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The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm

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D.E. NORWOOD AND A. GILMOUR. 2000. A constant-depth film fermenter (CDFF) was used to culture a steady-state multispecies biofilm consisting of one strain each of *Listeria monocytogenes, Pseudomonas fragi* and *Staphylococcus xylosus.* These bacteria were initially grown together in a conventional chemostat to achieve a steady state before being inoculated into the CDFF over an 18-h period. A dilute tryptone soya broth (TSB) medium was supplied to the CDFF and the biofilm allowed to develop over a 28-d period. This mature biofilm was then subjected to increasing levels of sodium hypochlorite solution to measure any antimicrobial effect. The three organisms were seen to reach a steady state after 6 d in the chemostat before being transferred to the CDFF where the mature multispecies biofilm reached steady state at 17 d. *Listeria monocytogenes* in both planktonic and biofilm growth stabilized at 1·8 and 1·5%, respectively, of the total plate counts, while *Ps. fragi* and *Staph. xylosus* were the predominant organisms in the biofilm at 59% and 39·5%, respectively, of the total microbial population. Steady-state biofilms in the CDFF were exposed to increasing strengths of sodium hypochlorite; 200, 500 and 1000 p.p.m. free chlorine, but a substantial two-log cycle drop in bacterial numbers was only achieved at 1000 p.p.m. free chlorine. In planktonic culture all three organisms were completely eliminated when exposed to 10 p.p.m. free chlorine for a 30-s period.

INTRODUCTION

Food has been shown to be the primary mode of transmission of *Listeria monocytogenes*, which has been implicated in numerous food-borne disease outbreaks (Schlech *et al.* 1983; Linnan *et al.* 1988; Farber and Peterkin 1991). This pathogen has been isolated from an extensive range of food products, including soft cheeses (Pini and Gilbert 1988), fish products (Eklund *et al.* 1995), vegetables (Breer and Baumgartner 1992), milk (Harvey and Gilmour 1992), paté and other cooked meat products (Gilbert *et al.* 1993). It is believed that contamination of these products by *L. monocytogenes* occurs during post-process procedures rather than survival during the processing itself (McLauchlin 1987). In at least one case, the same strain of *L. monocytogenes* had been isolated from both processed food and biofilms within the food plant implicated (Jacquet *et al.* 1993). The organism has been found in drains, standing water, residues, food-contact surfaces and floors (Cox *et al.* 1989).

The capacity of *L. monocytogenes* mono-cultures to adhere to surfaces has been well documented (Frank and Koffi 1990; Lee and Frank 1991; Kryinski *et al.* 1992; Hood and Zottola 1997a; Norwood and Gilmour 1999). Food processors have always relied on physical and chemical methods to eliminate micro-organisms from these surfaces. However, research in this area has indicated that adherent micro-organisms may be much more resistant to sanitizing compounds than planktonic cells. Frank and Koffi (1990) showed that *L. monocytogenes* adhering to glass survived more than 10 times longer than free-living cells when exposed to benzalkonium chloride, anionic acid sanitizer or heat. Also, Lee and Frank (1991) found that *L. monocytogenes* adhering for 8 d were 100 times more resistant to hypochlorite than those adhering for only 4 h. However, the vast majority of organisms in the natural environment (Costerton *et al.* 1987) and in the food-pro-

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cessing environment (Jeong and Frank 1994a; 1994b; Hood and Zottola 1995; 1997b) occur in multispecies biofilms in which the combined effect of bacterial shielding and increased production of extracellular polymeric substances, or glycocalyx, by adhering cells further increases their resistance to antimicrobial agents (Anwar *et al*. 1990, 1992).

A biofilm can either be studied as the natural system *in situ* or as a model system under controlled conditions in a laboratory. The controlled system is said to be at steady state. The merit of a biological system at steady state is that time has been removed as a variable. Parameters of interest can then be altered and cause-and-effect relationships determined. In the constant-depth film fermenter (CDFF), cells attach to the substratum surface and proliferate to fill a recessed 'plug', with excess material being removed from the upper surface of the biofilm using a scraper blade.

This study involved the co-culture of *L. monocytogenes, Pseudomonas fragi* and *Staphylococcus xylosus* to steady state using a chemostat. The steady-state output of the chemostat was then transferred to the CDFF and the growth of these three organisms into a multispecies biofilm achieved. This model was then used to determine the effect of increasing levels of sodium hypochlorite on the three micro-organisms.

MATERIALS AND METHODS

Micro-organisms

The three organisms used to form the biofilm were *L. monocytogenes* Scott A, *Ps. fragi* ATCC 4973 and *Staph. xylosus* DP5H. The *Staphylococcus* species had been isolated from a food-processing environment.

Growth medium and sterilization

Diluted Tryptone Soya Broth (TSB; 2 g l^{-1}; CM129; Oxoid, Basingstoke, UK) was the medium chosen to grow the cultures to steady state in the chemostat and to develop the biofilm in the CDFF (Jeong and Frank 1994b). The dilute TSB was sterilized by irradiation (30 kGy) in a 20-l plastic vessel (Nalgene, Milton Keynes, UK).

Chemostat inoculation and growth conditions

The chemostat used (LH 500; LH Engineering, Stoke Poges, UK) had a working volume of 500 ml. All three organisms were initially cultured from cryo-beads (−80 °C) in full-strength TSB (30 g l^{-1}) at 30 °C for 18 h before being inoculated (5 ml per organism) into 300 ml dilute TSB (2 g l^{-1}) within the chemostat. The sterile dilute medium was initially pumped into the chemostat at a rate of 15 ml h^{-1} until the working volume of 500 ml was attained. The media flow rate was then adjusted to 50 ml h^{-1} to give a working dilution rate of 0·1 h^{-1}. The chemostat was operated under aerobic conditions with sterile air being supplied at a rate of 200 ml min^{-1}. The pH value was maintained at 7·0 ± 0·5 by automatic addition of 1 mol l^{-1} NaOH. The whole experiment was carried out at a room temperature of 21 ± 1 °C (temperature-controlled laboratory).

