Factors influencing adhesion of bacteria Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus *and yeast* Pichia membranifaciens *to wooden surfaces*

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Factors influencing adhesion of bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and yeast *Pichia membranifaciens* to wooden surfaces

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Abstract

The aim of this study was to assess the potential of bacteria *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and yeast *Pichia membranifaciens* ZIM 2417 to adhere to wooden surfaces such as poplar (*Populus* sp.), Norway spruce (*Picea abies*), European beech (*Fagus sylvatica*), beech coated with the commercial Belinka oil food contact and disinfectant P3-oxonia active 150, and investigate their survival on the beech wood surface under different relative humidities (RH; 65%, 75%, 85%, 98%) and temperatures (10 °C, 20 °C, 27 °C/37 °C). To extend the research goals, the scanning electron microscopy (SEM) analysis was also performed. The adhesion was determined by the number of colony-forming units per mm^2 of sample (CFU/mm^2). Results showed that all tested bacteria and yeast were able to adhere to the wooden surfaces, although differences were observed according to strains and type of wood. It was evident that number of adhered cells of *S. aureus* was lower on spruce (3.62×10^3 CFU/mm^2) compared to poplar and beech (1.09×10^5 and 2.11×10^4 CFU/mm^2, respectively). Furthermore, oil and disinfectant promoted the adhesion of *P. aeruginosa* (155.93 and 130.50%, respectively) on the beech surfaces, while they had a strong inhibitory effect on the other tested microorganisms *E. coli* (87.44 and 88.44%, respectively), *S. aureus* (91.24 and 96.80%, respectively) and *P. membranifaciens* (92.45 and 87.24%, respectively). These findings are consistent with SEM micrographs. The current data also indicated that relative humidity and temperature significantly affected the adhesion of tested bacteria and yeast. The highest degree of adhesion was observed at a relative humidity of 98% and temperature of 20 and 37 °C for bacteria, or 20 and 27 °C for yeast. Thus, the knowledge of how these microorganisms adhere to wooden surfaces and which factors affect this phenomenon proves to be of great importance in order to avoid their colonization.

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Introduction

Wood has a long tradition as a natural material used by humans in the preparation, packaging and transport of food products. It is still frequently used in some traditional sectors around the world, such as the wine industry, cheese production, storage of fruits and vegetables, as well as the transportation of seafood and meat (Aviat et al. 2016; Fink et al. 2013). The fact is that with the green movements and "bio" approach it gains more and more attention with industry and consumers (Wyrwa and Barska 2017). In Europe, wood as a food contact material is subject to regulation (EC) no. 1935/2004 of the European Parliament and of the Council of 27 October 2004 on materials and articles intended to come into contact with food. This regulation refers to different materials that may be subject to specific measures and have been harmonized and adopted at the European level, but not yet for wood (Regulation of European Parliament, Council of the European Union 2004). The use of wood as packaging material will likely increase in the near future, due to public pressure on "plastic" materials (Wu et al. 2019). Wooden boards and barrels are essential in traditional cheese and wine production, as well as wooden crates for storing fruits, vegetables and fresh or smoked fish. There are many other examples where wood has been used as a lightweight and still rough or porous packaging material from natural sources. The use of wood in direct contact with food is usually considered less hygienic than other materials (Galinari et al. 2014) such as plastic, stainless steel (Bohinc et al. 2016) and glass (Bohinc et al. 2012; Jevšnik et al. 2017). However, replacing wooden utensils with other materials can change the characteristics of some products. In the production of cheese, these materials do not contribute to the syneresis of cheese and affect the traditional taste and texture. Sometimes, the biofilm formation on wooden utensils by microorganisms such as yeasts and bacteria that play a role in ripening cheese is required (Gaglio et al. 2019; Galinari et al. 2014; Lortal et al. 2009).

Several scientific studies have assessed the hygienic potential of wood compared to other materials and have shown that some types of wood have antimicrobial properties (Milling et al. 2005; Schönwälder et al. 2002). The ongoing trend is the reduction in waste packaging materials; hence, the users are stimulated to reuse packaging as many times as possible. In the past, wood was frequently treated with biocides; however, nowadays the use of biocides is extremely limited predominately due to changed user perception and introduction of harmonized legislation in the field of biocides (Regulations 2012). The main concern in respect of food contact surfaces is contamination by microorganisms, some of which can form biofilms (Aviat et al. 2016; Fink et al. 2013).

