ELSEVIER

Meat Science 57 (2001) 291–298

MEAT SCIENCE

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Survival and growth of *Escherichia coli* O157:H7, *Yersinia enterocolitica* and *Salmonella enteritidis* on decontaminated and untreated meat

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Received 8 May 2000; received in revised form 14 August 2000; accepted 14 August 2000

Abstract

Decontamination of meat or carcasses may have an effect in reducing the number of pathogens. Recontamination with other pathogens during cutting or packaging may, however, result in higher growth on decontaminated than on untreated meat due to the lack of competing non-pathogenic microorganisms. In this study we compared the growth of pathogens during storage at 10°C (worst case condition) on untreated meat and meat that had been decontaminated by steam vacuuming combined with spraying with 0.2 M lactic acid. *Salmonella enteritidis* inoculated on chicken multiplied quickly and reached log 7 cfu per cm^2 after 4 days of aerobic storage at 10°C, but growth was not significantly higher on decontaminated than on untreated chicken. The number of *Yersinia enterocolitica* inoculated on decontaminated pork skin reached log 9 cfu per cm^2 after 5 days of aerobic storage at 10°C. Overall, growth on vacuum-packed decontaminated and untreated pork under the same conditions was not significantly different, although there tended to be less growth on the untreated samples. The number of *Escherichia coli* O157:H7 on decontaminated beef increased by nearly 3 log cycles after 5 days of aerobic storage at 10°C compared to only a 1 log cycle increase on untreated beef. For the vacuum-packed beef, growth of *E. coli* O157:H7 on the fresh meat was very slow, while there was about a 3 log increase on the decontaminated beef. A higher average growth on the decontaminated beef was also found in an experiment with a very low inoculum (27 cfu per cm^2). During storage of vacuum-packed samples there was multiplication of *E. coli* O157:H7 on the decontaminated beef, but virtually none on the untreated beef. This study shows that multiplication of *S. enteritidis* on chicken and *Y. enterocolitica* on pork skin was not significantly higher on decontaminated compared to untreated meat. The increased multiplication of *E. coli* O157:H7 on decontaminated beef, especially when vacuum-packed, gives cause for concern. Preventive measures might be a strict HACCP approach to the handling of the decontaminated meat before packaging or use of a protective culture of lactic acid bacteria.

Keywords: Decontamination; Meat; Pathogenic bacteria

1. Introduction

Illness and death associated with ground beef containing *Escherichia coli* O157:H7 have prompted the meat industry to enhance its efforts to lower levels of *E. coli* O157:H7 and other pathogens in meat by monitoring food production more closely (MacDonald & Osterholm, 1993). The USDA Food Safety Inspection Service has proposed that all slaughter establishments should apply at least one antimicrobial treatment or other approved intervention procedure to livestock and poultry carcasses. Various treatments have been designed to decontaminate carcasses, including the use of sanitising agents such as hot water sprays (Powell & Cain, 1987), organic acid sprays (Smulders, 1995) or combinations of these treatments (Castillo, Lucia, Goodson, Savell & Acuff, 1998). Steam vacuuming has also been shown to be promising in reducing the number of pathogens on meat (Dorsa, Cutter & Siragusa, 1997; Kochevar, Sofos, Bolin, Reagan & Smith, 1997). These treatments as well as others, e.g. ionizing and non-ionizing irradiations, ultrasonics and high pressure, have been reviewed by Corry, James, James and Hinton (1995).

Not all microbiologists feel that decontamination of meat is required or even desirable. It has been argued by Jay (1995, 1996) that high levels of indigenous non-

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0309-1740/00/$ - see front matter
PII: S0309-1740(00)00104-2

pathogenic microorganisms may have a protective effect on meat and meat products by out-competing the pathogens. However, few, if any, studies have documented this. Another explanation for lower growth of the pathogens in such circumstances would be that higher numbers of background flora will mean a shorter shelf life of the meat. In addition to pathogens that have survived the decontamination, there will also be a risk of re-contamination of the meat during cutting and packaging. Since this risk cannot be completely removed, we believe it is important to examine the consequences of re-contamination of the meat by different pathogens by monitoring the possibility of growth during storage.

The meat used in this study had been decontaminated by steam vacuuming combined with spraying with lactic acid. The pathogen used for inoculation of beef was *E. coli* O157:H7, of pork *Yersinia enterocolitica* and of chicken *Salmonella enteritidis*. Growth of the pathogens on both decontaminated and untreated meat was monitored during storage at 10°C, a temperature chosen to represent "worst case" conditions.

