on glass discs on a rack above the studied material (Fig. 8). Other materials used were 30 ml sterile water, 1 ml α-pinene, and 1 ml limonene. Bacterial cultures were also incubated in an empty glass container as control. The glass discs with bacterial solution were first kept in sealed containers with silica gel for one hour.

Subsequently, the discs were moved into the containers with the studied material. After incubation at room temperature for 2, 4 and 24 hours, glass discs were dropped in test tubes with 7.5 ml physiologic NaCl solution, shaken vigorously, and 200 µl from the solution was spread on sheep blood or Drigalski-Conradi agar plates for *S. aureus* and *S. pneumoniae* or *S.* Typhimurium and *E. coli* respectively. Also 10^{-1} dilution of the solution was cultivated. After overnight incubation at 37 °C, the CFUs on the plates were counted. If the number of CFUs in the original broth could not be counted, the dilution results were used to calculate it whenever it was possible. The bacterial cultivations were made with three parallel samples and the average and standard variation were calculated.

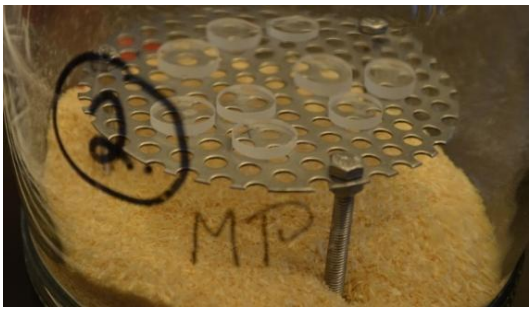

Figure 8. Cultivation set-up in Paper IV

3.6 Contact angle

Contact angle is measured by placing a drop on a surface and measuring the angle between the surface and the drop (Fig. 9). Contact angle can be used to measure the wettability of a surface over certain time (Cassie and Baxter, 1944) and in this thesis it was used to estimate whether the different drying rates of different surfaces had an effect on the antibacterial properties or not. The contact angle measurements (Paper II) were carried out at room temperature (20°C) at approximately RH 50% using sessile drop technique with a contact angle measuring instrument (KSV Instruments, model CAM200) on wood with different treatments and on glass. A drop of distilled water was placed on the surface and the contact angle determined with the help of a camera and CAM 200 software. Contact angle was measured every 10 s over 10 min. Five parallel specimens were measured except for glass, where two parallel specimens were measured.

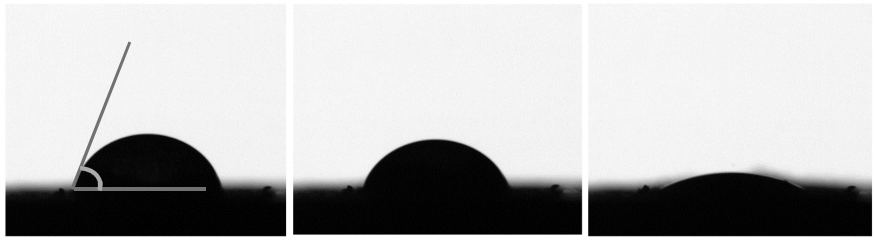

Figure 9. A drop of distilled water on pine HW after 0s, 40s and 390s. Contact angle is measured as shown in the picture.

3.7 Analysis of VOCs

The VOC evaluation was done according to EN ISO 16000 part 6 (2004). The air samples were taken directly from the glass containers used for the bacterial tests. The amount of wood particles in the container was 70 g with or without 30 ml water. The containers were closed and sealed with aluminum tape. For sampling, clean air supply was attached to the container and the VOCs were collected in sample tubes filled with Tenax TA (200 ng, 60 mesh to 80 mesh) by using a sampling pump with an electronic flow controller. A sample flow rate of 100 ml min^{-1} was used with a sampling time of 1 minute to give a sample volume of 100 ml to stay within the detection limits of the GC-MS (Agilent 7890A – Agilent 5975C, US.) procedure, but also to get the best analysis quality possible. Before sampling, each tube was spiked with 200 ng toluene-d_8 dissolved in methanol as the internal standard. Quantification was achieved through multipoint calibration with reference compounds. Detected peak areas were multiplied with the relative response factors of the internal standard. The VOCs were measured after 2 h, 4 h and 24 h and the containers were opened for five seconds after 2 h and 4 h measurements to simulate the conditions in the bacterial testing. The propane-equivalent concentration (ppm) of α-pinene and limonene was studied with a flame ionization detector device, FID 3006 by Sick-Maihak, Waldkirch, Germany. Empty containers were also measured to observe compounds from the system. Those compounds which were clearly emitted from the silicon tubes were subtracted from the results.

3.8 GC-MS analysis of the extractives

The extractives used as model surfaces (Paper V) were analyzed in Åbo Akademi with GC-MS according to Willför et al. (2003c). Identification of individual components was performed by GC-MS analysis of the silylated components with an HP 6890–5973 GC-MSD instrument. A crosslinked methyl polysiloxane (HP-1, 0.11 μm film thickness) 25 m × 0.20 mm i.d. column was used.

4 RESULTS AND DISCUSSION

4.1 Development of test methods to study antibacterial properties of wood

When choosing test methods for the various components of this study, the methods employed by earlier research which were discussed in the Background chapter were considered and partly used as a starting point. However, they did not directly suit our purposes. Here, the methods developed and used in this thesis are discussed.

4.1.1 Test method for solid wooden surfaces

In discussions on the hygienic properties of wood, there have been claims that the antibacterial properties observed are due solely to the adhesion of bacteria to the porous surface of the wood, and therefore not showing on the cultivations (Abrishami et al., 1994, Gough and Dodd, 1998). In this thesis, we investigated the antibacterial effect of wood independent of bacterial adhesion. Hence, the main goals when planning the test setup were to have a repeatable, precise method, which would also reveal bacterial cells that had possibly adhered to the wooden surface. The most common methods used in earlier studies are the contact plate method and the swabbing method, which have been discussed in section 2.3.1 These methods were specifically not used in order to avoid the effect of bacterial adherence.

Therefore, in Paper I, a method was developed for the purpose of studying the antibacterial properties of wood. This included utilizing small enough samples that could be vortexed in test tubes with nutrient broth after incubation and cultivating the resulting broth with bacteria on agar plates. The effectiveness of the procedure was evaluated by leaving the samples after final cultivation in the tubes overnight to see if there was turbidity in those tubes, which gave 0 CFU as a result of the plate count.

4.1.2 Test methods for studying extracts and structural components of wood

The extracts were studied by three different methods. The FAB-method was based on Rautio et al. (2007) and the sensitivity test was modified from the disk diffusion test. In the FAB-test, contrary to Rautio's studies, 50µl of the FAB was cultivated after incubation with bacteria and the extract, and the number of bacteria were counted instead of evaluating the turbidity. For the sensitivity test, instead of placing the extract drop on a paper disk, a drop was placed directly on the agar. This was done as the extracts were non-viscous and could not permeate the paper disk.

The third method of studying the extracts was not based on any previous microbiological method, rather an implementation of the method used for solid wood in Papers I and II. However, the method of preparing thin films of wood extracts was based on Tammelin et al. (2006). The structural components of wood were also studied with the same procedure.

