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Effect of quorum sensing agents on the growth kinetics of *Pseudomonas* spp. of raw milk origin

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Psychrotrophs, particularly strains of *Pseudomonas fluorescens*, dominate the microflora of refrigerated raw milk and secrete heat-stable extracellular enzymes (proteases and lipases) which survive pasteurisation and even UHT heat treatments and degrade the casein and fat components of raw milk causing a reduction in cheese yield, gelation of UHT milk and off flavours in many dairy products. These enzymes are usually produced in the late log/early stationary growth phases when the cell density is high. This fact indicated that induction of these enzymes may be a candidate for quorum sensing, a phenomenon by which bacteria can sense and respond to cell population size by means of chemical signals based on the homoserine lactone molecule. It is assumed that the production of these enzymes contributes to the selective advantage of psychrotrophs and hence have a significant effect on their growth kinetics. In the work reported here nine proprietary homoserine lactone compounds were screened, using water as a control, for their effect on the lag phase duration (LPD) and exponential growth rate (EGR) of three strains of *Pseudomonas fluorescens* isolated from refrigerated raw milk after 1, 3 and 5 days storage. Two compounds viz. N-benzoyloxycarbonyl-L-homoserine lactone and N-3-oxyhexanoyl-DL-homoserine lactone were found to significantly ($P<0{\cdot}001$) reduce the LPD and increase the EGR of the three strains. Further work with these compounds is warranted, monitoring growth in parallel with enzyme production, to determine the extent to which extracellular enzyme production is mediated by the quorum sensing phenomenon in this species.

Keywords: Quorum sensing, *Pseudomonas*, raw milk.

The advent of widespread refrigeration of raw milk has led to the creation of selective conditions for psychrotrophic bacteria, in particular, those of the genus *Pseudomonas*. These bacteria are ubiquitous in nature and hence strategies to completely exclude them from raw milk are problematical, if not impossible (Cousin, 1982). Pseudomonads, particularly *Pseudomonas fluorescens*, are important because they have a significant spoilage potential by virtue of their ability to elaborate heat stable enzymes (proteases and lipases) which can survive pasteurisation and even UHT heat treatments causing a reduction in cheese yield, gelation of UHT milk and off-flavours in many dairy products (Law, 1979). The inherent stability of these enzymes, although not of the producing organisms per se, means that once produced no method, consonant with the natural image of milk, can be applied without subsequent detectable deleterious changes to milk or dairy products manufactured from that milk.

These extracellular enzymes are usually produced in the late log/early stationary growth phase when the organisms have reached comparatively high cell densities ($>10^6$ cfu/ml; Rowe & Gilmour, 1982). The organisms presumably secrete these enzymes to degrade the protein and fat components in milk to supply amino acids/peptides and fatty acids, respectively, for catabolic purposes which, it is assumed, contribute to their selective advantage. Certainly there is evidence that either an adaptation or microflora succession process occurs (Jaspe et al. 1995).

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Table 1. Acyl homoserine lactone compounds used in growth kinetic experiments

Name	Side chain formula	Abbreviation
N-Butanoyl-DL-homoserine lactone	$-CO(CH_2)_2CH_3$	C4-HSL
N-Hexanoyl-DL-homoserine lactone	$-CO(CH_2)_4CH_3$	C6-HSL
N-Heptanoyl-DL-homoserine lactone	$-CO(CH_2)_5CH_3$	C7-HSL
N-Octanoyl-DL-homoserine lactone	$-CO(CH_2)_6CH_3$	C8-HSL
N-Decanoyl-DL-homoserine lactone	$-CO(CH_2)_8CH_3$	C10-HSL
N-Dodecanoyl-DL-homoserine lactone	$-CO(CH_2)_{10}CH_3$	C12-HSL
N-Tetradecanoyl-DL-homoserine lactone	$-CO(CH_2)_{12}CH_3$	C14-HSL
N-Benzoyloxycarbonyl-L-homoserine lactone	$-CO-O-CH_2(C_6H_5)$	Z-HSL
N-3-Oxyhexanoyl-DL-homoserine lactone	$-COCH_2CO(CH_2)_2CH_3$	3-oxo-C6-HSL

Microbial spoilage of raw milk and its subsequent impact on product quality is essentially an old but continuing problem. Research, in the light of developments in other areas of microbiology, such as the discovery of quorum sensing, together with improvements in techniques, for example monitoring growth kinetics by means of conductance measurements, presents the possibility of using new approaches to investigate this enigma.

