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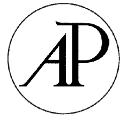

ORIGINAL ARTICLE

The effect of a competitive microflora, pH and temperature on the growth kinetics of *Escherichia coli* O157:H7

G. Duffy[1]*, R. C. Whiting[2] and J. J. Sheridan[1]

Growth curves were generated for Escherichia coli *O157:H7 in brain–heart infusion broth incubated at 37 or 15°C in the presence of individual and combinations of competing microflora. Broths were inoculated with* E. coli *O157:H7 (log_{10} 3·00 cfu ml^{-1}) and competitors (log_{10} 4·00 cfu ml^{-1}) and the initial pH of the broth was either neutral (7·0) or adjusted to 5·8 and then sequentially reduced to 4·8 over 10 h to simulate fermentation conditions. Growth curves were also generated for the competitors in these cultures, including* Pseudomonas fragi, Hafnia alvei, Pediococcus acidilactici *(pepperoni starter culture) and* Brochothrix thermosphacta*. Gompertz equations were fitted to the data and growth kinetics including lag phase duration, exponential growth rates and maximum population densities (MPD) calculated. In pure culture, the growth parameters for* E. coli *O157:H7 in neutral pH broths were significantly different from those recorded in simulated fermentation broths ($P < 0·05$). The presence of competitors in the broth also had a significant effect on the growth kinetics of the pathogen.* H. alvei *significantly inhibited the growth (lag phase, growth rate and MPD) of* E. coli *O157:H7 at 37°C, neutral pH and outgrew the pathogen under these conditions. In neutral pH cultures, two other competitors,* B. thermosphacta *and* P. acidilactici *also inhibited the lag phase of the pathogen but had no effect on the other growth parameters. In simulated fermentation broths, the growth rate of* E. coli *O157:H7 was consistently slower and the MPD lower in the presence of a competitive microflora than when grown individually. At 15°C, only one competitor,* P. fragi *significantly inhibited the lag phase of the pathogen. The implications of these findings for food safety are discussed.*
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[1] The National Food Centre, Teagasc, Dunsinea, Castleknock, Dublin 15, Ireland
[2] The Agricultural Research Service, Eastern Regional Research Centre, United State Department of Agriculture, East Mermaid Lane, Wyndmoor, Pennsylvania 19039, USA

Introduction

Verocytotoxigenic *Escherichia coli*, in particular serogroup O157, have emerged in the last 10 years as foodborne pathogens which can cause a severe and potentially fatal illness called haemorrhagic colitis, the symptoms of which include bloody diarrhoea and severe abdominal pain. It may lead to haemolytic uraemic syndrome which is the leading cause of kidney failure in children. This pathogen has caused severe public health and economic problems world-wide with numerous high-profile outbreaks (Centres for Disease Control and Prevention (CDC) 1993, CDC 1995, Swinbanks 1996). Undercooked beef burgers have been involved in many food-poisoning incidents associated with this pathogen (CDC 1993) but other foods including salami (CDC 1995), apple cider (Bisser et al. 1993), water

*Corresponding author.
E-mail: G.Duffy@NFC.Teagasc.ie

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(Swerdlow et al. 1992) and potatoes (Morgan et al. 1988) have also been implicated.

In terms of food safety, predictive modelling techniques are often used to predict the growth/survival of pathogens in food products or processes. These predictive models are usually developed in pure culture as it is often not feasible or economical to develop predictive models *in situ* for individual food products and processes. It is essential when developing these models that as many factors as possible are included in the model broth system to give an accurate prediction of growth in a real situation. While most models include factors such as pH, temperature and preservatives, it is unusual to include the effect of a competing microflora. It is however, well documented in the literature that a competing flora can have a significant effect on bacterial growth and survival (Davies et al. 1991, Lewus et al. 1991, Hinton et al. 1992, Rodriguez et al. 1994).

The aim of this study was to investigate the growth of *E. coli* O157:H7 in cultural conditions which would simulate some of the environmental conditions in raw and fermented meats. Growth was investigated at 37 and 15°C, at neutral pH and under simulated fermentation conditions (sequentially decreasing pH from 5·8–4·8). The effect of a competitive microflora common to raw and processed meat (*Pseudomonas fragi, Brochothrix thermosphacta, Hafnia alvei* and a *Pediococcus* starter culture) on *E. coli* O157:H7 growth was also investigated. Predictive modelling techniques were applied to describe the growth of the pathogen under the above described conditions.

