Journal of Dairy Research (2005) **72** 25–33. © *Proprietors of Journal of Dairy Research 2005*
DOI: 10.1017/S0022029904000524 *Printed in the United Kingdom*

Potential applications of high pressure homogenisation in processing of liquid milk

Maurice G Hayes, Patrick F Fox and Alan L Kelly*

Department of Food and Nutritional Sciences, University College, Cork, Ireland

Received 20 August 2003 and accepted for publication 3 June 2004

Studies of the potential of high pressure homogenisation (HPH) for the combined pasteurisation/homogenisation of raw bovine milk were undertaken. Raw milk was preheated to 45 °C and HPH-treated at 150, 200 or 250 MPa; milk outlet temperature at these pressures were 67, 76·8 and 83·6 °C, respectively, with a holding time of ~20 s. Raw and commercially pasteurized and homogenized (CPH) milk samples were analysed as controls. Fat globules in HPH samples were approximately half the size of those in CPH samples, although differences were not significant ($P>0·05$). β-Lactoglobulin was denatured at pressures ⩾150 MPa, although little denaturation of α-lactalbumin was observed. Numbers of psychrotrophic bacteria in raw milk were reduced by 2·73 log cycles by HPH at 150 MPa and were uncountable following HPH at 200 or 250 MPa. Mesophilic bacterial counts were reduced by 1·30, 1·83 and 3·06 log cycles by HPH at 150, 200 or 250 MPa, respectively. No viable *Staphylococcus aureus* nor coliform cells remained in any HPH milk samples. HPH did not affect the colour of milk and HPH samples did not cream during refrigerated storage. The activities of plasmin, alkaline phosphatase and lactoperoxidase in milk were all greatly reduced by HPH. *Pseudomonas fluorescens*, inoculated into milk (~10^6 cfu/ml), was reduced to undetectable levels by HPH at 200 MPa (milk inlet temperature, ~10 °C); however, *Ps. fluorescens* proteinase was quite resistant to HPH under such conditions. Overall, owing to the significant increase in temperature and the possibility of varying the holding time, there may be potential applications for HPH as a novel liquid milk processing technique, combining many advantages of conventional homogenization and pasteurization of milk in a single process.

Keywords: High pressure homogenisation, milk, pasteurization.

Processing of commercial liquid milk usually involves separation (of the cream and serum phases of milk and remixing to standardise the fat content), homogenization and pasteurization. During conventional homogenization, milk at 45–50 °C is passed through two consecutive small orifices at an approximate total pressure of 20 MPa; this creates small fat globules, which do not cream during milk storage. Pasteurization is a thermal process, typically performed in a plate heat exchanger, whereby milk is heated rapidly to 72–75 °C and held for 15–30 s, i.e., high temperature short time (HTST) pasteurization, followed by rapid cooling to refrigeration temperature and packaging under conditions that minimize contamination. This treatment is designed to destroy vegetative non-pathogenic and pathogenic micro-organisms (Kameni et al. 2002), and is used to obtain milk that, if subsequently refrigerated, is both safe for human consumption and has a reasonable shelf-life.

In recent years, several novel non-thermal food preservation processes have been developed, such as high pressure processing, and there have also been some novel developments of established technologies. For example, in the area of homogenization and emulsion formation, equipment with higher pressure capabilities has emerged, with two classes of technology, based on different principles. The principle of operation of high pressure homogenisers is similar to that of conventional ball-and-seat homogenizers, but operating at a far higher pressure, while microfluidization is based on the principle of collisions between high-speed liquid jets. The first applications of such technologies were in the pharmaceutical and biotechnology sectors (Chandonnet et al. 1985; Kleining & Middelberg, 1998). Possible applications of high pressure homogenization (HPH) include reduction in the molecular weight of hydrocolloids in solution (Floury

*For correspondence; e-mail: a.kelly@ucc.ie

et al. 2002), inactivation of bacteriophage (Moroni et al. 2002), destruction of bacteria (Wuytack et al. 2002) and the manufacture of shellac and lipid dispersions (Krause & Müller, 2001; Liedtke et al. 2000). Destruction of bacterial cells by HPH is due to several physical phenomena, e.g. pressure drops, cavitation, shearing, 0turbulence and collision (Moroni et al. 2002). Recent studies have demonstrated interesting effects of HPH on milk, including changes in the functional properties of skim milk, such as inactivation of enzymes and killing of bacteria (Adapa et al. 1997; Kielczewska et al. 2000; Hayes & Kelly, 2003a, b). There have also been studies of the effects of HPH on other dairy products, e.g., effects on the microbiological and textural characteristics of cheese (Guerzoni et al. 1998, 1999; Kheadr et al. 2002).

The objective of this study was to assess the possible application of HPH as a combined process for reducing the size of fat globules and inactivation of micro-organisms and enzymes in liquid milk (i.e., combined pasteurization/homogenization).

Materials and Methods

High pressure homogenisation (HPH) of milk

Raw whole bovine milk was obtained from a local creamery and subjected to two-stage HPH (90% of the pressure on the primary homogenising valve and the balance on the secondary valve) at 150, 200 or 250 MPa (model 'nm-GEN' 7400H, Stansted Fluid Power Ltd., Essex, UK) at an inlet temperature of ~45 °C. Exceptions to use of these conditions are indicated in the text. The homogenizer was thermostatted by circulating water at ~48–50 °C for 30 min through the entire system; the flow rate of milk through the homogenizer was constant at 300 ml/min. For trials at each pressure, 2·5 l milk were used, with the majority of this volume being processed through the homogeniser to ensure stable operating conditions (temperature and pressure), before a 100–150 ml sample was taken for analysis. Homogenized milk was cooled in a coiled stainless steel tube (length, 4·5 m; inner diameter, 3·5 mm) attached to the product outlet port of the homogenizer and submerged in ice water. The final temperature reached by the milk during HPH was monitored in separate experiments using a hand-held digital thermometer at the product outlet after the secondary valve. The primary homogenizing valve was surrounded by a constant flow of water at ambient temperature to minimise rapid expansion or contraction of this valve; the presence of this water supply had no effect on the temperature of the homogenized milk.

