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Growth kinetics and hydrolytic enzyme production of *Pseudomonas* spp. isolated from pasteurized milk

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Psychrotrophs, particularly *Pseudomonas* spp. are known to be the main determinants of the shelf-life of pasteurized milk and refrigerated raw milk. It is presumed that they mainly cause spoilage through the elaboration of proteinase and lipase enzymes. At the time of this research, under the relevant European Directive, one of the means of determining the quality of pasteurized milk was the pre-incubated count, which involves incubating the milk sample for 5 d at 6 °C followed by a plate count. Examination of numerous pre-incubated counts revealed a bimodal rather than a normal distribution indicating that the types of contaminants in pasteurized milk may be as important as their initial concentration. Pseudomonads that gave particularly high ($>5\times10^6$ cfu/ml) and low ($<10^3$ cfu/ml) pre-incubated counts were isolated (high and low count isolates respectively). After the organisms had been subjected to a cold shock no consistent trend between the groups of isolates was detected with respect to lag phase duration. However, the high count isolates consistently had a faster exponential growth rate. Unexpectedly, with the exception of one isolate, the low count isolates produced detectable proteinase and lipase earlier. In addition, with one exception, maximal proteinase and lipase production was observed with the low count isolates. These findings indicate that there is no causal relationship between selective growth advantage and ability to produce proteinase and lipase. It also indicates that the spoilage of pasteurized milk is a complex phenomenon and is worthy of further research.

Keywords: Psychrotrophs, pasteurized milk, spoilage.

Psychrotrophic bacteria, particularly *Pseudomonas* spp., are the main determinants of the shelf-life of pasteurized milk in Europe and gain access as post-pasteurization contaminants (Schroder, 1984). This is in contrast to the USA where spore-forming *Bacillus* spp., including psychrotrophic strains, are perhaps more important. The same strains of *Pseudomonas* spp. may also be responsible for the spoilage of refrigerated raw milk by virtue of their ability to elaborate heat-stable extracellular enzymes, particularly proteinases and lipases, which can survive pasteurization and even UHT heat treatments, causing a reduction in cheese yield, gelation of UHT milk and off-flavours in many dairy products (Law, 1979). It is presumed that pseudomonads spoil pasteurized milk in the same way except for those like *Ps. fragi*, which produce fruity taints (Gilmour & Rowe, 1990).

It is clear that pseudomonads have a significant selective advantage over the other microflora of refrigerated raw milk since they constitute up to 75% of the microflora (Thomas & Thomas, 1973 a, b). It is interesting to note, however, that even with that selective advantage the most frequently isolated pseudomonad species from refrigerated raw milk, *Ps. fluorescens* (Law, 1979), does not have the genetic competence to catabolize lactose (Krieg & Holt, 1984). This is against a background that bacteria, in general, tend to catabolize sugars preferentially ahead of other substrates such as proteins and fats. It is recognized, however, that since these enzymes may be produced primarily during the late log/early stationary growth phase (Rowe & Gilmour, 1982) when factors contributing to the selective advantage of the organisms would already have exerted their effect, the genetic competence

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to produce proteinase and lipase may only be a coincidental event. It is even conceivable that their production is detrimental to the selective advantage of psychrotrophs. However, it is attractive to link spoilage outcome with incipient changes that can be detected and which might form the basis of a rapid test to determine spoilage potential.

At the time this research was undertaken, one of the main statutory tests applied to gain an index of the quality of pasteurized milk was the pre-incubated test which entails incubating the milk sample for 5 d at 6 °C, thereby creating selective conditions for the growth of any psychrotrophs present, raising their numbers above the sensitivity of the plate count carried out subsequently at 21 °C. A count in excess of 100 000 constituted a failed test under EC Milk Hygiene Directive (85/397/EEC). Two main factors may determine the pre-incubated count value, namely the number of psychrotrophs gaining access after pasteurization and the type of contaminant. If the main determining factor is the initial contamination level then, if many results were collated, and a graph was plotted of $\log_{10}$ pre-incubated count (x axis) against frequency of count (y axis), a normal distribution should be observed. It is recognized that currently the relevant Directive (92/46) has changed to a three-class sampling plan regime but this still involves a pre-incubated count and in the authors' opinion does not invalidate the conclusions of the work reported here.