Materials and Methods

Bacteria

The bacterial strains used in this investigation included *E. coli* O157:H7 (380-94, salami food poisoning outbreak), *Pseudomonas fragi* (NCIMB 8542), *Pediococcus acidilactici* starter culture (HP 07044, Diversitech Inc., Alachua, Florida, USA), *Hafnia alvei* (ATCC 9760) and *Brochothrix thermosphacta* (ATCC 11509). Antibiotic resistant mutants of *E. coli* O157:H7, *P. fragi* and *B. thermosphacta* were used as the media available for detection of the wild-type strains of these bacteria were not sufficiently selective for recovery and enumeration of the target bacteria from a mixed bacterial culture.

Preparation of mutants

A wild-type colony was inoculated into 100 ml of nutrient broth, incubated for 18–24 h at optimum growth temperature and then transferred to 100 ml of nutrient broth containing 100 µg ml^{-1} of antibiotic. This broth was incubated for a further 18–24 h at optimum growth conditions. A 0·1 ml aliquot was placed in duplicate onto nutrient agar containing 50 µg ml^{-1} of the antibiotic and incubated for 18–24 h at optimum growth conditions. The resulting colonies were classed as single mutants to the antibiotic incorporated into the agar. In some cases a double mutant was made to ensure greater stability. This was prepared by transferring a colony of the single mutant to 100 ml broth containing a second antibiotic (2000 µg ml^{-1}). The procedure was repeated as above to the final stage where 0·1 ml aliquots of the broth were streaked onto nutrient agar containing both antibiotics. The colonies which grew on this agar were double antibiotic-resistant mutants. The mutants prepared were as follows: *E. coli* O157:H7 resistant to nalidixic acid (50 µg ml^{-1}) and streptomycin sulphate (1000 µg ml^{-1}); *P. fragi* resistant to novobiocin (50 µg ml^{-1}); and *H. alvei* resistant to acriflavin (50 µg ml^{-1}).

The growth kinetics (lag phase, growth rate and maximum population density) of each mutant bacterial strain was established in pure culture (BHI) and compared with the growth of the corresponding wild-type organism under similar growth conditions.

Growth of E. coli O157:H7 in broth

A colony of *E. coli* O157:H7 was inoculated into 100 ml BHI and incubated overnight at 37°C. The culture was then serially diluted to give a final concentration of approximately $\log_{10}$ 3·00 cfu ml^{-1} in fresh BHI (250 ml). Inocula of competitor organisms (*P. fragi, H. alvei, P. acidilactici* or *B. thermosphacta*) were prepared by inoculating colonies of these

organisms into BHI and incubating overnight at 25 (*P. fragi*) or 30°C. These overnight cultures were serially diluted and added to the BHI broth at a level of $\log_{10}$ 4·00 cfu ml^{-1}. Growth curves were carried out with *E. coli* O157:H7 in the presence of each individual competitor and then in combination with two and four competitors and incubated at two temperatures, 37 and 15°C for 26 and 80 h respectively. At various intervals during incubation, the broth was examined for the number and type of bacteria present. *E. coli* O157:H7 was isolated by plating 0·1 ml aliquots in duplicate onto sorbitol McConkey agar (SMAC) (Oxoid, Basingstoke, UK) containing streptomycin sulphate (Sigma, Dublin, Ireland) 1·0 gl^{-1} and nalidixic acid 0·05 gl^{-1} (Sigma) and incubating at 37°C overnight; *P. acidilactici* were plated onto De Man Rogosa, Sharpe agar (MRS) (Oxoid) and incubated at 30°C for 3 days; *P. fragi* were isolated onto cetrimide, fucidin cephaloridine agar (CFC) (Oxoid) with novobiocin 0·05 gl^{-1} and incubated at 25°C for 3 days; *H. alvei* were plating onto violet red bile glucose agar (VRBGA) (Oxoid) with acriflavin (0·05 gl^{-1}) and incubated at 30°C for 24 h; finally *B. thermosphacta* was isolated by plating onto streptomycin sulphate, thallous acetate, actidione agar (STAA) (Oxoid) and incubating at 30°C for 3 days.