All treatments were performed in triplicate and all analyses were also performed in triplicate. Commercially pasteurized and homogenized (CPH) full-fat milk was purchased from a local supplier on 3 occasions and subjected to the same analytical tests as HPH samples.

Bacterial enumeration

Immediately following HPH, serial dilutions of milk samples were prepared using Ringer's solution. The total bacterial population in all samples was enumerated using a pour plate method on plate count agar (Merck, Darmstadt, Germany) following incubation at 30 °C for 48 h or 4 °C for 8 d. *Bacillus cereus* was enumerated by spread plating on *B. cereus*-selective agar base (Oxoid, Hampshire, England) containing one vial of supplement SR99 (Oxoid) and 25 ml sterile egg yolk emulsion SR47 (Merck) per 500 ml agar base, following incubation at 30 °C for 18 h. Coliforms were enumerated using a pour plating on violet red bile lactose agar (Merck), following incubation at 35 °C for 24 h. *Staphylococcus aureus* was enumerated using spread plating on Baird-Parker agar base (Oxoid) containing 50 ml egg yolk-tellurite emulsion SR54/l (Difco Laboratories, Detroit, Michigan, USA), following incubation at 35 °C for 24 h.

After sampling for microbial analysis, sodium azide was added (0·5 g/l) to the remainder of each milk sample to prevent bacterial growth.

Determination of native β-lactoglobulin and α-lactalbumin

The levels of native β-lactoglobulin (β-lg) and α-lactalbumin (α-la) in the pH 4·6-soluble fraction of milk samples, prepared as described by O'Driscoll et al. (1999), were determined by reverse-phase high performance liquid chromatography (RP-HPLC). Samples were diluted 1 : 10 with solvent A [1 ml trifluoroacetic acid/l (TFA, HPLC grade, Sigma, St. Louis, MO 63178, USA) in deionised HPLC-grade water] prior to HPLC analysis. Samples were filtered through 0·45 µm cellulose acetate filters (SRP15, Sartorius, Goettingen, Germany) prior to injection.

RP-HPLC analysis was performed using a Shimadzu liquid chromatograph (Shimadzu Corp., Kyoto, Japan), consisting of a model LC-9A pump, a SIL-9A autosampler and a SPD-6A UV spectrophotometric detector. Nucleosil C_8 (300 Å; 5 µm particle size) guard (4·6 × 10 mm) and analytical (4·6 × 250 mm) columns (Machery-Nagel GmbH, Duren, Germany) were used. Eluates were monitored at 214 nm and data analysed using Varian software (Version 4.51). The chromatographic solvents were: Solvent A (described above) and Solvent B, 1 ml TFA/l acetonitrile (HPLC-grade, Labscan Ltd., Dublin, Ireland). Samples (100 µl) were injected onto the column and eluted at a flow rate of 0·75 ml/min by an eluent gradient as follows: 100% solvent A for 5 min, followed by a linear increase in the percentage solvent B to 45% over 15 min, then a linear increase to 60% solvent B over 15 min, holding at this level for 3 min, a linear increase to 95% solvent B over 5 min and holding at that level for 2 min. The gradient was then readjusted to 100% solvent A over 5 min and the system equilibrated at these conditions for 20 min before injection of the next sample.

https://doi.org/10.1017/S0022029904000524 Published online by Cambridge University Press

Determination of rate of creaming

Immediately after HPH, milk samples (100 ml) were placed in 100 ml graduated cylinders, which were hermetically sealed and stored at 5 °C. The volume of cream that separated from the milk was determined visually to the nearest 0·5 ml at 0, 4, 24, 48, 72, 96, 120 and 168 h after HPH and expressed as ml cream/100 ml milk.

Determination of residual activities of indigenous enzymes

Lactoperoxidase activity in milk samples was assayed by the method of Hernández et al. (1990), with modifications; samples were diluted 1 : 2 with 0·12 M-sodium phosphate buffer, pH 6·4, prior to analysis and 0·2 M-H_2O_2 was used. Alkaline phosphatase activity was determined as described by Ludikhuyze et al. (2000) and plasmin activity by the method of Richardson & Pearce (1981).

Analysis of milk composition, globule size, pH and colour

The gross composition of raw whole milk samples was determined using a Milkoscan FT 120 (Foss Electric, Hillerød, Denmark). The size distribution of fat globules was determined by the method of Hayes & Kelly (2003a), using the 3NAD presentation to calculate particle size parameters, including D [3,2] (surface-weighted diameter, or diameter of spheres of equivalent average surface areas to the particles in the sample). The pH of milk samples (containing 0·5 g NaN_3/l) was monitored during storage at 4 °C over 14 d. CIE L*, a* and b*-values of whole milk samples were also measured as described by Hayes & Kelly (2003a).

HPH of milk containing Pseudomonas fluorescens

A frozen (−80 °C) stock culture of *Pseudomonas fluorescens* AFT 36 (Culture Collection, Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork, Ireland) was thawed, streaked on tryptone soya agar (Oxoid) and incubated at 30 °C for 48 h. A sub-sample of the bacteria growing on this agar was then inoculated into 200 ml tryptone soya broth (Oxoid) in a 500 ml Erlenmeyer flask and incubated at 30 °C for 16 h, with shaking (80 rpm), to provide an inoculum. This culture was mixed with raw milk (10 °C) at a ratio of 1 : 10 and homogenized at conventional (18 MPa; Model APV 1000, APV homogenisers AS, Albertslund, Denmark) or high (50, 100, 150 or 200 MPa) pressure. The inlet temperature for conventional homogenisation was ∼50 °C while that for HPH was ∼10 °C. The increase in temperature during HPH was monitored at the product outlet; the milk was collected in containers and cooled in iced water.

