Active Anti-Microbial Effects of Larch and Pine Wood on Four Bacterial Strains

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Active anti-microbial effects of larch (*Larix decidua* Mill.) and pine (*Pinus sylvestris* L.) wood materials on *Staphylococcus aureus*, *Pseudomonas aeruginosa, Enterococcus faecium*, and *Bacillus subtilis* were tested. The agar-diffusion test, a method used in routine diagnostics, was implemented to detect anti-microbial effects of wooden discs and filter paper discs containing methanol extracts of different wood parts. The results showed that the bark of larch had an inhibitory effect on *Staphylococcus aureus*, and the heart wood of pine showed a significant anti-microbial effect on the gram-positive bacteria tested (*Staphylococcus aureus, Enterococcus faecium*, and *Bacillus subtilis*). These results were confirmed by using methanol-extracts. An anti-microbial activity against *Pseudomonas aeruginosa* was not found. Anti-bacterial effects of other parts of larch wood and of pine sapwood were also not found. The results of this study showed for the first time that certain parts of wood contain compounds that directly reduce microbial growth. These data are a further demonstration of the positive effects of specific wood species and could promote the usage of wood in hygienically sensitive areas.

Keywords: Agar-diffusion test; Anti-microbial effect; Pine; Larch; Bacteria; Bark; Knots; Methanol-extraction

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INTRODUCTION

Wood is an important building material and is used in many consumer products. However, its use in hygienic sensitive places, such as hospitals and the food industry, has been discussed over the past years. The use of wooden and plastic cutting boards in the meat industry has been studied in detail. From the fifties to the nineties, wood was described as unhygienic (Großklaus and Levetzow 1967; Gilbert and Watson 1971; Abrishami *et al.* 1994; Boucher *et al.* 1998) and was banned from hygienic sensitive places (Stiebing 2002). More recently, studies have shown that many wood species have better hygienic properties when compared to plastic surfaces and had been wrongly degraded as unhygienic (Schönwälder *et al.* 2002; Strehlein *et al.* 2004; Milling *et al.* 2005; Filip *et al.* 2010).

Anti-microbial effects could be divided into passive and active effects, while these terms have not been commonly differentiated in many publications.

Positive anti-microbial effects of wood are ascribed to passive effects, such as the hygroscopicity and the associated dehydration of bacteria (Schönwälder *et al.* 2000; Prechter *et al.* 2002; Gehrig *et al.* 2000). Moreover, some studies have shown that wood might contain anti-microbial substances, directly inhibiting the growth of micro-organisms (Johnston *et al.* 2001; Välimaa *et al.* 2007; Sequeira e*t al.* 2009).

Several studies have shown that, after different times of incubation, the growth of bacteria on wooden cutting boards was reduced, and this effect was dependent on the kind of wood. These effects were not observed on plastic cutting boards (Ak *et al.* 1994; Gehrig *et al.* 2000; Schönwälder *et al.* 2002; Strehlein *et al.* 2004). Moreover, Gehrig *et al.* (2000) showed that the humidity of wood plays an important role in the absorption and dehydration of bacteria: the drier the wood, the better the passive anti-microbial effects.

The active anti-microbial effect of pine wood is described in the literature by different research groups. Rauha *et al.* (2000) showed in their study that pine phloem extract has an effect on *S. aureus.* The positive effect of pine was confirmed in other studies (Strehlein *et al*. 2004; Milling *et al*. 2005; Wirmer 2005; Schuster *et al*. 2006; Fürst 2007; Boursillon and Riethmüller 2007; Välimaa *et al.*, 2007). Considering larch wood, very few investigations have been performed. Schönwälder (1999) showed that *Escherichia coli* growth could not be detected after 24 to 30 h of incubation on larch and pine chippings, whereas growth did occur on beech, maple, and spruce samples. Milling *et al.* (2005) showed that bacterial survival was shorter on pine sawdust than on sawdust of larch or maple, or on plastic chips.

In most studies, the passive and active effects were not differentiated, and the observed data are therefore a mixture of both. The aim of the current study was to determine the active effect of the chosen wood species, larch and pine, on four bacterial strains. In order to test these active effects, methods were used that are commonplace in the field of clinical microbiology. The choice was made to use the agar-diffusion test, a method that is also used for the analysis of antibiotic resistance or susceptibility. Larch and pine wood were chosen because of their durability against wood decay (larch) (Grosser and Zimmer 1998; Windeisen *et al*. 2002) and the known anti-microbial properties (pine).