In the production, processing, distribution and preparation of food items or simply along the food supply chain (Raspor and Jevšnik 2016), biofilms can be responsible for tremendous economic losses due to food spoilage and reduced quality of products or can cause harm to consumers' health because of their pathogenic properties. Due to the characteristic of adhesion, microorganisms serve as vehicles to transfer contamination vectors from place to place (Aviat et al. 2016;

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Frank 2001; Simões et al. 2010). If microorganisms survive on the surfaces, then the probability to transfer contamination to the next recipient is very high, which has a substantial impact on safety and quality of the final product or service (Fink et al. 2013; Tomičić et al. 2017). Besides, wooden packaging material can also be the vector for the transfer of several plant diseases (Payette et al. 2015). To the authors' knowledge, very few studies have focused on microbial survival on wooden surfaces (Hedge 2015; Lortal et al. 2009; Tomičić et al. 2017). It is well known that the adhesion of microorganisms to the wooden surfaces is a complicated process that is affected by various factors such as the wood species, the type of inoculated bacteria or yeasts and environmental conditions (Milling et al. 2005; Schönwälder et al. 2002; Soumya et al. 2011). However, data on the impact of these factors are still scarce. Therefore, there is a clear need for a better understanding of microbial survival on wooden surfaces that can play a significant role in controlling and developing strategies to reduce their adherence to this type of substratum.

The study is aimed to assess the adhesion of bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and yeast *Pichia membranifaciens* on different types of wooden surfaces such as poplar (*Populus* sp.), Norway spruce (*Picea abies*), European beech (*Fagus sylvatica*), and to evaluate whether Belinka oil food contact and disinfectant P3-oxonia active 150 can interfere with adhesion to beech surfaces. The scanning electron microscopy (SEM) analysis was also performed. To extend the research goals, the influence of different relative humidities (RH; 65%, 75%, 85%, 98%) and temperatures (10 °C, 20 °C, 27 °C/37 °C) on the degree of bacterial and yeast adhesion to beech surfaces was also examined.

Materials and methods

Strains and growth conditions

Two gram-negative bacteria *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, 1 g-positive bacteria *Staphylococcus aureus* ATCC 25923 and yeast *Pichia membranifaciens* ZIM 2417 were used to study the adhesion on different types of wooden surfaces. They were chosen because of their importance in food industry as the most frequent pathogens. Reference strains of bacteria were purchased lyophilized from the American Type Culture Collection (Manassas, USA), while the yeast strain was isolated from white cheese of cow's milk (Zlatibor, Serbia). All strains were obtained from the Collection of Industrial Microorganisms (ZIM) at the Biotechnical Faculty, Slovenia. The bacterial strains were preserved in tryptic soy broth (TSB, Oxoid CM0129, Hampshire, UK) with 15% glycerol as frozen stock at – 80 °C and revitalized on tryptic soy agar (TSA, Oxoid CM0131, Hampshire, UK) by overnight incubation at 37 °C. The yeast strain was preserved in Yeast Peptone Dextrose medium (Sigma-Aldrich, St. Louis, USA) (YPD) supplemented with 40% glycerol at – 80 °C as frozen stock and revitalized by cultivation on Malt Extract Agar for microbiology (MEA) (Merck KGaA, Darmstadt, Germany) for 24 h at 27 °C. After the incubation, a loopful of actively growing cells

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was suspended in the TSB medium for bacteria or Malt Extract Broth for microbiology (MEB) (Merck KgaA, Darmstadt, Germany) medium for yeast. The concentration of cells was determined and adjusted to 1×10^7 cells/mL by using the Bürker-Türk counting chamber (Brand, Wertheim, Germany). These cell suspensions were used immediately for adhesion assay.