The Milk Technical Laboratory in the Food Microbiology Unit of The Department of Agriculture and Rural Development for Northern Ireland, in the course of discharging its statutory responsibilities as a competent authority, routinely performs the pre-incubated test. Scrutiny of the results over a number of years reveals a bimodal rather than a normal distribution. This observation and subsequent research (Stevenson et al. 1996) indicates that the type of contaminant, as opposed to the initial post-pasteurization contamination level, is important. In the course of this research psychrotroph isolates were obtained from pasteurized milk that exhibited high pre-incubated counts ($>5\times10^6$ cfu/ml; high count isolates) and low pre-incubated counts ($<10^3$ cfu/ml; low count isolates).

In the work reported here growth kinetics, along with proteinase and lipase production, of two high count isolates and two low count isolates were monitored during incubation at 4 °C in UHT skim milk for up to 25 d. This was an attempt to determine whether proteinase and lipase production contributes significantly to the selective advantage of pseudomonads in pasteurized milk during refrigerated storage. This is an attractive prospect because it focuses attention on the relationship, if any, between selective advantage and spoilage. In addition, *Ps. fluorescens* strain B52, which has previously been used for research into psychrotroph activity in milk (Richardson, 1981) was included as a reference and to facilitate comparison of the results with those of other workers.

Materials and Methods

Culture preparation

Four isolates were obtained from Milk Agar (CM21, Oxoid, Unipath, Basingstoke, Hampshire, UK) after pasteurized milk had been subjected to the pre-incubated count (PIC; EU Directive 85/397/EEC92/46, 1985), two each from milk that exhibited high ($>5\times10^6$ cfu/ml) and low ($<10^3$ cfu/ml) counts respectively. Although in force when the work was carried out, it is recognized that this directive has been superseded by EU 92/46. Each isolate was from a different milk sample to reduce the likelihood that they were identical organisms. After checking for purity the organisms were identified to species level using API 20NE (API System, La Balme Les Grottes, 38390, Montalieu-Vercieu, France) along with other supplementary tests. These included the Gram reaction (Harrigan & McCance, 1966), oxidase test (Cowan, 1974), catalase test, motility test (Harrigan & McCance, 1966), oxidation/fermentation of carbohydrates (Hugh & Leifson, 1953), pigment production using King's A and B media (King et al. 1954), gelating liquefaction (Cowan, 1974) and levan production (Harrigan & McCance, 1966). The two high count isolates were identified as *Ps. fragi* (HC64), *Ps. fluorescens* (HC69) while the low count isolates were both strains of *Ps. fluorescens* (LC52 and LC56). The strains, along with *Ps. fluorescens* (B52) were checked for extracellular proteinase and lipase production competence using the methods of Harrigan & McCance (1966). The organisms were maintained in Protect bacterial preservers (Technical Services Consultants, Heywood, UK) and stored at −80 °C until required. A working culture was generated by inoculating a Protect bead into Nutrient broth (CM1, Oxoid) and incubating at 25 °C for 12 h (log phase culture). After the cell density of the culture had been determined by nephelometry it was used to inoculate UHT skim milk pretempered at 4 °C to give a final cell concentration of 10^2–10^3 cfu/ml. The inoculated cultures were incubated at 4 °C and samples removed at daily intervals for up to 25 d and subjected to a viable count using a spiral plater (Model D, Don Whitley Scientific Ltd., Shipley, UK) as described in BSI BS4285 (British Standards Institution, 1984) employing Milk Agar with incubation at 21 °C for 25 h. The samples were also subjected to a proteinase and lipase assay.