4.1.3 Test method for studying wood VOCs

As there were no previous studies found on the antibacterial properties of volatile compounds, a method was developed for the studies in Paper IV. The specific aims for the procedure were not to have any other volatiles than those coming from wood and to achieve a repeatable precise method. Additionally, the amount of VOCs needed to be high, because this was the first time their antibacterial properties had been studied and we wanted to make sure the effect would be found if there was such an effect. For these reasons, glass containers were chosen instead of plastic. Plastic is not an inert material and has the potential to emit other compounds. In order to achieve a high concentration of VOCs, wood was milled into particles. Glass cylinders with the bacterial titer on top were placed on a rack over the wood particles, in a similar manner to the situation in a desiccator, where the distance between them was very small but there was no direct contact. The sampling of the glass cylinders was carried out in the same way as in Papers I, II and V.

4.2 Antibacterial properties of solid wood surfaces

The results showed that pine HW had an antibacterial effect against *E. coli* and *L. monocytogenes* in contrast to glass surface. The turbidity results matched well with the plate count results (Table 4). If there had been viable bacteria adhered to the surface of the wood which would not have shown on the results, they should have been able to multiply when left overnight in the broth in 37°C. It could therefore be concluded that the test method revealed all viable bacteria on the surface of the wood.

Table 4. Occurrence of the bacterial growth of *L. monocytogenes* and *E. coli* placed on Pine HW surfaces for 2, 4 or about 24h in the two cultivations. The first + or − before the slash mark shows whether there was any bacterial growth in the plate count directly after vortexing and the second + or − after the slash mark shows whether there was bacterial growth one day later in the BHI broth incubated at +37°C overnight

Bacterial strain	Incubation time	Parallel samples				
		1	2	3	4	5
L. monocytogenes	2h	+/+	+/+	+/+	+/+	+/+
L. monocytogenes	4h	-/-	+/+	+/+	+/+	n.a.
L. monocytogenes	22h	-/-	-/-	-/-	-/+	-/-
E. coli	2h	+/+	-/-	-/-	+/+	+/+
E. coli	4h	-/-	-/-	-/-	+/+	+/+
E. coli	28h	-/-	-/-	-/-	-/-	-/-

+ = growth, - = no growth, n/a = not available

4.2.1 Antibacterial properties of Scots pine and Norway spruce surfaces

The HW and SW of Scots pine are visibly different and therefore can be divided and chosen according to end-use. Spruce HW and HW and SW of pine were therefore chosen for further investigation regarding their microbiological surface properties (Paper II). Because some extracts have exhibited antibacterial properties (Canillac and Mourey, 2001, Lindberg et al., 2004), the importance of the role of extractives in the antibacterial properties of wood was studied by actually removing them with acetone extraction from the sample.

All wood surfaces studied were found to exhibit antibacterial action, in contrast to glass surface which demonstrated none (Fig. 10). The differences between wood species and HW and SW were very small. The bacteria on pine SW were not viable after 2h, but also the amount of viable bacteria on pine HW and spruce samples was small and the variation large. The results of the extracted samples (Fig. 11) show a clear decrease in antibacterial properties. The difference between untreated and extracted samples was much larger than the differences between the wood species. It is probable that the extractives account at least partly for the inhibition of bacterial growth as the removal of the extractives also decreased the effect. The extractive contents of pine HW, pine SW and spruce were 5.0, 2.5 and 0.6 %, respectively.

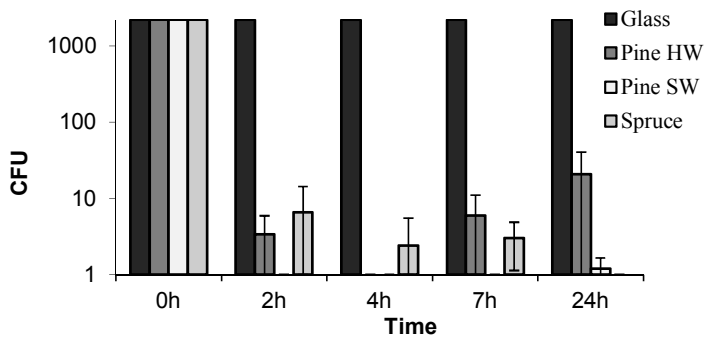

Figure 10. The amount of *E. coli* on glass, pine HW (PH), pine SW (PS) and spruce (S) after 0, 2, 4, 7 and 24 hours incubation.

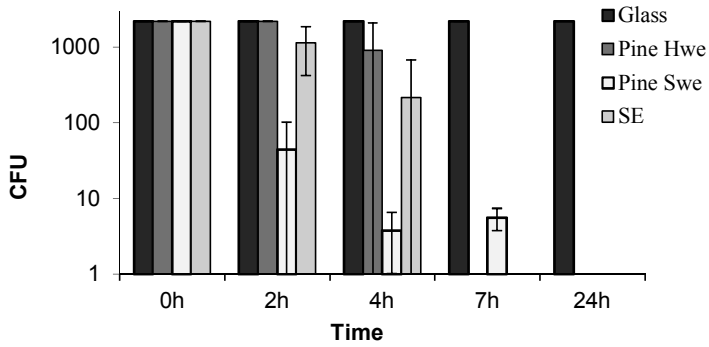

Figure 11. The amount of *E. coli* on glass, extracted pine HW (Pine Hwe), extracted pine SW (Pine Swe) and extracted spruce (SE) after 0, 2, 4, 7 and 24 hours incubation.

4.2.2 Fast drying of the wood surface and its effect on antibacterial properties

Bacterial cells need water to stay viable (Stanier et al., 1987) and wood has an ability to absorb a certain amount of water, leading to faster drying of the surface compared to a non-porous material. Water absorption properties vary between different types of wood and are also affected by extraction. Dynamic contact angle (DCA) method was used for measuring the water absorption rate. The fastest decrease of the contact angle was on pine SW, where the contact angle had decreased to 0 within 60 s. The drop volume reaches 0 at the same time as the contact angle. Hence, the contact angle can be used for describing the absorption of water into the tree. The evaporation rate can be seen from the contact angle curve of glass. Even during the measurement, the contact angle and drop volume seem to follow an approximately similar curve (Fig. 12). For both pine samples, water was found to absorb into wood faster in the non-extracted wood surfaces than in the extracted samples. The development of the contact angles of all samples over 10 min time are shown in Fig. 13.