Wooden surfaces

Since wood is still used to produce equipment for food manufacture (Aviat et al. 2016), the tested wood includes poplar (*Populus* sp.), Norway spruce (*Picea abies*), and European beech (*Fagus sylvatica*) as commonly used European tree species. Three types of wooden discs (poplar, Norway spruce and European beech with a length, width and thickness of 15.0, 7.0 and 5.0 mm^3, respectively) were obtained from Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana, Slovenia. All wooden discs were autoclaved at 121 °C for 15 min prior to use. One series of beech wood discs was surface treated with Belinka oil food contact, and one series was treated with disinfectant P3-oxonia active 150 to test the anti-adhesion effect. Belinka oil food contact (Belinka belles d.o.o., Ljubljana-Črnuče, Slovenia) and disinfectant P3-oxonia active 150 (Lenntech, Ecolab d.o.o., Maribor, Slovenia) were purchased from a commercial source. Belinka oil food contact is a bioimpregnation agent based on refined vegetable and mineral oils, with added aromatic oils, used for the protection and maintenance of wooden surfaces that come into contact with food. Belinka oils are made from natural ingredients and are not harmful to health nor the environment. P3-oxonia active 150 is applied in the food industry for a fast sanitizing of surfaces which are in contact with foods. It is a highly active bactericidal, fungicidal, sporicidal and virucidal agent based on hydrogen peroxide and peracetic acid for disinfection in the beer and beverage industry. P3-oxonia active 150 is environmentally friendly.

Adhesion assay

Compared to other materials such as plastic, stainless steel and glass, very little information is available on microbial adhesion to wooden surfaces. With this in mind, the aim of this study was to evaluate the adhesion of bacteria *E. coli*, *P. aeruginosa*, *S. aureus* and yeast *P. membranifaciens* on the surfaces of wooden discs made from three tree species. Adhesion assay was performed as previously described (Tomičić et al. 2017) with a few modifications. The course of the experiment is presented in Fig. 1. In the first part of the experiment, adhesion was formed on three types of wooden discs such as poplar, Norway spruce, European beech, as well as the European beech wooden discs coated with Belinka oil food contact and disinfectant P3-oxonia active 150. The three discs of each type of wooden samples were placed on the bottom of the Petri plates. Subsequently, 3 mL of each cell suspension (1×10^7 cells/mL) prepared as above was pipetted into the plate, covering the discs. The plates were incubated for 24 h at 37 °C

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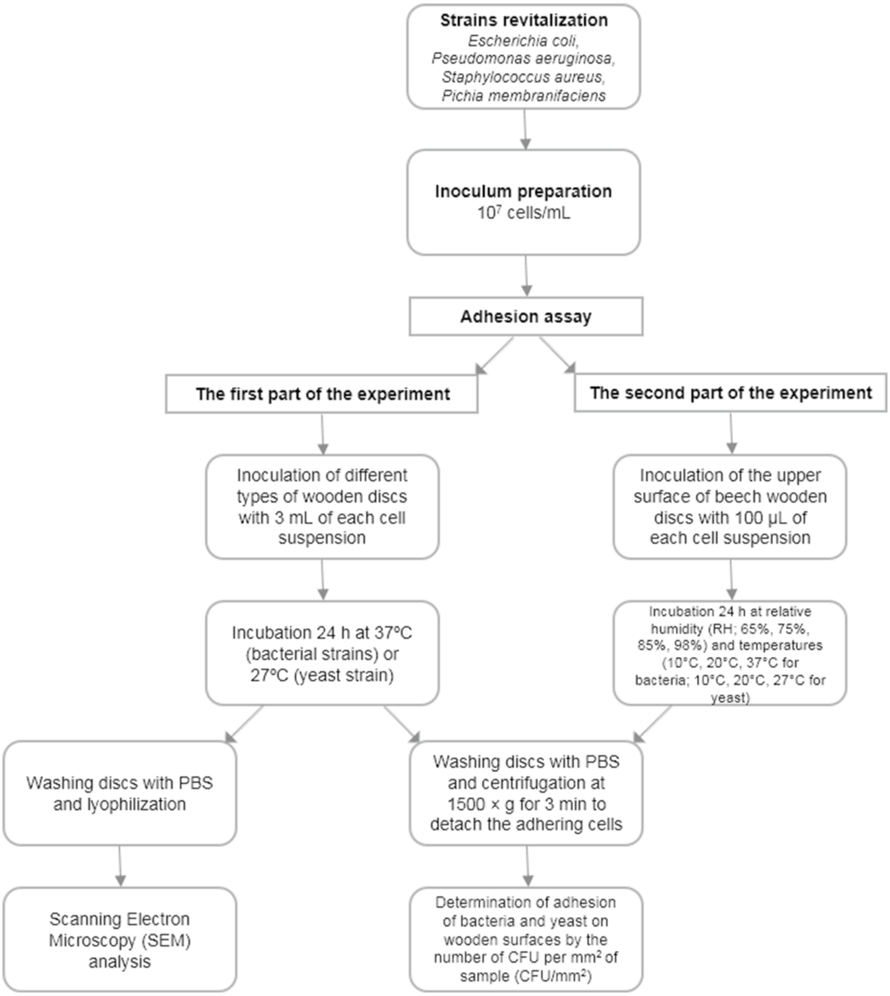