2. Material and methods

2.1. Design and operation of the decontamination rig

An experimental decontamination chamber was designed and constructed by the Food Refrigeration and Process Engineering Research Centre at the University of Bristol, UK. The experimental apparatus was based around a vacuum vessel (a bell jar of 30 cm diameter mounted via a rubber gasket on a stainless steel base plate) in which low pressure was achieved using a vacuum pump. Steam added at these low pressures (approx. 12 mbar) condensed at temperatures lower than 100°C. Control at the required pressure was achieved by opening and closing a solenoid valve in the vacuum line. When the valve was opened pressure was reduced, and when it closed the pressure rose due to steam introduction.

Organic acids could be sprayed into the vessel before or after steam treatment using a variable speed peristaltic pump. When steam and acid treatments were complete, the vessel could be further evacuated. Water in the vessel evaporated at low pressure/temperatures (e.g. 10°C), removing the heat added during the steam treatment. To prevent vapour and liquid entering the vacuum pump oil and to enable the required vacuum cooling pressures to be achieved, an ice-filled condenser was required in the vacuum line between the vessel and the pump.

2.2. Decontamination

Pieces of meat (approx. 10×25 cm) were placed in the pre-heated decontamination rig. The meat was heated to 75°C for 10 s. The temperature and the duration of the heat-treatment were controlled by monitoring the temperature probes on a PTFE block (a "mock" product to simulate a meat surface). When the temperature recorded by the probes reached 75°C, the steam valves and then the vacuum pump were shut, the acid pump started and 100 ml 0.2 M lactic acid heated to 55°C were sprayed on the meat. The pump was then stopped and the meat allowed to cool before being removed from the rig.

2.3. Inoculum preparation

For inoculation the following strains were used: *E. coli* O157:H7, NCTC (National Collection of Type Cultures, Colindale, London, UK) 1200 (nontoxigenic strain), *Y. enterocolitica,* GER, O3P (isolate from Dr. S. Bhaduri, Research Service, USDA Wyndmoor, PA, USA), both resistant to nalidixic acid at 50 μg/ml and streptomycin sulphate at 1000 μg/ml. *S. enteritidis* P125588 (Laboratory of Enteric Pathogens, Central Public Health Laboratory, Colindale, London, UK), resistant to 200 μg/ml nalidixic acid. All three antibiotic-resistant strains were supplied by the National Food Centre, Dublin, Ireland, as freeze-dried cultures. They were stored at −70°C on beads.

For inoculation all the pathogens were cultivated 3 days before the start of the experiment. The first day one bead from the frozen stock was added to 10 ml of TSYB (tryptone soya broth with 1% yeast extract added, CM129 and L21, Oxoid Ltd, UK) and incubated overnight at 37°C. The next day 0.1 ml of the bacterial supension was transferred to 10 ml fresh TSYB, again cultivated overnight and stored at 4°C until day 3. To determine numbers of colony-forming units (cfu) diluted samples were plated on TSYA [CM131 plus 1% yeast extract (L21), Oxoid Ltd, UK] and incubated at 30°C for 48 h. The refrigerated suspension of stationary-phase cells was diluted to a cell density of log 6 cfu per ml (chicken, pork and beef) or log 4 cfu per ml (beef only) and used as inoculum.

2.4. Treatment of chicken and inoculation with Salmonella enteritidis

Six poultry carcasses that had been frozen were thawed at refrigeration temperature overnight. Breast muscles with skin attached were removed from the carcasses (two pieces per carcass). Six pieces of breast were decontaminated as described in Section 2.2, while six were not. The pieces were then each cut into eight portions (approximately 3×4 cm). For inoculation, 100 μl of the pathogen inoculum were spread on each portion of meat to give ca. 4×10^3 cfu per cm^2. The samples were pooled and randomly assigned for packaging in small trays covered with food grade cling film (PVC, O_2-transmission $>10\,000$ cm^3 m^{-2} 27 h^{-1} at m^{-1} at 23°C) and stored at 10°C. Microbial analyses were done

by putting the chicken sample (and the cling film) into a stomacher bag with 100 ml peptone water (8.5 g NaCl, 1.0 g peptone/1000 ml water) and mixing in the stomacher (Stomacher 400, Lab Blender, A. J. Seaward Medical UAC House, London, UK) for 1 min. One hundred μl of 10-fold dilution series from the stomacher fluid was surface-plated on whole (90 mm diameter) or 20 μl aliquots on quarter plates of TSYA containing 200 μg/ml nalidixic acid (sodium salt, ICN, Biomedical INC, Auroras, OH, USA) for *S. enteritidis* and on quarter plates of TSYA (total count). The plates were incubated at 30°C for 2 days. Microbial analyses were done on the day of inoculation (day 0) and after 1, 2, 3, 4 and 5 days storage.

