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The use of wood in practice – a hygienic risk?

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Abstract The survival of bacteria on wood was investigated in laboratory experiments using naturally occurring and hygienically relevant bacteria and in a trial performed in a meat factory. Different types of wood dust (pine, larch, maple) and polyethylene chips were inoculated with chicken manure to study the interactions of bacteria and wood. The survival of the different bacteria from chicken manure was followed by plating on selective media. In addition, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA fragments from directly extracted DNA was used to precisely analyze changes in the relative abundance and composition of the manure-derived bacterial community on wood and plastic. Bands of DGGE-community profiles which reflected changes in the composition of the bacterial community on wood and plastic were excised, re-amplified, cloned and analyzed by sequencing. A rapid decrease of CFU of manure-derived bacteria was observed on pine-wood which correlated with the decreased amount of DNA extracted from pine-wood. Bacteria applied on larch, maple and plastic survived longer than on pine. Investigations have shown that some bacterial groups, e.g. enterobacteria, are more affected by wood and plastic than other bacteria. Furthermore, our results indicated that extractives from different wood types influenced the survival of bacterial species differently. Better hygienic characteristics of pine-wood compared to plastic as a material for transportation pallets were also observed in preliminary tests under practice conditions during a 4-week test in a meat processing factory.

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Stellt der praktische Einsatz von Holz ein hygienisches Risiko dar?

Zusammenfassung Das Überleben von Bakterien auf Holz wurde in Laborexperimenten mit natürlich vorkommenden und hygienisch relevanten Bakterien sowie im Rahmen eines Versuches in einer Fleischfabrik untersucht. Verschiedene Arten von Sägemehl aus Kiefer, Lärche und Ahorn sowie Chips aus Polyethylen wurden mit Hühnergülle inokuliert, um die Wechselwirkungen zwischen Bakterien und Holz zu studieren. Das Überleben der Bakterien aus der Gülle wurde durch Plattieren auf Selektivmedien verfolgt. Zusätzlich wurde die denaturierende Gradientengel-Elektrophorese von PCR-amplifizierten 16S rDNA-Fragmenten aus direkt extrahierter DNA angewendet, um Veränderungen in der Abundanz und der Zusammensetzung der bakteriellen Gemeinschaft aus der Gülle auf Holz und Kunststoff präzise zu analysieren. Banden in den DGGE-Profilen, die Änderungen in der Zusammensetzung der bakteriellen Gemeinschaft auf Holz und Kunststoff reflektieren, wurden aus dem Gel ausgeschnitten, kloniert und sequenziert. Eine rasche Abnahme der Keimzahl von Güllebakterien wurde auf Kiefernholz beobachtet, die mit einer abnehmenden Menge an aus Kiefernholz extrahierter DNA korrelierte. Die Bakterien überlebten länger auf Lärchen- und Ahornholz und auf Kunststoffchips als auf Kiefernholz. Die Untersuchungen haben gezeigt, dass einige Bakterienarten, z.B. Enterobakterien, sensitiver auf Trocknung und Holzinhaltsstoffe reagieren als andere. Des Weiteren lassen die Ergebnisse vermuten, dass Inhaltsstoffe verschiedener Holzarten Bakterienarten unterschiedlich beeinflussen. Die besseren Hygieneeigenschaften von Kiefernholz im Vergleich zu Kunststoff als Material für Transportpaletten wurden in einem 4-wöchigen Vorversuch unter Praxisbedingungen in einem Fleisch verarbeitenden Betrieb demonstriert.

1 Introduction

Although wood has been a traditional material for many applications in the food industry due to its stability, durability, elasti-

city at low temperatures, and slip resistance the debate about the hygienic eligibility of wood continues. Major reasons for the widespread negative attitude towards wood in the foodstuff sector are the risk of splinters, the porous structure of wood which can absorb and retain bacteria, and the insufficient power of cleaning and sanitation methods on wood. Several earlier studies demonstrated negative hygienic properties of wood since the bacterial load was always highest on wood compared to working surfaces made of metal (Kelch and Palm 1958) or cutting boards made of hard rubber (Großklaus and Levetzow 1967) and plastic (Rödel et al. 1994), respectively. Furthermore, when wood and plastic cutting boards were compared as to their bacterial adherence and removal potential, boards made of plastic were found to be more satisfactory (Gerigk 1966, Gilbert and Watson 1971, Borneff et al. 1988a, Borneff et al. 1988b, Schmidt 1989, Abrishami et al. 1994, Rödel et al. 1994). On the basis of these results EU regulations were developed that prohibit the use of wooden worksurfaces in areas where food is produced for public consumption, and only permit the transport of packed meat and poultry on wooden pallets. In contrast, American regulations permit the use of cutting boards made of maple or similar close-grained hardwood (Sanitation Performance Standards Compliance Guide 1999).

However, in the last decade several scientific studies concerning wood and hygiene have been performed which revealed new results. Experiments by Ak et al. (1994a) and (1994b) have been conducted with nine species of hardwoods, four plastic polymers, as well as hard rubber, and simulated conditions in a home kitchen. Results indicate satisfactory hygienic properties of wood because bacteria were not recoverable from wooden surfaces a short time after they were applied, unless very large numbers were used. Previous studies accomplished by our group with wooden boards and dust (Schönwälder 1999, Schönwälder et al. 2000, Schönwälder et al. 2002, Milling et al. 2005) showed that the hygienic properties of wood cannot be generalized because different wood types possess completely different hygienic characteristics. When wood was inoculated with *Escherichia coli* pIE639 and *Enterococcus faecium* the number of bacteria decreased fastest on pine and oak compared to larch, spruce, beech, maple, poplar and polyethylene. It was supposed that the antibacterial characteristics of pine and oak are caused by a combination of the hygroscopic properties of wood and the effects of wood extractives. Furthermore, the survival of the test bacteria was dependent on factors such as the moisture content of wood, humidity and ambient temperature, the type of the test bacteria and the initial inoculum size. The hygienic properties of wood, plastic and steel were also investigated in the study "Wood in the Food" initiated by the Nordic countries (Koch et al. 2002). Also this study revealed that bacteria survived considerably longer on plastic and stainless steel than on wood. Again, pine and oak displayed hygienic advantages compared to other wood species like beech, ash and spruce and plastic after contamination with *Pseudomonas fluorescens* and *Bacillus subtilis*.

