FCBA INFO

# TRANSFER OF MICROORGANISMS
## FROM WOODEN CRATES TO FOODSTUFFS : ASSESSMENT OF A TRANSFER THRESHOLD
## CARACTERISATION MICROBIOLOGIQUE DU SYSTÈME « MICROORGANISMES BOIS ALIMENT »

Wood is commonly used in contact with foodstuffs, for example for packaging fruits or vegetables. Most common species used are beech, pine, spruce and poplar.

Wood is often considered as being less safe than plastic because it is less easy to clean whereas several studies have demonstrated that microorganisms cannot survive easily on wood (Milling *et al.*, 2005; Revol-Junelles *et al.*, 2005). National recommendations for packaging fruits feel the necessity for clean materials, whether it be wood or plastic (Lurol *et al.*, 2007).

The analysis of microbial contamination is standardized only for paper and cardboard materials (Anonymous, 1998). No standard exists yet for wood packaging. A comparative analysis was managed to remove microorganisms from wooden surfaces used in the food industry. A grinding technique was developed on poplar (Ismaïl *et al.*, 2014).

On the basis of this method, the transfer of microorganisms from wood to foodstuffs was analysed. The model "poplar packaging/apple" was chosen. The two microorganisms *Escherichia coli* and *Penicillium expansum* were considered, as they are a well-known risk for apples.

In a first step of this work, the survival rate of the microorganisms on poplar crates was assessed, after 1h, 1 week and 3 weeks. The wood was artificially inoculated by different concentrations of the two microorganisms.

In a second step, the transfer rate of microorganisms from poplar to apple was quantified, after 1h and 1 week of contact, still using several inoculum concentrations.

Finally, a model was finalized using our results to describe a transfer threshold for both microorganisms from wooden packaging to apples. Analysis on wounded apples was also carried out.

Il existe très peu de données scientifiques sur la qualité microbiologique du bois au contact des aliments. Or sans ces données, la filière emballage bois s'expose à une forte concurrence des autres matériaux d'emballage (plastique par ex).

Au niveau européen, le règlement RCE 1935-2004 précise que les matériaux en contact avec les aliments ne doivent pas entraîner de danger pour la santé humaine, de modification de la composition des denrées ni d'altération des propriétés organoleptiques.

En France, la DGCCRF, en lien avec l'ANSES, a déclenché la révision de la fiche matériau bois relative à l'aptitude du bois au contact alimentaire. Ce travail est réalisé en prenant en compte le règlement RCE. L'aptitude du bois au contact alimentaire est revue sous ses aspects microbiologique, chimique (aspects traitements du bois) et organoleptique. FCBA est impliqué sur ces questions de par son expérience.

Sur le plan économique, l'emballage léger en France représente environ 45 PME, un chiffre d'affaire de 250 millions d'euros et une production d'environ 1 milliard d'emballages (unités) ce qui correspond à environ 2.3 millions de $m^3$ de bois (SIEL, 2009). Les cagettes sont principalement en peuplier. Les emballages légers peuvent également être en pin et en épicéa. Les clients sont principalement issus de la filière fruits et légumes. A noter que l'emballage léger génère directement et indirectement 6000 emplois.

Le Pôle emballage en Bois, représenté par le SIEL (Syndicat national des Industries de l'Emballage Léger), par ailleurs initiateur du Consortium scientifique EMABois, a engagé un programme de recherche pour répondre aux demandes de ses adhérents et de l'administration sur l'aptitude du matériau Bois au contact alimentaire, en vue de valoriser le bois au contact des aliments.

Les lignes directrices du projet ont été déterminées selon les besoins des industriels de l'emballage Bois et en intégrant l'expertise de chacun des partenaires. L'ONIRIS, Ecole Vétérinaire de Nantes, travaille sur un contaminant des viandes. ACTALIA a de l'expérience sur l'analyse de la flore microbiologique des planches d'affinage de fromage. FCBA a développé une méthode d'analyse microbiologique des emballages (type cagettes) (Le Bayon et al. 2010) et a de l'expérience sur l'analyse des microorganismes du bois.