Simulated fermentation conditions

Growth curves were also carried out under simulated meat fermentation conditions. The aim of these experiments was to test the effect of a sequentially decreasing pH on the survival and growth of *E. coli* O157:H7 in the presence of various competing micro-organisms. Inocula were prepared, added to BHI as described previously and incubated at 37°C for 26 h. At time 0, the pH was adjusted to 5·8, the typical pH of raw meat at the start of fermentation. At 2-h intervals over a 10-h period the pH was lowered by 0·2 using hydrochloric acid (1 N) until a pH of 4·8 was reached, which was taken as the end point of fermentation. pH measurements were determined using an Orion 210 pH meter (Orion Research Corp., Boston, Massachusetts; USA) with a combined electrode. All experiments were carried out in triplicate.

Statistics and modelling of data

The number of *E. coli* O157:H7 and competitive microflora ($\log_{10}$ cfu ml^{-1}) in BHI were plotted against time. A primary model was fitted to the data using Gompertz analysis in conjunction with ABACUS, an iterative nonlinear regression program developed at the Eastern Regional Research Centre by W. Damert (Buchanan and Phillips 1990). The Gompertz parameters (A, B, C and M) were used to calculate growth kinetics, including exponential growth rate (EGR) ($\log_{10}$ cfu ml^{-1} h^{-1}), generation time (GT) (h), lag phase duration (LPD) (h) and maximum population densities (MPD) ($\log_{10}$ cfu ml^{-1}).

Analysis of variance was carried out to compare the growth parameters of *E. coli* O157:H7 and the competitors under varied growth conditions.

Results

The growth kinetics of mutant strains of *E. coli* O157:H7, *P. fragi* and *P. acidilactici* were compared with their corresponding wild-type strains (data not presented). No significant difference in the lag phases, growth rates, or maximum population densities were detected between the mutant and wild type strains, indicating that the mutant is a good indicator of growth while overcoming problems associated with enumeration of these bacteria from a mixed culture.

When the data were modelled using the Gompertz equation, the fits obtained were generally good with the sum of squares less than 1 in all cases. Table 1 shows the growth parameters for *E. coli* O157:H7 in the presence of four competitors, at two temperatures (37, 15°C) at neutral pH (7·0) and under simulated fermentation conditions (pH 5·8 dropping to 4·8).

At 37°C and neutral pH, the lag phase of *E. coli* O157:H7 in pure culture was shown to be significantly shorter ($P < 0·05$) and the growth rate faster ($P < 0·05$) than under simulated fermentation conditions (Table 1, Fig. 1). However when competitors were present in the broth, the pH had no significant effect on the growth kinetics.

Table 1. Growth kinetics of *Escherichia coli* O157:H7 in the presence of competitors at two temperatures at neutral pH and under simulated fermentation conditions

Competitors	LPD (h)	s.e.	EGR ($\log_{10}$ cfu ml^{-1} h^{-1})	s.e.	MPD ($\log_{10}$ cfu ml^{-1})	s.e.
Temperature 37°C (neutral pH)						
Pure culture	1·10^{a}	0·13	1·47^{e}	0·19	9·36^{i}	0·10
P. fragi	1·76^{a}	0·63	1·29^{e}	0·25	9·07^{i}	0·42
B. thermosphacta	2·67^{b}	0·44	1·52^{e}	0·30	8·89^{i}	0·28
P. acidilactici	3·44^{b}	0·32	1·61^{e}	0·43	8·66^{i}	0·69
H. alvei	3·29^{b}	0·69	0·61^{f}	0·06	8·31^{j}	0·44
P. fragi, H. alvei	1·89^{a}	0·70	1·19^{e}	0·08	8·95^{i}	0·10
B. thermosphacta, P. acidilactici	1·38^{a}	0·25	0·94^{g}	0·13	8·40^{j}	0·17
P. fragi, H. alvei, P. acidilactici, B. thermosphacta	2·17^{b}	0·23	1·07^{e}	0·09	8·71^{i}	0·31
Temperature 37°C (simulated fermentation)						
Pure culture	3·01^{b}	0·63	0·59^{f}	0·12	9·52^{i}	0·54
P. fragi	2·05^{b}	0·27	0·89^{g}	0·09	8·31^{i}	0·58
B. thermosphacta	1·20^{a}	0·12	1·13^{e}	0·12	8·11^{j}	0·45
P. acidilactici	1·42^{a}	0·48	1·02^{e}	0·09	8·20^{j}	0·53
H. alvei	2·10^{a}	0·47	0·99^{g}	0·18	8·33^{j}	0·19
P. fragi, P. acidilactici, H. alvei, B. thermosphacta	3·47^{b}	0·25	1·12^{e}	0·10	8·20^{j}	0·45
Temperature 15°C (neutral pH)						
Pure culture	7·43^{c}	0·41	0·05^{h}	0·01	9·30^{i}	0·31
P. fragi	10·65^{d}	0·32	0·08^{h}	0·01	9·26^{i}	0·22
B. thermosphacta	8·04^{c}	0·25	0·09^{h}	0·01	9·53^{i}	0·11
P. fragi, B. thermosphacta	7·41^{c}	0·63	0·09^{h}	0·01	9·25^{i}	0·24