Serial dilutions of control and homogenised milk samples were prepared immediately using Ringer's solution. *Ps. fluorescens* was enumerated on *Pseudomonas*-selective agar base (Oxoid) containing 5 ml glycerol and one vial of C-F-C supplement SR103 (Oxoid; rehydrated in 2 ml of a sterile 1 : 1 mixture of distilled water and ethanol) per 500 ml, following incubation at 30 °C for 36 h.

Preparation of a cell-free supernatant of Ps. fluorescens *and determination of its proteinase activity*

Ps. fluorescens AFT 36 was grown in 10% reconstituted sterilised (110 °C, 10 min) skim milk powder at 8 °C for 9 d, with shaking (80 rpm). A cell-free supernatant (CFS) was prepared by centrifuging the grown culture at 20 000× ***g*** for 30 min; the supernatant was decanted and frozen at −20 °C. Thawed CFS was mixed 1 : 20 with raw whole milk and subjected to single-stage HPH at 50, 100, 150, 200 or 250 MPa, at an inlet temperature of 10–14 °C.

pH 4·6-Soluble supernatants of HPH inoculated milk samples were prepared as described by O'Driscoll et al. (1999). The pH of these supernatants was readjusted to 6·5 by the addition of 4 M-NaOH, and proteinase activity determined by the method of Twining (1984), using fluorescein isothiocyanate-labelled casein in 0·5 M-Tris–HCl, pH 6·5 as substrate.

Statistical Analysis

Statistical analyses were performed using a randomised block design, using Minitab version 12 (Minitab Ltd., Coventry, UK). The effect of treatment and block on the measured parameters was examined using the General Linear Model function, with Tukey's pairwise comparisons at the 95% confidence level. All results are means of data from 3 independent experiments. Analysis of residuals validated the assumptions of normality and constant variability.

Results and Discussion

Milk fat globule size

As expected, fat globules in CPH milk were significantly smaller, by all measured size parameters, than those of raw milk (Table 1). Fat globules in all HPH milks were numerically smaller than those in CPH samples but differences in globule size between those milks were not significant, except for D_v 0·5 (mean globule size) of samples HPH-treated at 150 or 200 MPa, values of which were significantly ($P<0·05$) lower than those for CPH milk. The globules in milk HPH at 150 or 200 MPa were of similar size and slightly smaller than those in samples homogenized at 250 MPa. All milk samples were processed at an inlet temperature of ∼45 °C, at which all milk fat was in the liquid state. Therefore, it seems that globules produced in this study at 150 or 200 MPa are the smallest achievable using this HPH system. The effects of HPH on the perceived mouthfeel (e.g., creaminess) of milk remains to be evaluated.

https://doi.org/10.1017/S0022029904000524 Published online by Cambridge University Press

Table 1. Temperature changes during high pressure homogenisation (HPH) and the effects of HPH at selected pressures on the fat globule size in raw whole milk. The fat globule size found in conventionally-pasteurised/homogenised commercial milk (CPH) is included for comparison

Values for globule size parameters are means of data from 3 independent experiments; the values for temperature are representative data from a single experiment

Treatment	Ti† (°C)	To‡ (°C)	D_v 0·9 (μm)	D_v 0·5 (μm)	D [4,3] (μm)	D [3,2] (μm)
Raw milk	—	—	$7·59 \pm 0·73^{a}$	$3·24 \pm 0·19^{a}$	$4·53 \pm 0·30^{a}$	$1·08 \pm 0·19^{a}$
150 MPa	45·1	67	$0·52 \pm 0·02^{b}$	$0·23 \pm 0·00^{c}$	$0·48 \pm 0·18^{bc}$	$0·18 \pm 0·01^{b}$
200 MPa	45	76·8	$0·50 \pm 0·01^{b}$	$0·26 \pm 0·03^{c}$	$0·42 \pm 0·05^{c}$	$0·22 \pm 0·03^{b}$
250 MPa	45·1	83·6	$0·71 \pm 0·12^{b}$	$0·29 \pm 0·01^{bc}$	$0·86 \pm 0·11^{b}$	$0·25 \pm 0·02^{b}$
CPH milk§	—	—	$1·37 \pm 0·03^{b}$	$0·52 \pm 0·04^{b}$	$0·88 \pm 0·10^{bc}$	$0·36 \pm 0·03^{b}$
P-value			<0·001	<0·001	<0·001	<0·001

Values with common superscripts in each column were not significantly different; $P < 0·05$
Significance of treatment is denoted by *P*-value
† Milk inlet temperature
‡ Milk outlet temperature
§ Conventionally-pasteurised/homogenised commercial milk

To cover the greatly increased surface area of fat globules in homogenized milk, part of the casein in milk is adsorbed onto the newly-formed globules. Dalgleish et al. (1996) concluded that casein micelle fragments, rather than intact micelles, are adsorbed on the globules during microfluidization of milk. They attributed disruption of the casein micelles to the forces encountered during the process (although Hayes & Kelly [2003a] found no reduction in micelle size in HPH skim milk), or to the surface forces (reduction of interfacial tension) which are experienced by micelles adsorbing at the fat/serum interface. The globule size parameters for HPH milk (Table 1) suggest that fat globules in such milk are also surrounded by a layer of casein micelle fragments rather than intact casein micelles; D_v 0·5 values for HPH milks would be considerably larger if intact casein micelles (the mean diameter of which is ~150 nm) were present at the fat/serum interface.