2.5. Treatment of pork and inoculation with Y. enterocolitica

Pork skin was obtained from six different carcasses of Norwegian Land Race pigs, 3 days post mortem. Pieces of pork skin 8–10 mm thick were removed, and two pieces from each carcass were decontaminated as described above (Section 2.2) while two were not. The skin pieces were cut into at least 10 samples of about 5×5 cm. The samples were inoculated, treated and packed in the same way as the chicken samples, but half of the samples were packed aerobically in small trays as for the chicken and the other half vacuum packed with an Intervac (IN30) chamber machine (Intervac Verpackungsmaschinen, Wallenhorst, Germany). The bags were of polyamide/polyethylene (Halvorsen & Larsen A/S, Oslo, Norway) and the samples were stored at 10 or 0°C. The inoculum used for the samples stored at 10°C was prepared as described above while the inoculum used for the samples stored at 0°C had been stored in the refrigerator for an extra day, i.e. for 4 days. The microbial analyses were done as described for the poultry, but TSYA plates with nalidixic acid 50 μg/ml and streptomycin sulphate (Sigma, Steinham, Germany) 1000 μg/ml were used as a selective medium for *Y. enterocolitica*. The plates were incubated at 25°C for 2–3 days. Microbial analyses were done on the day of inoculation (day 0) and after 2, 3, 4 and 5 days or 5, 10 and 15 days of storage in air at 10 or 0°C, respectively; and after 5, 10 and 16 days or 2, 3 and 4 weeks in vacuum at 10 or 0°C, respectively.

2.6. Treatment of beef and inoculation with E. coli O157:H7

Six loins were obtained 3 days post mortem from six different carcasses of Norwegian Red Cattle stored at 4°C. The meat was sliced manually to a thickness of approximately 10 mm. Two decontaminated slices from each animal and two untreated slices were cut into 10 samples of about 5×5 cm. For inoculation, 100 μl of a diluted culture (about log 6 bacteria/ml) of *E. coli* O157:H7 were spread on each sample. The samples were inoculated and packed as described for pork and stored at 10°C. The microbial analyses were done as described for the chicken, but TSYA plates with nalidixic acid 50 μg/ml and streptomycin sulphate 1000 μg/ml were used as selective medium for *E. coli* O157:H7. The plates were incubated at 30°C for 2–3 days. Microbial analyses were done on the day of inoculation (day 0) and after 1, 2, 3, 4 and 5 days of storage in air and after 7, 14 and 21 days of storage in vacuum.

In another experiment, an inoculum with a low cell density (ca. log 4 per ml) of *E. coli* O157:H7 was used. Except that the beef (from the same animals) had been frozen (ca. 3 months), decontamination, inoculation and packaging procedures were as above. To monitor the low number of *E. coli* O157:H7 only 50 ml of diluent were used per sample in the stomacher bag and 0.1 and 1 ml were plated on blood agar (Difco blood agar base, Detroit, MI, USA with defibrinated horseblood, TCS, Microbiology, Buckingham, 18 2LR) with 50 μg/ml nalidixic acid and 1000 μg/ml streptomycin added. At the lowest dilutions some antibiotic-resistant background flora of micrococci and yeast were observed, but they were recognised by their different colony form and in some cases by microscopy. In addition, 20 μl of the stomacher fluid were plated on quarter plates of TSYA (total count), MRS (de Man, Rogosa, Sharpe, CM359, Oxoid, Ltd, UK) pH 5.7 (lactic acid bacteria) and CFC (Pseudomonas agar base CM559 with SR102 supplement, Oxoid Ltd) for *Pseudomonads* spp.

2.7. Statistical analysis

For all data, counts were expressed as $\log_{10}$ colony-forming units (cfu) per cm^2. Mean values (over the six animals) are shown in the figures and tables. Data originally recorded as "less than x" (representing one colony from the lowest dilution) have been replaced by the arithmetic mean of 0 and x (i.e. $x/2$) before the logarithmic value was computed. This is based on the assumption that the true value behind this recording follows a rectangular (uniform) distribution, and substituting "less than x" with $x/2$ minimizes the expected error.

For each type of meat, temperature, storage condition (air/vacuum) and storage time, an analysis of variance (ANOVA) to compare the effect of decontamination vs no treatment was performed. The model was: Y = treatment + animal, or in statistical notation:

$$y_{ij} = \mu + \alpha_i + \beta_j + e_{ij} \quad i = 1, 2; j = 1, 2, \ldots, 6$$

where μ is the general mean, α represents the main effect of treatment, β the main effect of animal, and e is the error (residual) term.

3. Results

3.1. Chicken

S. enteritidis inoculated at about log 3.5 cfu per cm^2 on chicken breasts grew quickly and reached nearly log 7 cfu per cm^2 after 4 days of storage at 10°C (Fig. 1a). Growth was not significantly different on decontaminated and untreated chicken except after 3 days ($P < 0.05$), where there was significantly more growth on the untreated samples. The background flora was much lower on the decontaminated than on the untreated chicken at the start of storage (since most of the total number consisted of the inoculum) and after 4 days it was about 10 times higher than the number of salmonella, i.e. log 9 versus log 8 cfu per cm^2 (Fig. 1b).

