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Monitoring of bacterial load in terms of culturable and non-culturable cells on new materials placed in a delicatessen serve over counter

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ABSTRACT

The aim of this study was to determine how quickly the surface of a refrigerated supermarket serve over counter becomes loaded with bacteria. New material made of polyvinyl chloride or stainless steel was placed on the surface on which foodstuffs are displayed for sale. One to three samples per week for 7 weeks were collected on gauze pads. CFUs were counted and total cells were quantified by real-time PCR. "Viable" cells using real-time PCR following pre-treatment with ethidium monoazide were quantified on stainless steel. Attachment strengths were assessed at the end of the experiment by constructing detachment curves. Whatever the material, on day 1 the microbial load reached values near those observed in the following weeks i.e. 10^3–10^4 log total cells/cm^2. The number of cells deposited in one week was compensated for by the small reduction obtained by cleaning and disinfection (C&D). The mean difference between total and viable cells was 0.54 log CFUs/cm^2. A big drop in CFUs following C&D was observed at the beginning of the experiment, despite no visible decrease in the number of viable cells, but the CFU reduction decreased over time. Nevertheless, the low efficiency of C&D on the dominant microbiota did not indicate the fate of pathogenic bacteria on these materials. Our data suggest that dead cells do not adhere quite so well as viable cells. Although no growth was observed and the attached bacterial community cannot therefore be considered a biofilm, attached cells shared certain properties attributed to biofilms i.e. their resistance to C&D increased over time and they followed a biphasic detachment curve.

1. Introduction

The surfaces of equipment and rooms in shops selling foodstuffs are likely to contaminate the foodstuffs themselves either by direct contact or indirectly by aerosol transport, especially during cleaning (Holah and Thorpe, 1990). To limit such transfers, C&D is carried out regularly, but a fraction of the population on the surfaces remains (Mettler and Carpentier, 1998; Gibson et al., 1999). Numerous laboratory studies have focused on this issue, and particularly the formation of biofilms, resistance to disinfectants and bacterial transfers. There are significantly fewer field studies designed to find out what is happening on the surfaces. Most field studies only focus on the culturable population. However, within a bacterial population there are dead bacteria, culturable bacteria and viable but non-culturable bacteria (VBNC) in varying proportions. VBNC bacteria are found when the bacterial population is subject to stress, as observed during a study on surfaces subjected to alternate culturing in meat exudate and C&D for two species: *E. coli* O157:H7 (Marouani-Gadri et al., 2010) and *Pseudomonas fluorescens* (Peneau et al., 2007). The VBNC state is thought to be a survival strategy for bacteria in natural environments (Roszak and Colwell, 1987). Temperature (Oliver et al., 1991), limitation of nutriments (Jiang and Chai, 1996; Jorgensen et al., 1994), osmolarity (Roszak et al., 1984), humidity (Heidelberg et al., 1997) and aeration (Rollins and Colwell, 1986) are all major factors contributing to the occurrence of VBNC bacteria. Various methods of assessing viability have been successfully developed to detect the presence of VBNC cells, including the direct viable count (Kogure et al., 1979), fluorescence microscopy using a variety of markers such as cyanoditolyl tetrazolium chloride (CTC) (Rodriguez et al., 1992) or PCR, especially real-time quantitative PCR combined with ethidium monoazide (EMA-qPCR) (Nogva et al., 2003) or propidium monoazide (Cawthorn and Witthuhn, 2008; Nocker et al., 2006). EMA- and PMA-qPCR aim at quantifying cells with intact membranes which are widely assumed to be "viable" cells. The term "viable" will thus be used in the following to designate cells with intact membrane. Given that the microorganisms in food factories are subject to numerous stresses, including chemical treatments and water stress, it is likely that at least some of the non-culturable cells observed in situ are VBNC.

The research presented here aims to increase knowledge of the microbiology of surfaces in the field, where conditions are hostile to microorganisms. We sought in particular to establish how quickly a surface becomes bacterially loaded. We therefore studied the changes in bacterial load of new material (PVC and stainless steel sheets) placed in regularly cleaned and disinfected serve over counter for ready-to-serve food and fermented pork products. We counted culturable cells (CFU)

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over time and, for a broader overview, we also quantified "total" cells i.e. all cells, whether living or dead, and viable cells, which include both culturable and VBNC cells. Total cells were quantified using qPCR and viable cells by EMA-qPCR. Finally, we assessed the attachment strength of microorganisms on the two materials.

2. Material and methods

2.1. Refrigerated equipment, materials and sampling areas

New sheets of polyvinyl chloride (PVC) (Laque Folie, Auchan, France) and stainless steel (AISI 441, reference 713401, Bourgeat, Les Abrets, France) were placed on the surface on which food is displayed for sale in a serve over counter from a supermarket containing ready-to-serve food such as pâtés and quiches and fermented raw pork products such as sausage and salami. All food products were displayed on trays. Two areas of the counter were studied at the same time: one area where food is packaged and another where it is not.