# INTRODUCTION

Wood is widely used in contact with foodstuffs to make crates for vegetables or fruits, and boxes for cheeses and seafood products. Aromatic wood properties are used in wine barrels (Husson, 1996; Miserey, 1997; Barthelemy, 1998), and wooden boards are used for the maturation of surface ripened cheeses (Richard, 1997).

As is the case of other packaging materials, wooden packages have to keep the food in good condition until it is purchased and consumed. The most common species used for food packaging are poplar, pine, beech, and spruce. Poplar is particularly used for manufacturing light packaging.

Because it is a porous and absorbent material, wood is commonly considered as less safe and secure than plastic or stainless steel packaging (Jacobson, 1979; Lapping & Connor, 1991). However, several studies have demonstrated that microorganisms do not survive easily on wood (Schönwälder *et al.*, 2002; Milling *et al.*, 2005; Revol-Junelles *et al.*, 2005; DeVere & Purchase, 2007). The European regulation n° 1935/2004 of the 27th of October 2004 indicates that materials intended for safe food contact must not modify foodstuffs characteristics. National recommendations for harvesting and packaging fruits require the use of clean wooden, plastic or cardboard boxes (Lurol *et al.*, 2007).

In France, more than 20 wood species are authorized by the French decree of November, 1945 for direct contact with solid or liquid food products.

The French standardised method NFQ 03-070-1 (AFNOR 1998), which is a grinding based method, is suitable for analysing microbial contamination of paper and cardboard. Currently, no standard yet exists for wood. A comparative analysis was managed to remove microorganisms from wooden surfaces used in the food industry. A grinding technique was developed on poplar (Ismaïl *et al.*, 2014).

On the basis of this method, the transfer of microorganisms from wood to foodstuffs has been analyzed. The model “poplar packaging/apple” was chosen.

We chose two relevant hygienic risk models: *Escherichia coli* and *Penicillium expansum. E. coli* is a gram negative bacterium, usually used as an indicator of hygienic practice in food industry. Several studies have demonstrated that *E. coli* could grown on several fruits such as peaches, pineapples, melons, and also apples as well as on vegetables (Janisiewicz *et al.*, 1999; Leverentz *et al.*, 2004; Sivapalasingam *et al.*, 2004; Alegre *et al.*, 2010). *P. expansum* is an ascomycete, responsible of postharvest decay of several fruits like tomatoes, peaches, sweet cherry fruits and apples (Xiao *et al.*, 2011; Chatterton *et al.*, 2012; Yu *et al.*, 2012; Wang *et al.*, 2015). It is an substantial spoilage fungi for the fruit sector, in particular for pears and apples (Giraud *et al.*, 2001; Amiri *et al.*, 2005).

Firstly, the survival rate of the microorganisms on poplar specimens was assessed, after 1h and 1 week. The wood was artificially inoculated by different concentrations of the two microorganisms.

Secondly, the transfer rate of microorganisms from poplar to apple was quantified, after 1h or 1 week of contact, still using several inoculum concentrations.

Finally, a model has been finalized using our results to describe a transfer threshold for both microorganisms from wooden packaging to apples. Analysis on wounded apples was also carried out.

# EXPERIMENTAL METHODES

## Biological materials

### • Wood samples and apples

Poplar sapwood slats of *Populus euramericana*, freshly cut, were 0.4 cm thick (Bois Diffusion, Valanjou, France). Wood samples measuring 60x40 mm were cut within these slats (surface of 24 cm²). After 14 days of conditioning in a climatic chamber at 20°C and 65% of relative humidity (RH), specimens were sterilized by ionization prior to use (Ionisos, Dagneux, France).

Golden delicious apples from French suppliers were used in this study. Organic apples were stored at 4°C and disinfected with ethanol 70% prior to use. As a control, contact plates of PCA medium (tryptone 5g/L, yeast extract 2.5g/L, glucose 1g/L) were applied 10 seconds at the surface of apples and incubated at 22°C for 48h to ensure that disinfection was fully carried out.

### • Microorganism strains and inoculum preparation

*Escherichia coli* strain (ATCC-700926) was stored in cryotubes containing beads at -80°C. Two beads of a cryotube were suspended in 10 mL of PCA medium for 24h at 37°C and 180 rpm. 1 mL of this sub-cultured was then inoculated in 100 mL of PCA for 24h at 37°C and 180 rpm. The concentration of this culture was expected to be at $1x10^8$ CFU/mL. This solution was used to inoculate wood specimens and to prepare dilutions in sterile water with 0.9% NaCl.