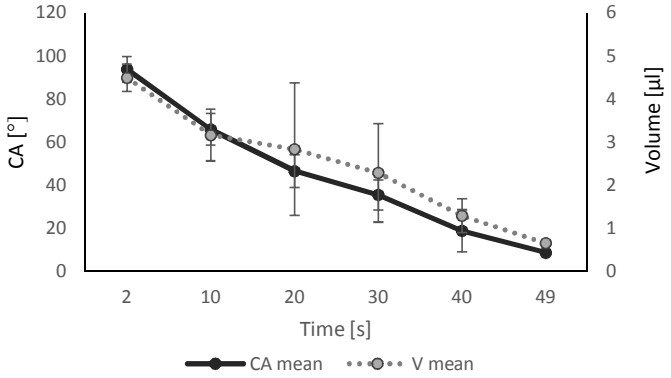

Figure 12. Contact angle (CA) and drop volume (V) on pine SW surface

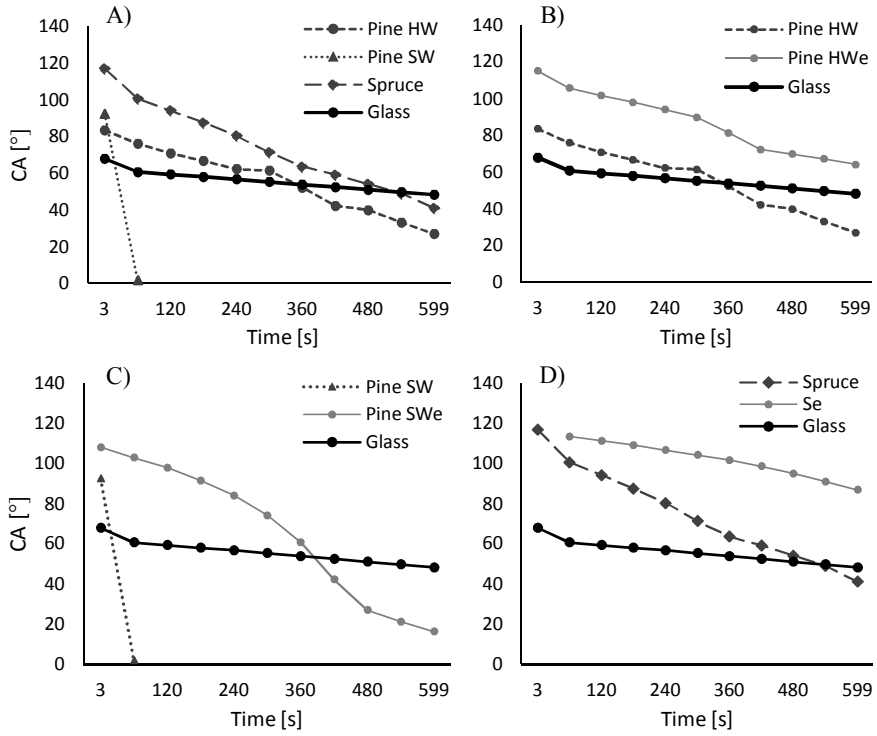

Figure 13. Contact angles of A) Pine HW, Pine SW, Spruce and glass, B) Pine HW and extracted pine HW (HWe), C) Pine SW and extracted pine SW (SWe) and D) Spruce and extracted spruce (Se).

It seems that the fast drying of the pine SW surface (Fig 13 A), compared to pine HW and spruce, correlates well with the antibacterial testing results, where pine SW was the most antibacterial of the tested surfaces. However, the difference between pine SW and pine HW

or spruce was not very large. The contact angles of untreated samples decrease faster than the contact angles of the extracted samples, being in the same order as the antibacterial results. Glass surfaces do not absorb water, so the glass curve demonstrates the effect of water vaporizing in the surrounding air. All the wooden surfaces show a steeper slope indicating at least some absorption into the surface.

It is not possible to directly compare the results from DCA with the bacterial test results as the RHs in the testing environments were different. The RH of the ambient air naturally affects the drying rate of a drop on a surface. DCA testing was done in a room with a regulated RH of 50%, whereas the RH in the Petri dishes was not regulated or measured. However, the DCA results give valuable information about the differences between different surfaces.

The contact angles were measured for only ten minutes and the exact time when the drop had dried is not defined for those samples that lasted longer, except for glass. The drop on the glass surface was evaporated in ca. 1h (unpublished data). It was observed that after one hour, most drops had disappeared, except on glass, which shows that the DCA measurements could only be used to estimate the water absorption properties of the samples. For other extracted samples, there was a minor amount of water left on the surface. In 24 h, all the drops had dried out. *E. coli* can stay viable on a dry surface for several days (Williams et al., 2005), so dryness alone does not explain the antibacterial effect of wooden surfaces. The decrease in the drop volume brings the bacterial cells into closer contact with the wood surface and, hence, allows the antibacterial components to reach the bacterial cells. Low wettability of a surface might therefore create a delay of the antibacterial effect. Also, the water soluble substances are able to act at an earlier time than the insoluble ones. The variation is quite large due to the non-homogeneous nature of wooden surfaces.

In Papers I and II, a small drop size (100 µl) was used in order to avoid the effect of the drop volume. However, as was seen in the results, the wettability of the surface and the evaporation into the surrounding air may affect the results, and, hence, the drop size was further decreased to 20 µl in Papers IV and V.

4.3 Antibacterial effects of wood extracts

The experiments with extracted samples supported the assumption that the extracts are one of the contributors to the antibacterial properties of wood. Since removing the extractives from wood decreased its antibacterial properties, the extracts were studied further in Papers III and V.

The cultures in FAB-media showed clear differences between different wood species and bacterial strains (Fig. 14). The growth of MRSA and VRE was inhibited by both pine extracts. With SW, some growth of VRE was still observed after 24h incubation, but after 48 hours, no viable bacteria were found. Spruce had an antibacterial effect on MRSA and a weak effect on VRE (Fig. 15 and 16). *S. pneumoniae* was the most sensitive to the presence of extracts. There were no viable bacteria after 24 or 48 hours of incubation with any of the extracts, even though the initial concentration of bacteria was higher with *S. pneumoniae* than with the other bacterial strains. Only pine SW had an effect on *E. coli* but even that was weaker than was seen with other bacterial strains.

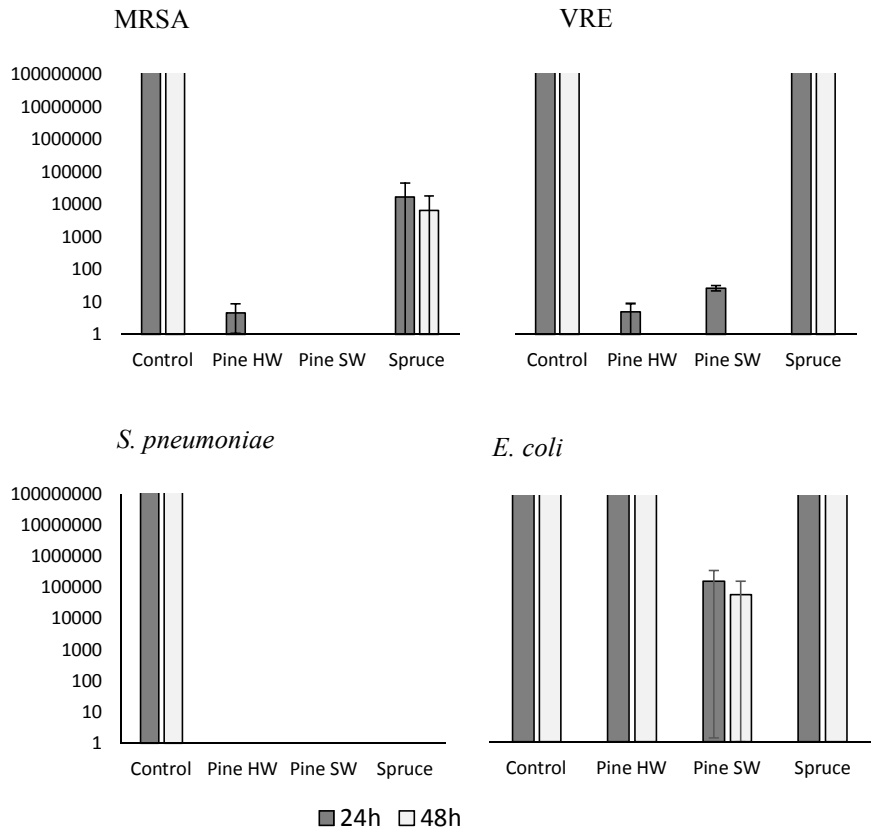

Figure 14. The number of viable bacteria after 24 and 48 hours incubation in FAB media with 100µl extracts or without extracts (control).

