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Short communication

Evaluation of biohazards in dehydrated biofilms on foodstuff packaging

Elisabeth Le Magrex-Debar, Jérôme Lemoine, Marie-Paule Gellé, Louis-Frédéric Jacquelin, Claude Choisy*

Laboratoire d'Adhésion Bactérienne, Biomatériaux, IFR 53, Biomolécules, 51 Rue Cognacq-Jay, 51096 Reims Cedex, France

Abstract

Plastic materials used for food packaging are clean but not sterile when the food is just packaged. Accidental wet contamination may occur at every moment between packaging and opening by the consumer: on polyethylene (PET), bacteria may adhere strongly and constitute a biofilm in less than 24 h. By rolling on themselves, PET sheets may contaminate food. We tried to show that contact with salted foodstuffs favoured microbial recovery. Four strains were chosen to perform biofilms on PET: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Escherichia coli*. Biofilms were dried up 24 h. Biofilm bacteria were stressed by adhesion, by starvation and by dehydration. However, they were capable of recovery in salted solutions or media, probably because one (or more) stress protected them against another stress. Stress was demonstrated by stress protein production, by mean of electrophoresis, and membrane lesions by mean of flow cytometry. Stress recovery was performed in aqueous salted solutions or salted brain–heart infusion with NaCl 9, 15, 20 and 30 g/l. *Staphylococci* were more sensitive to these stresses and recovery was a function of salt concentration. Gram-negative bacteria were little affected by stresses; salt effects were less important. If all these biofilms were capable of recovery from stresses in salted media, flexible PET could possibly lead to a health hazard when it is used for wet salt meats, e.g.

Keywords: Biofilm; Adherence; Dehydration; Stress; Foodstuff packaging

1. Introduction

Numerous bacterial species may adhere on polyethylene (PET), very frequently used for foodstuff packaging. Microbiological hazards, whenever water is present, generate biofilms, as a result of primary bacterial adhesion and coaggregation. Adhesion is a stress which favours stress protein production (Marshall and Goodman, 1994), such as heat (Oberg et al., 1998), cold (Bayles et al., 1996; Vodopyanov et al., 1997), pH changes (Brown et al., 1997; Flahaut et al., 1997), specific nutrient privation or starvation (Nystrom et al., 1992), toxic metal ions (Khare et al., 1997; Khassanova et al., 1998), and change in various parameters: osmolarity (Tripathi et al., 1998), a_w (Mugnier and Jung, 1985) and redox potential, or many successive stresses (Kenny et al., 1997). It seems that bacteria stressed with change in one of the previous parameters may resist many

*Corresponding author. Tel./fax: +33-26-913-595.
E-mail address: claude.choisy@univ-reims.fr (C. Choisy)

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other stresses (Nystrom et al., 1992). The synthesis of some virulence factors or toxins may be the consequence of one or more stresses (Carpentier et al., 1998). Since PET flexible sheets are often the closing pellicle of the packaging of many foodstuffs, and since these materials roll easily on themselves, the external face touching the food may contaminate it. CNERNA (France) has defined the concept of middle range water activity products (Multon, 1981). They have fixed the upper a_w to 0.84 (25°C). Nevertheless, according to Beuchat and Golden (1981), staphylococci may be able to grow at a_w = 0.8–0.87. On the contrary, colibacilli and *Pseudomonas* will not grow, except if they are protected by stress proteins or by their environment, such as amino acids or saline media.

In this paper, we showed that recovery of bacteria which were stressed with both adhesion and dehydration increased in saline solutions and hypersalted media. As a consequence, flexible PET sheets represent a potential danger for wet salt meats by microbiological hazards with staphylococci, colibacilli and *Pseudomonas*. Risk prevention of such hazards is quite difficult to achieve since the disinfection of biomaterial may only be transient (Jacquelin et al., 1994; Chumkhunthod et al., 1998). The treatment of PET materials in order to lower the surface energy (Carpentier et al., 1998) or the choice of a rigid packaging may be good alternatives.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains used were *Staphylococcus aureus* ATCC 9144, *Staphylococcus epidermidis* slime + *Pseudomonas aeruginosa* ATCC 15442 and *Escherichia coli* ATCC 8739.

Strains were chosen by reference to assay of biocidal activity of antiseptics and disinfectants or conservatives according to AFNOR, CEN, or Pharmacopeia regulation, cultured on brain–heart agar (BHA) according to AFNOR (1988) regulation NF T 72 140.

Planctonic bacteria were obtained from brain–heart-infusion (BHI) in glass bottles (250 ml).