EXPERIMENTAL

Materials

Wood species and materials

The experiments were performed using wood discs with a diameter of 10.00 (± 0.01) mm and a thickness of 5.00 (±2.00) mm and sawdust of the heart- and sapwood of Scots pine (*Pinus sylvestris* L.) and larch (*Larix decidua* Mill.). In addition, bark and knots materials from larch trees were also used for this study. The larch wood was obtained from different 110 to 140 year old trees of the Salzkammergut in Austria. The trees grew at 800 to 1300 m above sea level and were cut down in September 2012. The exact growing and cutting conditions of the pine wood are not known. Both wood types had a humidity of about 12% after drying. Test materials were produced under very clean but not sterile conditions.

Bacterial strains

In this study, four American Type Culture Collection (ATCC®) strains of three gram-positive and one gram-negative bacteria were used. All strains were chosen because of their importance in clinical diseases or their ability to sporulate. The bacteria used for this study were *Staphylococcus aureus* subsp. *aureus* (ATCC® 25923) (*S. aureus*), *Pseudomonas aeruginosa (*ATCC® 27853) (*P. aeruginosa*), *Enterococcus faecium* (ATCC® 6057) (*E. faecium*), and *Bacillus subtilis* subsp. *spizizenii* (Nakamura *et al.* 1999) (ATCC® CRM6633™) (*B. subtilis*). The frozen bacterial strains were thawed and grown on trypticase soy agar with 5% sheep blood (TSS, bioMérieux) for 18 to 24 h at 37 °C. The cultures were kept at room temperature and inoculated on new agar plates after one week. After a maximum of four weeks, the strains were discarded and a new aliquot was thawed and cultured.

Methods

Extraction

The use of methanol (MeOH) extracts was based on the study of Sequeira *et al*. (2009) with some modifications. In brief, MeOH-extracts of sawdust and wood plates were produced for each kind of wood. Only the wood samples that showed a positive effect in direct testing with wood discs were chosen for the extraction procedure. Extraction was performed by adding 1 g of wood (sawdust or plates) to 10 mL of MeOH for 24 h at room temperature. After incubation, the extract was pre-filtered using filter paper and then sterile filtered using 0.22 µm MILLEX® GV filters (MILLIPORE). This procedure was performed under a sterile laminar air flow bench. The extracts were kept shielded from light in the refrigerator at 4 to 7 °C until use.

Detection of the anti-microbial effects of wood materials

For the detection of the anti-microbial properties of larch and pine wood, the agar-diffusion test was used. The active effect was tested with wood discs and MeOH-extract placed on filter-paper discs. For testing the extracts, round discs with a diameter of 5.50 mm were perforated out of sterile blotting paper. Afterwards, different amounts of each extract (25 µL and 50 µL) were pipetted onto the discs, and these were than dried on an open, sterile petri-dish for 24 h at 37 °C in an incubator. After the drying process, the discs were directly used for the agar-diffusion tests.

For the agar-diffusion test, inoculums with an optical density of 0.53 (± 0.03) in 0.45% sterile NaCl were prepared via densitometry (DensiCHEK, bioMérieux). After inoculating the Müller Hinton agar (Bio-Rad), the wood or MeOH-extract discs were placed on the plate. Thereafter, the plates were incubated at 37 °C for 18 to 24 h. *B. subtilis* was plated out on Müller Hinton agar with horse blood (OXOID). After incubation, the plates were evaluated by measuring the zone of inhibition in mm. Each test was performed on three days and in triplicate on each day for each strain and test material (N=9). As a positive control, two antibiotic discs (OXOID) specific for each strain were used on each experimental day. Antibiotic discs of Penicillin (10 µg) and Teicoplanin (30 µg) were used for agar-diffusion tests with *S. aureus*. Meropenem (10 µg) and Gentamycin (10 µg) were used for testing with *P. aeruginosa* and Linezolid (30 µg) and Vancomycin (30 µg) were used in tests with *E. faecium*. As positive controls for *B. subtilis* the antibiotics Clindamycin (2 µg) and Ciprofloxacin (5 µg) were used. Additionally, dried filter paper discs with MeOH only were included as a negative control for the experiments with the extracts.

Data analysis

For statistical analysis the Chi-Quadrat-test, the Fisher-test and the Mann-Whitney-U-test were used. Detailed information about these statistical tests is given by Hartung *et al.* (2002). Statistics was performed using the Microsoft Office Excel 2007 Software.

RESULTS AND DISCUSSION

Wood discs and sawdust were tested for contamination before performing the agar-diffusion test. Germs were identified using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Vitek® MS, bioMérieux). As the identified bacterial strains were mostly ubiquitous environmental bacteria and skin commensals, interactions with the chosen ATCC® strains could be ruled out.