The results are the mean of three replicates.
LPD = lag phase duration; EGR = exponential growth rate; MPD = maximum population density.
Values with different letters are significantly different ($P<0·05$).

As expected, temperature had a significant effect on the growth kinetics (lag phase, growth rate) of *E. coli* O157:H7 with a lower growth temperature (15°C) significantly lengthening the lag phase ($P<0·001$) and slowing the growth rate ($P<0·001$) from that observed at 37°C (Fig. 2, Table 1). The pathogen did however reach an MPD of $\log_{10}$ 9·30 cfu ml^{-1} after 70 h incubation at 15°C.

The type of competitors present also had an effect on the growth kinetics. At 37°C (neutral pH) in the presence of *H. alvei*, the lag phase for *E. coli* O157:H7 was significantly longer ($P<0·01$), the growth rate significantly slower ($P<0·05$) and the maximum population density (MPD) ($P<0·05$) lower than in pure culture (Fig. 3, Table 1). In the presence of *B. thermosphacta* and *P. acidilactici* the lag phase of *E. coli* was lengthened but the growth rate and MPD were not affected. In the presence of all four competitors the only growth kinetic of the pathogen to be affected was the lag phase ($P<0·05$).

In simulated fermentation broths the effect of the competitors on the growth kinetics of *E. coli* O157:H7 was variable. In the presence of competitors the growth rate of the pathogen was consistently slower and the maximum population density lower, while alternatively the lag phase was shorter ($P<0·05$).

At 15°C and neutral pH, the competitors had little effect on the growth kinetics of the pathogen. The presence of *P. fragi* did lengthen the lag phase of *E. coli* O157:H7 by approximately 3 h ($P<0·05$) but it had no effect on either the growth rate or the maximum population density.

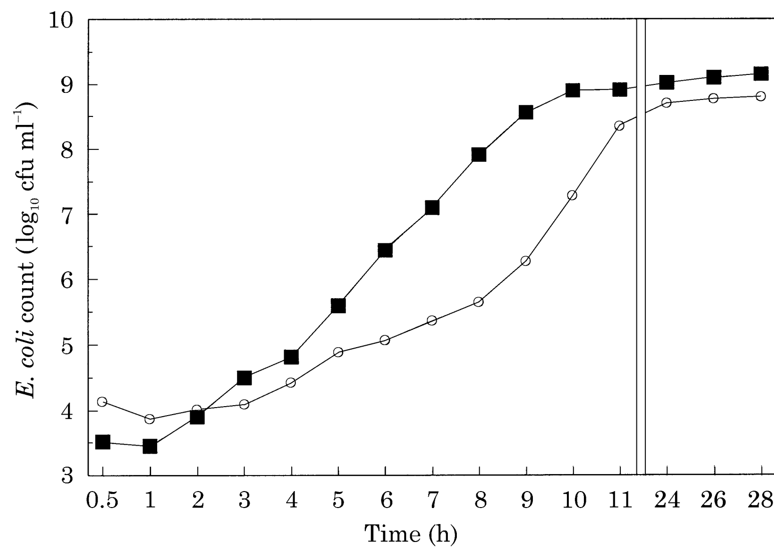

Figure 1. Growth curve for *E. coli* O157:H7 at 37°C in brain–heart infusion broth at either neutral pH (■) or under simulated fermentation conditions (acid pH) (○).

