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ORIGINAL RESEARCH

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Instant infusion pasteurisation of bovine milk. II. Effects on indigenous milk enzymes activity and whey protein denaturation

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Direct heat treatment of two milk types, skimmed and nonstandardised full-fat, was performed by instant steam infusion and compared with indirect heating. Infusion conditions were temperatures of 72–120°C combined with holding times of 100–700 ms, and indirect heat conditions were 72°C/15 s and 85°C/30 s. The activity of indigenous enzymes such as alkaline phosphatase, lactoperoxidase, xanthine oxidase and γ-glutamyl transpeptidase was evaluated. Infusion temperature was the main determinant of inactivation. Whey protein denaturation represented by β-lactoglobulin increased significantly with infusion temperature. The nonstandardised milk had a higher denaturation rate than skimmed milk. The effect of instant infusion on pH and milk fat globule size in relation to whey protein denaturation and association is discussed.

Keywords Instant infusion pasteurisation, Milk, Enzymatic activity, Whey protein denaturation.

INTRODUCTION

Drinking milk is processed by various methods to ensure the microbial safety of the product and to extend the shelf life compared with raw milk. Traditionally, this has been achieved by thermal pasteurisation using plate or tubular heat exchangers, i.e. through indirect heat transfer. Indirect heating may, however, affect the quality of the milk negatively, e.g. through protein denaturation and generation of off-flavours. To reduce such quality problems and still fulfil the legislative requirements of safety and shelf life of milk, alternative technologies such as membrane filtration (Punidadas and Rizvi 1998), high-pressure treatment (Adams *et al.* 2006; Rademacher and Hinrichs 2006), direct steam injection (Blake *et al.* 1995; Silveira and Abreu 2003), and steam infusion (deJong *et al.* 1994, 1996; Kiesner *et al.* 1996) have been developed and investigated in the last 15–20 years.

The use of direct heating by either steam infusion or injection has been suggested to provide a more gentle heat treatment in terms of reduced whey protein denaturation and improved sensory properties compared to ultra high temperature (UHT) and extended shelf life treatment as well as high-temperature short-time (HTST) pasteurisation (Fredsted *et al.* 1996; Marconi *et al.* 2004; Rysstad and Kolstad 2006). Previous studies have, however, been based on few or undefined heat treatments or only compared to UHT treatments. Furthermore, the effect of instant infusion treatment on the activity of the indigenous enzymes used to evaluate if sufficient heating has been applied to ensure microbiological safety of milk has not previously been investigated.

For almost 75 years, the inactivation of alkaline phosphatase (AP) in milk has been used as an indicator of proper pasteurisation (Kay and Graham 1935). It is inactivated by HTST heat treatment of at least 71.7°C for 15 s (Fox and Kelly 2006; Rysstad and Kolstad 2006). Inactivation of AP indicates that pathogens able to proliferate in milk have been killed during heating. The present standard within the EU sets an AP activity limit of 500 mU/L based on a fluorometric method (Harding and Garry 2005; CEN-European Committee for Standardisation 2006). Other indigenous milk enzymes are useful as time-temperature treatment indicators e.g. lactoperoxidase (LPO), which is used for distinguishing between HTST and high pasteurisation (> 20 s at 85°C) (Walstra *et al.* 1999). LPO is heat inactivated at 73°C for 10 min (Walstra *et al.* 1999) or 80°C for 20 s (Blel *et al.* 2002). The milk enzymes xanthine oxidase (XO) and γ-glutamyl transpeptidase (γ-GTP) are also heat labile (Cerbulis and Farrell 1977; Zehetner *et al.* 1995; Blel *et al.* 2002) and may be valuable indicators of the thermal impact caused by instant steam infusion and steam injection of milk.