Bacterial biofilms were obtained by biofilm development on PET from either 8-cm^2 samples or from 250-ml PET bags in adhesion medium:

BHI	100 ml	*Casamino acids Difco	1 g
Biofilm medium 10 × *	40 ml	Yeast extract	1 g
Sterile water	100 ml	$Na_2H\ PO_4$	12.5 g
Dextrose solution 10%	2.5 ml	$K\ H_2PO_4$	0.5 g
$MgSO_4 \cdot 7H_2O$ solution 20 g/l	2.5 ml	H_2O qsp 1000 ml	

Biofilm culture medium: the same medium diluted to 1/4 with sterile water, but with 1% of $MgS0_4$ solution.

2.2. Biofilm development

The third of successive cultures on BHA, 24 h apart, was harvested and resuspended in 10 ml sterile water, adjusted to an $O.D._{620}$ ranging between 0.20 and 0.40 (i.e., about 10^8 cells/ml) and inoculated in 250 ml medium. Adhesion was performed on PET bags (stress proteins) or on 8-cm^2 samples in Borrel's vials containing 15 ml of adhesion medium. After 45 min at + 37°C, the adhesion medium is replaced by similar volume of culture medium so that only bacteria which adhered to PET could grow. Biofilms were incubated at times consistent with optimal formation (24 h) and reference value (48 h) for biofilms ageing.

2.3. Biofilm quantification and recovery

Biofilm tests were rinsed three times, placed in test tubes containing 5 ml water, subjected to ultrasonic bath (Ultrasonik 300 Ney) for 60 s, homogeneized on a Vortex-mixer, and diluted for bacterial count, before seeding with Spiral (Interscience) on BHA.

All other borrels were emptied and placed upside down, PET samples left to dehydrate for 24 h at room temperature (regulated to 21°C).

Recovery was performed with saline solutions at

9, 15, 20 and 30 g/l in water or in nutrient broth. After 6 h all samples were treated as test biofilms.

2.4. a_w

a_w was measured on a Novasina apparatus, with reference saturated solutions: 0.931, KNO_3; 0.545, Mg $(NO_3)_2$; and 0.114, LiCl.

3. Results and discussion

Results are summarized in Fig. 1. The first column represents the maximal level that biofilms could reach at the moment of dehydration stress. The second, the maximal level in wet conditions. The third was left in dry conditions until the treatment of all biofilms. All others had various conditions of recovery.

Dehydrated staphylococci biofilms never reached the level of wet biofilms, either in nutrient broth or in saline solutions. *S. aureus* was able to increase by one log the number of viable bacteria in salted broth at 30 g/l NaCl versus nutrient broth without addition. *S. epidermidis* recovery increase was less important: 0.5 log, but the salted water solution effect was similar to that of salted nutrient broth, and reached a lower level that wet biofilms.

Gram-negative bacteria seemed protected from dehydration stress: variations in recovery are not significant with *P. aeruginosa*. We noted that the osmolarity of 30 g/l NaCl in water decreased the viable bacterial count. *E. coli* was little sensitive to dehydration stress, but salted nutrient broths favoured recovery, above the standard wet biofilm level. A light osmolarity effect was noted with NaCl 30 g/l solution.

However, hypersalted media favoured all four strains of this assay in recovery.

Bacterial stress in biofilms was proved by induced stress proteins synthesis (Gellé et al., 1995; Marshall and Goodman, 1994) and by flow cytometry, as demonstrated in a previous paper (Gellé et al., 1995). We pointed out that many stress proteins, such as β-galactosidase were synthesized by *E. coli* in a dynamic biofilm, grown in a medium without inducer (without lactose), planctonic bacteria did not show this production in the same medium. Heavy metal ions stress gave induced proteins with a similar pattern to the one observed with *E. coli* biofilm (Khassanova et al., 1998).

Moreover, bacteria labelled with propidium iodide (PI) and fluorescein diacetate (FDA) showed in biofilms a high ratio of double-labelled population versus planctonic bacteria. PI revealed membrane lesions and FDA revealed synthesis of acetylase. PI-labelled bacteria would be dead, and FDA-labelled bacteria would be viable. The double-labelled population (37% in a *E. coli* dynamic 5-day-old biofilm, 2% in planktonic *E. coli* 24 h) indicates that biofilm stress is consistent with membrane injury, but allows enzyme synthesis (Gellé et al., 1995)

In these experiments, stress had multiple origins: adhesion, starvation (diluted medium favoured biofilm development), dehydration, and perhaps osmolarity changes (with high NaCl concentrations). One of them, or all of them, protected biofilms cells, favouring recovery. Nystrom et al. (1992) showed that different stresses may produce these proteins in variable quantities at a rate that could be dependent on environment. Moreover, amino acids of the nutrient broth seemed able to protect specifically any strains (Hood and Zottola, 1997). It is quite possible than different ratios of stress proteins published by various authors differed chiefly on account of the delay after bacterial stress for analysis. Thus, bacteria could then restrict their synthesis to the most useful proteins as a function of their environment.

a_w of recovery media were measured, but their ranges were similar (0.97–0.99). So, differences observed in recovery were probably due to changes in osmolarity rather than in a_w.