Owing to the greater reduction in globule size by HPH than CPH, there is presumably a greater requirement for surface-active material to cover the greatly increased exposed fat interface. This suggests that a greater proportion of the casein in milk is adsorbed on the fat globules in HPH milk than in CPH milk. Cano-Ruiz & Richter (1997) reported that increasing the homogenization pressure from 30 to 90 MPa almost doubled the amount of casein adsorbed onto fat globules. Hence, at higher pressures it may be expected that a large proportion of the casein in milk is adsorbed onto the fat globules, although this was not measured in this study. However, at the highest pressure used (250 MPa), the amount of available casein may become limiting, which would account for the increase in fat globule size observed after HPH at 250 MPa; this may be due to partial agglomeration of very small globules insufficiently covered by surface-active material.

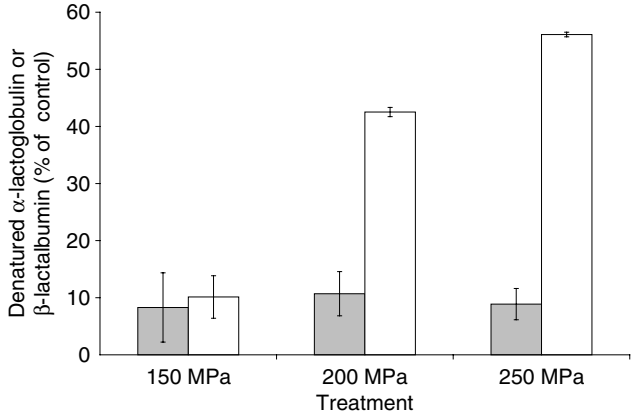

Fig. 1. Denaturation of α-lactalbumin (▩) and β-lactoglobulin (□) in high pressure homogenised (150–250 MPa) full-fat milk. Error bars represent standard deviations of data from triplicate trials.

Increase in temperature during HPH

A linear ($R^2 > 0·998$) temperature increase of 0·166 degrees C per MPa was observed for milk HPH at 150–250 MPa at an inlet temperature of ~45 °C (Table 1). The holding time for milk at the relevant outlet temperature was calculated to be ~20 s (volume of tubing from the primary valve to the cooling coil/flow rate). Thus, the thermal load to which the milk was exposed on HPH at 150, 200 or 250 MPa, was estimated to be less, slightly greater or substantially greater, respectively, than that encountered in normal HTST pasteurization.

Whey protein denaturation

Levels of α-la and β-lg denatured by HPH (150–250 MPa; inlet temperature, ~45 °C) are shown in Fig. 1;

https://doi.org/10.1017/S0022029904000524 Published online by Cambridge University Press

denaturation of β-lg was more extensive than that of α-la. The extent of denaturation of β-lg increased with pressure; 10·2, 42·5 or 56·1% was denatured in milk homogenized at 150, 200 or 250 MPa, respectively.

To determine if such an extent of denaturation was due to heating alone, values from the recent literature for denaturation of β-lg in milk were reviewed. Villamiel et al. (1997) reported that ∼8% of β-lg is denatured on heating milk at 72·5 °C for 15 s (normal HTST conditions). Oldfield et al. (2000) reported <20% denaturation of β-lg A in skim milk heated to 75 °C for <1 min, while Claeys et al. (2001) reported D-values of 12 900, 1662, 396 or 276 sec for denaturation of β-lg at 70, 77·5, 83 and 85 °C, respectively. All the above studies would indicate that the extent of denaturation on HPH was considerably higher than would be expected from purely thermal denaturation. Therefore, it is probable that the denaturation of β-lg on HPH observed in this study was due also to the physical forces experienced by the milk during HPH. Desrumaux & Marcand (2002) observed whey protein denaturation at homogenization pressures >210 MPa during the preparation of sunflower oil emulsions and attributed changes in protein conformation to the combined effects of homogenization forces and the increase in temperature at these pressures.

HPH in the range 150–250 MPa caused little, i.e. <10%, denaturation of α-la. Denaturation of α-la occurs >63 °C (DeWit et al. 1983); therefore, considering the outlet temperatures (Table 1), extensive denaturation of α-la would have been expected, especially in milk HPH at 200 or 250 MPa. However, α-la is quite resistant to heat-induced coagulation (Shulka, 1973); denaturation is 80–90% reversible after heating from 20 to 110 °C (Ruegg et al. 1977), which may explain the low level of denaturation of α-la observed in this study.

Changes in the pH of milk samples

Hayes & Kelly (2003a) reported a decrease of 0·3 pH units in HPH milk (inlet temperature ∼8 °C) stored at 4 °C for 24 h, which they attributed to the activity of lipoprotein lipase (LPL). In this study, the pH of milk samples HPH at 150 MPa decreased from 6·65 to ∼6·0 during 14 d of refrigerated storage (results not shown); no decrease in pH was observed in milk HPH at 200 or 250 MPa. These latter treatments were thermally more severe than HTST pasteurisation, which is known to cause extensive inactivation of LPL (Walstra et al. 1999); this supports the suggestion that LPL activity is responsible for the pH drop of less severely-treated samples.

Total bacterial count of milk

Microbial counts for raw, CPH and HPH milk samples are given in Table 2. Psychrotrophic bacteria (TBC at 5 °C) were not detectable in CPH milk nor in milk HPH at 200 or 250 MPa and were reduced by 2·73 log cycles in milk HPH at 150 MPa, relative to raw milk. HPH also reduced the total mesophilic bacterial population. All HPH samples had a lower mesophilic count than CPH milks. This reduction in mesophilic bacterial numbers was pressure-dependent, with lower numbers of bacteria surviving as homogenization pressure was increased. Thus, HPH had a greater lethal effect on psychrotrophic than on mesophilic microorganisms in milk. The raw milk contained low numbers of *Staphylococcus aureus* (Gram-positive) and coliforms (Gram-negative) but these were absent from all HPH milk samples; the raw milk did not contain *B. cereus*.