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The present work has investigated the hypothesis that instant infusion pasteurisation (IIP) may provide a more gentle heat treatment compared to indirect pasteurisation with respect to maintaining indigenous milk enzymes activities and reduce the degree of whey protein denaturation. The effect of IIP was studied using various combinations of holding times and temperatures evaluated by determination of pH, dry matter (DM), whey protein denaturation and indigenous enzymatic activities of AP, LPO, XO and γ-GTP in comparison with two conventional indirect pasteurisation regimes.

MATERIALS AND METHODS

Heat treatment and milk sampling

Four experiments were performed with IIP of skim milk (SM) and full-fat nonstandardised milk (NSTM), respectively, as described previously (Hougaard *et al.* 2009), and illustrated in Table 1. Briefly, the experiments were all conducted on a pilot instant infusion equipment (SPX APV, Silkeborg, Denmark) for pasteurisation at a flow rate of 500 L/h. Three holding times were examined: 100, 200 and ∼700 ms, which expanded the capacity of the pump at the holding cell. Furthermore, treatment temperatures from 72 to 120°C were applied. For reference treatments, a plate heat exchange pilot equipment was used for HTST pasteurisation at 72°C/15 s or for HTLT (high-temperature long-time) pasteurisation at 85°C/30 s. Also, samples of the raw milks were obtained. The milk batches used for each experiment were kept at 5 ± 1°C until heat treatment the same day.

For the experiments (1, 3 and 4a) with SM, samples were stored at 4°C overnight. Hereafter, aliquots of 1–15 mL were prepared and stored at −20°C until analysis. For the experiments (2 and 4b) with NSTM, samples were stored at 4°C overnight and skimmed the following day. A sample of 160 mL was centrifuged for 20 min at 1300 × *g* at 5°C, the cream layer was removed, the sample was centrifuged again for 10 min at 1300 × *g* at 5°C, the remaining cream layer was removed and skim milk fraction (SMF) aliquots of 1–15 mL were stored at −20°C until analysis.

Dry matter and pH

Dry matter content (%) was determined on ∼2 g sample that was heated to 198°C until equilibration for ∼10 min in a HR73 halogen moisture analyzer (Mettler Toledo, Schwerzenbach, Switzerland). The pH of milk samples was measured after equilibrating to room temperature (r.t.) using a MeterLab^{TM} PHM220 pHMeter equipped with a PHC2085-8 electrode (Radiometer Analytical, Copenhagen, Denmark). Both DM and pH were analysed in duplicate.

Activities of indigenous milk enzymes

Alkaline phosphatase (EC 3.1.3.1) activity was analysed by using *p*-nitrophenyl-phosphate (*p*-NPP) as substrate and measuring the amount of hydrolysed *p*-nitrophenol (*p*-NP) spectrophotometrically by UV absorbance units (AU) at 405 nm as described previously (Dinnella *et al.* 2004; Fadiloglu *et al.* 2004). In brief, 100 μL *p*-NPP was mixed with 980 μL 0.1 M diethanolamine buffer, pH 9.8, and measured as blank. Milk samples of 20 μL SM or SMF were added, mixed, and the $AU_{405\ nm}$ was followed for 30 min. The AP activity was calculated on basis of the slope of dAU/dt in the linear region. One AP activity unit (U) is expressed as the amount of enzyme, which produces 1 μmol/min of *p*-NP, by using p-NPs molar extinction coefficient ε = 18 500/M/cm at 405 nm.