The growth kinetics of the competitor bacteria was also recorded in each of the culture systems. It was noted that at low growth temperatures (15°C), the lag phase of all of the competitors was significantly longer and the growth rate slower than at 37°C ($P<0{\cdot}001$). Acidity of the environment and the presence of other competitors did not significantly affect the growth kinetics of any of the organisms.

When the growth kinetics of *E. coli* were compared with those of the competitive flora, it was observed that *H. alvei* had a significantly shorter lag phase and a significantly faster growth rate than *E. coli* at 37°C (neutral pH) (Fig. 4). It was previously noted that *E. coli* was inhibited by this organism under these growth conditions. In simulated fermentation broths, the lag phase of the competitors was shorter and the growth rate faster than that of *E. coli* O157:H7, which inhibited the growth of the pathogen in this environment.

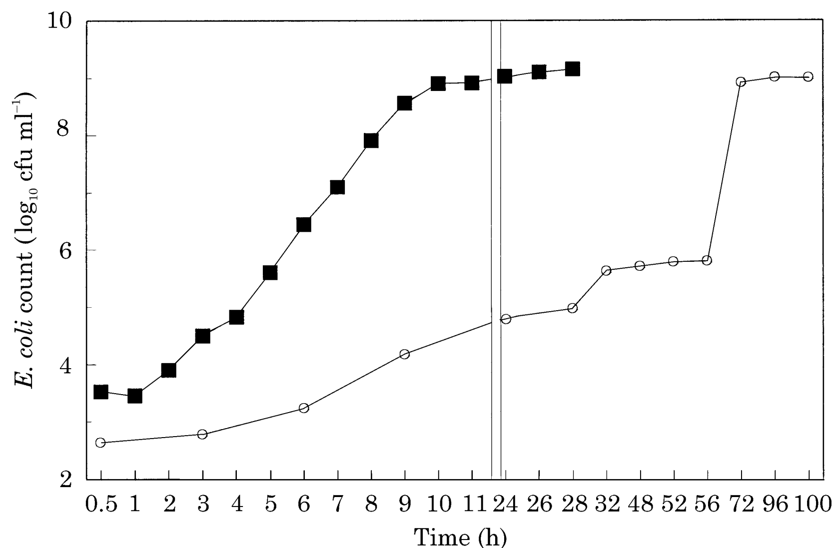

Figure 2. Growth curve for *E. coli* O157:H7 in brain–heart infusion broth at two temperatures, 37°C (■) and 15°C (○) and at neutral pH.

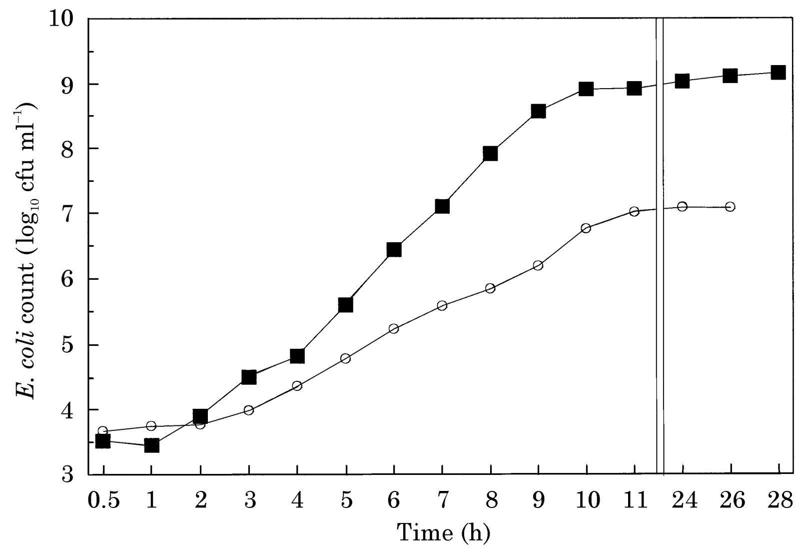

Figure 3. Growth curve for *E. coli* O157:H7 in brain–heart infusion broth in the presence (○) or absence (■) of *H. alvei* at 37°C.