Based on these more recent findings, this study aimed to investigate the microbiological status of wood under conditions closer to the practice. Contamination of dust from pine, larch or maple, and plastic chips with a pool of naturally occurring bacteria along with organic matter was simulated in the laboratory by inoculating those materials with chicken manure. The fate of manure-derived bacteria applied on sawdust from different woods or plastic chips was monitored using cultivation-dependent and cultivation-independent DNA-based methods. Furthermore, to evaluate the hygienic performance of pine-wood which exhibited strong antibacterial characteristics in previously performed lab experiments (Schönwälder 1999, Schönwälder et al. 2002, Milling et al. 2005) a first practical trial was carried out. Special transportation pallets made of pine-wood (HHP) were used in a meat processing factory and compared with pallets made of plastic. The degree of contamination of the different pallets used for four weeks and subsequently stored for 5 days was determined by agar contact plates.

2 Material and methods

2.1 Test of wooden dust and plastic chips in the laboratory

Material. Scots pine (*Pinus silvestris* L.), European larch (*Larix decidua* Mill.) and Sycamore maple (*Acer pseudoplatanus* L.) were tested. The coniferous species represent commonly used European tree species. Maple was selected as a representative of deciduous species frequently used for cutting board production. Furthermore, referring to previous studies performed with single laboratory strains pine was chosen as representative of wood with excellent antibacterial properties, larch showed variable antibacterial characteristics and experiments with maple revealed no antibacterial potential (Schönwälder et al. 2002, Milling et al. 2005). Samples derived from trees of a diameter of 15 to 25 cm at breast height (dbh) grown in forests in the immediate vicinity of Braunschweig in Northern Germany. To obtain results that are more independent of the physical properties of wood, such as capillary properties, orientation of wood fibres or the water retention capacity, we prepared sawdust of the appropriate woods. The trees were cut into 2 cm thick boards longitudinally, air-dried at ambient temperatures for 6 to 12 months and converted into sawdust using a circular saw. Experiments were made with mixed samples of heartwood and sapwood in heartwood-forming species (pine and larch) and with mixed sapwood samples taken across the wood diameter in maple. The proportion of heartwood averaged 60%–70% in pine and larch samples. Polyethylene chips (5 × 10 mm, 0.5 mm thick) served as reference material.

Bacteria. Wooden sawdust and plastic chips were inoculated with chicken manure representing a pool of hygienically relevant bacteria. Manure was prepared by resuspending chicken faeces with sterile saline to obtain a liquid suspension of the faeces subsequently called chicken manure. Chicken faeces originated from a small biological farm close to Braunschweig.

Inoculation. The sawdust was dried and disinfected for at least 12 h at 103 °C, polyethylene chips were treated under UV light for 2 h prior inoculation. The cell density of the inoculum was adjusted by diluting the chicken manure with 0.85%(w/v) NaCl. Fifty g of each wooden sawdust and polyethylene chips were

treated by spraying with 0.5 $ml g^{-1}$ of an appropriately diluted manure to achieve an initial inoculum level of approximately 5×10^6 CFU g^{-1}. The samples were incubated at room temperature. The experiment was performed with two replicates per treatment.

Extraction of bacterial cells and bacterial counts. Wooden sawdust and plastic chips (3 g) were transferred to sterile plastic bags, mixed with extraction buffer (0.85%(w/v) NaCl, 0.1%(w/v) Bacto-Tryptone, 0.1%(v/v) Tween 20 [Merck, Darmstadt, Germany]) in a 1:10 relation, and treated in a Stomacher Blender (Seward Medical, London, UK) for 3 min at 260 rpm to dislodge the adhering bacteria. To determine the titer of the total colony forming units per gram of sample (CFU g^{-1}), serial dilutions were plated onto Plate Count Agar (Merck). In addition, selective media were employed to follow the survival of different hygienically relevant groups of bacteria such as enterobacteria on MacCONKEY agar, enterococci on Membrane-filter enterococcus agar base according to SLANETZ and BARTLEY, and streptococci on Streptococcus selective agar (Merck). To suppress growth of fungi all media contained 100 $\mu g ml^{-1}$ cycloheximide. After an incubation period of 24–72 h at 37 °C the bacterial colonies were counted. Colonies grown on MacCONKEY agar were differentiated as described by Merck, and only colonies with morphology typically of enterobacteria were included.

DNA extraction and purification. The cell suspension obtained after Stomacher treatment was centrifuged at $10\,000 \times g$ for 30 min. Total community DNA was extracted from the bacterial pellet by the method of van Elsas and Smalla (1995) originally developed for DNA extraction from soil and successfully used for DNA extraction from wooden sawdust (Milling et al. 2005). The crude DNA was purified by glass milk (GeneClean Spin, Qbiogene, Carlsbad, CA) according to the manufacturers' instructions.

PCR amplification of 16S rDNA fragments for DGGE analysis (DGGE-PCR). Amplification of the bacterial 16S rDNA fragment was performed from the purified DNA extracts using the primer pair F984GC and R1378 as described by Heuer et al. (2001). The reaction mixture consisted of 1 µl of template DNA (1–5 ng) and a 24 µl aliquot of a PCR mix according to Heuer et al. (2001).