The activity of LPO (EC 1.11.1.7) was analysed using the previously described method with 2,2′azino-bis-(3-ethyl-benxothiazoline-6-sulphonate) (ABTS) as co-substrate with H_2O_2 (Hernandez *et al.* 1990; Marks *et al.* 2001; Trujillo *et al.* 2007). The LPO catalyses the oxidation of ABTS in the presence of H_2O_2 forming $ABTS^+$, which is a greenish coloured soluble compound with UV absorption at 412 nm. Freshly prepared 1 mM ABTS (used as diammonium salt) solution in 10 mM phosphate buffer pH 6.8, was heated to 35°C in a water bath. Fifty microlitre of 10 mM H_2O_2 prepared in 10 mM phosphate buffer, pH 6.8, was subsequently added in excess with respect to the enzyme to 1150 μL ABTS. Hereafter, 25 μL 20-fold diluted SM or SMF samples in 10 mM phosphate buffer pH 6.8 was added. The sample was thoroughly mixed and UV absorbance readings were made at 412 nm for 10 min at 35°C. The 10 mM phosphate buffer was used as blank. One enzyme activity unit (U) is defined as the amount of enzyme that catalyses the oxidation of 1 μmol/min of ABTS (Hernandez *et al.* 1990). The oxidation rate was calculated on the basis of the dAU/dt in the linear region. The U was calculated using the molar extinction coefficient ε = 32 400/M/cm of the oxidised ABTS at 412 nm.

γ-glutamyl transpeptidase (EC 2.3.2.2) activity in milk samples was analysed by a previously described method (McKellar and Emmons 1991; Blel *et al.* 2002; Pandey and Ramaswamy 2004). The principle is based on the enzyme transferring the glutamyl group from L-γ-glutamyl-*p*-nitroanilid to a receptor molecule; glycylglycin. The released *p*-nitroaniline reacts with nitrite forming a diazonium ion, which couples with naphthylethylenediamine to form an azo-compound that can be quantified by UV $AU_{540\ nm}$. A standard curve was generated in the range of 0.04–0.15 mM *p*-nitroaniline, where one γ-GTP activity unit (U)

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Table 1 Overview of the four performed experiments with different milk types (SM, skim milk; NSTM, nonstandardised milk), holding time and temperature combinations in instant infusion pasteurisation (IIP) together with reference treatment conditions in a plate heat exchanger

Exp. no.	*Milk type*	*Holding time (ms)*	*IIP temperature (°C)*							*Reference treatment (°C/s)*
			72	*80*	*85*	*90*	*100*	*110*	*120*	
1	SM	100				x	x	x	x	72/15
		200			x	x	x	x	x	
		700			x	x	x	x	x	
2	NSTM	100		x		x	x	x	x	72/15
		700		x		x	x	x	x	
3	SM	200	x	x			x		x	72/15
4a	SM	200	x	x		x	x	x	x	72/15
										85/30
4b	NSTM	200	x	x		x	x	x	x	72/15
										85/30

is defined as the amount of enzyme that releases 1 μmol *p*-nitroaniline/min at 37°C. SM or SMF was diluted in 0.1 M Tris–HCl, pH 9.0, 10-fold for IIP and HTLT-treated samples and 50-fold for raw and HTST samples. Ten microlitre of diluted sample was added to 250 μL substrate and incubated at 37°C for 20 min. The reaction was stopped by adding 1 mL 10% acetic acid. Hereafter, 500 μL 0.1% $NaNO_2$ was added, mixed and incubated at r.t. for 3 min, 500 μL 1% ammonium sulfamate was added, mixed and incubated at r.t. for 3 min, and 500 μL 0.052% (w/v) naphthylethylene diamine was added, mixed and incubated at r.t. for 3 min. The UV $AU_{540\ nm}$ was measured immediately using 0.1 M Tris–HCl as blank.

Xanthine oxidase (EC 1.17.3.2) activity of the SM and SMF was analysed as the rate of urate formed from xanthin catalysed by xanthin oxidase as previously described (Cerbulis and Farrell 1977; Steffensen *et al.* 2004). Samples of 50 μL were mixed with 450 μL 50 mM phosphate buffer, pH 7.4, and 250 μL xanthine (0.02 g/L) was added. The mixture was incubated at 25°C for 5 min and the reaction was stopped by adding 500 μL 20% trichloroacetid acid (TCA). The samples were centrifuged at 2000 × *g* for 5 min and the amount of urate in the supernatant was quantified by UV $AU_{285\ nm}$. The activity was expressed in enzymatic units U = μmol xanthin oxidised/min using the molar extinction coefficient for urate of ε = 12 200/M/cm. All enzymatic analyses were performed in triplicate.