Inoculation of the constant-depth film fermenter

Upon reaching steady state, the 500 ml culture was inoculated into the CDFF over a period of 18 h after which sterile dilute TSB (2 g l^{-1}) was supplied at 30 ml h^{-1} for the remainder of the experiment.

Operation of the constant-depth film fermenter

The CDFF used for this study was the same as that described by Wimpenny *et al*. (1993). The biofilms grew on 4·75-mm diameter stainless steel (grade 304) plugs in 15 polytetrafluoroethylene (PTFE) sample pans which were inserted into a rotatable stainless steel turntable. Each pan contained six plugs recessed to a depth of 300 μm, making a total of 90 plugs in the CDFF. The biofilm was allowed to develop over 28 d, by which time it had reached this thickness, which was maintained by the action of a PTFE scraper bar. The turntable rotated at a speed of 3 rev min^{-1} and the fermenter was again operated at room temperature with air being allowed to diffuse through a sterile air filter.

Medium dripped onto the turntable just in front of the scraper blade hand at a rate of 30 ml h^{-1} and was then evenly spread over the sample pans. Waste medium flowing off the turntable was collected in a 20-l vessel below, via an outlet port in the base plate of the fermenter. Sample pans were removed aseptically over time through a sample port in the top plate of the fermenter.

Estimation of biomass

Chemostat viable counts. Daily samples of chemostat suspension were taken, serially diluted in maximum recovery diluent and plated out on a range of selective media. *Listeria* Selective Agar Base (Oxford Formulation; CM856; Oxoid) plus *Listeria* Selective Supplement (SR140E) was used for counting *L. monocytogenes* Scott A while *Pseudomonas* Agar Base (CM559; Oxoid) plus *Pseudomonas* C-F-C Selective Supplement (SR103E) was used for counting *Ps. fragi*. Tryptone Soya Agar plus 40 μg ml^{-1} erythromycin was used to select for *Staph. xylosus*. Agar selectivity was confirmed before experimental use. Tryptone soya agar plates were used to obtain total viable counts.

Constant-depth film fermenter viable counts. Samples were

obtained periodically by removing a sample pan from the fermenter and carefully pushing the six plugs out of the pan with biofilm still intact on the surface. A pair of plugs was then placed into each of three 30-ml sterile plastic universals (Bibby-Sterilin, Stone, UK) together with sterile glass beads (0·7 g universal^{-1}; G-4649, 106 m; Sigma, St Louis, MO, USA) and 10 ml (per universal) phosphate-buffered saline (PBS). The biofilm was then removed and dispersed by vortexing for 1 min at maximum intensity on a Genie II (Scientific Industries, Bohemia, NY, USA) vortexer. Plate counts were then made on the range of selective media described above and the geometric mean together with log S.D. at each time point calculated.

Constant-depth film fermenter protein estimation. Samples were again obtained periodically by removing a sample pan from the fermenter as described above. Protein estimation was determined for each plug using a modified Lowry method (Kinniment *et al*. 1996). The protein on each plug was assayed separately by boiling in 0·5 ml 1 mol l^{-1} NaOH for 5 min within a 1·5-ml microfuge tube and diluting 0·125 ml of the boiled solution into a further 0·375 ml 1 mol l^{-1} NaOH. A solution (2·5 ml) containing 96 ml 5% (w/v) Na_2CO_3 and 4 ml 0·5% $CuSO_4.5H_2O$, 1% (w/v) sodium potassium tartrate was then added to the samples. After 10 min, 0·5 ml 1 mol l^{-1} Folin & Ciocalteu's Phenol Reagent (F-9252; Sigma) was added to the tube, which was immediately shaken and left for 30 min before the A_{750} was measured (8625 UV/VIS Spectrometer; Unicam, Cambridge, UK). Protein standards containing 2–160 μg protein (Albumin Standard no. 23209; Pierce, Rockford, IL, USA) were prepared each time the assay was performed and a standard curve prepared. Mean protein values and S.D. for the protein levels of the biofilms at each time point could then be calculated. The experiment was repeated in duplicate, thus using 12 plugs per time period.

Treatment of steady-state biofilms with sodium hypochlorite

Steady-state biofilms at 28 d in the CDFF were treated with increasing strengths of sodium hypochlorite, a sanitizer commonly used in dairy plants for cleaning in place procedures (Chumkhunthod *et al*. 1998). Increasing levels of 200, 500 and 1000 p.p.m. free chlorine were used. The sodium hypochlorite solutions (Chlorus; William Clements Chemicals, Belfast, Northern Ireland) were released onto the biofilms over a 20-min period using six 1-s pulses min^{-1}. Immediately following treatment, the biofilms were neutralized over 10 min under the same pulsing system, using 2·22 mmol l^{-1} sodium thiosulphate in 0·011% peptone (Lee and Frank 1991). Viable counts were then made by removing sample pans and following the procedure outlined above. The media flow of dilute TSB was suspended during the pulsing treatment. The free chlorine concentration was measured on each occasion using the direct iodometric titration method (Case *et al*. 1985). The experiment was carried out in triplicate, thus using 18 plugs per time period.