Table 2. Effects of high pressure homogenisation on some microbial populations in raw milk. Results shown are means of data from 3 independent experiments

	Microbial counts (log cfu/ml)			
Treatment	TBC† 5 °C	TBC 30 °C	Staphylococcus aureus	Coliforms
Raw milk	4·48	4·59	1·12	2·71
150 MPa	1·75	3·29	ND	ND
200 MPa	ND‡	2·76	ND	ND
250 MPa	ND	1·53	ND	ND
CPH milk§	ND	3·49	ND	ND

† Total Bacterial Count
‡ Indicates that colonies could not be detected following these treatments
§ Conventionally-pasteurised/homogenised commercial milk

Possible mechanisms for the destruction of bacteria by HPH have been discussed in the literature (e.g., sudden pressure drops, torsion and shear stresses, cavitation shock waves, turbulence, viscous shear and high velocity collisions; Kleining & Middelberg, 1998; Wuytack et al. 2002; Hayes & Kelly, 2003a). The high temperatures reached by milk samples in this study may also have had a destructive effect on the bacterial population. Overall, HPH samples had similar, or lower, microbial counts than CPH samples.

Obviously, the survival of specific individual microorganisms is more significant for full evaluation of the stability and safety of HPH milk than total levels of microbial inactivation; in particular, mesophilic bacteria (which were effectively killed by HPH) have less effect on the keeping quality of refrigerated milk than psychrotrophs. Since psychrotrophic bacterial counts were greatly reduced in samples HPH at 150 MPa and not detectable in milk HPH at higher pressures, it is likely that the storage stability of HPH milk would be at least as good as that of CPH samples.

Colour of homogenised milk samples

Small but significant differences in L*-values were observed between raw, CPH and HPH milk samples (results not shown). For example, CPH milk was significantly whiter ($P<0·05$) than raw milk, as expected; CPH and

https://doi.org/10.1017/S0022029904000524 Published online by Cambridge University Press

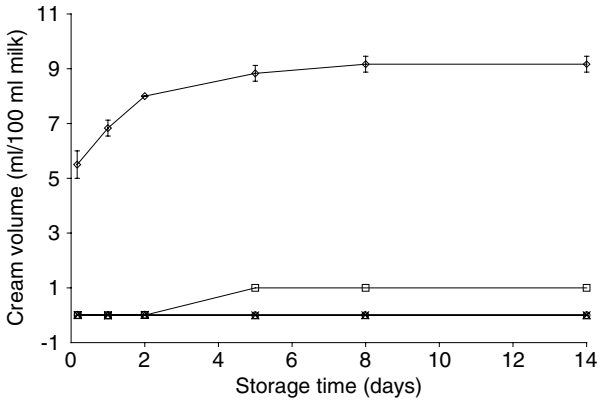

Fig. 2. Effect of storage time (days) at 5 °C on the volume of cream separated during storage of raw (—◇—), commercially pasteurised/homogenised (—□—) or high pressure (150 MPa, —△—; 200 MPa, —×— or 250 MPa, —○—) homogenised milks. Results for all high pressure homogenised samples were essentially identical and therefore the symbols are overlaid. Error bars represent standard deviations of data from triplicate trials.

HPH samples had similar L*-values. Differences in a* and b*-values following treatments were very small. Overall, differences in instrumental colour measurements between CPH and HPH milks were not visually obvious.

Creaming of homogenized milk samples

Rapid creaming occurred initially in raw milk, e.g., the volume of cream was 5·5 ml/100 ml after 4 h refrigerated storage and increased in a curvilinear fashion to 9 ml/100 ml after 5 d; no further increase was observed on continued storage (Fig. 2). A low level (1%, v/v) of creaming was evident in CPH samples after 5 d storage, but the volume of cream remained constant during the remainder of the 14 d storage period. No creaming was evident in any of the HPH samples.

Four factors reduce creaming in homogenized milk; inhibition of 'cold agglutination', decreased fat globule diameter, decrease size distribution of fat globules and increased density of the globules (bringing them closer to that of the continuous phase) owing to adsorption of protein to the fat/serum interface. For these reasons, creaming was not expected in HPH samples. The larger fat globules observed in CPH milk compared with HPH samples may have caused the limited creaming observed in the former.

Effects of HPH on milk enzymes

The activity of plasmin, alkaline phosphatase and lactoperoxidase in samples of raw, CPH and HPH (150–250 MPa; inlet temperature ~45 °C) milk are shown in Table 3. Plasmin activity in CPH milk was 30% of that in raw samples. Loss of plasmin activity in milk on heating at 75 °C for 15 s (pasteurization) has been reported by Richardson (1983) and Koryacka-Dahl et al. (1983) to be ~17%. Conventional homogenization also reduces plasmin activity in raw milk (Hayes & Kelly, 2003b). Thus, both homogenization and pasteurization contribute to the inactivation of plasmin in CPH milk. Approximately 85 and 95% of the plasmin activity in raw milk was inactivated by HPH at 150 or 250 MPa, respectively. Hayes & Kelly (2003b) observed inactivation of plasmin by HPH of milk at lower outlet temperatures (maximum of 54 °C), with the highest degree of inactivation being 65%. Metwalli et al. (1998) reported that 65 °C was the minimum temperature required for thermal inactivation of plasmin, which suggests that shear or other forces may have been involved in inactivation of plasmin by HPH at lower temperatures. In the present study, outlet temperatures were much higher than those in the study of Hayes & Kelly (2003b) and therefore the greater degree of inactivation of plasmin observed may have been due to thermal denaturation combined with the other forces to which the enzyme was subjected. Milk treated by HPH at a high inlet temperature would have very little indigenous plasmin activity, which may have implications for physicochemical changes such as age gelation of UHT milk (Enright et al. 1999).