Fig. 1 Flowchart of the experiment

(bacterial strains) or 27 °C (yeast strain). In blank control plates, the wooden discs were inoculated identically to 3 mL of bacteria-free TSB and yeast-free MEB broth. The second part of the experiment aimed to examine whether adhesion of test bacteria and yeast on beech wooden discs is dependent on the environmental conditions such as relative humidity and temperature. Saturated salt solutions were used for control of relative humidity (RH; 65%, 75%, 85%, 98%) at temperatures between 10 °C and 37 °C. The salt solutions included $Ca(NO_3)_2$ (65% RH at 10 °C), NH_4NO_3 (65% RH at 20 °C and 27 °C), KI (65% RH at 37 °C), NaCl (75% RH at all temperatures), KCl (85% RH at all temperatures) and K_2SO_4 (98% RH at all temperatures). The three beech wooden discs were placed on the bottom of Petri plates, and the upper surface of the discs was inoculated with 100 μL

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of each cell suspension (1×10^7 cells/mL) prepared as above. Subsequently, the discs were exposed to 4 different relative humidities (65%, 75%, 85%, 98%) and 3 temperatures (10 °C, 20 °C, 37 °C for bacteria; 10 °C, 20 °C, 27 °C for yeast) for 24 h. After the incubation period in both experiments, non-adherent cells were removed by washing three times with phosphate-buffered saline (PBS; Oxoid, Hampshire, UK), and the discs were transferred to 2-mL Eppendorf tubes with 1 mL of PBS. Wood samples were centrifuged at $1500 \times g$ for 3 min to detach the adhering cells. The cell suspensions were then serially diluted and plated on TSA (bacterial strains) or MEA (yeast strain) plates and incubated for 48 h at 37 °C (bacterial strains) or 27 °C (yeast strain). After the incubation period, the grown colonies were counted in order to define the number of colony-forming units (CFU). The adhesion of bacteria and yeast on the wooden discs was determined by the number of CFU per mm^2 of sample (CFU/mm^2).

Scanning electron microscopy (SEM)

To achieve the research goals, the adhesion of bacteria *E. coli*, *P. aeruginosa*, *S. aureus* and yeast *P. membranifaciens* on the three types of wooden discs, poplar, Norway spruce, European beech, as well as the European beech wooden discs coated with Belinka oil food contact and disinfectant P3-oxonia active 150, was analysed by scanning electron microscopy (SEM). The wooden discs were inoculated, at the same levels as described above, with 3 mL of each cell suspension (1×10^7 cells/mL) and incubated for 24 h at 37 °C (bacterial strains) or 27 °C (yeast strain). After cultivation of microorganisms on wood, the discs were washed 3 times with PBS, and the specimens were immediately frozen in liquid nitrogen and lyophilized to obtain a state as intact as possible. After lyophilization, the SEM micrographs were then taken at a low voltage (10 kV) and in low vacuum (50 Pa) conditions with a large field detector (LFD) in a FEI Quanta 250 SEM microscope at a working distance of 10 mm in native condition, without any coating with metals or carbon. The flowchart of the experiment is presented in Fig. 1.