Sodium hypochlorite inactivation of planktonic cells

The three organisms were grown up from cryo-beads (−80 °C) in full-strength TSB (30 g l^{-1}) at 30 °C for 18 h and washed three times in PBS. A volume (5 ml) of a cell suspension (approximately 1×10^9 cfu ml^{-1}) was mixed with 45 ml PBS to give a stock solution for each micro-organism; 5 ml of this stock solution for each organism was removed and mixed with 5 ml of the neutralizing solution (2·22 mmol l^{-1} sodium thiosulphate containing 0·011% peptone) and the resulting plate count used as a control. A volume of the stock solution (20 ml) for each organism was then added to 20 ml sodium hypochlorite to give the desired concentration of free chlorine. Solutions containing three different concentrations of free chlorine were used (5, 10 and 20 p.p.m.). Continuous mixing then took place using a magnetic flea with 5-ml samples being removed at 30 s and 5 min after the addition of the free chlorine. Each sample was then mixed immediately with 5 ml neutralizing solution. Plate counts of the resulting suspensions were made. The free chlorine concentration was again measured using the iodometric titration method (Case *et al*. 1985). The experiment was carried out in triplicate, giving nine observations per free chlorine concentration per organism.

Data analysis

Mean bacterial numbers, the achievement of steady state and the differences in mean values for chemostat and CDFF runs were compared by analysis of variance (ANOVA) and the Student's *t*-test using $\log_{10}$-transformed data. Evaluations were based on a 5% significance level unless otherwise stated.

RESULTS

Chemostat growth

A steady state for all three organisms was not reached until 6 d after inoculation (calculated using ANOVA at the 5% significance level). Although the viable count of *L. monocytogenes* fell slightly further and levelled off in the following 4 d, these counts were not significantly different to that at 6 d growth. Following inoculation at time 0, the viable counts of all three organisms rose one to two log cycles within 24 h before stabilizing for a further 24 h (Fig. 1). Further growth occurred and *Ps. fragi* reached steady state at 3 d and *Staph. xylosus* at

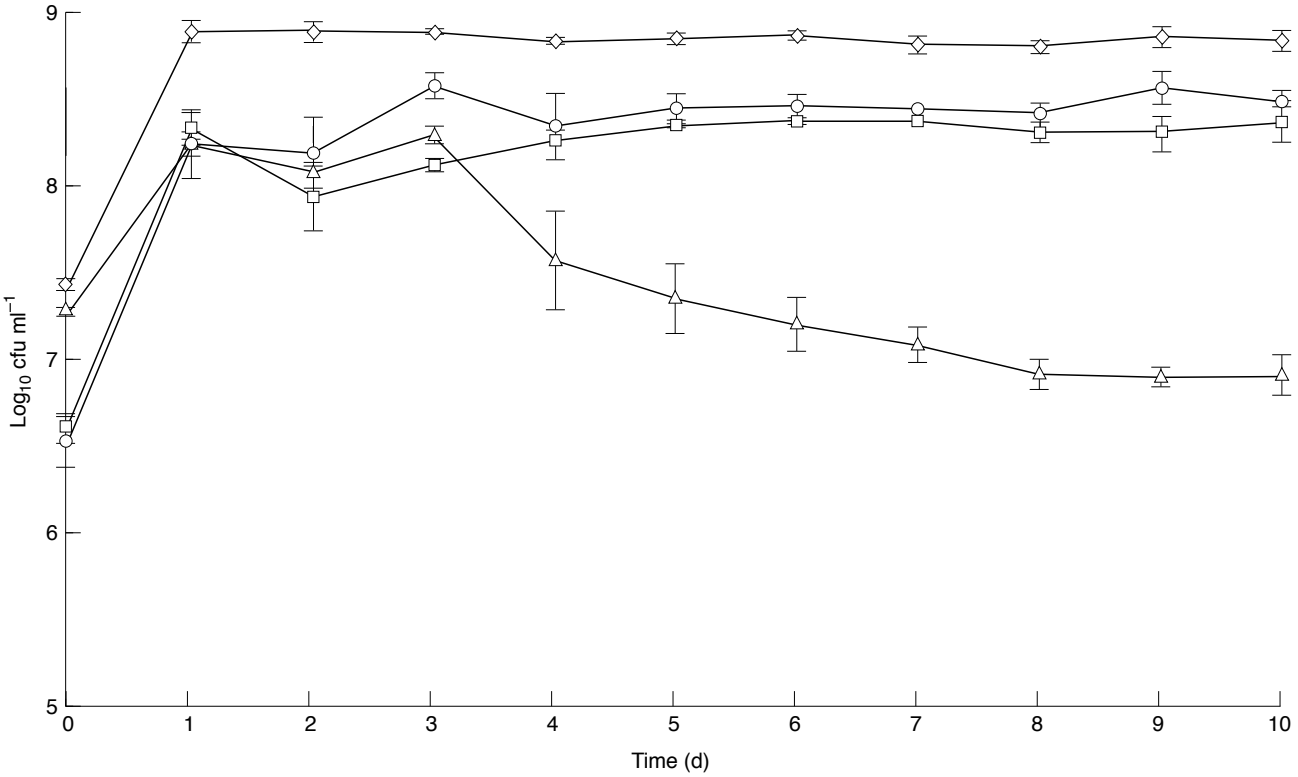

Fig. 1 The growth of *Listeria monocytogenes, Staphylococcus xylosus* and *Pseudomonas fragi* to steady state in a 500-ml chemostat over 10 d at a temperature of 21 ± 1 °C. △, *L. monocytogenes*; □, *Staph. xylosus*; ○, *Ps. fragi*; ◇, total count. $n = 12$