3.2. Pork

Y. enterocolitica inoculated at about log 3.8 cfu per cm^2 on pork grew even faster than salmonella on chicken, and reached log 9 on the decontaminated pork after 5 days of storage in air at 10°C (Fig. 2a). Growth was a little higher after 4 and 5 days of storage on the decontaminated than on the untreated pork, but not significantly so. The background flora was quite low after decontamination (most of the total number consisted of the inoculum), and was for the first 2 days about 1 log higher on the untreated pork (Fig. 2b). On the vacuum-packed pork, growth of *Y. enterocolitica* was a little slower than in air, reaching nearly log 8 cfu per cm^2 on the decontaminated pork after 5 days (Fig. 2c). Total numbers on decontaminated and untreated pork were about the same, except for day 14 (Fig. 2d).

For the pork incubated at 0°C, growth of *Y. enterocolitica* was just as high on the decontaminated as on the untreated pork, except in the vacuum packs after 21 days where growth was a little higher on the decontaminated samples (not shown). However, this may be due to a higher inoculum of *Y. enterocolitica* at 0 than at 10°C (log 5–6 cfu per cm^2 versus 3.8 cfu per cm^2).

3.3. Beef

The number of *E. coli* O157:H7 (initial inoculum about log 3.6 cfu per cm^2) did not differ much between decontaminated and untreated beef during the first few days in aerobic storage, but by 5 days the number of *E. coli* had increased by nearly 3 log cycles for the decontaminated beef but only 1 log cycle for the untreated beef (Fig. 3a). There was, however, great variation among the replicates. Thus, after 4 days of incubation there were three replicates with high and three with low numbers. The total number of microbes was significantly higher ($P < 0.05$) on the untreated meat during the whole storage period except for day 3 (Fig. 3b). For

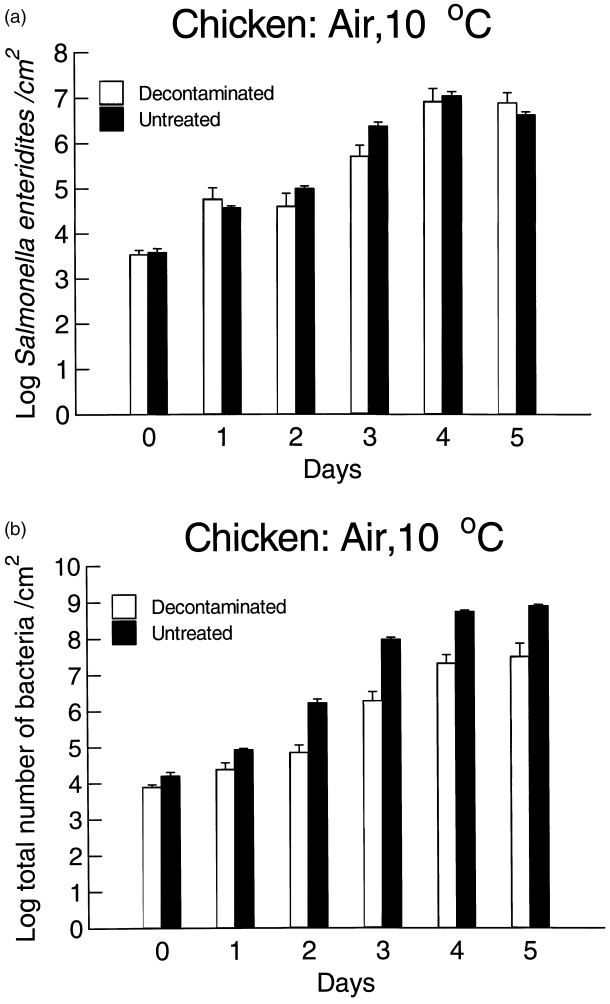

Fig. 1. Microbial growth (log cfu per cm^2) on decontaminated and untreated chicken when stored at 10°C: (a) *S. enteritidis* when stored in air; (b) total counts when stored in air. Means + S.E. ($n = 6$).

the vacuum-packed beef (Fig. 3c) growth of *E. coli* O157:H7 on the untreated meat was slow while there was about a log 3 increase on the decontaminated beef, although there was again considerable variation between the samples. In both cases the bacterial counts decreased somewhat after the first week. Contrary to what was found for the aerobic samples, the total number of microbes on the vacuum-packed sample was at least as high on decontaminated as on untreated meat (Fig. 3d). However, *E. coli* O157:H7 constituted a higher percentage of the total number of bacteria on the decontaminated beef (Fig. 3c vs. 3d).