Discs of Various Wood Materials

Inhibitory zones were detected by a novel method using direct measurements on the plates. Images were taken from all plates. Figure 1 shows a representative example of *S. aureus* exposed to pine wood discs.

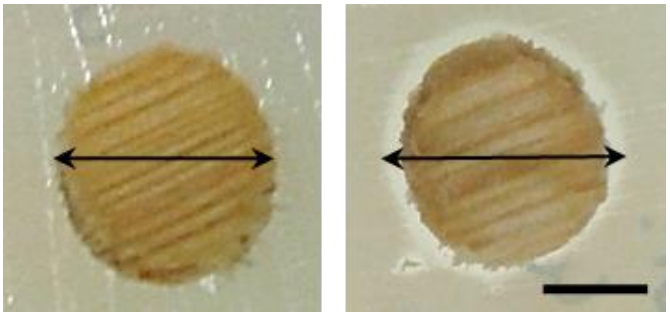

Fig. 1. Representative photographic results of the effects of pine sapwood (left image) and pine heartwood (right image) on the growth of *S. aureus*. Arrows indicate the measured diameter used for data analysis and statistics. Bar = 5.0 mm.

The results of agar-diffusion test with sap-, heart- and knot-wood of larch trees showed no anti-microbial effects against any of the four bacterial strains tested. Larch bark discs did not inhibit the growth of *B. subtilis*, *E. faecium*, or *P. aeruginosa.* In contrast, larch bark inhibited the growth of *S. aureus*, but the detected inhibiting zones varied strongly in size.

Not all bark discs affected the growth of *S. aureus*. In addition, some incomplete inhibition zones could be detected, whereas complete inhibition zones also were observed. An explanation for the variety of reactions might be the different concentrations of active substances in the larch trees used (Windeisen *et al.* 2002). The concentration of substances in a single larch tree also fluctuates depending on the part used (Windeisen *et al.* 2002). Despite this variation in the strength of the anti-microbial effect, larch bark showed a highly significant anti-microbial effect against *S. aureus* ($p=0.0003$, Fisher-test).

In tests with pine sapwood no anti-microbial effect against any of the four bacterial strains was detected. In contrast, the use of pine heartwood resulted in definite anti-microbial reactions for *S. aureus*, *B. subtilis*, and *E. faecium* (Fig. 2). It is expected

that the multi-resistant variants of *S. aureus* and *E. faecium* (MRSA and VRE) react in a similar manner when exposed to pine heartwood.

Pine heartwood had no effect on *P. aeruginosa*. The only gram-negative bacterial strain tested was *P. aeruginosa.* Therefore, the preliminary data presented might indicate that there is a difference in the response to certain substances in the wood between gram-positive and gram-negative, non-fermenting bacteria. Further studies, including more bacterial strains, should be performed to test the validity of this hypothesis.

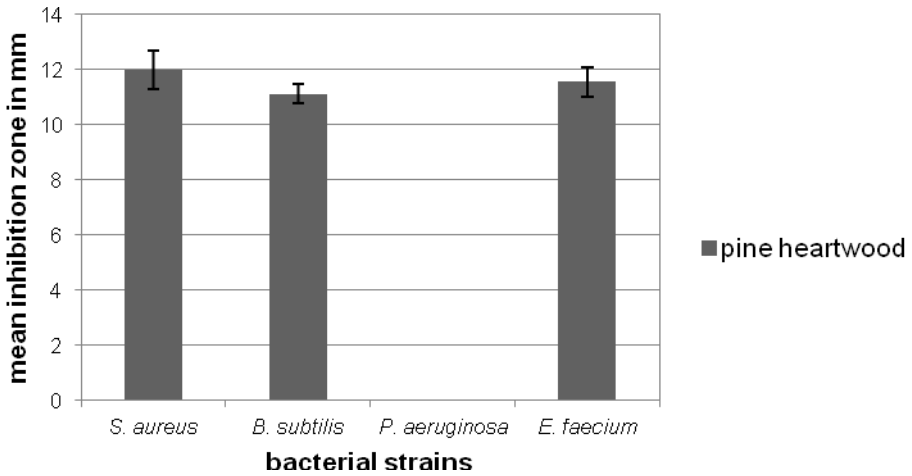

Fig. 2. Mean zones of inhibition (+/- SD) caused by exposing the four bacterial strains to pine heartwood (N=9)

As direct agar-diffusion tests with wooden discs showed anti-microbial effects for specific wood parts, additional tests were performed with MeOH-extracts. The direct comparison of the wood discs with the MeOH-extracts is a novel approach. These tests were applied to determine whether the detected effects with pine heartwood and larch bark were due to a diffusion of active anti-microbial substances from the wood into the agar. The hypothesis was that using MeOH-extract discs would eliminate the possibility that hygroscopic (passive) effects of the wood induced the inhibitory zones. Similar effects caused by the blotting paper itself were excluded by testing blotting paper that did not contain MeOH-extracts.