4 d. Both organisms then remained at around 8·3 $\log_{10}$ cfu ml^{-1} for the remainder of the run. At all times following 24 h growth, however, the *Ps. fragi* remained at a slightly higher level than the *Staph. xylosus,* although this was not statistically significant. The *L. monocytogenes* strain, however, pursued a very different path following the initial growth. While, initially, it followed a growth path roughly similar to the other two organisms, from day 3 its viable count began to fall sharply, reaching a statistically steady state from day 6 and stabilizing at around 7·1 $\log_{10}$ cfu ml^{-1}. At steady state *L. monocytogenes* constituted 1·8% of the population (Fig. 2), while *Ps. fragi* and *Staph. xylosus* were the predominant organisms, constituting 52·0% and 46·2%, respectively, of the populations determined by total plate counts. The steady-state value of the *L. monocytogenes* growth curve showed a significant difference from that of both other organisms with $P < 0{\cdot}01$ (Student's *t*-test, two-tailed). The total count reached a steady-state value of 8·8 $\log_{10}$ cfu ml^{-1} within 24 h and remained approximately at this level throughout the remainder of the run. These results were obtained from four separate chemostat runs with plate counts being made in triplicate for each time period.

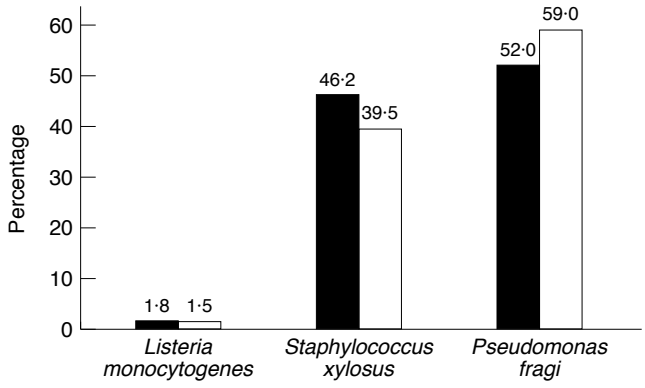

Fig. 2 A comparison of the steady-state proportions of *Listeria monocytogenes, Staphylococcus xylosus* and *Pseudomonas fragi* in the ■, chemostat and □, constant-depth film fermenter

Constant-depth film fermenter growth

Steady states in total viable counts were reached for *Staph. xylosus*, *Ps. fragi* and *L. monocytogenes* at 21, 8 and 4 d, respectively (Fig. 3). In terms of protein production, levels increased

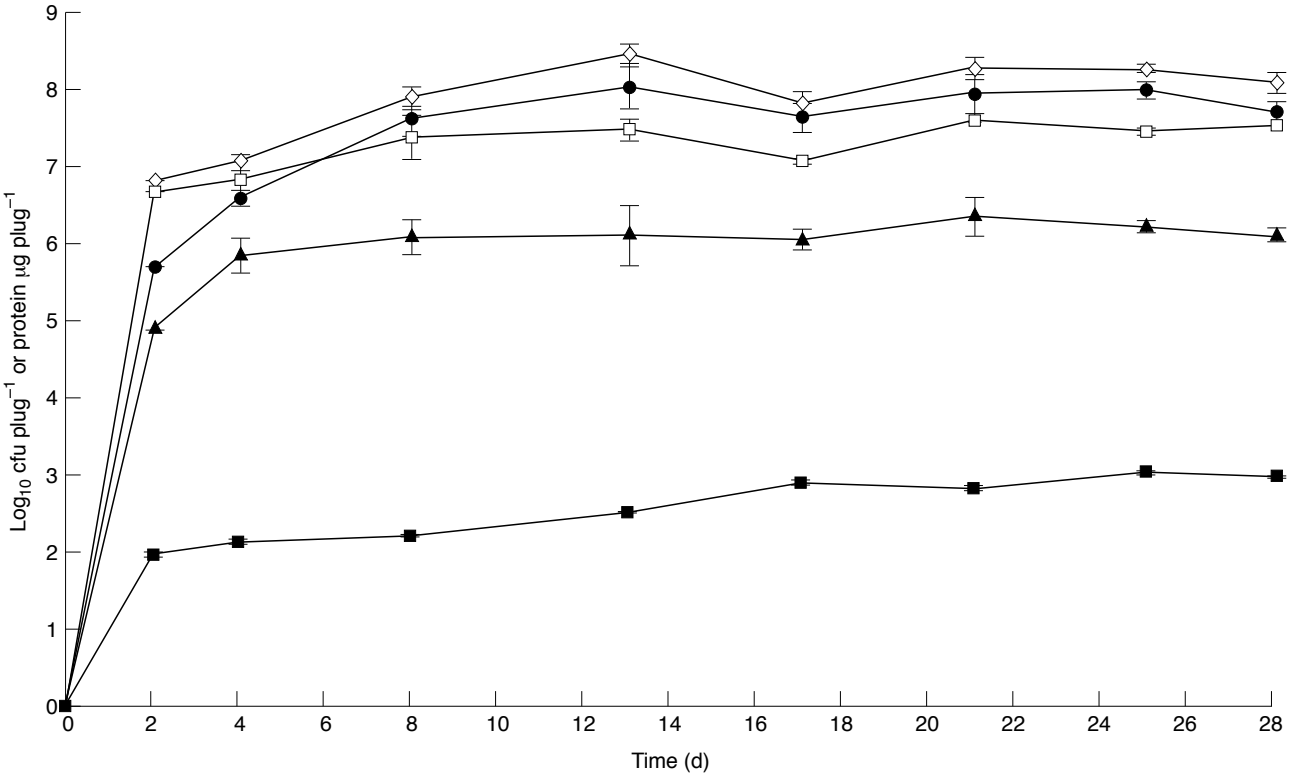

Fig. 3 The biofilm growth in a constant-depth film fermenter of *Listeria monocytogenes, Staphylococcus xylosus* and *Pseudomonas fragi* over 4 weeks in terms of cfu plug^{-1} ($n = 9$) and μg protein plug^{-1} ($n = 12$). ▲, *L. monocytogenes*; □, *Staph. xylosus*; ●, *Ps. fragi*; ◇, total count; ■, protein assay