*Penicillium expansum* strain (ATCC-7861) was propagated on malt/agar medium (Malt 40g/l; agar 20g/L). Spores suspension was generated by culturing *P. expansum* on 4 malt/agar plates for 2 weeks at 22°C. 10 mL of water with 0.9% NaCl and Tween® 0.05% was added to each plate and spores were then collected. After filtration through sterile gauze, spores suspension was rinsed three times in 10 mL of sterile water by centrifuging 20 min at 2000 g. Spores were then counted on a Malassez cell. This solution was used to inoculate wood specimens after dilutions in sterile water with NaCl 0.9%.

Concentrations of the inocula of $8x10^7$, $8x10^6$ and $8x10^5$cells/mL were chosen

## Methods

### • Wood moisture content

A moisture content of 37% was used in this study as it represents wet packaging storage conditions. To obtain these targeted moisture conditions, samples were soaked in sterilized water for 1 min. and weighed. They were then put in an oven at 103°C for 48h to determine their dry mass. The moisture content was then determine as already described in Ismail et al, 2014 to ensure that specimens were at 37% of moisture content.

### • Viability control of inocula

A viability control was carried out to determine the concentration of viable microorganisms in tested solutions.

For *P. expansum*, inoculum viability was assessed by sowing 100 µL of serial dilutions on malt/agar plates. After incubation 48h at 22°C, enumeration of colonies was undertaken to determine strain viability.

For *E. coli*, inoculum viability was assessed by sowing 1 mL of serial dilutions on PCA plates. After incubation 24h at 37°C, enumeration of colonies was undertaken to determine strain viability.

### • Inoculation of wood specimens

Sterile wood specimens were inoculated with 300 µL of inocula, at several concentrations. After 15 minutes of inoculum static impregnation, specimens were grinded, incubated alone or in contact with an apple for 1h at room temperature, 1 week or 3 weeks at 10°C and 85% RH. These conditions correspond to optimum storage conditions in cold room (http://www.fruits-legumes.net/revue_en_ligne/point_sur/fich_pdf/ECO/StockageFLEco.pdf).

### • Microbial enumeration

After incubation, microorganisms from wood specimens were recovered by grinding as already described (Ismail et al, 2014). After incubation, apples were peeled fruitlessly using a stainless steel potato peeler to extract microorganisms. 5 cm² of peel corresponding to the surface contact with boards were blend using a Stomacher® 80 (Seward, United-Kingdom) in 6 mL of sterile water with NaCl 0.9% for 2 min.

The recovery solutions were inoculated sowing 1 mL of serial dilutions on PCA (for *E. coli*) or 100µL of serial dilutions on malt/agar (for *P. expansum*). After incubation at 22°C for *P. expansum* or 37°C for *E. coli*, enumeration of colonies was undertaken.

### • Analysis on wounded apples

Wood specimens were inoculated with inoculum of *P. expansum* at $8x10^7$ and $8x10^5$ cells/mL. To mimic wounds on apples and associated necrosis linked to *P. expansum*, apples were wounded with 4 nails prior to contact with wood specimens. Controls on uninoculated wood and on plates covered with *P. expansum* cultures (positive control) were carried out. Necroses on apples were measured after 12 days of incubation at 10°C and 85% RH. Microbial enumeration was also performed on wood specimens, and on necrotized peels in contact with wood.

## Statistical analysis

The experiments, described above, were performed in triplicates, except for wounded apples, where 30 replicates were used. Results of microbial enumeration, expressed in CFU/cm² and transformed in log10 scale, correspond to the mean value ± standard deviation. Results were analyzed using a Student t-test ($p<0.05$) with the Minitab® statistical software 16 (Minitab® Inc.). A transfer rate (%) was expressed as follow: (CFU/cm² recovered on apples after incubation) x 100 / (CFU/cm² inoculated on wood specimens at T0).