Proteinase assay

Aliquots of 100 μl of culture were incubated with 1 ml of 1% isoelectric casein (Analytical standard; United States Biochemical Corporation, Cleveland, Ohio, USA) in 0·1 M-sodium phosphate buffer (pH 7·0) containing 0·5 g sodium azide/l for 30 min at 30 °C. The reaction was stopped by the addition of 1 ml 50% trichloroacetic acid (Sigma Chemical Co. Ltd., Gillingham, UK) before being mixed vigorously in a vortex mixer and centrifuged at 1355 ***g*** for 10 min. The supernatant was filtered (Whatman no. 1,

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Whatman International Ltd., St Leonards Road, Maidstone, Kent, UK) and 25 μl filtrate was added to 2 ml 0·1 M-sodium phosphate (pH 7·5; Sigma) and 1 ml fluorescamine (10 mg/100 ml in acetone; Sigma) in a cuvette of 10-mm light path. Fluorescence ($\lambda_{excitation}$ 390 nm, $\lambda_{emission}$ 475 nm) was measured 5 min after addition of the reagents using a spectrofluorimeter (Model LS-5, Perkin Elmer Ltd., Beaconsfield, Buckinghamshire, UK). The fluorescence of samples at time zero was subtracted from each of the readings and all determinations were made in duplicate. For each assay run L-leucyl-L-leucine (Sigma) was used as a calibrant.

Lipase assay

The method was based on that of McKellar & Cholette (1986). The assay mixture contained in a final volume of 2 ml: 1·43 ml 50 mM-N,N′-bis [2-hydroxyethyl]-2-aminoethane sulphonic acid buffer (BES; Sigma) pH 7·2; 500 μl culture supernatant; 50 μl 4% trypsin (Sigma) in BES buffer and 20 μl 300 mM-β-naphthyl caprylate (Sigma) in dimethyl sulphoxide (DMSO, Sigma). After 1 h incubation of the other reagents at 40 °C, 20 μl β-napthyl caprylate was added to the mixture to start the reaction before incubation for 30 min. After 30 min incubation with β-napthyl caprylate Fast Blue (20 μl; 100 mM, Sigma) in DMSO was added and incubated for a further 5 min. The reaction was terminated by the addition of 200 μl 0·72 M-trichloroacetic acid. The coloured complex was extracted by vigorous shaking in a vortex mixer with 5 ml ethyl acetate (Rathburn Chemicals Ltd., Walkerburn, Scotland). The resulting layers were separated by centrifugation (1355 ***g*** for 10 min) and the absorbance of the top layer was measured at 540 nm. Controls in triplicate, lacking enzyme, were subtracted from the means of triplicate samples and converted to β-naphthol released/ml crude enzyme/h using β-naphthol standards.

Statistical analysis

The viable counts of each experimental run for each isolate were fitted separately using the sigmoidal Gompertz relationship to produce growth curves in addition to enabling two sets of growth kinetic parameters, exponential growth rate (EGR) and lag phase duration (LPD) to be calculated. An analysis of variance was performed on the two replicate runs to compare the means of the individual isolates and then the means of the high and low count isolates.

Results and Discussion

UHT skim milk was chosen as the growth medium because McKellar & Cholette (1986), using a similar lipase assay, showed that the presence of as little as 2% butterfat causes a 92% reduction in detectable lipase activity attributable to substrate competition between the butterfat and β-naphthol caprylate, the latter being the substrate used in the lipase assay. Although the data are not presented here, there was no significant difference ($P>0{\cdot}05$) in either LPD or EGR when the growth kinetics of the isolates were compared in whole and skim UHT milk (Stevenson, 2001).

When the LPD means of the high and low count isolates were compared, no consistent trend was observed (Table 1). Taking into consideration that the organisms received a cold shock (from 25 to 4 °C) this is in contrast to the findings of Stevenson et al. (1996) who observed that the high count isolates had a significantly shorter lag phase when subjected to such a shock. As regards the EGR, the high count isolates had significantly ($P<0{\cdot}05$) greater values than the low count isolates (Table 1) which indicates that this parameter may differentiate between the two groups of isolates. Interestingly *Ps. fluorescens* B52 had an EGR value more similar to the low count isolates (Table 1).