gradually until a steady state was reached by 17 d at 2·8 $\log_{10}$ μg plug^{-1}. As in the chemostat, *Ps. fragi* and *Staph. xylosus* became the predominant organisms at steady state, this time at 59% and 39·5% of the total viable counts, respectively. *L. monocytogenes* constituted 1·5% of the total viable count (Fig. 2). At 2 d biofilm growth, *L. monocytogenes*, at 4·9 $\log_{10}$ cfu plug^{-1}, was already lagging behind *Ps. fragi* at 5·7 $\log_{10}$ cfu plug^{-1}and *Staph. xylosus* at 6·7 $\log_{10}$ cfu plug^{-1}. By day 8 *Ps. fragi* had overtaken *Staph. xylosus* at 7·6 $\log_{10}$ cfu plug^{-1} and 7·4 $\log_{10}$ cfu plug^{-1}, respectively, but *L. monocytogenes* was much lower at 6·1 $\log_{10}$ cfu plug^{-1} and consistently remained so throughout the rest of the run. The values of all three organisms at steady state showed a significant difference to each other at the 5% significance level. The total count reached a steady state by 8 d at 7·9 $\log_{10}$ cfu plug^{-1}. The results presented were obtained from three separate CDFF runs with plate counts being made in triplicate for each time period.

Sodium hypochlorite treatment

The effects of sodium hypochlorite treatment on the three organisms within the biofilms are illustrated in Fig. 4. Using 200 p.p.m. free chlorine, only *Staph. xylosus* showed a significant, though small, fall in numbers when compared with the steady state. At the 500 p.p.m. free chlorine level all three organisms showed a statistically significant fall ($P < 0{\cdot}05$). The drop in *Staph. xylosus* was such that it was significant even at the $P < 0{\cdot}001$ level. At 1000 p.p.m. all three organisms showed a significant fall in numbers ($P < 0{\cdot}001$) from the steady state, of 1·75, 2·56 and 2·84 log cycles for *L. monocytogenes, Staph. xylosus* and *Ps. fragi*, respectively.

The planktonic cells showed much greater sensitivity to the hypochlorite. The 20 and 10 p.p.m. free chlorine levels totally eliminated the planktonic cells of all three organisms following 30 s exposure, while 5 p.p.m. also completely eliminated the cells of *L. monocytogenes* and *Ps. fragi* at 30 s exposure. Only *Staph. xylosus* showed any surviving cells at 5 p.p.m. free chlorine, with a mean of $2{\cdot}3 \times 10^2$ cells ml^{-1} surviving at 30 s exposure and $4{\cdot}3 \times 10^1$ cells ml^{-1} after 5 min exposure.

DISCUSSION

While *Ps. fragi* is a well-known food-spoilage organism (Jay 1996) and *Staph. xylosus* has been found to produce entero-

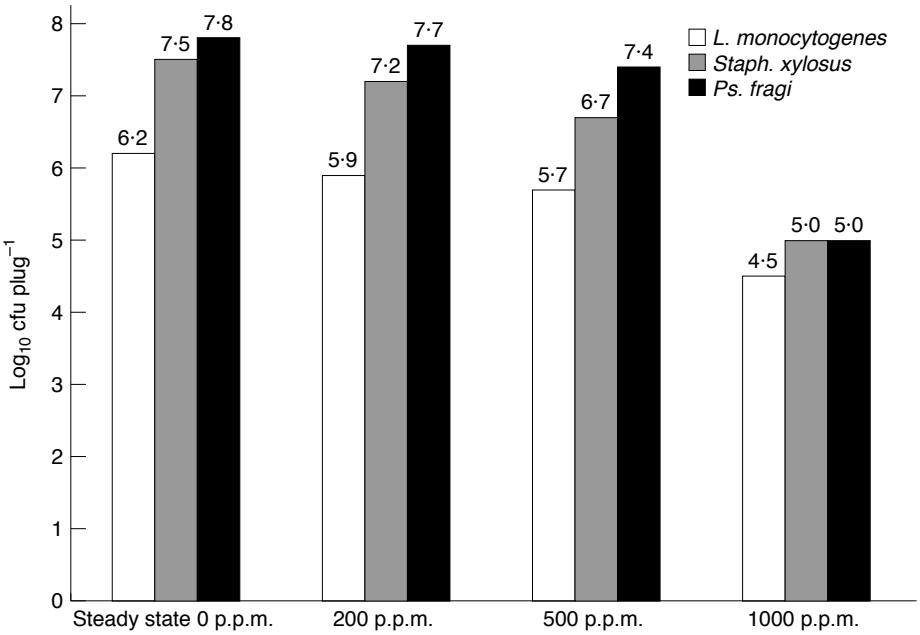

Fig. 4 The survival, after 20 min sodium hypochlorite treatments, of *Listeria monocytogenes, Staphylococcus xylosus* and *Pseudomonas fragi* within the steady-state biofilms. $n = 18$

toxins (Valle *et al*. 1990), the main interest in these organisms in this study was as co-colonizing organisms for *L. monocytogenes*. Sasahara and Zottola (1993) found that, when *L. monocytogenes* was grown in mixed culture with *Ps. fragi*, attachment and microcolony formation by *L. monocytogenes* was enhanced. Pseudomonads and staphylococci are commonly found in the food-processing environment (Jeong and Frank 1994a, 1994b; Hood and Zottola 1997b) and have been previously documented as good producers of extracellular polymeric substances (EPS) or glycocalyx (Das *et al*. 1998; Zoltai *et al*. 1981; Sasahara and Zottola 1993). Staphylococci have also been found to be good indicator micro-organisms for the presence of *Listeria* spp. in the dairy processing industry (Frank *et al*. 1990). They are, therefore, very suitable organisms with which to investigate the growth of *L. monocytogenes* within multispecies biofilms.