When investigating the extracts as thin films, the results were very similar to the results in the FAB and the sensitivity tests, as could be expected (Paper V). All the extract films studied displayed an antibacterial effect against MRSA, similar to the extracts studied in FAB (Fig. 14 and 15). However, the only one that had any effect against *E. coli* in the FAB test was pine SW, whereas pine HW was the only extract film displaying an effect against

E. coli. There was a large difference between the sampling times of the two tests. The extracts in FAB broth were tested after 24 and 48 h and the extract surfaces were tested after 2, 4 and 24 h. Therefore, smaller differences between the antibacterial effectivenesses of the extracts could be observed in the tests made with extract surfaces where the amount of bacteria was clearly lower and there was no viable bacteria left after 24 h even on the empty glass surface.

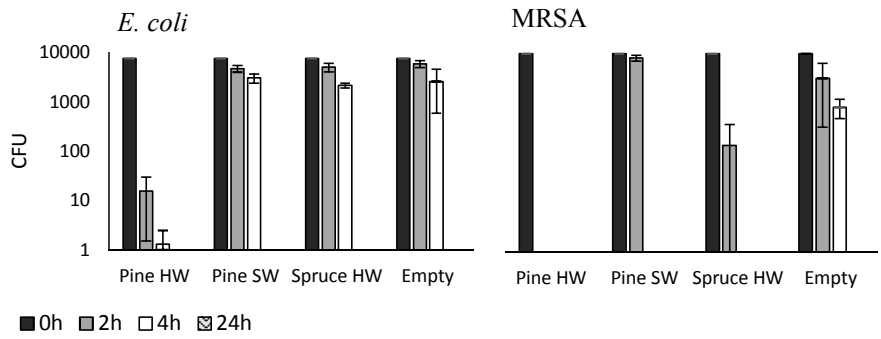

Figure 15. The number of CFU on the extract surfaces after 0, 2, 4 and 24 h incubation

In the FAB-broth, small differences could not be seen in the results because the broth was shaken before cultivation. The amount in those where some effect could be seen after cultivation but before shaking (Fig. 16) was so high that the plate count showed no difference to those where there was no effect at all.

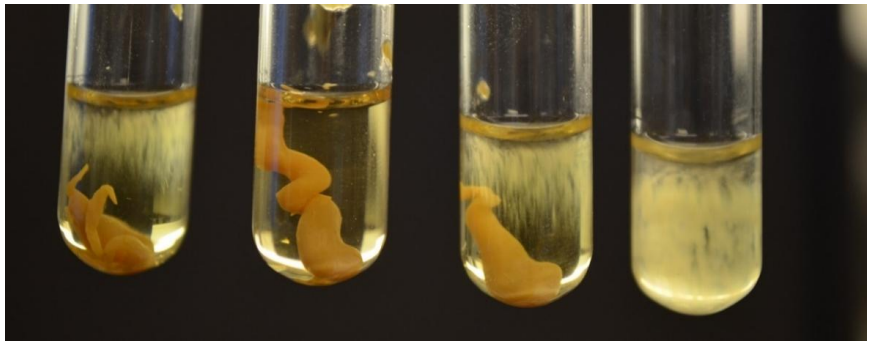

Figure 16. Spruce extract drops (3 on the left) and control (on the right) in FAB-media + VRE after 24 h incubation. Bacterial growth is seen as turbidity.

For the diffusion test, the extractive drops were placed on agar seeded with bacteria. Based on the differing viscosity of the extracts, the resulting drops were not perfectly round or the same size. For these reasons, the results are shown as an area of inhibition (Fig. 17), where the size represents the bacteria-free area, without the drop, contrary to conventional sensitivity testing. Some areas were very uneven and in these cases the results show the range measured around the drop (Table 5).

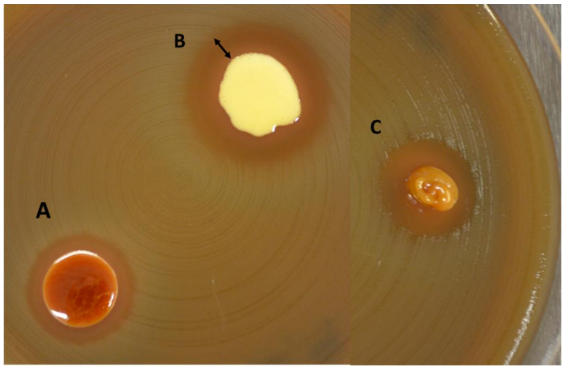

Figure 17. Pine HW (A) and SW (B) extracts and spruce extract (C) and the areas of inhibition on *S. pneumoniae.* The double headed arrow shows how the area of inhibition was measured.

Table 5. The area of inhibition (mm) around the extractive drops on Müller-Hinton II (*S. aureus, E. faecalis* and *E. coli*) or Müller-Hinton II-F horse blood agar (*S. pneumoniae*).

	Bacterial strains			
Wood species	*S. aureus*	*E. faecalis*	*S. pneumoniae*	*E. coli*
Pine HW	2	0.5	2	0
Pine SW	1-3	0-2.5	4	0
Spruce	0.5	0	3	0

On the plates with *S. aureus*, there was an area of inhibition around all extracts. The largest areas were around both pine extracts, followed by spruce. With *E. faecalis*, both pine extracts formed inhibition areas, whereas spruce did not cause any inhibition. Also in these tests, *S. pneumoniae* was the most sensitive strain. Pine SW had the largest area of inhibition followed by spruce. Pine HW had the smallest areas of inhibition with *S. pneumoniae*. None of the extracts formed any inhibition area with *E. coli*. The solubility of the substances in agar had more of an effect on these results than the results of the FAB-test, as the test tubes in the FAB-test were vortexed. This may explain why the results differ from each other. For example, both pine HW and spruce showed stronger antibacterial effect in the FAB-tests. Also, the extract of pine SW did not form any area of inhibition with *E. coli* but showed a clear antibacterial effect in the FAB-test. The FAB-test was more sensitive, showing also weaker antibacterial effects, unlike the diffusion test.