Proteinases and lipases were first detected when the organisms under test were in their late log or early stationary growth phases (Table 2) which is in agreement with previous reports (McKellar, 1982; Rowe & Gilmour, 1982, 1983; Driessen, 1983). If a causal relationship exists between high and low count isolates and the time of onset of detectable production of extracellular hydrolytic enzyme, then it might be expected that the high count isolates would produce proteinase and lipase earlier than the low count isolates. However, with the exception of lipase production by *Ps. fluorescens* LC56, the opposite

Table 1. Mean growth kinetic data for each isolate grown in UHT skimmed milk at 4 °C ($n=2$)

Isolate	Mean Growth Kinetic Parameters	
	LPD†	EGR‡
Ps. fragi HC64	0	1·42
Ps. fluorescens HC69	1·21	1·48
Ps. fluorescens LC52	2·04	1·25
Ps. fluorescens LC56	0·61	1·25
Ps. fluorescens B52	0·39	1·29

† Lag Phase Duration (LPD) units in days
‡ Exponential Growth Rate (EGR) units $\log_{10}$ cfu/ml per d

Table 2. Mean times (d) to detectable enzyme production for each isolate in UHT skimmed milk at 4 °C ($n=2$)

Isolate	Lipase (d)	Proteinase (d)
Ps. fragi HC64	12	13
Ps. fluorescens HC69	11	11
Ps. fluorescens LC52	10	10·5
Ps. fluorescens LC56	11·5	9·5
Ps. fluorescens B52	11	13

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Table 3. Mean maximum enzyme activity level for each isolate in UHT skimmed milk at 4 °C ($n=2$)

Isolate	Lipase activity†	Proteinase activity‡
Ps. fragi HC64	2·32	4·09
Ps. fluorescens HC69	1·91	4·27
Ps. fluorescens LC52	2·27	4·51
Ps. fluorescens LC56	2·31	4·58
Ps. fluorescens B52	1·91	4·13

† Lipase activity units, $\log_{10}$ (μmol β-napthol equivalents released $\times 10^3$)
‡ Proteinase activity units, $\log_{10}$ (μmol leucyl-leucine equivalents released $\times 10^3$)

was the case. It is recognized that differences in time to detection of proteinase as compared with lipase production may be a simple reflection of the relative sensitivities of the respective assays and may not represent a valid comparison. In respect of time to detectable enzyme production, *Ps. fluorescens* B52 responded more like a high count isolate than a low count isolate (Table 2).

If a contributory factor to the more rapid growth rate of the high count isolates is the production of extracellular hydrolytic enzymes then it might be expected that they would produce more proteinase and lipase than their counterparts. In effect, with the exception of lipase production by *Ps. fragi* LC64 the opposite was the case (Table 3). In contrast to previous observations *Ps. fluorescens* B52 responded similarly to the high count isolates (Table 3).

In summary, a number of factors are pertinent. Firstly, if the proteinase and lipase enzymes are produced when the organisms are in their late log or early stationary growth phase then they would have minimal impact on the early stages of the growth kinetics of the isolates, whether of high or low count origin. Secondly, if the organisms do not have the genetic competence to catabolize lactose, what do they catabolize and apparently so efficiently? If such component(s) could be identified and detected rapidly and simply, then perhaps a more meaningful index of the spoilage potential of pasteurized milk might be devised. Since psychrotrophs, particularly *Pseudomonas* spp, also predominate in refrigerated raw milk (Bramley & McKinnon,1990) such an assay might also have application in raw milk. Finally if proteinase and lipase contribute significantly to the spoilage of pasteurized milk then the work reported here calls into question the value of the preincubated test as a reliable index of the quality and shelf-life of the product. It should be recognized, however, that these conclusions are based on only five isolates and might not be indicative of pasteurized milk psychrotrophs as a whole.

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