# RESULTS AND DISCUSSION

## Viability on wood

To assess the viability of the two microorganisms on wood, suspensions of 4.14 log CFU/cm² for *E. coli* and 5.57 log CFU/cm² for *P. expansum* were inoculated on wood specimens at 37% of moisture content. Immediately after inoculation (T0), after 1h of incubation at room temperature or 1 week at 10°C and 85%, microbial enumerations were performed. Results are presented in Table 1.

| Incubation time | *E. coli* | *P. expansum* |
|---|---|---|
| T0 | 3.95 (0.23) | 5.36 (0.15) |
| 1 hour | 1.85 (0.23) * | 5.00 (0.02) |
| 1 week | 0.02 (0) * | 4.94 (0.63) |

Table 1: Viability of *E. coli* or *P. expansum* on wood after 1 h or 1 week of incubation.

Results correspond to Mean (standard deviation).* means significantly different from result obtained at T0 ($p<0.05$, Student t-test).

Our results demonstrate that *P. expansum* conidia can survive on wood specimens even after 1 week, as from 4.94 to 5.36 log CFU/cm² are recovered whatever the incubation time. However they do not grow. For *E. coli*, results after 1 hour and 1 week are significantly different from results obtained at T0 and demonstrate that *E. coli* do not seem to survive on poplar specimens.

We can observe a different behavior between *E. coli* and *P. expansum*. Poplar contains phenolic compounds able to inactivate microbial adhesins and to complex with microbial cell wall (Cowan, 1999). *E. coli* is a gram negative bacterium, with a peptidoglycan layer whereas conidia of *P. expansum* are resistance shape, with a thick cell wall. These phenolic compounds could complex with the cell wall of *E. coli* leading to a lower viability.

Physiological differences exist between *E. coli* cells and *P. expansum* conidia. *E. coli* is a gram negative bacterium whereas *P. expansum* conidia are resistant cells. *E. coli* requires a temperature range of 7°C to 37°C a minimum water activity (aw) of 0.95 and a pH optimum of 6 to 7 (Anonymous, 1996; Presser *et al.*, 1997) . *P. expansum* is more tolerant as its conidia require a temperature range of 0°C to 35°C, a minimum aw of 0.82 and a pH of 2 to 10 (Piit & Hocking, 2009; Anonymous, 2011).

A hypothesis could be that poplar exhibits unfavorable conditions for *E. coli* survival such as a low water activity, a pH of 5.8 to 6.4 (Balatinecz *et al.*, 2014). Revol-Junelles et al. (2005) results indicated that the decrease of *E. coli* survival was probably linked to the desiccation of bacterial cells on poplar surfaces because of its hygroscopic properties and of its wood natural extractives (Revol-Junelles *et al.*, 2005).

*P. expansum* conidia survival on poplar is constant whatever the incubation time at 10°C and 85% RH. These results suggest that the conditions on poplar such as surface moisture content and pH allowed *P. expansum* conidia to survive but not to grow on wood (Piit & Hocking, 2009; Anonymous, 2011).

## Transfer rates from poplar to apples

To assess transfer rates of *E. coli* and *P. expansum* from wood to apples, suspensions of 4.14 log CFU/cm² and 5.57 log CFU/cm² respectively, were inoculated in sterile conditions on wood specimens at 37% of moisture content. An apple was then put in contact with wood samples. After incubation 1h at room temperature or 1 week at 10°C and 85% RH, microbial enumerations were performed on wood samples and on apples. Results are presented in Fig 1.