It is quite interesting that pine SW was so effective, since the extractive compounds that have been reported to have antibacterial properties including, terpenoids (Raman et al., 1995), fatty acids (Desbois and Smith, 2010), resin acids (Söderberg et al., 1990, Smith et al., 2005) and stilbenes (Plumed-Ferrer & al., 2013), are generally more abundant in pine HW than SW. Yet, SW was the only extract showing antibacterial activity against *E. coli* in the FAB-test. This is, however, in accordance with the results in Paper II with solid pine SW and HW samples, where the *E.coli* mortality rate was higher on SW than on HW.

Gram-positive *S. aureus* was more susceptible to wood components than Gram-negative *E. coli*. This is similar to several studies on wood components and bacteria (Himejima et al., 1992, Mourey and Canillac, 2002, Välimaa et al., 2007, Rautio et al., 2007, Plumed-Ferrer et al., 2013). The results in Paper III were very similar to these as could be expected. However, the effect of pine SW extract in FAB on *E.coli* was stronger than that of pine HW extract, whereas the results in studies mentioned above were the opposite. This could be a result of the differences in water solubility of the extracts. Most of the extracts are insoluble in water, however some are slightly soluble. The amount of water-soluble substances, for example some terpenoids and fatty acids, among those identified with GC-MS, is higher in pine HW than in pine SW. Hence HW would also be expected to have a greater antibacterial effect in an aqueous solution, which was not the case. Possibly there could be a synergistic effect explaining the stronger antibacterial effect of pine SW in the FAB.

The antibacterial properties of the extracts could originate from various single compounds or from a synergistic effect of several compounds. Some of the most common fatty acids found in all samples (Table 6.), like palmitoleic ($C_{16:1}$), linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids, have been reported to have antibacterial properties (Kabara et al., 1972, Desbois and Smith, 2010), though some of them only against Gram-positive strains. Most of the resin acids found in the samples, such as abietic acid, isopimaric acid, neoabietic acid, pimaric acid and palustric acid, have been reported to have antibacterial effects (Söderberg et al., 1990, Smith et al., 2005) only against Gram-positive strains. Pine HW had the greatest amount of almost all of these acids, followed by spruce HW, although in spruce HW there was more isopimaric acid than in pine HW. Even stilbenes, pinosylvin and pinosylvin monomethylether, were found exclusively in pine HW. The chemical composition explains the results for extract surfaces as pine HW was found to be rich in antibacterial components and also the most antibacterial of the samples tested.

Table 6. The main component groups and the most abundant compounds (>5mg/g) in wood extracts analyzed by GC-MS. The amount is shown as (mg/g dry weight)

	Spruce	Pine SW	Pine HW
Terpenoids			
a-terpineol	2	0	13
thunbergol	8	0	0
cis-abienol	6	0	0
Others	1	2	5
∑	17	2	18
Fatty acids			
acid 18:3	29	2	37
acid 18:2	42	5	108
acid 9-18:1	12	6	59
Others	26	6	28
∑	109	19	232
Stilbenes			
monomethyl-pinosylvin	0	0	139
pinosylvin	0	0	35
Others	0	0	0
∑	0	0	174
Resin acids			
pimaric acid	4	18	36
sandaracopimaric acid	7	3	5
isopimaric acid	23	13	14
palustric acid	26	16	37
levopimaric acid	5	18	0
dehydroabietic acid	48	43	62
abietic acid	18	10	104
neoabietic acid	9	10	29
Others	1	1	2
∑	141	133	290
Hydroxy resin acids	6	12	9
Lignans			
isolariciresinol	5	0	0
HMR	84	0	0
conidendrin	15	0	0
Others	26	0	0
∑	129	0	0
Sterols			
campesterol	8	0	0
sitosterol	17	3	4
Others	3	2	1
∑	28	5	5
Monosaccharides	27	4	35
Steryl esters	97	40	34
Diglycerides	39	36	26
Triglycerides	22	414	3
Sum	614	665	827

Although the antibacterial properties of spruce were comparable to pine HW and SW when studied as solid surfaces (Papers I and II), spruce extracts had less antibacterial effect on, especially, *E. faecalis* (Paper III) but also on MRSA (Papers III and IV). The extractive content of spruce is lower than of pine, so there is also less extractives available on the spruce surface. The results with spruce extracts were similar to Sipponen's (2013) studies on spruce resin, except in the case of VRE. Spruce resin was found to have an antibacterial effect against MRSA, VRE and all other Gram-positive bacteria tested, but not against *E. coli*. Spruce resin contains many of the same components (Sipponen, 2013) as spruce extract, namely resin acids such as dehydroabietic acid, isopimaric acid and palustric acid and lignans. All extracts and spruce resin contain antibacterial resin acids (Table 1), isopimaric, abietic and dehydroabietic acid. The amount of all these was even higher in spruce extract than pine SW extract (Table 6) yet pine SW extract was more antibacterial. The extracts were produced by acetone extraction whereas spruce resin is collected directly from trees without extraction, so it is possible that there are some antibacterial substances in spruce resin that are not found in the extract, which could explain the difference with VRE.

The pine and spruce HW extracts seem to be similar in various ways. The extracts from the HWs have both a higher amount of terpenoids, fatty acids and monosaccharides than pine SW, but also lower amounts of triglycerides. SW, contrary to HW, contain living parenchyma cells until kiln drying. Hence it is understandable that the HWs resemble each other. SW cells, on the other hand, have had metabolic activity which might have produced water-soluble substances, that are still available after drying, and are able to penetrate the outer membrane of Gram-negative strains (Conner and Kotrola, 1995, Alakomi et al., 2000). This could explain why pine SW extracts in the broth in Paper III were the most effective against *E. coli*, whereas in Paper V the HW extracts were the only ones effective against the same strain. In the broth, the effect was caused by water-soluble substances since the extracts and bacteria were in water-based broth. On the model films, the contact area between the extract surface and the bacterial solution was larger in relation to the solution volume.

4.4 The antibacterial properties of the structural components of wood

To further investigate the antibacterial properties of the structural components of wood, thin films of cellulose, hemicellulose and lignin were prepared on glass cylinders. The antibacterial properties of the surfaces were studied by cultivating bacterial solution on top and evaluating the number of viable bacteria after each incubation time.

The structural wood component surfaces did not decrease the number of *E. coli* O157:H7 (Fig.18). On CNF and especially on the GGM surfaces, the bacteria stayed alive longer than on the control, and the number of viable bacteria on the MWL surface was similar to the control at all sampling times. PS, 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 MWL and extractives films, where PS was used as an anchoring layer, as MWL, pine SW and spruce HW extracts caused no difference to the control (Figs. 15 and 18). Also, all the films covered the surfaces well, which was evaluated by AFM and XPS (Paper V).