Statistical analysis

Descriptive statistical analyses for calculating the means and the standard error of the mean were performed using Microsoft Excel software (Microsoft Office 2013). The mean value and standard deviation of 3 readings were recorded ($n = 3$), and all results were expressed as the mean ± standard deviation (± SD). In this article, analysis of variance (ANOVA) and the post hoc Tukey's HSD test were conducted by StatSoft Statistica, ver. 10 to show the significant effects of independent variables on the responses and to show which responses were significantly affected by the varying combinations. A P-value of < 0.05 was considered as statistically significant.

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Results and discussion

Wood is well known as a renewable natural resource and widely used along the food supply chain including food processing and preparation. For centuries, wood has been used as a traditional material for the ripening of various food products and was considered a key factor for the organoleptic features, especially cheese (Aviat et al. 2016), wine or vinegar production (Raspor and Goranovic 2008) among others. In all fermentation processes, the microbial biofilm represents starting microflora for successful conversion of substrate to final product. If this is illustrated in cheese-making process, the wooden vat surfaces allow the development of microbial biofilms that include dairy lactic acid bacteria (LAB). In particular, investigations carried out by Italian and French groups showed that LAB biofilm present on wooden vat surfaces counters the adherence of many dairy pathogens (Lortal et al. 2009; Cardamone et al. 2020; Gaglio et al. 2016). However, each type of wood has a unique anatomy and chemistry, which leads to a specific action against microorganisms (Munir et al. 2019). Several studies have indicated that the survival of bacteria and yeast can be different on different wood species (Milling et al. 2005; Schönwälder et al. 2002; Soumya et al. 2011). To the authors' knowledge, not enough work has been done in this area of research. For that reason, this study was carried out to provide a better understanding of the influence of various factors on the adhesion of bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and yeast *Pichia membranifaciens* to wooden surfaces as indicative microbes who can be present along the food supply chain as natural contaminants connected to the hygienic status of the particular production or processing step.

A very different adhesion of the test bacteria and yeast on the different wood surfaces such as poplar, Norway spruce, European beech as well as European beech coated with the Belinka oil food contact and disinfectant P3-oxonia active 150 was found in this study (Fig. 2). The bacteria cells noticeably had a higher ability to adhere to wood in comparison with yeast, confirming the previous findings (Aviat et al. 2016; Filip et al. 2012; Soumya et al. 2011). In addition, evaluation of adhesion in the present study revealed that the gram-negative bacteria *E. coli* ATCC 35218 and *P. aeruginosa* ATCC 27853 exhibited a much better ability for adherence to wooden surfaces than gram-positive bacteria *S. aureus* ATCC 25923 (Fig. 2). Differences in the composition and structure of the cell wall of the tested microorganisms might explain these results (Milling et al. 2005; Schönwälder et al. 2002). On the other hand, the research showed that *S. aureus* ATCC 25923 and *P. membranifaciens* ZIM 2417 had less adhesion on spruce when compared with poplar and beech (Fig. 2), suggesting that this wooden species possesses substantially better hygienic characteristics. This observation contradicts the fact that the majority of the wooden packaging is made of poplar wood. Poplar is chosen predominantly due to economic and technological reasons. Regarding the use of wood in dairy processing, the specific investigation conducted by Cruciata et al. (2018) revealed that the wood type influenced the levels of beneficial lactic acid bacteria who inhibit the adhesion of harmful microorganisms