Community fingerprints by DGGE. DGGE analysis for 16S rDNA products was done with the DCode System DGGE apparatus from Bio-Rad, Inc. (Germany) as previously described by Heuer et al. (2001). Polyacrylamide gels were composed with a denaturating gradient of 26% to 58% and a gradient of acrylamide-*N*, *N*'-methylenbisacrylamide (37.5:1) of 6%–9%, 0.19% (v/v) TEMED, 0.053% (w/v) ammonium persulfate, and 1× TAE (Sambrook et al. 1989). Aliquots of PCR products were applied on the gel. DGGE was performed at 220 V in 1× TAE at 58 °C for 6 h. Gels were air-dried and scanned transmissively (EPSON Expression 1680 Pro, EPSON, Germany). The fingerprints were analyzed by the GelCompar 4.0 programe (Applied Maths, Belgium) with a slight modification of normalization settings (Smalla et al. 2001). A dendrogram was generated by using the Pearson correlation index for each pair of lanes within a gel and cluster analysis by the unweighted pair group method using arithmetic averages (UPGMA).

Cloning and sequencing of DGGE bands. Differentiating bands selected for further analysis by sequencing were excised from SYBR green (FMC, Valensbaek Strand, Denmark) stained DGGE gels containing *N*, *N*′-bis-acryl(yl)cystamine instead of *N*, *N*'-methylenbisacrylamide to improve the efficiency of DNA recovery, reamplified and cloned according to Heuer et al. (2001). Inserts of clones matching the selected bands of the DGGE bacterial community patterns were sequenced with the standard primers SP6 and T7. Each two clones with the same migration behaviour obtained from one band were sequenced to confirm the result. Data analysis was done with ARB software (Ludwig et al. 2004).

Nucleotide sequence accession numbers. Nucleotide accession numbers of the partial 16S rDNA sequences determined in this study can be accessed as NCBI gene bank entries (http://www.ncbi.nlm.nih.gov, bacterial sequences: AJ639857-AJ639859).

2.2 Hygienic properties of transportation pallets made of pine or plastic compared in use

Material and experimental setup. Two special hygienic pallets made of pine-heartwood (HHP) were prepared exclusively for the trials by "Gustav Wilms Holzverpackungen" (Bad Essen-Barkhausen, Germany) according to DIN 15146 (Euro-Pallet). In order to improve the absorption efficiency and the hygienic properties the pine-wood was treated by a special wash and dry procedure (European Patent: EP 1005964) of the above company (Schönwälder et al. 2002). Each of these pallets was compared with two randomly selected standard pallets made of plastic (H1) commonly used in that factory. The pallets were used in parallel for four weeks to transport products of a meat processing factory, mainly hackled raw meat packed in plastic boxes, and for their storage. The temperatures were adjusted to 10 °C in the processing area and 4 °C in the storage rooms. While the plastic pallets were regularly cleaned by water sprinkling, the pallets made of pine-wood were used without intermediate cleaning.

Sampling. Two pallets of both pine-wood and plastic were sampled by means of agar contact plates, once a week during their use and at three different times (3 h, 24 h, and 120 h) after their withdrawal from the production process. To prepare the contact plates Petri dishes were filled completely with Plate Count Agar (Merck) containing 100 $\mu g ml^{-1}$ cycloheximide, and a contact area (10 cm^2) slightly curved upwards developed. Bacteria were recovered by pressing the agar plate directly onto the surface of the tested areas for 10–20 s. A maximum of 400 single colonies per plate could be recorded. The bacterial titer was determined in four different areas of each pallet (1: at the top, 2: area in contact with the floor, 3: at a side, 4: below the top).

Table 1 Taxonomic distribution of bacteria isolated from used transportation pallets made of pine and plastic
Tabelle 1 Taxonomische Verteilung der von den Holz- und Kunststoffpaletten isolierten Bakterien

Taxonomic affiliation (most frequent genus or species of group)	Percentages of strains isolated			
	Pine 3 h	Pine 120 h	Plastic 3 h	Plastic 120 h
γ-Proteobacteria				
Pseudomonas (*P. putida*)	26.8	-	13.2	2.8
Stenotrophomonas (*S. maltophilia*)	19.5	36.7	35.8	19.4
Moraxellaceae (*Psychrobacter immobilis*)	2.4	4.1	3.8	8.3
Enterobacteriaceae (*Serratia sp.*)	4.9	-	5.7	-
Gram-positive high G+C (*Rhodococcus*)	19.5	14.3	11.3	27.8
Gram-positive low G+C (*Bacillus*)	17.1	20.4	7.6	13.9
α-Proteobacteria (*Ochrobactrum anthropi*)	-	4.1	1.9	2.8
Uncertain affiliation	9.7	20.4	20.7	25
No. of isolates	41	49	53	36

Fatty acid methylester (FAME) analysis. Aerobic isolates were identified by cellular fatty acid methylester (FAME) analysis. For each sample type (pallets made of pine and plastic, respectively) 50 colonies were randomly picked from the contact plates prepared 3 h and 120 h after the pallets' use in the factory. Bacterial isolates were purified by several transfers to Plate Count Agar, not all of them could be re-cultivated (Table 1). Pure cultures were streaked onto plates containing 30 g per litre of trypticase soy broth (BBL, Becton Dickinson Microbiology systems, Cockeysville, MD) supplemented with 15 g per litre of Bacto Agar (Difco Lab., Detroit, MI) in four quadrants. The plates were incubated for 24 h at 28 °C. Cell pellets from the streaks of the third quadrant of each plate were harvested, saponified, and methylated, and FAME profiles were analyzed by gas chromatography according to the standard procedure of the Microbial Identification system (MIS; MIDI Inc., Newark, NJ).