MeOH-extracts

MeOH-extracts were prepared only from the wood samples that induced an anti-microbial effect on at least one bacterial strain. The results showed that all of the anti-microbial effects found when testing solid wood could be confirmed by using the extracts. The included positive controls (antibiotic discs as mentioned above for each strain) were within the range of reference. Negative controls (discs with MeOH only) did not induce an inhibitory zone. A representative example of bacteria exposed to MeOH extract discs is shown in Fig. 3.

Since the growth of *S. aureus* was shown to be inhibited by incubation with larch bark and pine heartwood, this bacterial strain was tested with sawdust- and wood discs-extracts of both kinds of wood.

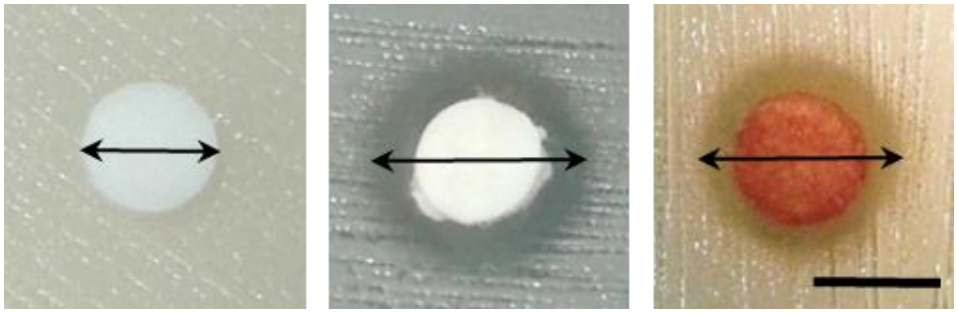

Fig. 3. Representative photographs of the agar-diffusion test with different MeOH-extracts on *S. aureus.* Left image is the negative control (MeOH only), the middle image depicts the results obtained with 50 µl of pine heartwood sawdust-extract. The image on the right side shows the results obtained with 50 µl of larch bark sawdust-extract. Arrows indicate the measured diameter used for data analysis and statistics. Bar = 5.5 mm.

Testing with larch bark wood disc-extracts did not affect *S. aureus* growth at all, independent of the amount of extract used. In contrast, using 25 µL of larch bark sawdust-extract resulted in an inhibition zone of 7.00 mm (SD=0.00). The use of a double volume of extract increased the mean inhibition zone to a value of 8.39 mm (SD=0.49) (Fig. 4). These preliminary results of the experiments show that the way the wood is processed plays an important role for a successful extraction of anti-microbial active substances out of larch bark. Due to the large freely available surface of sawdust, anti-microbial substances were dissolved more easily. In addition, the results obtained with the extract was more consistent when compared to those using wood discs, because the inhomogenous distribution of anti-microbial compounds in the wood does not play a role when the extracts are tested.

The active anti-microbial effects of pine heartwood on *S. aureus* showed that sawdust- and wood disc-extracts did not induce statistically significant differences in the inhibitory zones. Inhibitory zones were increased when using sawdust-extracts from 8.50 mm (SD=0.50) when using 25 µL to 10.11 mm (SD=0.78) when using 50 µL of extract (Fig. 4). Inhibitory areas increased when testing with wood disc-extracts from 8.22 mm (SD=0.44) for 25 µL extract to 9.94 mm (SD=0.17) for 50 µL of extract.