For each area studied, we successively used a PVC sheet in the middle of which we defined an 80×40 cm sampling zone and two stainless steel sheets, each measuring 60×40 cm, placed next to each other. Samples were taken all over the stainless steel. Supermarket staff clean and disinfect (C&D) the display cabinet on a Saturday evening after the supermarket has closed, once a week at most. They spray an alcohol-biguanide mixture, which is a cleaner and disinfectant product, over the surfaces (Divosan S5, Johnson-Diversey, Fontenay-sous-bois, France) and then wipe them with a clean single-use cloth. The supermarket is shut on Sundays.

2.2. Temperature and relative humidity

The temperature and humidity of the air used to refrigerate the display were measured at the cold air discharge and warm air return for 7 days, one measurement being taken every 5 min with a time-temperature-relative humidity data logger (Proges Plus, Willems, France).

2.3. Sampling

Samples were taken in the morning before the supermarket opened. On Mondays sampled surfaces were visually clean except when C&D had not been performed (fat was visible). Between 1 and 3 samples were taken per week, one always on a Monday, for 7 weeks. The surface of each material was wiped with a zigzag motion in two perpendicular direction with sterile gauze pads (40×40 cm) kept in a stomacher bag and impregnated with 10 ml of a multi-purpose neutraliser (reference 757.001800.02, Humeau, La Chapelle sur Erdre, France). The area chosen for sampling on each material was swabbed 5 times on all sampling days, always in the same place and with a new sterile gauze pad each time. The 1st and 5th swabs, plus the ten swabs of the last day, were submitted for analysis within 6 h of collection. Twenty-five millilitres of a 1 g/l peptone solution (Merck, VWR, Fontenay sous Bois, France) were added to the bag containing a gauze pad then homogenised for 30 s using a Stomacher 400 Lab Blender (AES Chemunex, Bruz, France). The bacteria found in the resulting suspensions were counted using different methods.

2.4. CFU counts

Colony-forming units were counted on a Tryptone Soy Agar (Oxoid, Dardilly, France) supplemented with 6 g/l of yeast extract (Oxoid) (TSA-Ye). To count the CFUs, 100 µl of each bacterial suspension was spread over the agar at an appropriate dilution then incubated in aerobic conditions at 25 °C for 72 h.

2.5. Quantification of the bacterial population by real-time quantitative PCR

Bacterial pellets were obtained from 1 ml of each suspension from samples after centrifuging at 15,000 *g* for 3 min. The pellets were then stored at −20 °C until use to quantify either total cells (qPCR) or viable cells (EMA-qPCR).

2.5.1. Extraction of total bacterial DNA

The total bacterial DNA was extracted using a "MagNa Pure LC DNA isolation kit" (Roche Diagnostics) in accordance with the manufacturer's instructions. This DNA extraction method was chosen since preliminary comparisons with 3 other methods showed that it yielded the best extraction efficiency and produced solutions with no PCR inhibitors. The resulting DNA solutions were stored at −20 °C prior to analysis.

2.5.2. qPCR

A LightCycler 480 (Roche Diagnostics) was used for the real-time quantitative PCR. 96-well plates were used for amplification and detection with SYBR-Green® PCR 2X Master Mix (Applied-Biosystems). Each reaction was performed twice in a final volume of 25 µl with a final concentration of 0.2 µM for each primer F_Bact1369 (CGGT GAATACGTTCCCGG) and R_Prok1492 (TACGGCTACCTTGTTACGACTT) (Suzuki et al., 2000) and 10 µl of appropriate dilutions of DNA solutions. Amplification ramping conditions were 1 cycle at 95 °C for 10 min, then 40 cycles at 95 °C for 30 s, followed by 60 °C for 1 min.

2.5.3. Standard curves and quantification

We established a proportionality relationship between the CFUs and threshold cycle values (Ct) determined by qPCR following the method proposed by Lyons et al. (2000). Twenty strains belonging to the dominant bacterial flora were used (Table 1). These strains were isolated six months before the present study from the same refrigerated serve over counter that was already devoted to display the same food products. Each strain was cultured on Tryptone Soy Agar slants supplemented with 6 g/l of yeast extract (TSA-Ye, Oxoid, France) for 20 h at 25 °C. The cultures were then suspended in 5 ml of saline buffer and the concentration of resulting suspensions adjusted to approximately 10^8 CFU/ml using optical density measurements at 600 nm. The number of CFUs was determined after spreading on TSA-Ye and incubation at 25 °C for 72 h. The bacterial genomic DNA of each suspension was extracted as previously indicated. Real-time

Table 1
Strains belonging to the dominant flora of the serve over counter used to establish the standard reference line for qPCR.

Genus	Species	CCL[a] reference
Acinetobacter	*lwoffii*	646
Arthrobacter	*bergeri*	647
Bacillus	*simplex*	621
Carnobacterium	*viridans*	625
Corynebacterium	*propinquum*	651
Empedobacter	*brevis*	656
Kocuria	*varians*	658
Lactococcus	*lactis*	659
Microbacterium	*testaceum*	649
Micrococcus	*luteus*	655
Propionibacterium	*avidum*	654
Pseudomonas	*fluorescens*	638
Pseudomonas	*putida*	637
Psychrobacter	*aquimaris*	645
Staphylococcus	*capitis*	636
Staphylococcus	*lugdunensis*	650
Staphylococcus	*saprophyticus*	657
Staphylococcus	*sciuri*	641
Staphylococcus	*xylosus*	624, 635

[a] Collection CNEVA LERPAC.