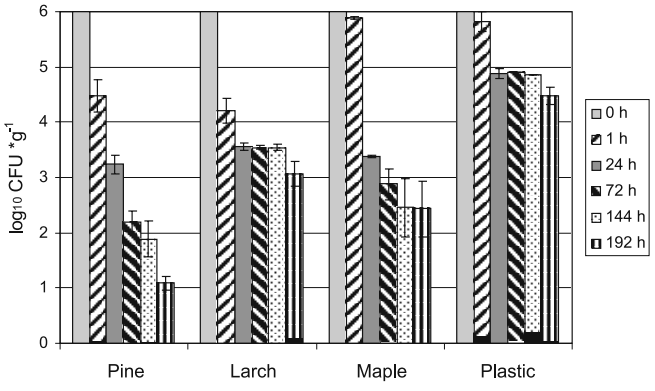


Fig. 1 Survival of manure-derived bacteria on wooden sawdust and plastic chips. Determination of total bacterial numbers. Incubation at RT = 21 °C and RH = 55% (RH: relative humidity)
Abb. 1 Überleben von Bakterien aus Hühnergülle auf Sägemehl und Plastikchips. Bestimmung der Gesamtkeimzahl. Inkubation bei RT = 21 °C und RH = 55% (RH: relative Luftfeuchte)

3 Results

3.1 Survival of manure bacteria on wooden sawdust and plastic chips

Wooden sawdust or plastic chips were inoculated with chicken manure to simulate the contamination with a pool of naturally occurring bacteria along with organic matter. Total plate counts on Plate Count Agar showed a drastic decrease in bacterial numbers on the wooden dust compared to the plastic chips (Fig. 1). The decline of the bacterial titer was fastest on pine followed by maple and larch. After eight days only 10 CFU g^{-1} were detected on pine sawdust corresponding to a log 5 reduction in bacterial numbers. The bacterial titer recovered from maple and larch was one to two orders of magnitude higher than on pine. Only little decline of bacterial numbers corresponding to log 1.5 reduction was observed on the plastic chips where also eight days after the inoculation 4.5×10^4 CFU g^{-1} were detected. In addition, the survival of enterobacteria, enterococci and streptococci that are hygienically relevant bacteria was followed by plating on selective media. Initial cell densities after inoculation derived from manure were determined and corresponded to 5×10^4 CFU g^{-1} for enterobacteria on MacCONKEY agar, 1×10^4 CFU g^{-1} for enterococci on Membrane-filter enterococcus agar base according to SLANETZ and BARTLEY and 5×10^5 CFU g^{-1} for streptococci on Streptococcus selective agar. Plate counts determined for pine-sawdust are presented in Fig. 2. Different bacterial species showed a completely different survival on the different materials tested. Enterobacteria showed the lowest survival rates on wooden samples followed by enterococci and streptococci. Even on plastic chips the bacterial titer of the enterobacteria decreased fastest, followed by streptococci. No enterobacteria could be cultured from pine and larch after 24 h and from maple after 72 h. In contrast, on plastic the decrease of culturable enterobacteria corresponded to a log 2.5 reduction and about 10^2 CFU g^{-1} were still recovered after eight days. Moreover, after 72 h no bacteria were cultured on media selective for enterococci from pine and larch, and after six days no bacteria were grown on media selective for streptococci, respectively. A decline in streptococci numbers corresponded to log 3.5 on maple and log 2 on plastic revealed after eight days. While

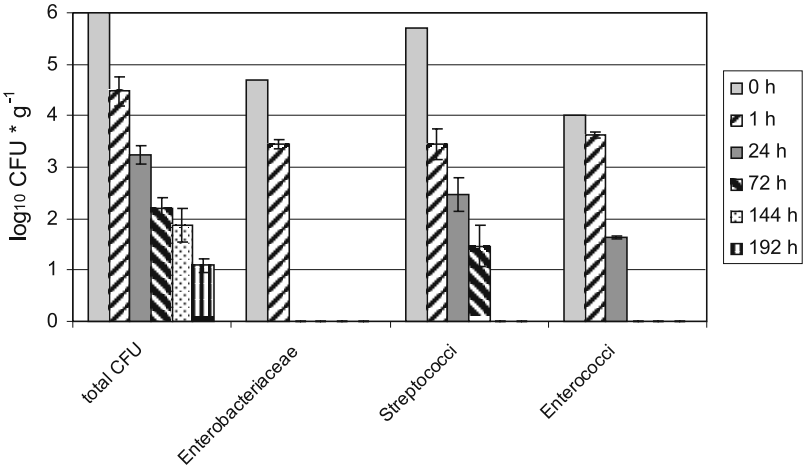


Fig. 2 Survival of manure-derived bacteria on pine-wood sawdust. Comparison of total and selective plate counts. Incubation at RT = 21 °C and RH = 55%
Abb. 2 Überleben von Bakterien aus Hühnergülle auf Sägemehl von Kiefernholz. Vergleich der Gesamtkeimzahl und der Keimzahl auf Selektivmedien, Inkubation bei RT = 21 °C und RH = 55%

the titer of culturable enterococci decreased on maple, and after eight days only 1.5×10^1 CFU g^{-1} were recovered, the titer of enterococci on plastic remained almost constant at a high level of 10^4 CFU g^{-1} over the whole sampling time of eight days (data not shown).