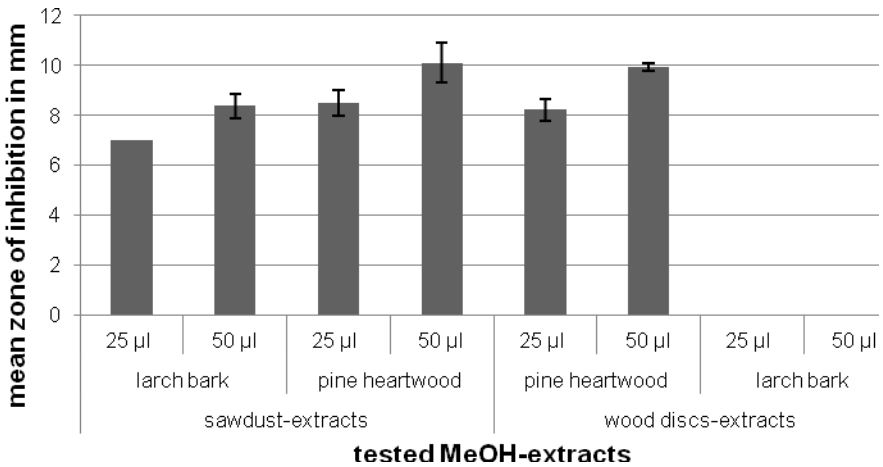

Fig. 4. Results (mean +/- SD) of the agar-diffusion tests with *S. aureus* and different MeOH-extracts (N=9).

Extracts of pine heartwood also had an active anti-microbial effect on *E. faecium* and *B. subtilis* (Fig. 5). No statistically significant differences between sawdust- and wood disc-extracts were found for both germs. Concerning *E. faecium*, a mean inhibitory zone of 6.89 mm (SD=0.22) (25 µL) and 7.42 mm (SD=0.47) (50 µL) when using sawdust-extracts was found (Fig. 5). Using wood disc-extracts showed an inhibitory zone which increased from 6.67 mm (SD=0.25) to 7.33 mm (SD=0.50). Using 25 µL extract, *B. subtilis* showed an inhibition area of 6.31 mm (SD=0.24) for sawdust-extract and 6.17 mm (SD=0.25) for wood disc-extract. Doubling the extract amount to 50 µL resulted in an increase of the diameters of inhibition to 7.08 mm (SD=0.48) (sawdust-extract) and 7.28 mm (SD=0.34) (disc-extract).

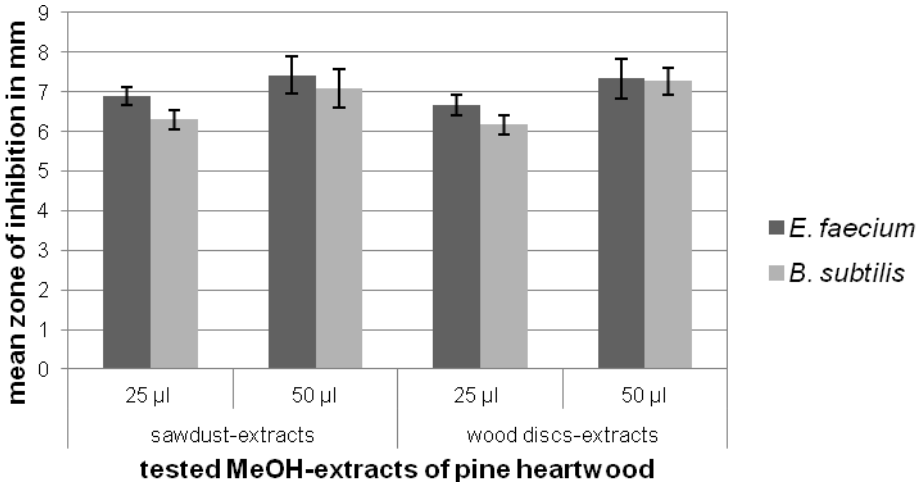

Fig. 5. Results (mean +/- SD) for the analysis of inhibitory zones when testing *E. faecium* and *B. subtilis* with MeOH-extracts of pine heartwood

The data showed that the anti-microbial effects of the wood extracts were concentration dependent in all cases. Moreover, the relative inhibition zones found for the extracts were larger than those found for the solid wood discs. This is most likely due to an easier diffusion of the anti-microbial compounds into the aqueous agar after extraction with MeOH. The data presented show that compounds in certain parts of wood are responsible for the inhibition of the bacterial growth. Analysis of these compounds will be part of future studies.

The results of the study prove that specific parts of pine and larch wood have an active anti-microbial effect on specific bacterial strains.

CONCLUSIONS

1. Heartwood of Scots pine has an active anti-microbial effect against the chosen gram-positive bacteria: *E. faecium, B. subtilis*, and *S. aureus*.
2. Sapwood of Scots pine did not show significant active anti-microbial effects on the bacteria investigated.
3. Larch bark has anti-microbial properties against *S. aureus*, but these effects are strongly dependent on the chosen piece of wood.

4. The data obtained with the MeOH extracts combined with the data obtained with the wood discs showed that the observed effects are due to an active effect of wood compounds. Therefore, the agar-diffusion test is an appropriate method to analyze these active anti-microbial effects.

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