Discussion

The growth kinetics of *E. coli* O157:H7 in pure culture was inhibited in simulated fermentation broths (pH 5·8–4·8) with a longer lag phase and a slower growth rate in this environment. It has previously been noted that an initial pH of less than 5·5 significantly inhibited the growth of *E. coli* O157:H7 in pure culture (Buchanan and Klawitter 1992, Conner and Kotrola 1995). However, this study showed that in the presence of a competitive microflora, *E. coli* O157:H7 was not inhibited by an acidic environment. It is also widely reported in the literature that *E. coli* O157:H7 can survive in low pH foods (Glass et al. 1992, Zhao et al. 1993, Abdul-Raouf et al. 1993 and Riordan et al. 1998). This study therefore indicates that the increased bacterial

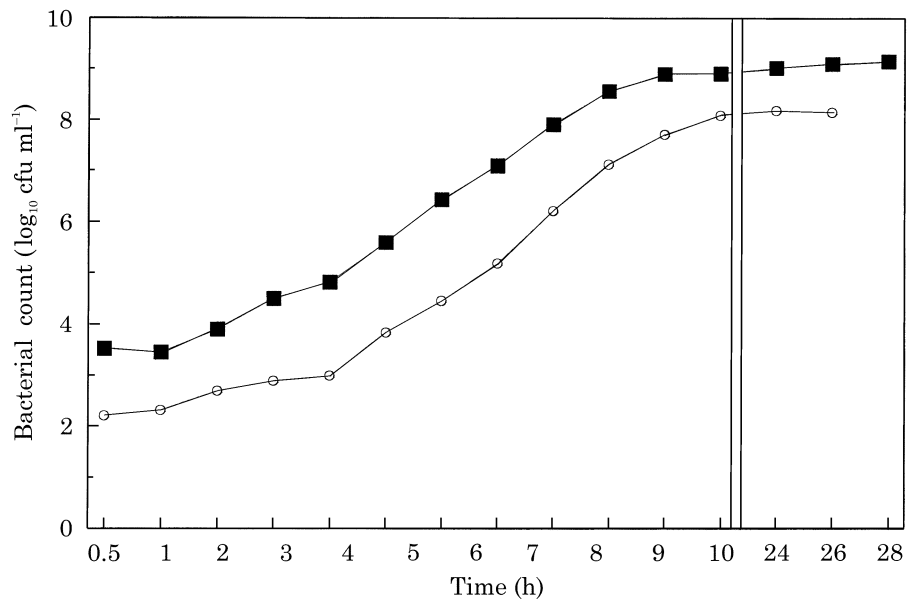

Figure 4. Growth curve for *E. coli* O157:H7 (■) and *H. alvei* (○) in brain–heart infusion broth at 37°C.

numbers in the environment protected the pathogen against the inhibitory effects of the acid. This is in agreement with previous studies which have reported that the presence of a competitive microflora can protect pathogens against other stress in particular thermal inactivation (Duffy et al. 1995).

It should be noted that in this study an inorganic acid (HCL) was used to manipulate the pH of the broth as opposed to an organic acid. The reason for choosing HCl was related to the stability of the acid in broth culture, HCl is widely reported to hold a given pH over a long period of time, while organic acids tend to be stable only at their pKa value (Perrin and Boyd, 1974). This presents difficulties in an experiment where the pH is constantly being lowered over the course of a long experiment (up to 3 days incubation) particularly in broth culture. Some authors have reported that HCl may be more inhibitory to pathogen growth than organic acids (Brackett 1987) while others have reported it to be less inhibitory due to the lack of associated anion effects (Buchanan and Golden 1994). This should be taken into account in the application of this data to a food product.

SMAC agar was employed in this experiment for the recovery of *E. coli* O157:H7. Although some workers have reported that the use of TSA with a SMAC overlay gives better recovery of heat-injured cells (Doyle and Schoeni 1984) other studies on acid-injured cells in pepperoni standard formulation (final pH 4·69–4·86) reported no significant differences between SMAC and TSA–SMAC for the recovery of *E. coli* O157:H7, except in very low pH product (pH 4·4–4·59), where significant differences between the two recovery media were reported (Riordan et al. 1998). This indicated that for the lowest pH values achieved in this experiment (pH 4·8) the use of a recovery media was unnecessary.