In parallel, DNA was extracted from wooden sawdust and plastic chips to detect the manure-derived bacteria independent from cultivation. The crude DNA was loaded on an agarose gel to check the quality and quantity of the DNA extracted. It became apparent that the DNA content of the manure-derived bacteria on the wooden material rapidly decreased. Already 24 h after inoculation no DNA signals were observed for pine, and after 72 h on larch and maple, respectively, because the DNA content fell below the detection limit of agarose gel electrophoresis. In contrast, clear DNA signals were obtained from the plastic chips at all sampling times (data not shown). To analyze changes in the composition of the bacterial community of manure on wooden sawdust and plastic chips, denaturing gradient gel electrophoresis (DGGE) fingerprints of 16S rDNA fragments amplified from total community DNA with universal eubacterial primers were generated. PCR products obtained from total community DNA after 1 h, 72 h and 216 h were compared, running 2 replicates of each treatment in parallel on two DGGE gels. The similarity of the DGGE patterns of the dominant Eubacteria was relatively low when comparing pine, larch, maple and plastic indicating that bacterial communities characteristic for the different materials tested were established (Fig. 3). Although variations between the replicates became apparent the majority of samples of pine, larch, maple and plastic, respectively, formed a separate cluster when the DGGE profiles were compared by UPGMA (Fig. 3b). At all sampling times, the patterns of larch, maple and plastic consisted of about 8 to 10 strong bands and a lower number of weak bands. DGGE profiles of pine obtained directly after inoculation were very similar to that of larch and maple. However, the DGGE patterns of pine DNA revealed clear shifts in the relative abundance of bacterial populations after 72 h. The intensity of bands and the diversity of bacterial populations were strongly reduced, and a change in the composition of the bacterial community was observed. Separate clusters were formed when the DGGE profiles of bacterial communities on pine sawdust were compared by UPGMA analysis one hour after inoculation with those after 72 h and 216 h. However, shifts of the relative abundance of bacterial populations were also detected on plastic chips. Especially the intensity of one band was increased over the time and the UPGMA analysis generated a separate cluster of DGGE patterns after 72 h and 216 h, respectively, compared to the profiles made immediately after inoculation. In contrast, no clear shifts of relative abundance over the time were observed in the DGGE profiles of maple and larch, but this can be partly also attributed to a relatively large variation between the replicates. Bands of DGGE community profiles reflecting the bacterial change on wood were excised from the gel and analyzed by sequencing (Fig. 3a). The sequence of band A showed the highest similarity to an uncultured gram-positive bacterium with high G+C content (98.9%) isolated from the cecum of broiler chickens (Zhu et al. 2002). This band was excised from pine-wood bacterial patterns 1 h after inoculation, because its intensity was strongly reduced 72 h after inoculation. Moreover, after 216 h this band was no longer detected in the DGGE profiles for pine. In contrast, a band with the same migration behaviour was observed in the DGGE profiles of larch, maple and plastic at all samplings analyzed. While band B represented a dominant band in the patterns of maple, larch and plastic at all sampling times this band was detected in the bacterial community patterns of pine only immediately after inoculation. The sequence obtained from band B showed the highest similarity to *Brachybacterium tyrofermentans* (99.3%) belonging to the Actinobacteria. The abundance of band C was drastically enhanced in the DGGE profiles obtained for plastic after 72 h and 216 h, respectively, and was not or much weaker observed in the patterns for pine, larch and maple. The sequence of the DNA from this band showed 100% similarity to *Moraxella* sp. (*Psychrobacter glacinola* 100%) belonging to the group of γ-Proteobacteria.

3.2 The microbiological status of transportation pallets in use

The hygienic status of the surface of transportation pallets made of pine-wood or plastic was compared under the conditions in a meat processing factory. Although a very high variability in the

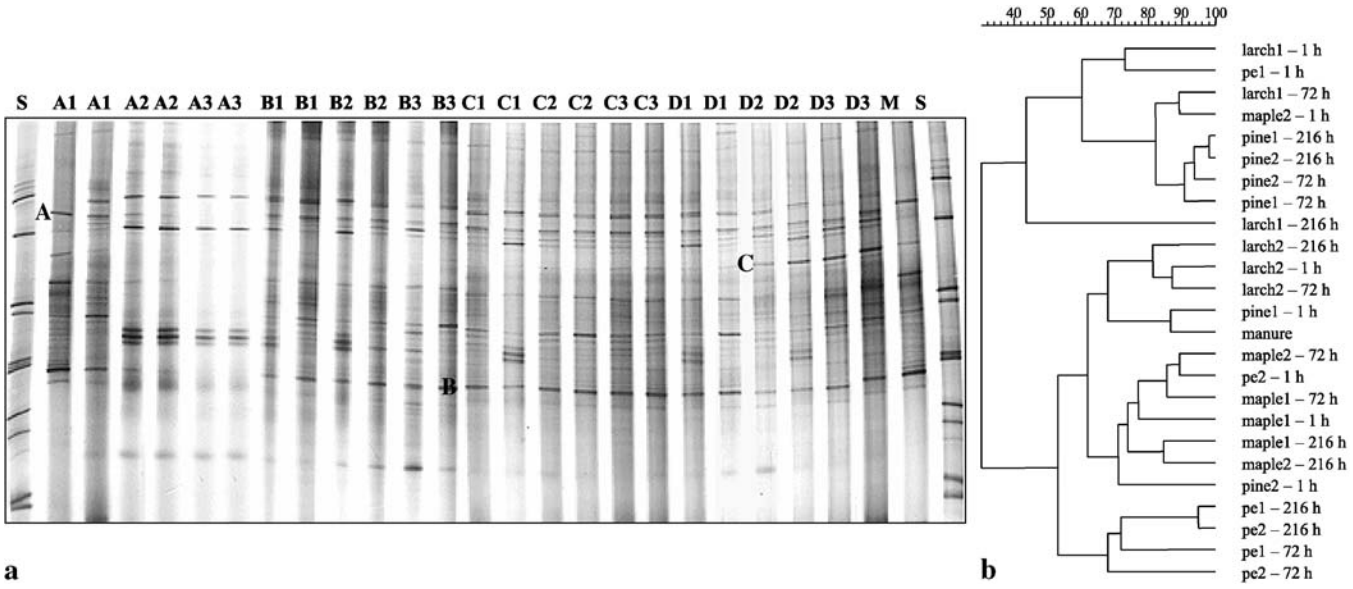
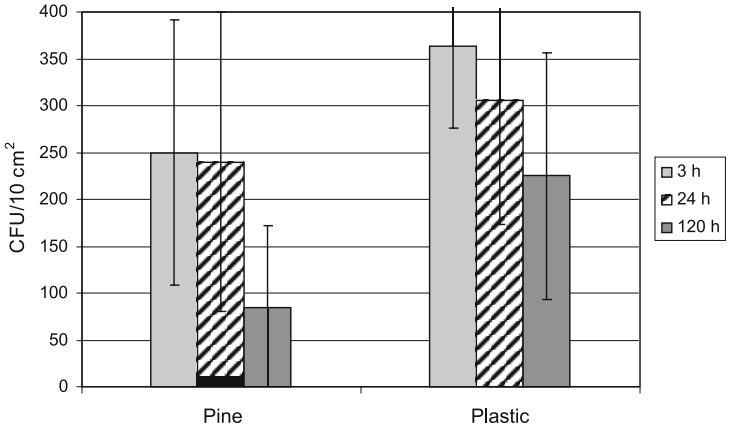