Raw milk is a liquid menstruum and subject to intermittent agitation in large milk silos to prevent separation of the skim and cream fractions. In the authors' opinion therefore it is likely that because of the initial low numbers of bacteria the site of action of extracellular lipases and proteases and their consequent products would not be sufficiently proximal to be absorbed and hence catabolised by the enzyme-producing organisms. There is therefore a selective advantage for such organisms to produce these extracellular enzymes only when at high cell densities, thereby maximising the chance of the enzyme products being sufficiently close to be absorbed, albeit perhaps not as a result of enzyme action initiated by the recipient bacterium. It is this premise which formed the basis of the work reported here.

Many Gram negative bacteria, including members of the genus *Pseudomonas* (Chapon-Herve et al. 1997; Pearson et al. 1997), regulate gene expression in response to their population size by sensing the level of acyl-homoserine lactone (HSL) signal molecules which they produce and liberate into the environment (Shaw et al. 1997). This phenomenon is termed quorum sensing and appears to be a conserved process amongst prokaryotes since many bacteria have been shown to exploit cell-cell communication devices to regulate diverse physiological processes (for reviews see Salmond et al. 1995; Fuqua et al. 1996; Shapiro & Dworkin, 1997). Hence the supposition that induction of extracellular enzyme production may be, at least partly, under control of the quorum sensing phenomenon seems plausible.

In the work reported here a number of commercially-available HSL signal molecules were screened to determine if they had any significant effect on the growth kinetics of pseudomonad organisms which had previously been isolated after 1, 3 and 5 days refrigerated storage of raw milk. These organisms were chosen to reflect the changing microflora of refrigerated raw milk. The experimental design, it was realised, represented an indirect approach but it was hoped that the results generated would identify particular signal compounds that would warrant further investigation in respect of a possible causal link between such compounds, induction of extracellular hydrolytic enzyme production and selective advantage.

Materials and Methods

Isolation of psychrotrophs

Three strains of psychrotroph isolates viz. 29, 36 and 41 were isolated from refrigerated (6 °C) raw milk after 1, 3 and 5 days storage respectively as described by Rowe et al. (2001). The strains were isolated from different milk samples each originating from a separate processing establishment and hence the chances of them being the same strain are considered remote. The significance of the 1, 3 and 5 day refrigerated storage was to take account of the effect, if any, of the natural microflora succession of raw milk during such storage. They were subsequently identified using APIZ0NE (bio Merieux UK Ltd, Basingstoke, UK) as strains of *Ps. fluorescens*. The cultures were maintained in Protect Vials (Technical Service Consultants Ltd, Heywood, UK) and stored at −80 °C in order to maintain genetic integrity.

Homoserine lactone compounds tested

These are shown in Table 1 and were obtained from Sigma-Aldrich (Poole, Dorset, UK). Water was used as the control. It is recognised that *N*-benzoyl carbonyl-L-homoserine lactone (Z-HSL) has, to date, not been shown to mediate in the quorum sensing phenomenon but it was considered to have such a similar chemical structure to the other HSLs that it warranted inclusion.

Some of the HSL compounds proved difficult to dissolve and in these cases the compounds were dissolved initially in the minimum of ethanol and the solution made to volume with water. All solutions were observed to be clear and hence the compounds were deemed to be completely dissolved.

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Growth kinetics experiment

Indirect impedance methodology as described by Bolton (1990) was used for determining growth kinetics. All impedance measurements were carried out using a Rapid Automated Bacterial Impedance Technique (RABIT; Don Whitley Scientific Ltd, Shipley, UK). This involves using an alkaline agar plug consisting of equal volumes of agar no. 1 (20g/l; L11; Oxoid Ltd, Basingstoke, UK) and potassium hydroxide (7g/l). The molten mixture (0·75ml) was dispensed into each impedance tube, which was tightly stoppered and allowed to stabilise in the dark overnight, after which only tubes that produced a steady minimum conductivity reading of 6000 μS at 42 °C were used for further study. Glass tubes (12 × 75 mm external dimension) containing 4·5 ml Whitley Impedance Broth (WIB;GS001, Don Whitley) were sterilised according to the manufacturer's instructions. Working cultures of individual isolates were generated by inoculating a Protect Bead into WIB and incubating at 24 °C for 18 h. The culture was subsequently centrifuged at 6000 ***g*** for 10 min at 6 °C and the pellet resuspended in Maximum Recovery Diluent (MRD; CM733, Oxoid) to give a concentration of 10^6–10^7 cfu/ml as determined by nephelometry. Glass tubes containing 4·5 ml of WIB/HSL mixture were inoculated with the cultures to give a final cell concentration of approximately 10^2 cfu/ml and inserted into the RABIT tubes and located in RABIT blocks at 6 °C. The conductance was measured at 30 min intervals for up to 8 days. Duplicate tubes were used for each run for each isolate and three independent runs were performed on each isolate. The three respective HSL final concentrations used in the study were 1, 10, 100 μg/ml. It is recognised that HSLs are very susceptible to lactonolysis which would be likely to occur if they came into contact with the alkaline agar in the plug. This problem was obviated by using indirect impedance where the culture is present in a small inner tube so that the culture mentruum never comes in contact with the plug material. All conductance values were transferred to a Microsoft Excel Spreadsheet (Excel 97, Microsoft Corp., Washington DC, USA.) for manipulation and subsequent statistical analysis.