In another experiment with a lower inoculum of *E. coli* O157:H7 (27 cfu per cm^2), there was some growth of *E. coli* O157:H7 both on the decontaminated and the untreated samples stored aerobically, but on average the number of *E. coli* O157:H7 was higher on the decontaminated beef (Fig. 4a). As in the experiment above, the total number of microbes was significantly higher on the untreated samples (Fig. 4b). When stored in vacuum

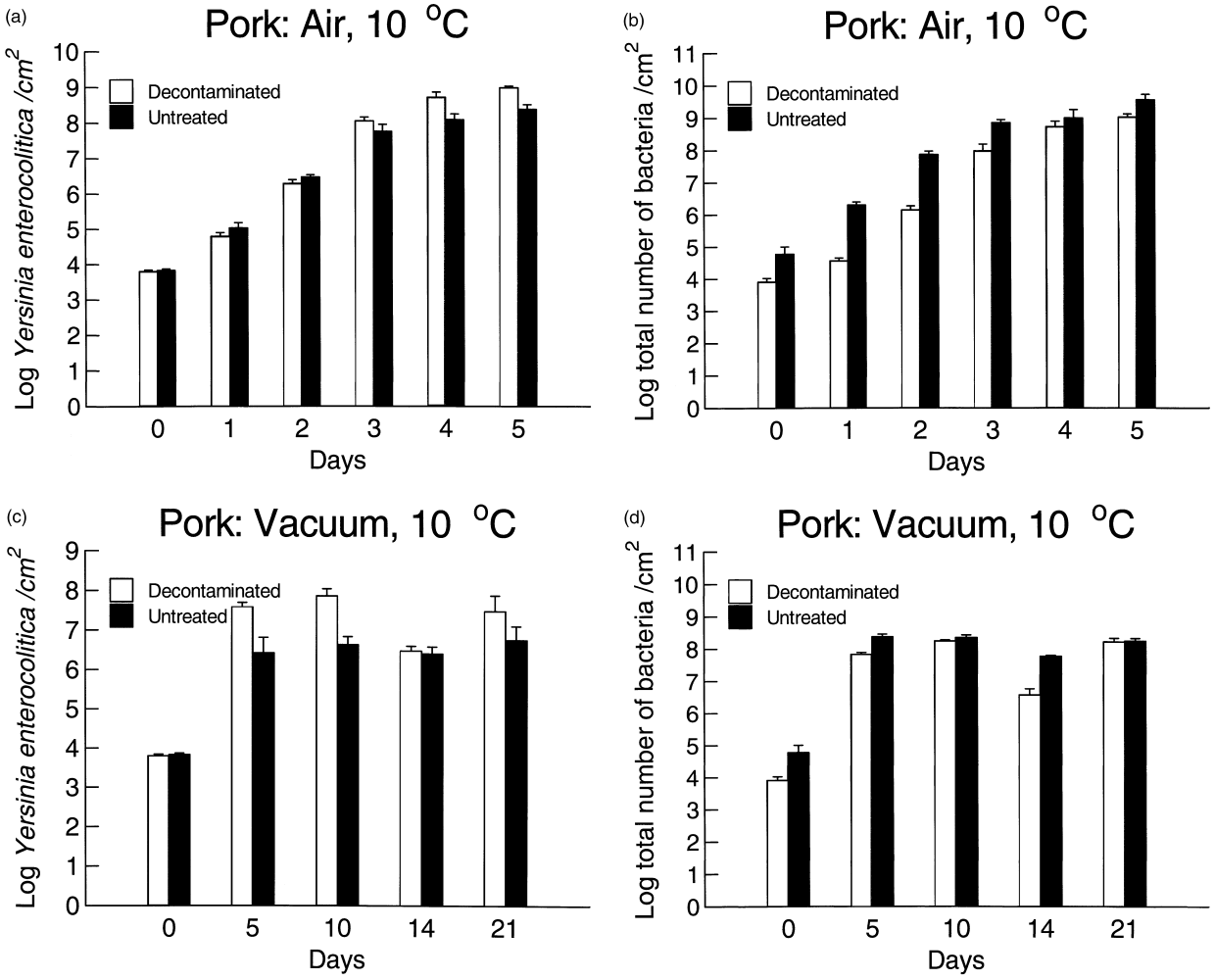

Fig. 2. Microbial growth (log cfu per cm^2) on decontaminated and untreated pork skin when stored at 10°C; (a) *Y. enterocolitica* when stored in air; (b) total counts when stored in air; (c) *Y. enterocolitica* when stored in vacuum; (d) total counts when stored in vacuum. Means + S.E. ($n = 6$).