Fig. 3a,b DGGE profiles of amplified 16S rDNA fragments with primers F984GC and R1378 of total bacterial communities from wooden sawdust and plastic chips. The letters indicate bands that have been cut, sequenced and phylogenetically identified as A: uncultured gram-positive bacterium (98.9%), B: *Brachybacterium tyrofermentas* (99.3%), C: *Moraxella* sp. (100%). **a** (gel picture) The standard (S) was composed of 16S rDNA products generated from (in order of the migration distance): *Clostridium pasteurianum* DSM 525, *Erwinia carotovora* DSM 30168, *Agrobacterium tumefaciens* DSM 30205, *Pseudomonas fluorescens* R2f, *Pantoea agglomerans*, *Nocardia asteroides* N3, *Rhizobium leguminosarum* DSM 30132, *Actinomadura viridis* DSM 43462, *Kineosporia aurantiaca* JCM 3230, *Nocardiopsis astra* ATCC 31511, and *Actinoplanes philippiensis* JCM 3001, M: manure (inoculum), A: pine, B: larch, C: maple, D: polyethylene; samples were analyzed after 1: 1 h, 2: 72 h , 3: 216 h. **b** (dendrogram) The DGGE profiles were analyzed by the GelCompar 4.0 programme (Applied Maths, Belgium) and a dendrogram was generated by using the Pearson correlation index (UPGMA)

Abb. 3a,b DGGE-Profile von 16S rDNA-Fragmenten der gesamten Bakteriengemeinschaft von Sägemehl und Plastikchips amplifiziert mit den Primern F984GC und R1378. Die Buchstaben markieren ausgeschnittene Banden, die sequenziert und phylogenetisch identifiziert worden sind als A: unkultiviertes gram-positives Bakterium (98,9%), B: *Brachybacterium tyrofermentas* (99,3%), C: *Moraxella* sp. (100%). **a** (Gel) Der Standard (S) besteht aus den 16S rDNA-Produkten von (geordnet nach der Laufstrecke): *Clostridium pasteurianum* DSM 525, *Erwinia carotovora* DSM 30168, *Agrobacterium tumefaciens* DSM 30205, *Pseudomonas fluorescens* R2f, *Pantoea agglomerans*, *Nocardia asteroides* N3, *Rhizobium leguminosarum* DSM 30132, *Actinomadura viridis* DSM 43462, *Kineosporia aurantiaca* JCM 3230, *Nocardiopsis astra* ATCC 31511, and *Actinoplanes philippiensis* JCM 3001, M: Gülle (Inokulum), A: Kiefer, B: Lärche, C: Ahorn, D: Polyethylen; Probenanalyse nach 1: 1 h, 2: 72 h , 3: 216 h. **b** (Dendrogram) Die DGGE-Profile wurden mit GelCompar 4.0 (Applied Maths, Belgium) ausgewertet und ein Dendrogram unter Verwendung des Pearson-Korrelations-Index (UPGMA) erzeugt

Fig. 4 Average survival of bacteria on transportation pallets made of pine-heartwood and plastic in a meat processing factory. Recovery of bacteria via agar contact plates

Abb. 4 Überleben von Bakterien auf Transportpaletten aus Kiefernkernholz und Kunststoff in einem Fleisch verarbeitenden Betrieb (Mittelwerte). Detektion der Bakterien mittels Abklatschplatten



contamination degree of different areas of the pallets became apparent total contact plate counts made from pine-wood pallets were clearly lower on average compared to plastic pallets during the 4-week use for transportation (data not shown) and after the storage at room temperature without further contamination (Fig. 4). From plastic pallets on average 36 CFU cm^{-2} were recovered 3 h after withdrawal of the pallets from the production process. In contrast, bacterial numbers obtained from pine-wood pallets resulted on average in 25 CFU cm^{-2} only. The decline in bacterial numbers continued within the storage time on both, the wooden and the plastic pallets. However, the decrease of bacteria was more efficient on pallets made of pine, and clearly different numbers of bacteria were recovered from the different types of pallets after 120 h storage. While on the plastic pallets still

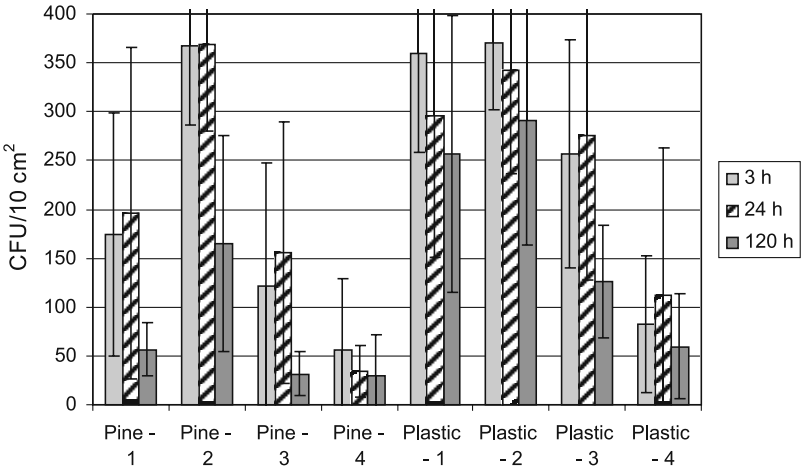


Fig. 5 Survival of bacteria on transportation pallets made of pine-heartwood and polyethylene in a meat processing factory. Recovery of bacteria via agar contact plates from the surfaces 1: at the top, 2: area in contact with the floor, 3: at a side, 4: below the top
Abb. 5 Überleben von Bakterien auf Transportpaletten aus Kiefernkernholz und Kunststoff in einem Fleisch verarbeitenden Betrieb. Detektion der Bakterien mittels Abklatschplatten von den Oberflächen 1: der Oberseite, 2: dem Fußbereich, 3: dem Seitenbereich, 4: unterhalb der Oberseite