Lactoperoxidase activity was significantly (90%) lower in CPH milk than in raw milk (Fig. 3). Milk samples HPH at 150, 200 or 250 MPa retained 91, 34 or 0%, respectively, of the lactoperoxidase activity of raw milk. Kameni et al. (2002) observed partial inactivation of lactoperoxidase on heating of milk at 74–77 °C for 1 min and complete inactivation at >78 °C for 1 min; complete inactivation of lactoperoxidase in milk occurs on heating at 70 °C for 50 min, 72 °C for 1·5 min or 80 °C for 1 s (Davis, 1963). Villamiel et al. (1997) reported 42% inactivation of lactoperoxidase in HTST-pasteurised milk. Farkye & Imafidon (1995) concluded that lactoperoxidase is very sensitive to temperatures around 80 °C. The outlet temperature of milk HPH at 200 or 250 MPa was

Table 3. Effects of high pressure homogenisation on indigenous plasmin, alkaline phosphatase and lactoperoxidase activities in raw milk

Results shown are means of data from 3 independent experiments

Treatment	Plasmin (AMC units/ml)	Alkaline phosphatase (AU)	Lactoperoxidase (AU/min)
Raw milk	$0{\cdot}188 \pm 0{\cdot}026^{a}$	$1{\cdot}57 \pm 0{\cdot}05^{a}$	$0{\cdot}249 \pm 0{\cdot}024^{a}$
150 MPa	$0{\cdot}028 \pm 0{\cdot}010^{bc}$	$0{\cdot}458 \pm 0{\cdot}058^{b}$	$0{\cdot}227 \pm 0{\cdot}010^{a}$
200 MPa	$0{\cdot}018 \pm 0{\cdot}005^{b}$	$0{\cdot}032 \pm 0{\cdot}065^{c}$	$0{\cdot}085 \pm 0{\cdot}015^{b}$
250 MPa	$0{\cdot}008 \pm 0{\cdot}003^{b}$	ND†^{c}	ND^{c}
CPH milk‡	$0{\cdot}068 \pm 0{\cdot}027^{c}$	ND^{c}	$0{\cdot}028 \pm 0{\cdot}014^{c}$
P-value	<0·001	<0·001	<0·001

Values with common superscript were not significantly different, $P<0{\cdot}05$

† Indicates that enzyme activity could not be detected following these treatments

‡ Conventionally-pasteurised/homogenised commercial milk

https://doi.org/10.1017/S0022029904000524 Published online by Cambridge University Press

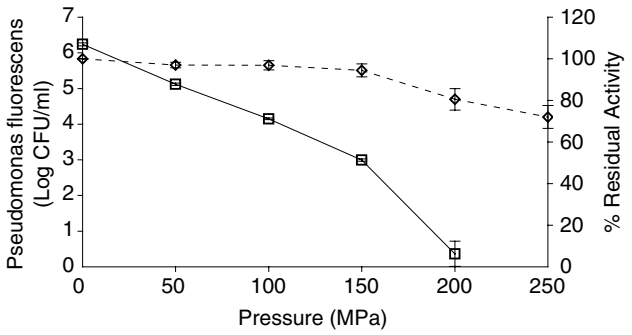

Fig. 3. Survival (log cfu/ml) of *Pseudomonas fluorescens* AFT 36 (full line) in raw milk subjected to high pressure (150–200 MPa) homogenisation. The broken line represents residual protease activity in a cell-free supernatant of a culture of this micro-organism, diluted in raw milk and high pressure (50–250 MPa) homogenised. Error bars represent standard deviations of data from triplicate trials.

~77 and 84 °C, respectively. With a residence time of ~20 s, inactivation of lactoperoxidase due to HPH may therefore be largely a result of heating of the milk.

Using a standard rapid test (Fluorophos; International Dairy Federation, 1992), CPH milk and milk HPH at 200 or 250 MPa were phosphatase-negative, as expected from the temperature reached during processing, while samples treated at 150 MPa were phosphatase-positive (results not shown). Using a quantitative assay, samples HPH at 150, 200 or 250 MPa retained 29, 2 or 0%, respectively, of the alkaline phosphatase (ALP) activity of raw milk (Fig. 3); CPH samples had no measurable ALP activity. The time/temperature conditions encountered during HPH of milk at 200 or 250 MPa (i.e., heating to 77 or 84 °C, respectively, for 20 sec) are clearly in excess of those practised during conventional HTST pasteurization of milk. This fact was confirmed by the negative ALP results (and low assayed activities) for these samples. The contribution of factors encountered during HPH other than heating (i.e., shear forces) to inactivation of ALP is not clear; however, the thermal load applied alone would account for the inactivation measured.

This may then suggest that HPH of milk at high inlet temperatures may destroy pathogenic bacteria that are inactivated by HTST, or by heating to 76·8 or 83·6 °C for 20 sec (e.g. *Coxiella burnetti, Mycobacterium tuberculosis* and *Listeria monocytogenes*), if present in raw milk, and yield a product safe for consumption, if refrigerated. However, due to the profound public health implications of the introduction of any new processing treatment for liquid milk, much research will be required to evaluate this possibility.

Effect of HPH on Ps. fluorescens *and its proteinase*

The lethal effect of HPH on *Ps. fluorescens* was pressure-dependant, with the number of viable bacteria decreasing as homogenization pressure was increased (Fig. 3). HPH at 50 or 200 MPa reduced the population of *Ps. fluorescens* by ~1 or 6 log cycles, respectively; at intermediate pressures, a reduction in viable cell count between these two extremes was observed. Outlet temperatures in this trial for milk HPH at 50, 100, 150 or 200 MPa were ~29, 38, 47 and 56 °C, respectively. Since the sample volume collected was very small (~5 ml) and this was cooled immediately in ice water, the residence times for each sample at the relevant temperatures was estimated to be ~1 min. In separate experiments, these time/temperature combinations, e.g., 29, 38, 47 or 56 °C for 1 min, did not significantly reduce (<1 log cycle) bacterial numbers (results not shown). Thus, reductions in viable bacterial numbers observed in this study were probably due to HPH-induced shear forces or a combination of these forces and heating effects. Elaamadi et al. (1996), who subjected *Ps. fluorescens* ATCC 948 in reconstituted 10% (w/v) sterilised skim milk powder to microfluidization at pressures between 50 and 120 MPa, observed a similar lethal effect.