Statistical analysis

The RABIT results were averaged over the duplicate values for the three runs. Sigmoidal (Gompertz) curves were fitted for each of the nine homoserine lactone compounds, three concentrations and three isolates.

In general the Gompertz provided a good fit with 114 out of 162 cases having R^2 greater than 0·99 and 18 having R^2 less than 0·90. Missing values were inserted for these latter cases.

Lag phase durations (LPDs) and exponential growth rates (EGRs) were derived from the computed Gompertz parameters. The LPD and EGR values were compared by analysis of variance to test for significant differences between the means of the LPD and EGR values.

Results and Discussion

Three strains of *Ps. fluorescens* were chosen which had been isolated from refrigerated raw milk after one, three and five days. This was in order to determine the relative importance, if any, of quorum sensing effects on microbial adaptation and/or microflora succession processes during refrigerated storage. These strains have been used previously in other related growth kinetic studies (Rowe et al. 2001). Strains of *Ps. fluorescens* were chosen since this is the species most implicated in the spoilage of refrigerated raw milk (Law, 1979) and to minimise the impact of species but not strain differences.

Given the large number of species known to release autoinducing compounds (Swift et al. 1993) and the imperfect ability of bacteria to distinguish between them (Eberhard et al. 1986) coupled with the fact that, like *Ps. aeruginosa*, they may have more than one quorum sensing system (Latifi et al. 1996) as wide a range of HSLs as commercially available were screened. Unfortunately of the three HSL which have previously been shown to induce quorum sensing in a strain of *Ps. fluorescens* (Laue et al. 2000) only *N*-hexanoyl-DL-homoserine lactone (C6-HSL) was included; neither *N*-(3-oxotetradecanoyl)-L-homoserine lactone nor *N*-(3-hydroxytetradecenoyl)-L-homoserine lactone were available.

It was considered that if a compound or compounds were successfully identified this would facilitate a more practical investigation of the effect of the chosen compounds on extracellular enzyme production by pseudomonads and hence help to determine the role, if any, of quorum sensing as an induction system for these enzymes. It is recognised that this is rather an indirect approach i.e. as opposed to monitoring enzyme production directly at the same time as viable numbers, but was adopted because of the large number of parameters involved *viz.* water (control) and 9 compounds each at 3 concentrations with 3 organisms. Such a wide range of variables made, in the authors' opinion, monitoring of growth by conductance measurements via the RABIT apparatus the method of choice. This was for two main reasons. Firstly, each RABIT block can monitor the growth of at least 32 tubes simultaneously thus speeding up the acquisition of the requisite data. Secondly, the machine monitors conductance every 30min throughout the whole incubation period, logistically impractical using conventional plate counting techniques. This means that an error in any individual datum is less likely to skew the overall results and subsequent statistical analysis. It should be noted that conductance values reduce as an organism grows and hence the growth curves generated are a mirror image of those produced by conventional viable counting techniques in which $\log_{10}$ transformation of viable counts are plotted against time.