The chief purpose of culturing the three organisms in a chemostat before transferring them to the CDFF for biofilm formation was to allow the organisms to be presented to the stainless steel plugs of the CDFF in steady-state proportions, thus approaching the scenario that would be most likely to take place in a natural food-processing environment. Figure 2 compares the steady-state proportions of the three organisms in the chemostat and CDFF. It appears to support the hypothesis that, under uniform environmental conditions, biofilm numbers will follow the proportions of organisms in the feeder vessel, as there is no significant difference between the percentage levels of all three organisms in the chemostat and the CDFF. This was also found to be the case in a study by Jeong and Frank (1994a). The only difference between the two systems in this study is the liquid state in the chemostat and the biofilm state in the CDFF. However, while the environmental conditions in both the chemostat and CDFF in this study were identical, other studies, such as that by Kinniment *et al*. (1996), have altered conditions between the chemostat and CDFF, resulting in significant differences in bacterial growth in the respective vessels.

In the first few days of the chemostat culture all three organisms reached similar levels of growth. However, following day 3 *L. monocytogenes* was not able to compete with *Ps. fragi* and *Staph. xylosus*, falling from 26·3% to 1·8% of the total population by day 10, while *Ps. fragi* and *Staph. xylosus* competed on roughly equal terms, with the former being at a slightly higher (not significant) level throughout. In the CDFF the steady-state population of *L. monocytogenes* was 1·5% of the total count, but the *Ps. fragi* level was significantly greater than that of the *Staph. xylosus,* showing a greater competitive advantage for the pseudomonad in the biofilm compared with the chemostat. The level of *L. monocytogenes* in this multispecies biofilm is comparable with the results of Jeong and Frank (1994a), whose *L. monocytogenes* strain maintained itself at 1% of the total population in biofilms containing a mixture of four competitive cultures. Also in this same work (Jeong and Frank 1994a), *L. mono-*

cytogenes in broth culture experienced a 100-fold decline around the fifth day. In the present study *L. monocytogenes* numbers in the chemostat culture fell almost two log cycles from day 3 to day 8.

While the percentage of each of the steady-state bacterial counts of the three organisms showed no significant difference between the chemostat and the CDFF, there were differences before achieving steady state. While in the chemostat the three organisms grew to roughly the same levels for the first 3 d (differing by only 0·5 log on day 3), in the CDFF the organisms already exhibited large differences from the second day. This is undoubtedly largely influenced by the steady-state levels of the three organisms transferred to the CDFF from the chemostat.

Farrag and Marth (1991) observed that the presence of *Pseudomonas* spp. had either a slight negative effect or no effect on growth of *L. monocytogenes* in tryptose broth, but Villani *et al*. (1997) found that a number of strains of *Staph. xylosus* produced antagonistic compounds that inhibited *L. monocytogenes*. Therefore, in addition to the competition for nutrients and unequal growth rates, this antagonistic effect of *Staph. xylosus* could be a reason for the decreasing competitiveness of *L. monocytogenes* in the chemostat and CDFF cultures.

While *Staph. xylosus, Ps. fragi* and *L. monocytogenes* reached steady states in the chemostat at 4, 3 and 6 d, respectively, the total count reached steady state by day 1. In the CDFF, however, the total count only reached steady state by day 8, while it was reached for *Staph. xylosus, Ps. fragi* and *L. monocytogenes* at 21, 8 and 4 d, respectively. It can be seen, therefore, that there was a general lengthening of time to reach steady state in the biofilm compared with the chemostat, which is not surprising as biofilm takes some time to form. The only exception was *L. monocytogenes* which actually reached steady state 2 d earlier in the biofilm than in the chemostat.

A second indicator of biofilm growth was protein determination. One of the predominant constituents of EPS is protein and, while plate counts will measure any change in cell numbers, protein determinations are a worthwhile measure of 'total' biofilm growth as they measure the protein of cells and that of EPS. Fletcher (1980) reported that EPS from a marine pseudomonad contained 50–80% protein (dry weight). It is, therefore, interesting to note that, while total cell count steady state was reached in the biofilm by day 8, protein steady state was only reached by day 17. It can, therefore, be surmised that the biofilm continues to develop at least until day 17, presumably by producing increased amounts of EPS. The main limiting factor of biofilm growth in the CDFF was the removal of excess material from the upper surface of the biofilm using the scraper blade, which presumably occurred around the 17 d mark from when no further significant protein increase occurred. In ecological terms a 'climax' population was achieved at this stage, which then entered a dynamic equilibrium with the surrounding environment.

With respect to the treatment of the biofilm with sodium hypochloride, only *Staph. xylosus* showed a significant, although small, drop at 200 p.p.m. free chlorine, while at 500 p.p.m. all three organisms showed a significant fall in numbers. Only at 1000 p.p.m. free chlorine, however, was there a greater than 2 log cycles fall in bacterial numbers. In comparison, when planktonic cells (10^8 cfu ml^{-1}) were subjected to only 10 p.p.m. free chlorine for 30 s, an 8 log cycle reduction in numbers occurred. This study clearly demonstrates the vast increase in protection afforded to organisms within multispecies biofilms from antimicrobial agents, compared with the planktonic state. These results therefore agree with the many studies demonstrating the shielding ability of biofilms (Anwar *et al*. 1990, 1992).