In this study, the ratio of each competitor to the pathogen was in general 10:1. When a mixture of two or four different competitors were present, the overall ratio of competitors to the pathogen increased to 20:1 or 40:1, which would mimic the situation in raw meat where levels of *E. coli* O157:H7 are generally low ($<5{\cdot}0$ cfu g^{-1}) and detectable only by enrichment, while *P. fragi, H. alvei* and *B. thermosphacta* have been reported to be present at levels of $\log_{10}$ 2·00 cfu g^{-1}, $\log_{10}$ 1·5 cfu g^{-1} and $\log_{10}$ 1·00 cfu g^{-1} respectively on minced beef (McMahon 1997). However, starter cultures may be present at levels as high as $\log_{10}$ 7·00 cfu g^{-1} in fermented meat (The National Food Centre, unpublished observations).

Some interesting observations can be made from this study on the effect of the competitive microflora on the growth of *E. coli* O157:H7. In pure culture at neutral pH, the growth kinetics (lag phase, growth rate and MPD) of *E. coli* O157:H7 were significantly inhibited by *H. alvei*. In simulated fermented broths the growth rate and MPD of the pathogen were similarly affected. This type of competitive interaction between enteric bacteria has previously been noted by Abbiss (1986) and Stavric et al. (1992). This may have important implications for the isolation of the pathogen from enrichment samples containing high levels of enteric bacteria such as faecal specimens. It may also have positive applications in competitive exclusion of pathogens from poultry (Stavric et al. 1992).

This study has shown that in neutral pH cultures grown at 37°C, the presence of a starter culture (*Pediococcus acidilactici*) lengthened the lag phase but had no effect on the growth rate of *E. coli* O157:H7. Previous studies have also reported that the presence of a lactic acid culture will inhibit the growth rate of many pathogens (Nielsen et al. 1990, Hutton et al. 1991, Lewus et al. 1991) but have little effect against *E. coli* O157:H7 (Farag et al. 1992). In simulated fermentation conditions, the growth rate of the pathogen and its MPD were inhibited by the presence of any competitors in the broth. This is most likely related to the faster growth rate of the competitors in this environment.

E. coli O157:H7 incubated at 15°C had a growth rate of 0·05 $cfu^{-1} ml^{-1} h^{-1}$ and reached its maximum population density within 70 h. In the context of fermented meats these results may have considerable implications. Fermented sausages are dried at 15°C for approximately 7 days (Riordan et al. 1998), indicating that if low levels of *E. coli* O157:H7 contamination are present after the fermentation step, they may have the potential to survive/grow to high levels during the drying phase, which increases the risk

of cross-contamination, particularly in light of the low infectious dose.

At 15°C, only one competitor, *P. fragi* had any inhibitory effect on the pathogen. In the presence of this competitor, which is an important spoilage organism on meat and can grow rapidly at refrigerated temperatures, the lag phase of *E. coli* O157:H7 was lengthened but growth rate and MPD were unaffected. The interaction between *Pseudomonas* spp. and food pathogens has been widely reported in the literature but the results obtained have been contradictory. Some studies have shown inhibition of the Gram-positive food pathogens such as *Listeria monocytogenes* by *Pseudomonas* (Freedman et al. 1989), while others (Farrag and Marth 1989) indicate that growth of the pathogen was enhanced in the presence of these spoilage bacterium. In this study, the inhibitory effect of the *P. fragi* on *E. coli* O157:H7 was minimal and was completely absent when *B. thermosphacta* was also present, indicating that if *E. coli* O157:H7 is present on foods stored at abuse storage temperatures it may be capable of growing to very high numbers before the food is visually spoiled.

Overall, this study shows that predictive modelling in pure culture shows a significantly different growth profile from that observed in the presence of a competitive microflora. It clearly indicates that there is an inherent danger in applying predictive models, developed for pathogen growth in pure cultures, directly to foods. The results of this study suggest that growth curves developed in mixed culture may provide a better predictive model for food.

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