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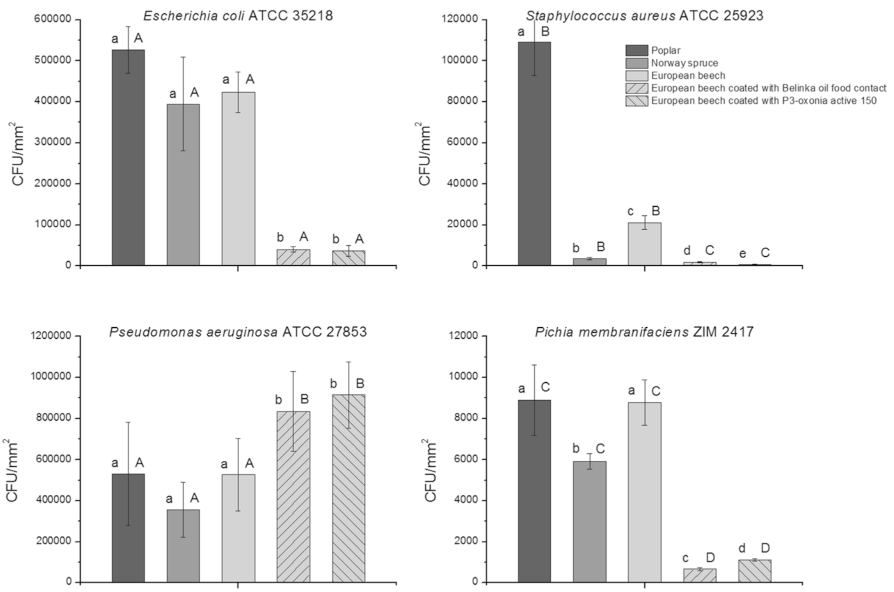

Fig. 2 Adhesion of *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Pichia membranifaciens* ZIM 2417 to different types of wooden surfaces obtained by the number of colony-forming units (CFU/mm^2). Each bar represents the mean ± standard deviation (SD). Different letters (a, b, c, d, e) mark significant differences among wooden surfaces for each strain, while different letters (A, B, C, D) indicate that there is significant difference among strains at the same type of wooden surface ($P < 0.05$)

such as *E. coli*, *Listeria monocytogenes*, *Salmonella* spp. and *S. aureus*. The high levels of LAB were registered on the surfaces of cedar, ash, walnut and poplar vats. In addition, Schönwälder et al. (2000, 2002) performed two studies concerning *Escherichia coli* and *Enterococcus faecium* survival on different wood species and showed that pine wood boards exhibited better hygienic performance than other wooden boards made of spruce, beech, and poplar or plastic boards. The study indicated that the absorption potential of wood can cause desiccation conditions for microbes, and the presence of extractives can inhibit microorganisms. Many authors have also claimed that an antimicrobial effect of wood causes the reduction in microbial numbers on the wood surfaces based on the compounds present in wood as well as several physical-properties of wood (Laireiter et al. 2013; Milling et al. 2005; Munir et al. 2019). The compounds such as phenols, lignans, tannins, stilbenes, flavonoids and terpenoids could be responsible for an antimicrobial effect of different wood species (Lee et al. 2005; Plumed-Ferrer et al. 2013; Rauha et al. 2000; Välimaa et al. 2007). Among the wood species tested, Norway spruce wood possesses the highest portion of biologically active extractives (Burčová et al. 2018; Haman et al. 2019). Besides, it should be considered that some of the biologically active extractives are volatile and consequently evaporate from wood during the service life (Fengel and Wegener 1989; Liu et al. 2012; Munir et al. 2019). The studies have also shown that extractives from different parts of wood have different effects on microbes (Vainio-Kaila et al. 2015,

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2017). However, several reports have repeatedly pointed out that due to the porosity, wooden materials are supposed to be harder to clean and decontaminate (Aviat et al. 2016; Gough and Dodd 1998). Nevertheless, Ak et al. (1994) mentioned that with appropriate cleaning, new or used wooden surfaces can be safely used with reduced risks of cross-contamination of food. In the present contribution, it is noteworthy that a drastic decrease in the number of adhered cells for *E. coli* ATCC 35218, *S. aureus* ATCC 25923 and *P. membranifaciens* ZIM 2417 became apparent when the beech wood surfaces were coated with oil and disinfectant, while in the case of *P. aeruginosa* ATCC 27853, adhesion is not suppressed; it is even significantly stimulated (Fig. 2). With this, previous studies were confirmed, where *Pseudomonas* survived cleaning and disinfection concerning the other bacteria (Liu et al. 2013; Møretrø and Langsrud 2017). The resistance of *P. aeruginosa* to disinfectants could be partly explained by rapid changes in gene expression after bacterial contact with surfaces (Balasubramanian and Mathee 2009; Davies et al. 1993; Sagripanti and Bonifacino 2000). Such implication indicates the importance of the tested agents against certain microorganisms in hygiene measures in the food industry.