22 CFU cm^{-2} were found only 8 CFU cm^{-2} were detected on the pallets made of pine. Obviously, very different contaminated areas were determined on the pallets. Areas in contact with the floor and at the top of the pallets showed the highest degree of contamination whereas areas at the side and below the top were less contaminated on both types of pallets (Fig. 5).

In order to get a first idea of the culturable bacterial species that are contaminating the pallets, 50 isolates were randomly picked per sample type and sampling time from contact plates (3 h after their last use in the factory and after 120 h storage at room temperature) and identified based on their FAME profiles. Isolates were considered positively identified by FAME analysis because of a similarity index of 0.3 or above. A diverse spectrum of species from the α- and γ-subdivision of Proteobacteria, and high- and low-G+C gram-positive bacteria was obtained from the wooden and the plastic pallets. However, not all the isolates picked could be identified by FAME analysis. The taxonomic distribution of all strains recovered from the pallets is shown in Table 1.

The strains isolated 3 h after the withdrawal of the pallets from the factory and after a 120 h storage of the pallets without further contamination most frequently belonged to the γ-Proteobacteria (for pine: 53.6% and 40.8% after 3 h, and for plastic: 58.5% and 30.5% after 120 h, respectively).

Although statistically significant differences in the species or genus composition of strains retrieved from pine and plastic pallets were not observed due to limited numbers of isolates analyzed, results indicate changes in the species or genus abundance of strains recovered from pine and plastic during the storage of the pallets without further contamination. In contrast to the samples taken 3 h after the last use of the pallets culturable *Enterobacteriaceae* could not be recovered from pine as well as from plastic pallets after 120 h of storage at room temperature. *Pseudomonas* sp. were not detected on pallets made of pine and only rarely found on plastic pallets after 120 h storage. The proportion of *Moraxellaceae* mainly represented by *Psychrobacter immobilis* was increased after 120 h whereas more isolates of this group were cultured from plastic pallets (8.3%) than from pallets made of pine (4.1%). In contrast, the survival of *Stenotrophomonas maltophilia* seemed to be not or only little influenced on the wooden and the plastic pallets. The proportion of gram-positive bacteria during storage was very stable on pine (36.6 and 34.7%, respectively) but the percentages of gram-positive bacteria on the plastic pallets increased noticeably during storage from an average of 17% after 3 h to 44.4% after 120 h.

4 Discussion

In this study we compared the hygienic properties of pine, larch and maple wood with plastic under conditions close to the practice. In contrast to previous studies which tested mainly the survival of axenic cultures, a complex mixture of bacteria with high titer and organic matter content was added to the test material. The good hygienic characteristics of pine previously observed in a number of studies (Schönwälder 1999, Schönwälder et al. 2002, Milling et al. 2005) could be also confirmed in this study. The titer of chicken manure bacteria decreased fastest on pine-sawdust compared to larch, maple and plastic. However, more time was required for the decline of the bacterial titer than in tests performed with single bacteria. While a log 8 decrease in CFU numbers was observed for *E. coli* pIE639 cells applied on pine-wood sawdust and a log 6 decrease in numbers of *E. faecium* cells within 24 h, respectively, in previous experiments (Milling et al. 2005), the decline in bacterial numbers on pine dust after inoculation with chicken manure resulted in a log 3 reduction after 24 h only in the present study. Micro-aggregates and nutrients might protect the bacteria from desiccation (Abrishami et al. 1994, Ak et al. 1994a, Ak et al. 1994b, Rödel et al. 1994, Gehrig et al. 2000) and the effect of wood extractives.

Interestingly, the CFU counts on enterobacterial selective medium decreased faster for all sample types than on the enterococcal and streptococcal selective media indicating that different members of the manure-derived bacterial community are differently affected. These data confirm the findings of earlier studies which showed a longer survival of the gram-positive *Enterococcus faecium* compared to the gram-negative *Escherichia coli* pIE639 on boards made of pine and plastic (Schönwälder et

al. 2002), and on pine-wood dust and plastic chips (Milling et al. 2005). We assume that the gram-positives are better protected against desiccation and attacks of antibacterial compounds due to differences in the cell wall of gram-negative and gram-positive bacteria. Also the study performed by Strehlein et al. (2004) demonstrated a faster decline in bacterial numbers of the gram-negative strains *P. aeruginosa* and *E. coli* on boards made of specially treated pine-heartwood (Schönwälder et al. 2002) compared to the survival rates of the gram-positive strains *E. faecium* and *S. aureus*. Contrary to these findings, results by Koch et al. (2002) indicated slightly higher survival rates of the gram-negative *Pseudomonas fluorescens* compared to the gram-positive *Bacillus subtilis* on boards made of ash, beech, oak, plastic and stainless steel. Biofilm formation and thus a protection of *Pseudomonas fluorescens* cells due to a polymeric matrix could be a possible explanation (Korber et al. 1994, Robleto et al. 2003).