Wuytack et al. (2002) concluded from studies of HPH (100–300 MPa) inactivation of 11 species of bacteria that Gram-positive bacteria were significantly ($P<0·05$) more resistant than Gram-negative species, possibly due to differences in cell wall structure. However, Gram-negative bacteria vary in their resistance to HPH (Wuytack et al. 2002), indicating that all micro-organisms present in raw milk may not be as susceptible to HPH as *Ps. fluorescens*.

The effect of HPH on the activity of the extracellular proteinase of *Ps. fluorescens* is also shown in Fig. 3. Homogenization at 50–150 MPa had no effect on this proteinase but 20 or 30% inactivation was observed at 200 or 250 MPa, respectively. Elaamadi et al. (1996) reported that *Ps. fluorescens* proteinase fully survived 10 successive microfluidization treatments at 100 MPa. Since the proteinase of *Ps. fluorescens* is very heat stable (Stepaniak & Sorhaug, 1995), the inactivation observed in this study may be due to the many stresses experienced by the proteinase during HPH.

Conclusion and requirements for further study

Ps. fluorescens, *Staphylococcus* and coliforms in milk were extremely vulnerable to HPH; other bacteria in milk may also be susceptible to HPH, as demonstrated by the reductions in TBC. The results obtained suggest that it might be expected that certain HPH treatments may also have a destructive effect on other pathogenic microorganisms present in raw milk. Further studies are required to confirm this, and to determine the effects of HPH on spore-forming bacteria in milk, such as *Bacillus* and *Clostridium*. The effects of HPH on indigenous milk enzymes varied considerably; the proteinase of *Ps. Fluorescens* was quite resistant to inactivation.

In conclusion, HPH could be used as part of a hurdle system to destroy bacteria in milk, minimizing the intensity of heating required and reducing adverse effects of heat

https://doi.org/10.1017/S0022029904000524 Published online by Cambridge University Press

on food properties or constituents. HPH may also have potential as a combined pasteurization/homogenization step in the industrial treatment of beverage milk. Milk treated thus may have a shelf-life similar to, or longer than, conventional market milk since it produces milk stable to creaming and with a bacterial load similar to, or lower than, that of CPH milk samples.

At present, the most common way to evaluate the effect of heat treatment on milk is to select a chemical, biochemical or enzymatic indicator sensitive to a given time/ temperature treatment. However, no such indicator for HPH is obvious. Obviously, HTST-like conditions are achievable by HPH; however, due to the additional contribution of shear forces, it may be possible to produce safe milk by HPH involving heating conditions less than those (e.g., 72–74 °C for 15–30 sec). HPH is a continuous process, and may facilitate heat regeneration to reduce energy consumption by preheating incoming cold milk; however, capital and maintenance costs, as well as product quality and safety, will ultimately determine industrial potential of HPH.

Further investigation is also required to evaluate the effects of HPH on the sensory and nutritional aspects of milk and to determine the minimum pressure required to produce milk safe for human consumption. Also, additional information is required on the shelf-life of HPH milk.

This research was funded by the Irish Government under the National Development Plan 2000–2006. The authors would like to acknowledge Mr Dan Walsh, Department of Microbiology, University College, Cork, for his assistance with bacterial cultures.

References

Adapa S, Schmidt KA & Toledo R 1997 Functional properties of skim milk processed with continuous high pressure throttling. *Journal of Dairy Science* **80** 1941–1948

Cano-Ruiz ME & Richter RL 1997 Effect of homogenisation pressure on the milk fat globule membrane proteins. *Journal of Dairy Science* **80** 2732–2739

Chandonnet S, Korstvedt H & Siciliano AA 1985 Preparation of microemulsion by microfluidisation. *Soap Cosmetics Chemical Specialities* **(61)** 37–38

Claeys WL, Ludikhuyze LR, Van Loey AM & Hendrickz ME 2001 Inactivation kinetics of alkaline phosphatase and denaturation kinetics of β-lactoglobulin in raw milk under isothermal and dynamic temperature conditions. *Journal of Dairy Research* **68** 95–107

Dalgleish DG, Tosh SM & West S 1996 Beyond homogenisation: the formation of very small emulsion droplets during the processing of milk by a microfluidizer. *Netherlands Milk and Dairy Journal* **50** 135–148

Davis JG 1963 A Dictionary for Dairying, 2nd Edn. London: Leonard Hill Ltd

Desrumaux A & Marcand J 2002 Formation of sunflower oil emulsions stabilised by whey proteins with high pressure homogenisation (up to 350 MPa): effect of pressure on emulsion characteristics. *International Journal of Food Science and Technology* **37** 263–269

DeWit JN, Klarenbeek G & Hontelez-Backx E 1983 Evaluation of functional properties of whey protein concentrates and whey protein isolates. 1. Isolation and characterisation. *Netherlands Milk and Dairy Journal* **37** 37–49

Elaamadi L, Turcotte G & Goulet J 1996 High pressure homogenisation of skim milk containing *Pseudomonas fluorescens*. In *IDF Symposium, Heat Treatments and Alternative Methods*, pp. 214–221. Vienna, September 1995

Enright E, Bland AP, Needs EC & Kelly AL 1999 Proteolysis and physicochemical changes in milk on storage as affected by UHT treatment, plasmin activity and KIO_3 addition. *International Dairy Journal* **9** 581–591