Additionally, since wood is well known as a material of high roughness, porosity and able to entrap microbial cells within corrugated fibres (Lortal et al. 2009; Siroli et al. 2017), the SEM analysis was performed on inoculated wooden surfaces with

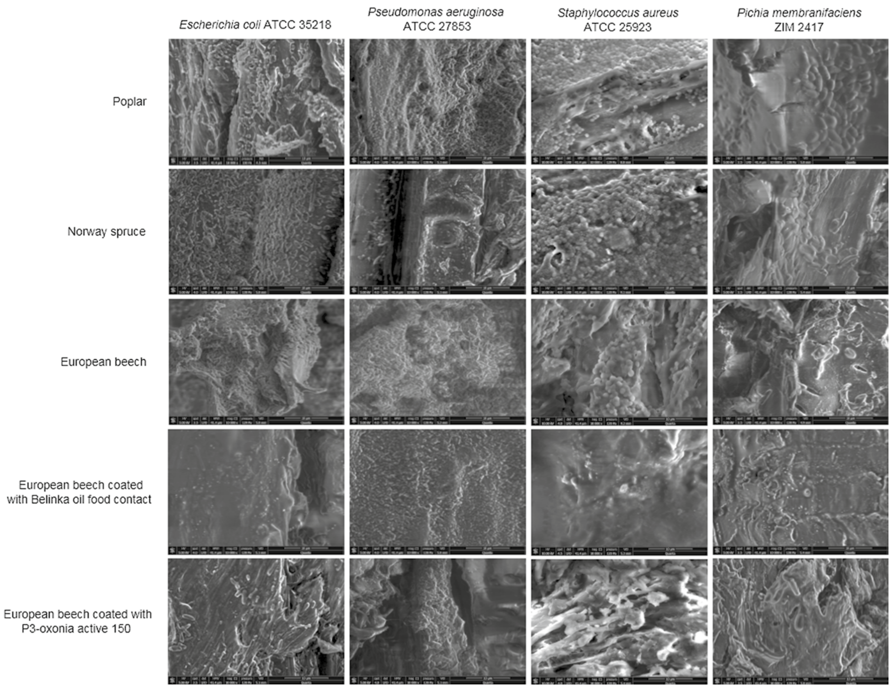

Fig. 3 Scanning electron microscopy (SEM) images of adhesion of *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Pichia membranifaciens* ZIM 2417 to different types of wooden surfaces

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bacteria and yeast (Fig. 3). Although this work was not done to quantify microbial cells in the biofilm, the micrographs showed the diversity of *E. coli*, *S. aureus* and *P. membranifaciens* adherence, while the presence of *P. aeruginosa* was found on all investigated surfaces, which is consistent with the results obtained by plate counting (CFU/mm^2) (Fig. 2).

The storage conditions such as relative humidity (RH) and temperatures are other factors which are known to play an important role in the biofilm formation of pathogenic and spoilage microorganisms (Møretrø and Langsrud 2017; Siroli et al. 2017; Tomičić and Raspor 2017; Zoz et al. 2016). In the present study, it was evident that these factors affected the adhesion of tested bacteria and yeast on beech wooden surfaces (Fig. 4). The highest counts were noted at a relative humidity of 98%. In some treatments, the adhesion of the bacteria and yeast was observed in traces, in particular at 65% and 75% relative humidity, while in some cases adhesion did not occur at all, indicating that particular relative humidity is the best storage condition to prevent microbial adhesion and growth. Increase in relative humidity of storage has also been known to lead to an increase in microorganisms count (Onilude et al. 2010). Additionally, evaluation of adhesion in a further trial revealed that at the relative humidity of 65%, 75%, 85% and 98%, there was a different succession of