When considering the effects of the quorum sensing agents tested on LPD it was evident firstly that, overall, they had a significant ($P<0·001$) effect (Table 2). Secondly,

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Table 2. Effect of distilled water (control), *N*-hexanoyl-DL-homoserine lactose (C6-HSL), *N*-Benzoyloxycarbonyl-L-homoserine lactone (Z-HSL), *N*-3-Oxyhexanoyl-DL-homoserine lactone (3-oxo-C6-HSL), *N*-Dodecanoyl-DL-homoserine lactone (C12-HSL), *N*-Decanoyl-DL-homoserine lactone (C10-HSL), *N*-Octanoyl-DL-homoserine lactone (C8-HSL), *N*-Tetradecanoyl-DL-homoserine lactone (C14-HSL), *N*-Butyryl-DL-homoserine lactone (C4-HSL) and *N*-Heptanoyl-DL-homoserine lactone (C7-HSL) on the lag phase duration and exponential growth of three *Pseudomonas fluorescens* strains isolated from refrigerated raw milk after 1, 3 and 5 days respectively

	Acyl homoserine lactones											
	Control (H2O)	C6-HSL	Z-HSL	3-oxo-C6-HSL	C12-HSL	C10-HSL	C8-HSL	C14-HSL	C4-HSL	C7-HSL	SE of means	Significance
LPD† (min)	6341	4901	3035	4560	6081	7327	6524	6599	6334	5738	224·9	***
EGR‡ (ms/min)	0·68	1·034	1·435	2·476	0·99	1·17	1·071	1·165	0·621	1·054	0·11	***

† Lag Phase Duration
‡ Exponential Growth Rate

Table 3. Overall effect of homoserine lactone agents on the individual lag phase durations and exponential growth rates of three *Pseudomonas fluorescens* strains isolated from different refrigerated raw milk samples after 1, 3 and 5 days storage

	Isolate				
	Day 1	Day 3	Day 5	SE of Means	Significance
LPD† (min)	6064	5017	6151	123	***
EGR‡ (ms/min))	0·949	1·162	1·398	0·06	***

† Lag Phase Duration
‡ Exponential Growth Rate

that there was a significant difference ($P<0·001$) in response to the agents overall amongst the individual isolates (Table 3). Some significant ($P<0·05$) interactions were detected between isolates, chemicals and concentrations. However, on examination these were mainly a matter of degree of difference and not a reversal of trend, hence only main effect means are presented in the tables of results.

When the effects of the agents are considered individually it is apparent that *N*-benzoylcarbonyl-L-homoserine lactone (Z-HSL) and *N*-3-oxyhexanoyl-DL-homoserine lactone (3-oxo-C6N-HSL) stood out as having marked influences on both parameters.

The decrease in LPD with Z-HSL was not surprising since it has previously been shown to reduce the LPD, though not significantly so, of two strains of *Ps. fluorescens* this time isolated from pasteurised milk (Whan et al. 2000). However, in the work of Whan et al. (2000) Z-HSL was also found to reduce the EGR with the same organisms. No explanation can be advanced for the disparity presented by this latter finding except the possibility of strain differences. It is recognised that Z-HSL may only be analogous to the true quorum sensing agent produced by *Ps. fluorescens* since media conditioned by the prior growth of *Ps. fluorescens* has been shown to activate the phenazine synthesis genes of *Ps. aureofaciens* (Dunlap, 1997).

3-Oxo-C6-HSL has been reported to act as a signalling molecule in several species for antibiotic synthesis, bioluminescence and exoenzyme production (Whitehead et al. 2001).

The fact that concentration overall had no significant effect (data not shown) indicates perhaps that, even at the lowest concentration used (1 μg/ml), the compounds had reached saturation levels. It is interesting to note that both Z-HSL and 3-oxo-C6-HSL, unlike the other agents, possess a polar end that could be expected to inhibit translocation across the cytoplasmic membrane thus preventing these HSL molecules from performing as other HSLs and activating transcriptional regulator proteins which would drive the expression of target genes (Joint et al. 2002).

It was hoped that the day 3 isolate would give an intermediate response compared with the day 1 and 5 isolate. This was observed with respect to EGR (Table 3) but, LPD for day 3 gave a lower value than either the day 1 or day 3 isolate. The difference in both LPD and EGR were reduced between the day 3 and 5 isolates compared with day 1 and 3 indicating that the change in microflora of refrigerated raw milk may be exponential rather than linear which has been observed with these isolates previously (Rowe et al. 2003).

This work has clearly identified Z-HSL and 3-oxo-C6-HSL as worthy of further research to determine their effect, if any, on extracellular enzyme production by pseudomonads. If they are found to induce protease and/or lipase production then heat resistance studies would indicate the extent to which high populations of the organism in raw milk may predispose post-pasteurisation pseudomonad contaminants to induce premature spoilage.

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