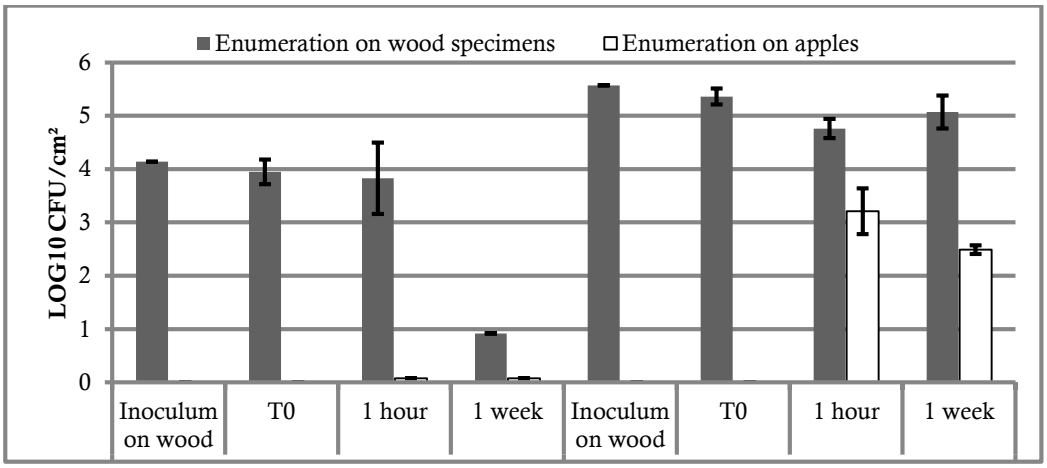


Figure 1: Enumeration of *E. coli* (on the left) or *P. expansum* (on the right) on wood samples and apples after 1 hour or 1 week.

Analyses were first performed on wood specimens. For *E. coli*, only 0.92 $\log_{10}$ CFU/cm² are recovered after 1 week. For *P. expansum*, more than 4 $\log_{10}$ CFU/cm² are recovered on wood specimens, whatever the incubation time. These results, coupled with results of Table 1 strongly suggest that recovery of these two microorganisms on wood specimens is not affected by contact with apples.

Analyses were then performed on apples. For *E. coli*, obtained recoveries were lower than the detection level of the method, suggesting that *E. coli* is not transferred from wood to apples. Calculated corresponding transfer rates (0.008%) after 1 hour or 1 week confirm this hypothesis. For *P. expansum*, 3.21 $\log_{10}$ CFU/cm² are recovered after 1 hour and 2.49 after 1 week. Corresponding transfer rates after 1 hour or 1 week are equal to or lower than 0.25%. These results demonstrate that *E. coli* is not transferred from wood to apples and that the transfer rate of *P. expansum* from poplar to apples is very low.

Results with contact plates were negative, demonstrating that disinfection was fully carried out on apples prior to use (data not shown).

This study, leading to low transfer rates, was carried out using very high contamination levels on wood. Such levels are higher than possible contaminations of wooden packages. Thus, taking into consideration both a lower package contamination and a low transfer rate, the apple contamination would be considered to be insignificant.

In our study, transfer rates are very low, whatever the considered microorganism. This result is fully in accordance with several studies, dealing with the transfer of *Listeria monocytogenes* from wood specimens to chicken meat or cheeses (Goh *et al.*, 2014; Ismaïl *et al.*, 2014), of several foodborne pathogens from stainless steel surfaces to food (Kusumaningrum, 2003). However, transfer rates of microorganisms could vary according to considered foodstuffs (Silagyi *et al.*, 2009; Abadias *et al.*, 2012; Jensen *et al.*, 2013; Goh *et al.*, 2014). Materials, depending on their topography and properties, could also influence transfer rates and recovery (Gough & Dodd, 1998; Midelet & Carpentier, 2002; Amiri *et al.*, 2005; Dawson *et al.*, 2007; Knobben *et al.*, 2007). In this sense, one perspective could be to assess transfer rates of *E. coli* and *P. expansum* from wood to several fruits or vegetables and also from several materials to apple.

## Impact of inoculum concentration

As only transfer rate of *P. expansum* was above detection level of our method, analysis with several inoculum concentrations was carried out. Wood samples were inoculated with $8x10^7$, $8x10^6$ or $8x10^5$ cells/mL, incubated in contact with an apple for 1 hour, 1 week or 3 weeks. Enumeration on apples was then performed. Results are presented in Figure 2.