packs there was growth of *E. coli* O157:H7 on the decontaminated beef, but virtually none on the untreated beef (Fig. 4c). The total number of micobes (Fig. 4d) consisted mostly of background flora even on the decontaminated samples. The average number of lactic acid bacteria in the background flora was about log 5 cfu per cm^2 on the decontaminated and log 7 cfu per cm^2 on the untreated beef after 5 days of storage, while the number in vacuum was about log 6 cfu per cm^2 for the decontaminated and log 7 cfu per cm^2 for the untreated beef (Table 1).There were, however, no obvious correlations between a high number of lactic acid bacteria and low number of *E. coli* O157:H7 on the individual replicate samples (not shown). At day 3, considerable growth of pseudomonads was observed both on the decontaminated and the untreated beef (Table 1). Surprisingly there was also growth of pseudomonads on the vacuum-packed beef. Pseudomonads are aerobic, but there may have been some residual air in the packages or the growth may have been caused by other non-pseudomonads species that will grow on the CFC medium.

4. Discussion

In all the experiments described above, the bacterial culture had been stored in the refrigerator for at least 1 day before being used for inoculation. The reason for this was to mimic a real life situation since the chances of contamination with exponentially growing bacteria are very small. Also, studies have shown that stationary-phase cultures are much more resistant to acid and other stresses than are cultures in the log phase of growth (e.g. Sheridan & McDowell, 1998). To monitor growth of a low inoculum of the pathogens, antibiotic-resistant mutants were selected since most methods for selective detection of these bacteria are based on an enrichment step and antibiotic mutants are easy to detect by direct plating. The growth of the mutants was comparable with their parent strains at different pH, water activities and temperatures.

The present study shows that re-contamination, after decontamination, of pork with *Y. enterocolitica* did not present a much higher risk of increased growth on