While numerous studies have demonstrated the increased antimicrobial resistance of mono-culture biofilms (Frank and Koffi 1990; Lee and Frank 1991; Mosteller and Bishop 1993), relatively little work has been done on the shielding effect of multispecies biofilms, containing food pathogens, to antimicrobial agents. Multispecies biofilms are undoubtedly the 'norm' within food-processing environments, making research into their resistance to antimicrobials all the more pertinent. Lee and Frank (1991), working with mono-culture *L. monocytogenes* biofilms attaching to stainless steel, found that 8-d adherent cells were over 100 times more resistant than a 4-h adherent cell population when exposed to 200 p.p.m. free chlorine for 30 s. Furthermore, after 5 min exposure to 200 p.p.m. free chlorine no adherent cells remained viable. Mosteller and Bishop (1993) also observed a 2 log cycle reduction in *L. monocytogenes* cells adhering to stainless steel after only 30 s exposure to 200 p.p.m. free chlorine. In contrast, a 2 log cycle reduction in *L. monocytogenes* cells in the multispecies biofilms of the present study was only achieved when the biofilm was exposed to 1000 p.p.m. free chlorine for a full 20 min. The work presented here appears to demonstrate the increased protective properties of multispecies biofilms containing *L. monocytogenes* compared with those of mono-culture biofilms of the same micro-organism. This may be attributed to two main factors: the shielding effect of increased numbers (or aggregation) of micro-organisms and the production of greater amounts of EPS (Carpentier and Cerf 1993).

In most food-processing environments, the daily use of sanitizers at correct concentrations preceded by detergent-aided cleaning, will remove the vast majority of adhering organisms or biofilms. However, the maximum concentration of sodium hypochlorite to be used on non-porous surfaces recommended by manufacturers is 200 p.p.m. free chlorine (Mustapha and Liewen 1989; Kryinski *et al*. 1992). Although this is more than sufficient to eliminate planktonic organisms

it is wholly unsuitable for the elimination of *L. monocytogenes* in the multispecies biofilms cultured on stainless steel plugs used in this study. In every food-processing environment there will exist certain surfaces and areas where cleaning is difficult to accomplish, such as bends in pipes, rubber seals, cracks in surfaces, etc. and it is from these locations that the main danger of cross-contamination of pathogens from mature biofilms will exist. It may well be necessary, therefore, to use much higher concentrations of sanitizer than the manufacturer suggests for these particular areas in the food-processing environment.

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REFERENCES

Anwar, H., Dasgupta, M. and Costerton, J.W. (1990) Testing the susceptibility of bacteria in biofilms to antibacterials. *Antimicrobial Agents and Chemotherapy* **34,** 2043–2046.

Anwar, H., Strap, J.L. and Costerton, J.W. (1992) Establishment of ageing biofilms: Possible mechanism of bacterial resistance to antimicrobial therapy. *Antimicrobial Agents and Chemotherapy* **36,** 1347–1351.

Breer, C. and Baumgartner, A. (1992) Occurrence and behaviour of *Listeria monocytogenes* on salads, vegetables, and in fresh vegetable juices. *Archiv fur Lebensmittelhygiene* **43,** 108–110.

Carpentier, B. and Cerf, O. (1993) Biofilms and their consequences, with particular reference to hygiene in the food industry. *Journal of Applied Bacteriology* **75,** 499–511.

Case, R.A., Bradley, R.L. Jr and Williams, R.R. (1985) Chemical and physical methods. In *Standard Methods for the Examination of Dairy Products*, 15th edn. ed. Richardson, G.H. pp. 327–404 Washington, DC: U.S. Public Health Service.

Chumkhunthod, P., Schraft, H. and Griffiths, M.W. (1998) Rapid monitoring method to assess efficacy of sanitizers against *Pseudomonas putida* biofilms. *Journal of Food Protection* **61,** 1043–1046.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C. Dasgupta, M. and Marrie, T.J. (1987) Bacterial biofilms in nature and disease. *Annual Reviews in Microbiology* **41,** 435–464.

Cox, L.J., Kleiss, T., Cordier, J.L., Cordellana, C., Konkel, P., Pedrazzini, C., Beumer, R. and Siebenga, A. (1989) *Listeria* spp. in food processing, non-food and domestic environments. *Food Microbiology* **6,** 49–61.

Das, J.R., Bhakoo, M., Jones, M.V. and Gilbert, P. (1998) Changes in the biocide susceptibility of *Staphylococcus epidermis* and *Escherichia coli* cells associated with rapid attachment to plastic surfaces. *Journal of Applied Microbiology* **84,** 852–858.

Eklund, M.W., Poysky, F.T., Paranjpye, R.N., Lashbrook, L.C., Peterson, M.E. and Pelroy, G.A. (1995) Incidence and sources of *Listeria monocytogenes* in cold-smoked fishery products and processing plants. *Journal of Food Protection* **58,** 502–508.

Farber, J.M. and Peterkin, P.I. (1991) *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Reviews* **55,** 476–511.

Farrag, S.A. and Marth, E.H. (1991) Behaviour of *Listeria monocytogenes* in the presence of *Pseudomonas fluorescens* at 7 or 13°C in skim milk. *Journal of Food Protection* **52,** 852–855.

Fletcher, M. (1980) Adherence of marine microorganisms to smooth surfaces. In *Bacterial Adherence* ed. Beachey, E.H. pp. 347–373. London: Chapman & Hall.

Frank, J.F. and Koffi, R.A. (1990) Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. *Journal of Food Protection* **53,** 550–554.