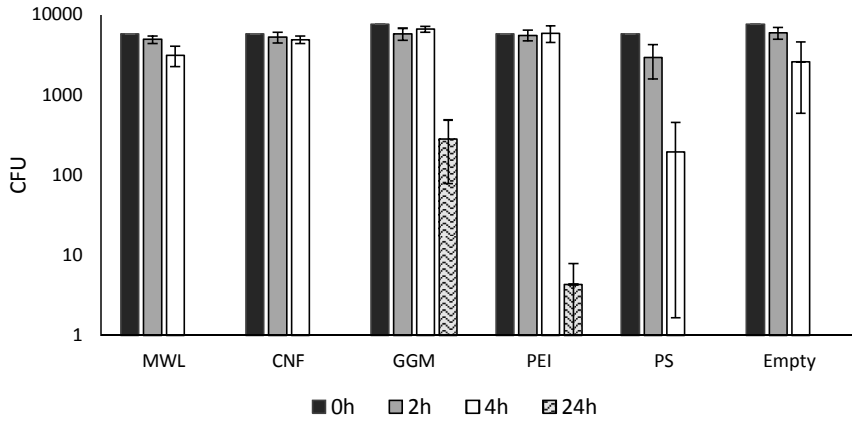

Figure 18. The amount of colony forming units (CFU) after 0, 2, 4 and 24 hours incubation of *E. coli* O157:H7 on different model surfaces of the structural compounds of wood and the glues used for preparing the surfaces. Clean glass surface is used as a control.

MWL had an antibacterial effect on MRSA (Fig. 19), but on the GGM and CNF surfaces, the number of bacteria decreased at a much slower rate than that of the control. On CNF, GGM and PEI surfaces there were viable bacteria left after 24 hours, but on the empty control surface there were no viable bacteria left. No difference could be observed between PS and the control when studied with MRSA even though PS showed a weak antibacterial effect against *E. coli*.

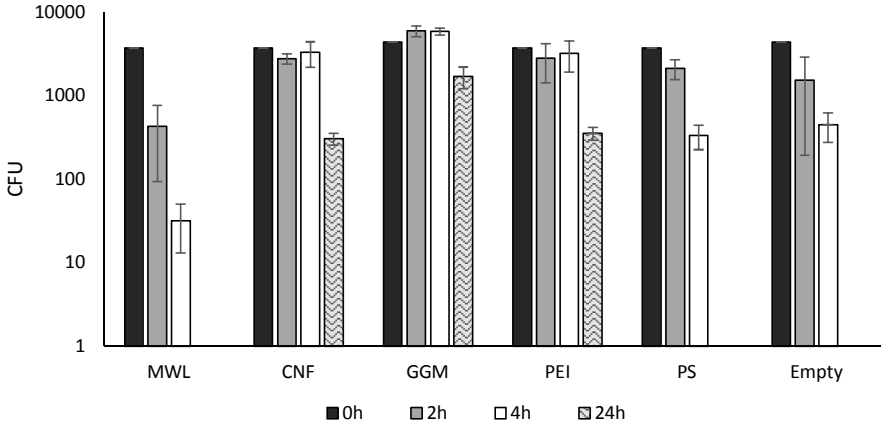

Figure 19. The amount of colony forming units (CFU) after 0, 2, 4 and 24 hours incubation of MRSA on different model surfaces of the structural compounds of wood and the glues used for preparing the surfaces. Empty glass surface is used as a control.

On the GGM and CNF surfaces there are available nutrients. GGM consists of shorter chains of galactose, glucose and mannose and is more easily accessible than CNF, which is paracrystalline. Some hemicelluloses are also available on the CNF surface. This accessibility leads to GGM providing easier nutrition for the bacterial cells. This explains the higher amount of viable bacteria on the GGM surface compared to CNF.

The extractives are clearly one of the main factors in the antibacterial properties of wood, but even lignin was found to contribute to these properties. Lignin is more stable on wooden surfaces, not degrading or dissolving easily. Its antibacterial effect could therefore be considered more permanent than the effect of extractives.

Comparing MWL and the extracts is nevertheless somewhat complicated. The differences in the thicknesses of the films are large and the form of the materials are different. The extract surfaces were around 200 nm, whereas MWL surfaces were only 6 nm thick. Most of the acetone extracts are not water-soluble, but in Paper III, it was shown that they could affect bacteria in a water-based broth which means that some active components of the extracts have been dissolved in the broth. However, the MWL covered the whole surface and its thickness should not make any difference to the results. Also, these two materials differ in form, as the extracts are softer and stickier and MWL is harder.

4.5 Significance of volatile compounds

Wood, especially when it is new, emits large amounts of VOCs into the air. VOCs which are emitted by other materials have been discussed in scientific literature, mainly in relation to their harmful effect on human health (Englund and Nussbaum, 2000, Hodgson et al., 2002, Kim et al., 2010, Steckel et al., 2010, Chaudhary and Hellweg, 2014, Widhalm et al., 2016). In Paper IV, these volatile components were investigated to ascertain whether or not they could also have an antibacterial effect similar to solid wood and its components. In order to analyze the results, the VOCs were analyzed by GC-MS.

The antibacterial effect of VOCs was studied against four human bacterial pathogens, MRSA, *E. coli, S. pneumoniae* and *S.* Typhimurium. MRSA, which had shown to be sensitive in the presence of pine and spruce extracts in Papers III and V, was not very sensitive to VOCs from the same wood species. For this reason, the experiment was continued for an additional two days (Fig. 20), except for the container with water, as the amount of viable bacteria was clearly already increasing after 24 h. The first differences between the containers with wood particles and the empty control could be seen only after 3d incubation. In the pine HW container, the number of bacteria had decreased from an original starting point of about 2000 CFU/plate to about 200 CFU/plate, while the control showed no clear decrease after 3d incubation. Also, in spruce HW and pine SW, a clear decrease could be seen after 3d. The effect of α-pinene was also only seen after 3d, however the effect at that point was stronger than that of pine HW. These results agree well with earlier literature, where methicillin-resistant *S. aureus* has also been found to be persistent on hard surfaces (Makison and Swan, 2006).

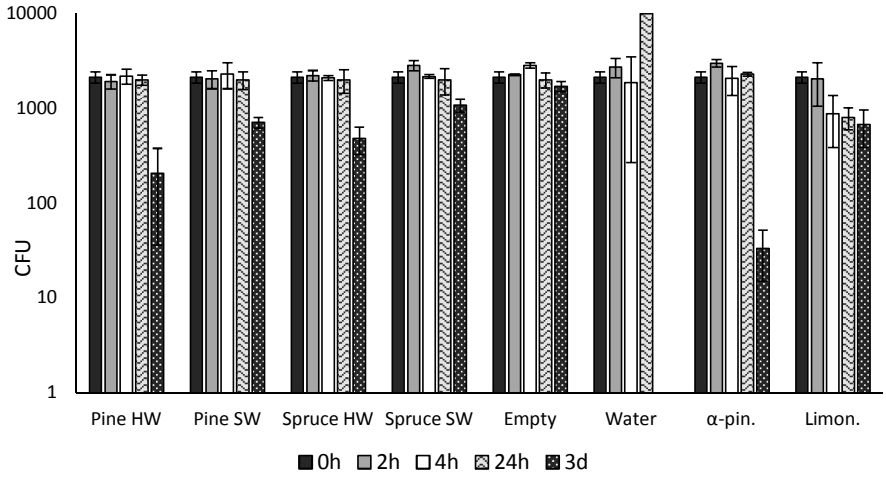

Figure 20. The amount of viable MRSA on the containers with different materials after five different incubation times.