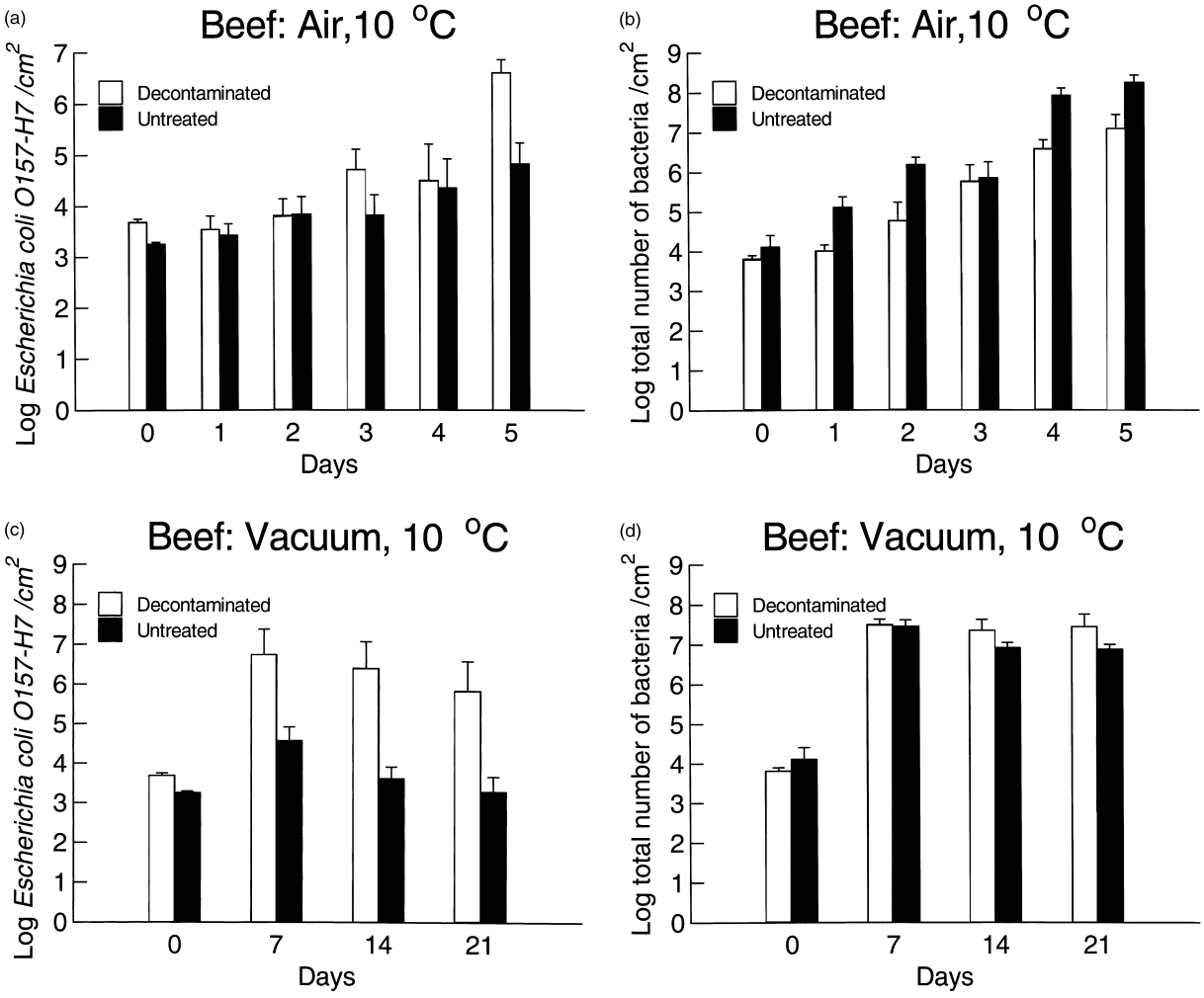

Fig. 3. Microbial growth (log cfu per cm^2) on decontaminated and untreated beef when stored at 10°C: (a) *E. coli* O157:H7 when stored in air; (b) total counts when stored in air; (c) *E. coli* O157:H7 when stored in vacuum; (d) total counts when stored in vacuum. Means + S.E. ($n = 6$).

decontaminated than on untreated pork when the background flora was quite low (ca. log 5 cfu per cm^2) compared to the inoculated bacteria (ca. log 4 cfu per cm^2). The difference was somewhat larger when the samples were vacuum packed. On chicken, *S. enteritidis* grew well both on the decontaminated and the untreated meat, but the chicken was not vacuum-packed. With chicken and pork there is, however, the danger that although the pathogens may not grow significantly faster on the decontaminated samples, less background flora may give a prolonged shelf life which in turn may allow the number of pathogens to increase more.

The consequences of growth of *E. coli* O157:H7 on beef are particularly serious. In this study we used a storage temperature of 10°C as a "worst case" of inadequate refrigeration. When a high inoculum of log 3.6/cm^2, i.e. the same order of magnitude as the background flora of the untreated beef, was used, growth was significantly higher ($P < 0.05$) on the decontaminated beef in air by day 5, and during the whole storage period when vacuum-packed. Growth was also higher on decontaminated vacuum-packed beef when a low inoculum (ca. 27 bacteria/cm^2) was used, but the variation between the samples was even higher than in the first experiment with *E. coli* O157:H7 when a higher inoculum was used. This was also the case for two other experiments with low inocula (10–20 cfu per cm^2) (results not presented). The lack of growth in the vacuum packs may be due to a combination of anaerobic metabolism with somewhat higher or different background flora. Microbial variations in the packages of beef could have been caused by remnants of lactic acid on the meat, and it has been shown that organic acids may influence the growth of *E. coli* O157:H7 (Dorsa, Cutter & Siragusa, 1998; Smulders & Woolthuis, 1985; Woolthuis & Smulders, 1985). In addition, the nature of the surface of the meat can play a role in the attachment of the bacteria (Selgas, Marin, Pin & Casas, 1993). The moisture content of the meat may also be important (Dickson, 1990).