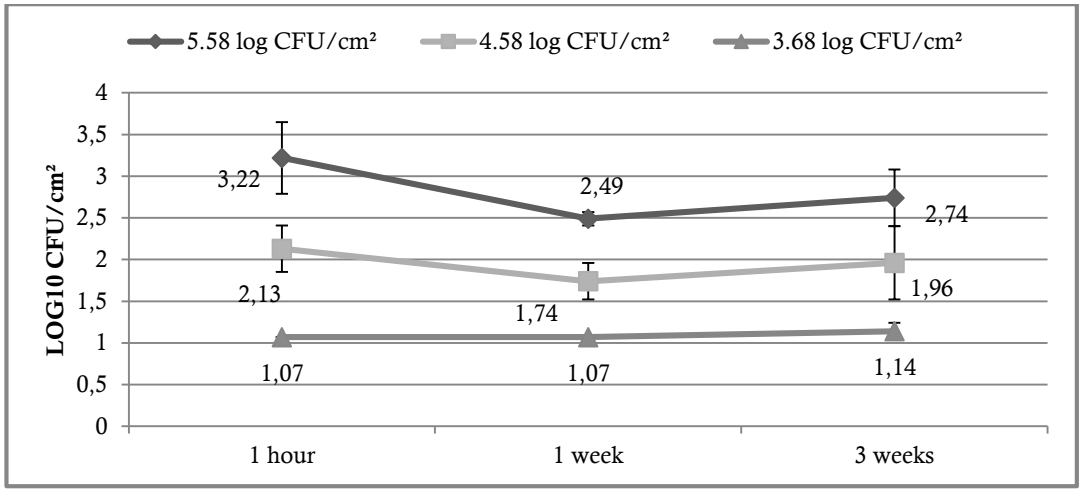


Figure 2. Enumeration of *P. expansum* on apples after 1 hour, 1 week or 3 weeks of incubation, according to inoculum concentration dropped off on wood samples at T0.

Three concentrations corresponding to 5.58, 4.58 and 3.68 $log_{10}$ CFU/cm² were inoculated on wood specimens at T0. For the highest concentration, results obtained after 1 hour to 3 weeks are steady, with values ranging from 2.49 to 3.22 $log_{10}$ CFU/cm². For the median concentration, results are steady again, ranging from 1.74 to 2.13. For the lowest concentration, results are at the detection level (1.07 log). Our results demonstrate that a relationship exists between inoculated concentration and recovered concentration on apples. This correlation is independent of incubation time. A log loss of approximatively 3 is observable between inoculation on wood samples and recovery on apples, whatever the tested concentration. Transfer of *P. expansum* from wood to apple peel is very low (≤ 0.25%) and proportional to inoculum concentration on wood.

## Analysis of wounded apples

*P. expansum* is an important spoilage ascomycete for the fruit sector, and in particular pears and apples. To mimic wounds on apples and analyse necrosis linked to *P. expansum*, apples were wounded with 4 nails. Wood specimens were inoculated with 5.67 or 3.66 log CFU/cm². After incubation at 10°C and 85% RH for 12 days, necrosis analysis was performed. Negative and positive controls (apples put in contact with plates invaded with *P. expansum*) were also carried out. Number of apples with necrosis, and number of wounds having necrotized have been estimated. Results are presented in Table 2.

| Condition | Apples with necrosis (%) | Wounds having necrotized (%) |
|---|---|---|
| Uninoculated wood | 0 | 0 |
| 5.67 log CFU/cm² on wood | 30 | 14.17 |
| 3.66 log CFU/cm² on wood | 33.33 | 17.65 |
| Positive control | 59.26 | 37.04 |

Table 2: Percentage of apples with necrosis and percentage of wounds having necrotized after 12 days of incubation, according to inoculum concentration.

Analyses were first performed on apples in contact with non-inoculated wood. No necrosis was observed, confirming that *P. expansum* is necessary for necrosis development. When wood was inoculated with conidia, around 30 % of apples had necroses and between 14 and 17% of wounds had necrotized. Results were identical for both concentrations. On agar plates covered with *P. expansum* mycelia, 59 % of apples had necroses and between 37% of wounds had necrotized. These results demonstrate that contact with wood reduces percentages of necrosis, compared to the positive control on agar plates. Inoculum concentration does not affect necrosis.

# CONCLUSION

Our results demonstrate that:

- *P. expansum* conidia can survive on wood specimens even after 1 week but do not grow, whereas *E. coli* do not survive on poplar specimens;
- *E. coli* does not seem to be transferred from poplar specimens to apple peels whereas transfer rates for *P. expansum* after 1 hour or 1 week are equal to or lower than 0.25%;

This study contributes to demonstrate that wood is a safe material with regards to microbial contamination of foodstuffs.

Cette étude a démontré que la méthode FCBA est performante pour l'analyse microbiologique des emballages bois et que le bois est un matériau apte au contact alimentaire.

# Pour en savoir plus

✓ *Conférence IRG47 du 15 au 19 mai 2016 à Lisbonne, Portugal*


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