Frank, J.F., Gillet, R.A.N. and Ware, G.O. (1990) Association of *Listeria* spp. contamination in the dairy processing plant environment with the presence of staphylococci. *Journal of Food Protection* **53,** 928–932.

Gilbert, R.J., McLauchlin, J. and Velani, S.K. (1993) The contamination of paté by *Listeria monocytogenes* in England and Wales in 1989 and 1990. *Epidemiology and Infection* **110,** 543–551.

Harvey, J. and Gilmour, A. (1992) Occurrence of *Listeria* species in raw, milk and dairy products produced in Northern Ireland. *Journal of Applied Bacteriology* **72,** 119–125.

Hood, S.K. and Zottola, E.A. (1995) Biofilms in food processing. *Food Control* **6,** 9–18.

Hood, S.K. and Zottola, E.A. (1997a) Growth media and surface conditioning influence the adherence of *Pseudomonas fragi, Salmonella typhimurium*, and *Listeria monocytogenes* cells to stainless steel. *Journal of Food Protection* **60,** 1034–1037.

Hood, S.K. and Zottola, E.A. (1997b) Isolation and identification of adherent gram-negative microorganisms from four meat-processing facilities. *Journal of Food Protection* **60,** 1135–1138.

Jacquet, C., Rocourt, J. and Reynaud, A. (1993) Study of *Listeria monocytogenes* contamination in a dairy plant and characterization of strains isolated. *International Journal of Food Microbiology* **20,** 13–22.

Jay, J.M. (1996). *Modern Food Microbiology*, 5th edn. London: Chapman & Hall.

Jeong, D.K. and Frank, J.F. (1994a) Growth of *Listeria monocytogenes* at 10°C in biofilms with micro-organisms isolated from meat and dairy processing environments. *Journal of Food Protection* **57,** 576–586.

Jeong, D.K. and Frank, J.F. (1994b) Growth of *Listeria monocytogenes* at 21^0C in Biofilms with micro-organisms isolated from meat and dairy processing environments. *Food Science and Technology-Lebensmittel Wissenchaft und Technologie* **27,** 415–424.

Kinniment, S.L., Wimpenny, J.W.T., Adams, D. and Marsh, P.D. (1996) Development of a steady-state oral microbial biofilm community using the constant-depth film fermenter. *Microbiology* **142,** 631–638.

Kryinski, E.P., Brown, L.J. and Marchisello, T.J. (1992) Effects of cleaners and sanitizers on *Listeria monocytogenes* attached to product contact surfaces. *Journal of Food Protection* **55,** 246–251.

Lee, S. and Frank, J.F. (1991) Inactivation of surface-adherent *Listeria monocytogenes* using hypochlorite and heat. *Journal of Food Protection* **54,** 4–6.

Linnan, M.J., Mascola, L., Lou, X.D., Goulet, V., May, S., Salmi-

nen, C., Hird, D.W., Yonkura, M.L., Hayes, P., Weaver, R., Andurier, A., Plikaytis, B.D., Fannin, S.L., Kleks, A. and Broome, C.V. (1988) Epidemic listeriosis associated with Mexican-style soft cheese. *New England Journal of Medicine* **319,** 823–828.

McLauchlin, J. (1987) *Listeria monocytogenes*, recent advances in taxonomy and epidemiology of listeriosis in humans. *Journal of Applied Bacteriology* **63,** 1–11.

Mosteller, T.M. and Bishop, J.R. (1993) Sanitizer efficacy against attached bacteria in a milk biofilm. *Journal of Food Protection* **56,** 34–41.

Mustapha, A. and Liewen, M.B. (1989) Destruction of *Listeria monocytogenes* by sodium hypochlorite and quaternary ammonium sanitizers. *Journal of Food Protection* **52,** 306–311.

Norwood, D.E. and Gilmour, A. (1999) Adherence of *Listeria monocytogenes* strains to stainless steel coupons. *Journal of Applied Microbiology* **86,** 576–582.

Pini, P.N. and Gilbert, R.J. (1988) The occurrence in the UK of *Listeria* spp. in raw chickens and soft cheeses. *International Journal of Food Microbiology* **6,** 317–326.

Sasahara, K.C. and Zottola, E.A. (1993) Biofilm formation by *Listeria monocytogenes* utilizes a primary colonizing organism in flowing systems. *Journal of Food Protection* **56,** 1022–1028.

Schlech, W.F., Lavigne, P.M., Bortulussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S. and Broome, C.V. (1983) Epidemic listeriosis: Evidence for transmission by food. *New England Journal of Medicine* **308,** 203–206.

Valle, J., Gomez-Lucia, E., Piriz, S., Goyache, J., Orden, J.A. and Vadillo, S. (1990) Enterotoxin production by staphylococci isolated from healthy goats. *Applied and Environmental Microbiology* **56,** 1323– 1326.

Villani, F., Sannino, L., Moschetti, G., Mauriello, G., Pepe, O., Amodio-Cocchieri, R. and and. Coppola, S. (1997) Partial characterization of an antagonistic substance produced by *Staphylococcus xylosus* 1E and determination of the effectiveness of the producer strain to inhibit *Listeria monocytogenes* in Italian sausages. *Food Microbiology* **14,** 555–566.

Wimpenny, J.W.T., Kinniment, S.L. and Scourfield, M.A. (1993) The physiology and biochemistry of biofilm. In *Microbial Biofilms: Formation and Control* ed. Denyer, S.P., Gorman, S.P. & Sussman, M. Society for Applied Bacteriology Technical Series, no. 30. pp. 51-94. Oxford: Blackwell Scientific.

Zoltai, P.T., Zottola, E.A. and McKay, L.L. (1981) Scanning electron microscopy of microbial attachment to milk contact surfaces. *Journal of Food Protection* **44,** 204–208.