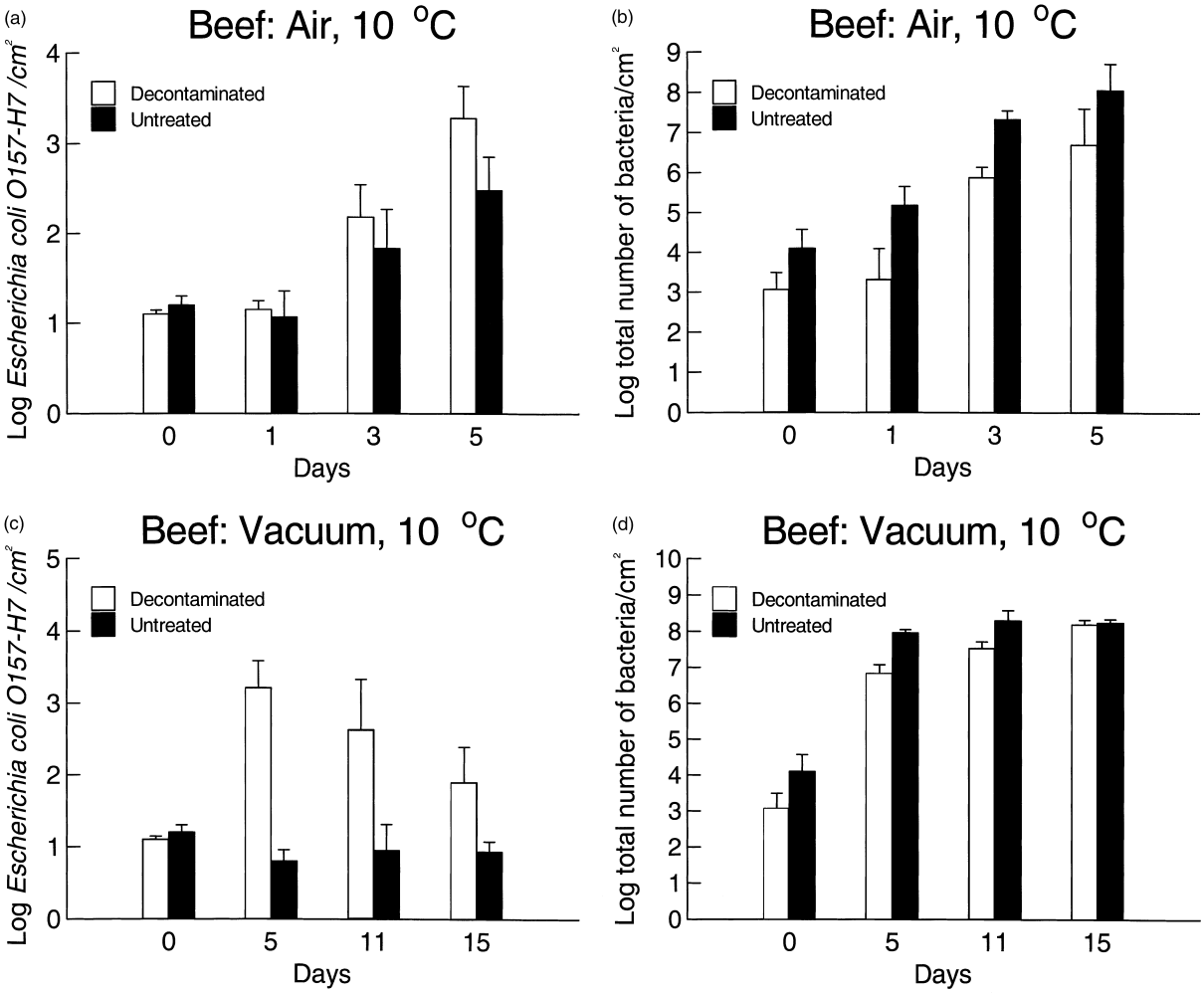

Fig. 4. Microbial growth (log cfu per cm^2) on decontaminated and untreated beef (inoculated with a low inoculum of *E. coli* O157:H7) when stored at 10°C: (a) *E. coli* O157:H7 when stored in air; (b) total counts when stored in air; (c) *E. coli* O157:H7 when stored in vacuum; (d) total counts when stored in vacuum. Means + S.E. ($n = 6$).

Table 1
Number of lactic acid bacteria (log_{10} cfu per cm^2 on MRS medium) and pseudomonads (log_{10} cfu per cm^2 on CFC medium) on decontaminated and untreated beef (see Fig. 4)

	MRS				CFC			
	Decontaminated		Untreated		Decontaminated		Untreated	
Days	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Air								
0	2.1	0.4	3.3	0.4	1.8	0.1	2.4	0.3
1	2.6	0.6	4.9	0.3	1.8	0.0	2.5	0.4
3	5.2	0.5	6.3	0.1	4.7	0.5	6.2	0.4
5	4.8	0.3	7.0	0.3	5.0	0.4	8.4	0.3
Vacuum								
0	2.1	0.4	3.3	0.4	1.8	0.1	2.4	0.3
5	6.2	0.3	7.0	0.2	3.3	0.8	4.9	0.3
11	5.8	0.4	7.3	0.1	2.9	0.1	4.8	0.4
15	8.0	0.1	7.6	0.3	2.9	0.2	5.2	0.7

5. Conclusions

Decontamination of raw meat may be a useful precaution, especially for high risk products such as meat from herds that have been diagnosed positive for *E. coli* O157:H7. There is, however, a risk that decontamination, especially in combination with vacuum packaging, will allow increased multiplication of the pathogen because of the extended shelf life. Further studies would be needed to determine the number of pathogens for different decontamination and storing conditions at the point of spoilage. Preventive measures might be a strict HACCP approach to the handling of the decontaminated meat after treatment and before packing.To satisfy the concern that pathogen reduction strategies such as decontamination will reduce the background flora, the meat could be sprayed with a protective culture of lactic acid bacteria (Jay, 1995). In this way, a harmless microflora would be restored, even if it might cause a shorter shelf life for the product.

Acknowledgements

We thank Janina Berg and Anne Gulliksen for able technical assistance. We also thank Richard H. Dainty and Janet Corry for stimulating discussions and comments on the manuscript. The study has been carried out with financial support from the Commission of the European Communities, Agriculture (FAIR) specific RTD programme, CT 1027.

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