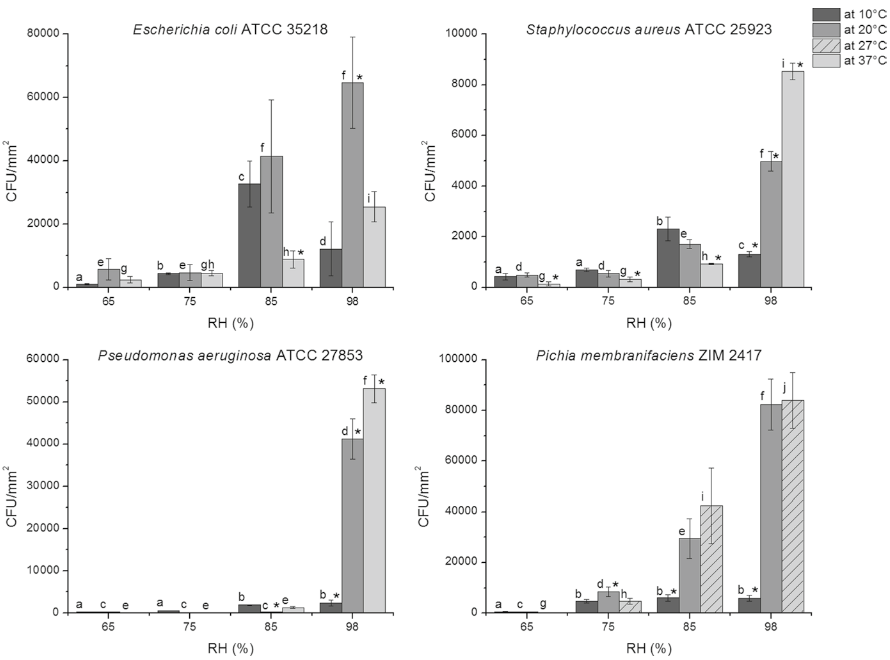

Fig. 4 Effects of relative humidity (RH) and temperature on adhesion of *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Pichia membranifaciens* ZIM 2417 to European beech surface obtained by the number of colony-forming units (CFU/mm^2). Each bar represents the mean ± standard deviation (SD). The asterisks (*) mark significant differences among temperatures at the same RH, while different letters (a, b, c, d…) mark significant differences among RH at the same temperature for each strain ($P < 0.05$). Unmarked terms are not significant

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organism with temperature. The ability of bacteria and yeast to form a biofilm at the tested temperatures between 10 and 37 °C characteristic in the food industry during processing and storing is therefore a highly important virulence trait of a pathogen. Considering this observation, the presented results could also have a practical application in the food industry and also in other sections of the food supply chain in order to reduce the risks of cross-contamination as it has been shown that microbial biofilms could develop over a wide range of temperature and humidity regimes.

Conclusion

The problem of contamination with microbes via contact surfaces is permanent and persistent. In the food supply chain, we try to avoid microbes harmful to health, but we also search for efficient prevention of food spoilage organism in processing beside pathogenic microbes. This study showed that the bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and yeast *Pichia membranifaciens* possess the ability to adhere to wooden surfaces. Based on the results obtained in this study, it is evident that the type of wood, disinfectant, relative humidity (RH) and temperature regime had a significant impact on the microbial adhesion to wood surface. Hence, it should be pointed out that wood species like Norway spruce had antimicrobial effect against *S. aureus* and *P. membranifaciens* compared to poplar and European beech. Agents used in the food industry to maintain food contact surfaces such as the Belinka oil food contact and disinfectant P3-oxonia active 150 also showed antimicrobial properties and consequently lower adhesion of microbes when exposed to experimental colonization. The lowest relative humidity and temperature had repressive effects on the adherence of all tested bacteria and yeast, suggesting that these findings offer great potential applicability to the food production, storage and distribution as well as final preparation of food dishes because proper selection of wooden surfaces would give operators the possibility to manage and control biofilm formation. Nevertheless, further research needs to be conducted under conditions closer to practice (e.g. organic matter and other naturally occurring microbial communities) to assess whether the hygienic situation in several areas, such as private households or food supply chain, could be enhanced by careful selection of the suitable wood species and appropriate handling based on the results obtained.

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