Farkye NY & Imafidon GI 1995 Thermal denaturation of indigenous milk enzymes. In *Heat Induced Changes in Milk*, pp. 331–348 (Ed. PF Fox). Brussels: International Dairy Federation

Floury J, Desrumaux A, Axelos MAV & Legrand J 2002 Degradation of methylcellulose during ultra-high pressure homogenisation. *Food Hydrocolloids* **16** 47–53

Guerzoni ME, Suzzi G, Lanciotti R, Vannini L & Chaves Lopez C 1998 Effects of high pressure homogenisation on biochemical and texture characteristics of yoghurt. In *Proceedings of the IDF Symposium, Texture of Fermented Milk Products and Dairy Desserts*, Vicenza, Italy, 5–6 May 1997, pp. 172–180. Belgium: International Dairy Federation

Guerzoni ME, Vannini L, Chaves Lopez C, Lanciotti R, Suzzi G & Gianotti A 1999 Effects of high pressure homogenisation on microbial and chemico-physical characteristics of goat cheese. *Journal of Dairy Science* **82** 851–862

Hayes MG & Kelly AL 2003a High pressure homogenisation of raw whole bovine milk (a) Effects on fat globule size and other properties. *Journal of Dairy Research* **70** 297–305

Hayes MG & Kelly AL 2003b High pressure homogenisation of milk (b) Effects on indigenous enzymatic activity. *Journal of Dairy Research* **70** 307–313

Hernández MCM, Van Markwijk BW & Vreeman HJ 1990 Isolation and properties of lactoperoxidase from bovine milk. *Netherlands Milk Dairy Journal* **44** 213–231

International Dairy Federation 1992 *Milk and milk-based drinks. Determination of alkaline phosphatase activity using a fluorometric method*. Brussels: IDF (*FIL–IDF Standard* no. 155)

Kameni A, Imele H & Mbanya NJ 2002 An alternative heat treatment for milk pasteurisation in Cameroon. *International Journal of Food Technology* **55** 40–43

Kheadr EE, Vachon JF, Paquin P & Fliss I 2002 Effect of dynamic high pressure on microbiological, rheological and microstructural quality of Cheddar cheese. *International Dairy Journal* **12** 435–446

Kielczewska K, Haponiuk E & Krzyżewska A 2000 Effect of high pressure homogenisation on some physicochemical properties of milk. *Medical Faculty Landbouww University of Gent* **65/3b** 603–606

Kleining AR & Middelberg APJ 1998 On the mechanism of microbial cell disruption in high-pressure homogenisation. *Chemical Engineering Science* **53** 891–898

Koryacka-Dahl M, Ribadeau Dumas B, Chene N & Martal J 1983 Plasmin activity in milk. *Journal of Dairy Science* **66** 704–711

Krause KP & Müller RH 2001 Production of aqueous shellac dispersions by high pressure homogenization. *International Journal of Pharmaceutics* **223** 89–92

Liedtke S, Wissing S, Müller RH & Mäder K 2000 Influence of high pressure homogenization equipment on nanodispersions characteristics. *International Journal of Pharmaceutics* **196** 183–185

Ludikhuyze L, Claeys W & Hendrickx M 2000 Combined pressure-temperature inactivation of alkaline phosphatase in bovine milk: a kinetic study. *Journal of Food Science* **65** 155–160

Metwalli AAM, De Jongh HHJ & Van Boekel MAJS 1998 Heat inactivation of bovine plasmin. *International Dairy Journal* **8** 47–56

Moroni O, Jean J, Autrent J & Fliss I 2002 Inactivation of lactococcal bacteriophages in liquid media using dynamic high pressure. *International Dairy Journal* **12** 907–913

O'Driscoll BM, Rattray FP, McSweeney PLH & Kelly AL 1999 Protease activities in raw milk determined using a synthetic heptapeptide substrate. *Journal of Food Science* **64** 606–611

Oldfield DJ, Singh H, Taylor MW & Pearce KN 2000 Heat-induced interactions of β-lactoglobulin and α-lactalbumin with the casein micelle in pH-adjusted skim milk. *International Dairy Journal* **10** 509–518

Richardson BC & Pearce KN 1981 The determination of plasmin in dairy products. *New Zealand Journal of Dairy Science and Technology* **16** 209–220

Richardson BC 1983 The proteinases of bovine milk and the effect of pasteurisation on their activity. *New Zealand Journal of Dairy Science and Technology* **18** 233–245

Ruegg M, Moor V & Blanc B 1977 A calorimetric study of the thermal denaturation of whey proteins in simulated milk ultrafiltrate. *Journal of Dairy Research* **44** 509–520

Shulka TP 1973 Chemistry and biological function of α-lactalbumin. *CRC Critical. Reviews in Food Technology* **3** 241–312

Stepaniak L & Sorhaug T 1995 Thermal denaturation of bacterial enzymes in milk. In *Heat induced changes in milk*, pp. 349–363 (Ed. PF Fox). Brussels: International Dairy Federation

Twining SS 1984 Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Analytical Biochemistry* **143** 30–34

Villamiel M, López-Fandiño R, Corzo N & Olano A 1997 Denaturation of β-lactoglobulin and native enzymes in the plate heat exchanger and holding tube section during continuous flow pasteurisation of milk. *Food Chemistry* **58** 49–52

Walstra P, Geurts TJ, Noomen A, Jellema A & Van Boekel MAJS 1999 Milk components. In *Dairy Technology. Principle of Milk. Properties and Proteinases*, pp. 471–483. New York: Marcel Dekker, Inc

Wuytack EY, Diels AMJ & Michiels CW 2002 Bacterial inactivation by high-pressure homogenisation and high hydrostatic pressure. *International Journal of Food Microbiology* **77** 205–212

https://doi.org/10.1017/S0022029904000524 Published online by Cambridge University Press