Several reports concluded that the decrease in bacterial numbers detected on wood is exclusively due to the absorption of bacteria into the wood. Entrapped bacteria are closely attached to the porous wood structure and thus difficult to recover (Kampelmacher et al. 1971, Abrishami et al. 1994, Rödel et al. 1994, Lorentzen et al. 2000). Furthermore, bacteria can survive stressful conditions caused by desiccation by entering a viable but nonculturable (VBNC) state (Roszak and Colwell 1986, Winfield and Groisman 2003). Those cells are metabolically active, do not grow directly on standard laboratory media but can be revitalized in suitable environments (Kampelmacher et al. 1971). Therefore, a cultivation-independent approach employing DNA extraction and DGGE fingerprinting of 16S rDNA fragments amplified from directly extracted DNA was applied (Heuer and Smalla 1997, Heuer et al. 2001). The detected decrease of bacterial numbers on wooden dust correlated with the decrease of DNA extracted from wood. Especially the rapid decline of DNA on pine-wood indicates that the bacteria applied were efficiently killed on pine due to interaction with wood comparable to the results obtained in previous experiments (Milling et al. 2005). In contrast, the titer of culturable manure-derived bacteria as well as the amount of DNA recovered from plastic chips was very stable at all sampling times.

The relative abundance of the most dominant manure bacteria on the different sample specimen was assessed in this study by DGGE of 16S rDNA fragments amplified from DNA. While DGGE fingerprints provide information on the relative abundance of bacterial population they do not give insights into their titer. Shifts in the relative abundances of several populations became apparent on pine and plastic over the time. Moreover, the diversity of several bacterial populations, e.g. Firmicutes and Actinobacteria, was strongly reduced on pine only compared to larch, maple and plastic. Interestingly, the relative abundance of one band, which showed a high similarity to *Moraxella* sp. based on the 16S rDNA sequence, was strongly enhanced on plastic after 72 h. Moraxella species can cause severe systemic infections such as pneumonia, meningitis, and endocarditis (Morse 2004). In contrast, a weak band with the same electrophoretic mobility was found on pine and larch only directly after inoculation while at all samplings on maple. Furthermore, results indicate that wood extractives from different wood types influence the survival of the bacterial community members differently because the DGGE profiles showed that bacterial communities characteristic for the different wood types as well as for plastic were established.

Industrial packaging material and pallets made of wood play an important role in the distribution chain of food products. There is an increasing pressure on manufacturers to use food packaging material that is environmentally friendly as well as hygienic (Guðbjörnsdottir et al. 2002). Again, there are serious concerns whether products made of wood are safe enough in terms of hygiene when wood pores are clogged as observed in practice (Kampelmacher et al. 1971). Several authors proved a delay in germ reduction due to fat, proteins and serum and wooden surfaces (Rödel et al. 1994, Ak et al. 1994a, Ak et al. 1994b). Scott and Bloomfield (1990) showed that the presence of soil leads to growth and multiplication on laminated working surfaces which became dirty. In addition, higher levels of humidity, higher moisture contents of wood and lower ambient temperatures cause a delay in germ reduction (Ak et al. 1994b, Gehrig et al. 2000, Koch et al. 2002, Milling et al. 2005). Nevertheless, the results of this study indicate that transportation pallets made of pine-heartwood seemed to have a better or at least comparable hygienic performance compared to pallets made of plastic when these were tested in use including conditions such as cold temperatures, humid air, contamination with fat, proteins and high numbers of naturally occurring bacteria from meat. Determination of CFU numbers on the surface of the pallets clearly showed a lower degree of bacterial contamination of the pine-wood pallets compared to plastic pallets during use and after storage without cleaning. In the frame of the Nordic wood project different pallets made of pine, spruce, beech and plastic were tested in a supermarket and in a salt fish warehouse. After two months in a dry (RH 25%–30%) and warm (16 °C) environment the microbial count on the wooden and the plastic pallets was comparable. Moreover, when the pallets were used for salt fish storage in a wet (RH $>$ 80%) and cold ($<$ 3 °C) environment the number of bacteria was comparable on pallets made of pine, spruce and polyethylene. Cleaning with high-pressure cold water was satisfactory for all types of pallets tested (Guðbjörnsdottir et al. 2002).

The identification of bacterial isolates recovered from pine or plastic pallets by FAME analysis provided information on the kind of bacteria which can be found on pallets under practical conditions. Overall a high variability of the composition depending not only on the material but also on the sampling site and time was observed. In addition to the high variability, the limited number of samples taken and isolates identified prevented to detect any statistically significant difference in the composition of cultured bacteria from pine or plastic pallets. Interestingly, the total contact plate counts indicated a faster decrease of enterobacteria on both the pine-wood and the plastic pallets compared to other bacterial populations as already observed in the laboratory experiments. Furthermore, the very first results indicate high

survival rates of *Stenotrophomonas maltophilia* on pallets made of pine as well as of plastic. This fact might be a serious hygiene problem, the number of *S. maltophilia* diseases in humans having dramatically increased in recent years. The strain has become important as a nosocomial pathogen associated with significant case/fatality ratios particularly among individuals who are severely debilitated or immunosuppressed (Denton and Kerr 1998).

The application test was repeated with a total of 500 transportation pallets made of pine-heartwood (HHP) by Steinkamp (2002). The hygienic performance of those pallets was compared with that of pallets made of mixed woods and plastic over six months in fourteen factories of the food processing industry. Again, the results confirmed the good hygienic properties of pallets made of pine-heartwood. The microbial growth on those pallets was approximately 50% lower than that of its counterparts made of standard woods (mix of spruce, poplar, birch) and plastic. However, no clear differences in the composition of microbial communities grown on the different types of pallets were observed (Steinkamp 2002).

5 Conclusions

The central question of this study was whether the use of wood in the foodstuff sector might pose a serious hygienic risk. The results of this study support the thesis that a general evaluation of wood to be unhygienic is not justified. Especially pine showed excellent antibacterial characteristics also under unfavourable practice conditions, and displayed clear hygienic advantages compared to other woods and plastic. Results indicate that the hygienic situation in domestic kitchens or during the transportation of foodstuff could be improved by a careful selection and use of suitable wood types with antibacterial properties, and the use of these wood varieties could thus be recommended from our point of view.

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