E. coli, on the other hand, was found, in Papers III and V, to be less sensitive to wood extracts compared to MRSA. In the FAB-broth tests, the only extract inhibiting the growth of *E. coli* was pine SW. When studied as films, the only inhibiting extract was pine HW. In contrast to the studies with the extracts, the VOCs from all the wood particles showed some inhibitory effect on *E. coli* (Fig. 21). Pine HW had the strongest effect of the wood samples followed by spruce HW, pine SW and spruce SW. Limonene had a very strong effect, with no viable bacteria left after 2h. α-pinene clearly demonstrated less of an antibacterial effect than that of the wood particles on the amount of viable bacteria. Antibacterial effects of both limonene and α-pinene against *E. coli* have been reported earlier in the literature. However, it was observed only when they were in direct contact with the bacterial solution (Himejima et al., 1992, Dorman and Deans, 2000). Water caused a small decline in the number of viable cells of *E. coli* after 2h or 4h, but later the count surpassed the original number of bacterial cells. This is in agreement with literature, where RH 96 % has been found to be the minimum level at which bacterial cells can grow (Scott, 1953). The RH in the containers with water reached 94 – 98 % after 24h.

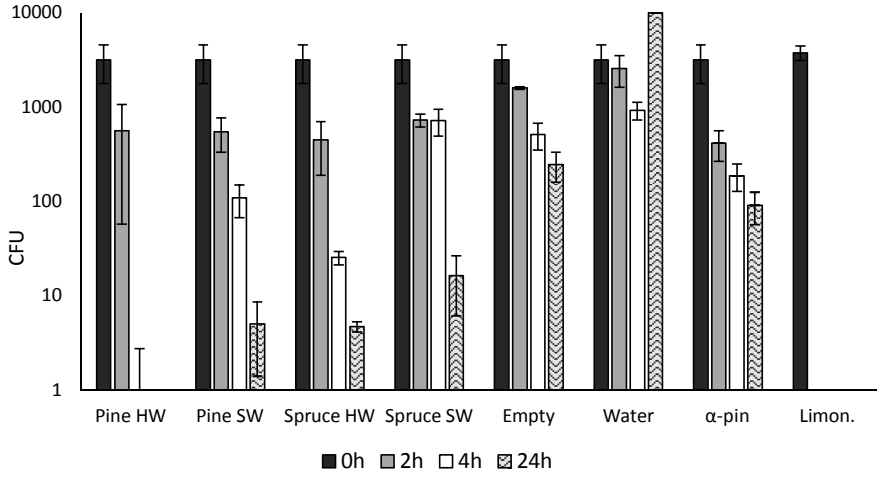

Figure 21. The amount of viable *E. coli* O157:H7 on the containers with different materials after four different incubation times.

S. pneumoniae, which was found to be very susceptible to all the wood extracts in Paper III, was also found to be susceptible to VOCs (Fig. 22). Pine SW and spruce HW and SW showed a higher rate of decline in bacterial numbers than the empty container. However, the bacterial count decreased at almost the same rate as in the container with water. Even though water is essential for bacteria to multiply, it has been shown that very low and very high RH decrease the time bacteria stay viable on a surface (Palumbo and Williams, 1990, Jawad et al., 1996, Makison and Swan, 2006), which could possibly explain these results.

The RH was clearly higher in the containers with wood particles than in the empty container, which could also affect the results. α-pinene and limonene, on the other hand, were very effective. With α-pinene there were no viable cells after the 0h measurements. Limonene caused the CFU count to decrease after only 2h to under ten colonies per plate.

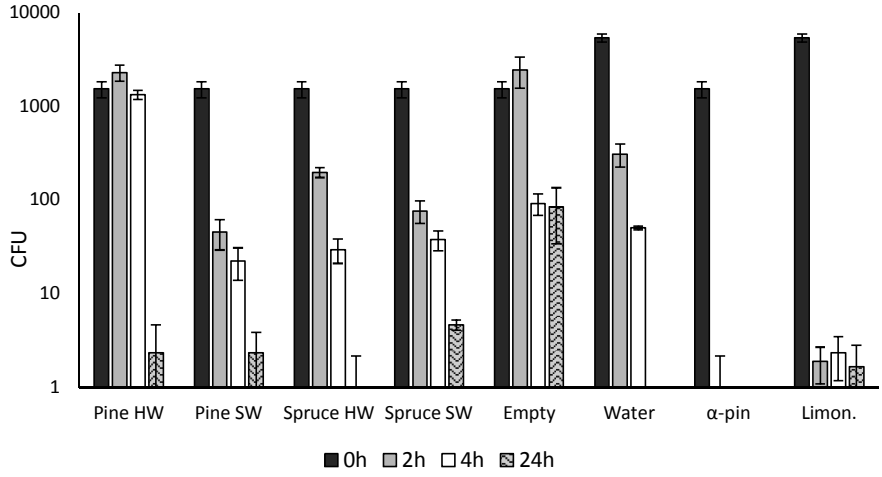

Figure 22. The amount of viable *S. pneumoniae* on the containers with different materials after four different incubation times.

S. Typhimurium was used only in the study of its susceptibility to the VOCs. It was quite resistant to the VOCs emitted from wood (Fig. 23). Only after 24h could clear differences between the empty container and the sample containers with both pine HW and spruce HW, be seen. Neither SWs showed any difference to the empty container. In the container with water, the bacterial count had actually increased between the 4h and 24h measurements, similar to the results for *E. coli*. The most antibacterial effect on *S.* Typhimurium was seen with α-pinene, for which the bacterial count decreased to less than 100 CFU/plate in four hours. Limonene had no antibacterial effect on *S.* Typhimurium.

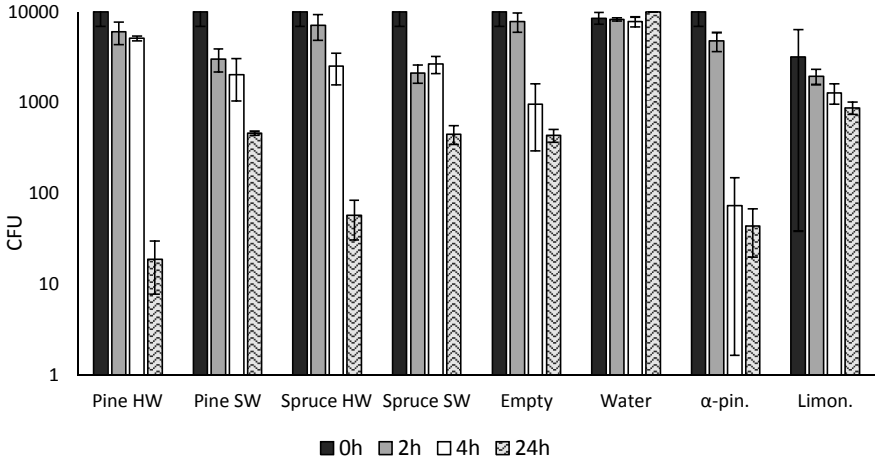

Figure 23. The amount of viable *S. Typhimurium* on the containers with different materials after four different incubation times.