4. Conclusion

The resistance of bacteria in biofilms, as seen frequently in foods, is potentially dangerous for health and allows one to think that their presence may be especially hazardous on foodstuff packaging of wet salt meats (e.g., sliced ham). Moreover PET films, by rolling on themselves, may touch the food and thus contaminate it. As a consequence, soft PET foils represent a microbial hazard with staphylococci, colibacilli and *Pseudomonas*, since NaCl is not an inhibitor in an open packaging.

Risk prevention of such hazards is quite impossible to carry out well, since the disinfection of a

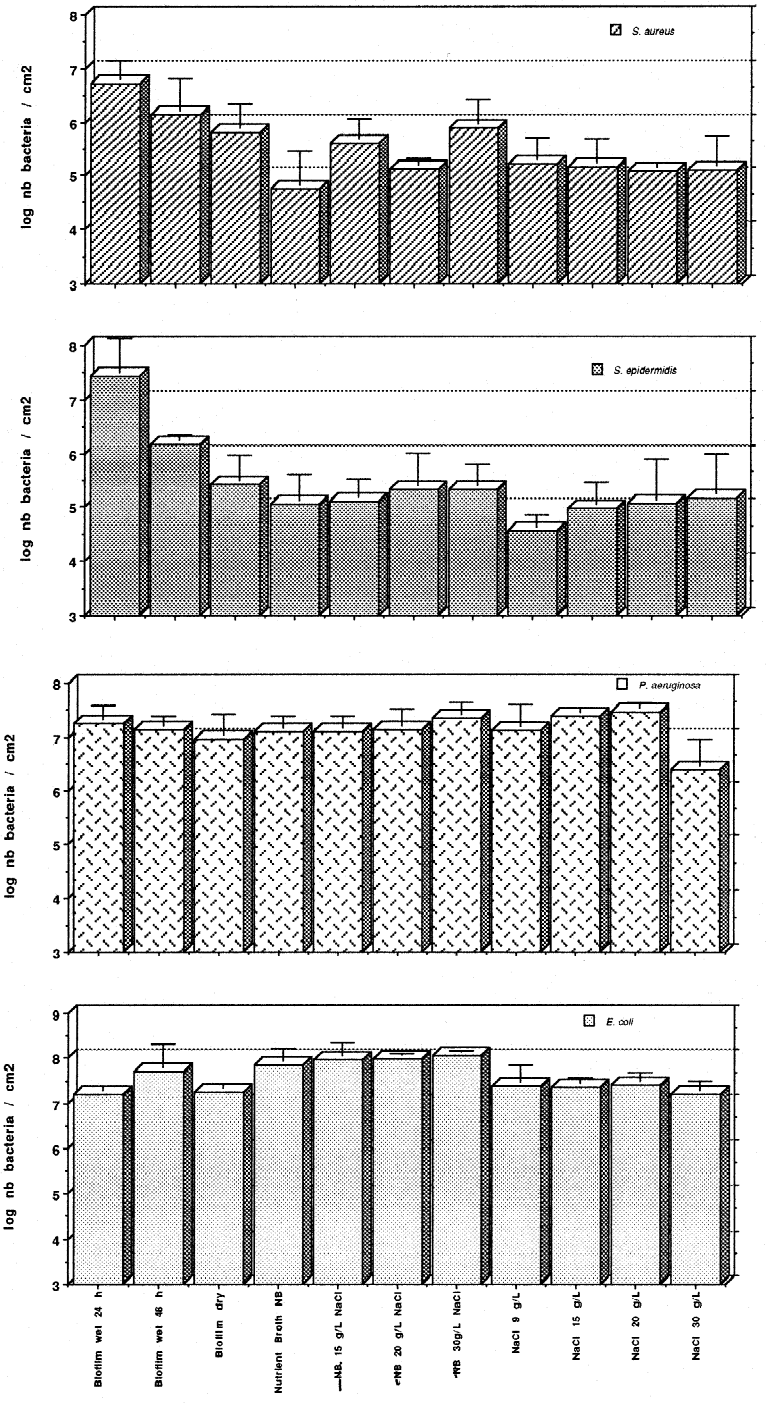

Fig. 1. Quantification of viable biofilms bacteria after recovery in various salted media (conditions of recovery are listed under *E. coli* versus 24- or 48-h fed biofilms).

biomaterial may only be transient (Jacquelin et al., 1994; Le Magrex et al., 1994; Chumkhunthod et al., 1998). The surface treatment of PET materials, in order to reduce the surface energy (Carpentier et al., 1998), or the choice of a rigid packaging may be good alternatives.

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