The overall amount of VOCs in the containers was very high. Monoterpenes were the largest group for all wood species at all sampling times. α-pinene was the dominant terpene in all samples and the aldehydes consisted mostly of saturated aldehydes, but also benzaldehyde was found in all samples. These results are similar to those reported earlier (Englund, 1999, Englund and Nussbaum, 2000, Wajs et al., 2006, Steckel et al., 2010). Acetic acid was the dominant acid in all samples. Ethanol was found in larger quantities in both SWs than in the HWs, where it was found only in trace amounts. This is understandable, as ethanol is synthesized in the cytoplasm via alcoholic fermentation (Kreuzwieser et al., 1999), which is possible only in living cells. The amount of α-pinene in the pine HW samples at 24 h exceeded the detection limit of GC-MS and those results are therefore not included. Since the amount of pure α-pinene and limonene exceeded the detection limit of the GC-MS equipment, in the containers with pure compounds, their amount was measured with FID and the results are given in Table 8.

Table 7. The main component groups of the VOCs from the wood particles.

		Pine HW		Pine SW			Spruce HW			Spruce SW		
		2h	**4h**	**2h**	**4h**	**24h**	**2h**	**4h**	**24h**	**2h**	**4h**	**24h**
Acids	Acetic acid	287	726	323	134	387	50	286	865	161	351	294
	4-Hxdroxymandelic acid, ethylester, di-TMS	79	78	83	82	33	68	68	27	69	69	23
	Hexanoic acid	0	0	0	0	0	0	10	23	0	0	0
Alcohols	Ethanol	0	34	421	397	453	31	34	29	742	794	752
	1-Propanol	0	0	0	0	771	397	796	0	651	0	0
	1-Octen-3-ol_1-Pen	0	0	0	24	0	0	0	0	0	0	0
Aldehydes	Benzaldehyd	20	44	17	27	25	15	17	39	26	32	33
	Hexanal	11	20	52	167	259	8	16	17	21	75	46
	Pentanal	12	14	96	210	292	0	11	13	38	114	84
	Nonanal	15	30	14	15	16	9	11	14	12	13	14
	Butanal	14	23	90	88	122	0	0	0	59	81	79
	Propanal	0	35	236	155	0	33	30	0	93	99	0
	Heptanal	13	0	0	0	0	0	0	0	0	0	0
Terpenes	a-Pinene	11282	23167	1363	2928	3644	508	1166	871	316	797	486
	3-Carene	675	1219	54	94	221	25	32	32	41	66	49
	Camphen	82	226	17	33	22	12	32	17	0	0	0
	b-Pinene	90	210	8	18	28	7	16	13	22	62	36
	Tricyclen	43	116	9	20	15	5	12	0	0	0	0
	Limonene	33	68	5	9	12	0	0	0	0	0	0
	Phellandren	6	12	0	0	0	0	0	0	0	0	0
	Myrcen	9	20	0	0	0	0	0	0	0	0	0
	Thujen	15	38	0	0	0	0	0	0	0	0	0
	Terpinolen	22	28	0	0	0	0	0	0	0	0	0

Table 8. The amount of VOCs as propane-equivalent concentration (ppm) in the containers as detected by GC/FID

Time (h)	α-pinene	Limonene
2	1 900	200
4	2 000	220
24	3 400	510

For all bacteria studied, either α-pinene or limonene (or both) were found to be effective in reducing bacterial survival. They have also been reported to have antibacterial effects in literature, however, only in liquid form (Dorman and Deans, 2000, Mourey and Canillac, 2002). As the amount of both of these monoterpenes was highest in pine HW, they might partly explain the superior antibacterial properties of pine HW VOCs compared to VOCs from other wood types.

Some aldehydes present in essential oils have been reported to have antibacterial properties (Moleyar and Narasimham, 1992, Kim et al., 1995, Kubo et al., 1995). The synergistic effect of single aldehydes on bacteria have been found, for example, with cinnamic aldehyde and eugenol (Moleyar and Narasimham, 1992), and this could also be an important factor in these results. The aldehydes found in the wood VOCs are mostly different substances, but they could likewise contribute to the antibacterial effects. Ethanol, which is commonly known to be very effective against bacteria, was found in the emissions of both SWs. Nevertheless, the SWs were not found to have as great an antibacterial effect as the HWs, where no ethanol was found.

5 CONCLUSIONS

The hygienic properties of wood are important in the interior environment and in developing wood products for hygienically challenging end-uses. The antibacterial properties of Scots pine and Norway spruce were studied to determine the causal agents for these properties. Wood was studied both as a solid surface and as separate components, such as extracts, lignin, cellulose, hemicellulose and VOCs, in order to separate the effects caused by each component.

The methods for achieving the aims were developed partly based on methods used in earlier studies. The novel features of this study were the use of small enough sample sizes to be able to shake samples in a test tube and comparing wooden surfaces with glass surfaces. Also, the use of the model surfaces in bacterial testing has not been done earlier. Finally, the test setup for VOC testing was developed specifically for these studies.

Both pine and spruce surfaces were found to have an antibacterial effect. As we expected, the extractives were found to be one of the main causes of this antibacterial effect, however not the only one. Even extracted wood surfaces showed antibacterial properties. It is possible that some extractives were left on the surface but the effect could also be caused by lignin, which showed antibacterial properties as a model surface. Cellulose and hemicellulose showed no antibacterial properties.

The wood extracts were studied separately against several bacterial strains. The results showed promising effects against, among other things, two nosocomial strains, MRSA and VRE, but only pine SW had any effect on *E. coli*, and that only very weakly. The effect against MRSA was also found when studying the extracts as surfaces. The extracts consist of a large combination of compounds. The antibacterial effect could derive from various compounds with antibacterial properties or from a synergistic effect of several compounds.

The antibacterial effects of VOCs were clear for some bacterial strains but negligible for others. The amount of VOCs employed in this study is clearly higher than that normally occurring in indoor air. In terms of this study of VOCs in general, however, it is a new approach to studying the positive effects of wood VOCs on indoor air.

In all studies, pine was more antibacterial than spruce, although spruce was also found to have an antibacterial effect against most bacterial strains tested. Pine heartwood and sapwood differed from each other, pine heartwood had stronger antibacterial effects in the tests with VOCs and as extract surfaces. Somewhat contrary results were obtained in the tests with solid wood surfaces and extracts studied in the broth where sapwood was more

antibacterial or equal to heartwood. Spruce heartwood and sapwood were studied separately only in the VOC tests and the differences between the two were small, spruce heartwood being slightly more antibacterial.

The results of this thesis open several interesting areas for future studies. As the tests were only made with non-surface treated wood, further research could investigate whether it is possible to apply surface treatment without losing the antibacterial properties, or possibly develop surface treatments with antibacterial components from wood. In this thesis, only new wood was utilized and further research is needed to determine how aging affects antibacterial properties. Wooden surfaces degrade with time, the volatile matter evaporates into the surrounding air and its chemical composition changes. Hence, it is not known how long wood surfaces remain antibacterial. The methods developed and the results with the extracts provided a good ground for future studies in exploiting the promising antimicrobial properties found in this study. Nosocomial bacteria are a growing problem in hospitals and other public areas where the most vulnerable of society's members congregate, such as care homes and daycares. Further research into substances and materials that can provide more hygienic working and living environments will become more critical, especially with the rise of bacterial pathogens with antimicrobial resistance.

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