Gel permeation of whey proteins

Protein denaturation was analysed using size exclusion chromatography by gel permeation FPLC (fast protein liquid chromatography) as described previously (Law *et al.* 1993, 1994; Law and Leaver 2000). SM or SMF samples of 2 mL were acidified to pH 4.6 by adding 2 mL of 0.83 M acetic acid:0.2 M sodium acetate. The acidified samples were stirred for 20 min at r.t. before centrifugation at 1000 × *g* for 10 min at 20°C. The supernatant was filtered through a 0.45-μm cellulose filter and analysed immediately by FPLC in triplicate. Samples of 200 μL were injected to the FPLC unit and gel permeation was carried out on a Superdex 75 10/300 GL column (GE Healthcare, Hillerød, Denmark) with 0.1 M Tris–HCl/0.5 M NaCl buffer pH 7.0 at a flow rate of 0.75 mL/min. The area under curves (AUCs) of β-lactoglobulin were used for evaluation of whey protein denaturation and the relative (%) denaturation degree (DD) was calculated in relation to the AUCs of the raw samples.

Statistical analysis

Data were subjected to statistical analysis by the Generalised Linear Models procedure of SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). For each treatment combination, the variance homogeneity was analysed by the Bartlett's test (Bartlett 1937) and data distribution by the Probit Analysis (Blæsild and Granfeldt 1995) respectively. To obtain normality, the data on AP activity were transformed by the inverse square root function, XO and LPO activities were transformed by the log(10) function, and the γ-GTP activity data was transformed by the square root function. All other data were distributed according to the normality function. The first model (eqn 1) was used to test the hypothesis of no differences between all treatments within each experiment:

$$Y_{ik} = a_i + e_{ik} \quad (1)$$

where a = treatment i (raw, HTST, HTLT, IIP1, IIP2, ..., IIPn), e = replicate k (1,…, n) of each treatment combination.

Second, a two-way model (eqn 2) for testing the hypothesis of no differences between IIP holding times and temperatures was used for data in

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experiment 1 and 2, where holding time was included as experimental variable:

$$Y_{ijk} = a_i + b_j + c_{ij} + e_{ijk} \quad (2)$$

where a = holding time i (ms) (100, 200, 700), b = temperature j (°C) (72, 80, 85, 90, 100, 110, 120), c_{ij} = interactive effects between holding time and temperature, and e = replicate k (1,….n) of each treatment combination. Where no significant interactions between the class variables were found the interactive part was excluded from the models and the reduced model was used for analysis of data.

The LS-means were calculated and differences regarded as significant at minimum 95% level ($P < 0.05$). Differences were classified by the Ryan-Einot-Gabriel-Welsch (REGW) multiple range test (SAS Institute Inc.).

RESULTS AND DISCUSSION

Dry matter and pH

The DM content of the milk samples was markedly affected by the IIP treatments (Table 2). For experiments with SM, the DM of IIP-treated milk decreased on average 0.5% units ranging from 0.2% to 1.1% units compared with the raw milk, and this effect was more pronounced as IIP temperature increased. One treatment in particular, at 120°C for 700 ms (experiment 1) had a very high DM reduction >2.0%. However, for the indirect heat treatment by HTST the DM only decreased ∼0.2% units, ranging from 0.05% to 0.3% units. For the NSTM, the milk DM increased on average 0.4% units ranging from 0% to 0.6% units as function of IIP treatment in experiment 2. In contrast, DM decreased by 1.1% unit ranging from 0.6% to 1.6% units in experiment 4b, indicating that the removal of excess water introduced as steam during the infusion heating was difficult to control in the flash cooling. In contrast to IIP, the indirect heating resulted in a DM decrease of ∼0.4% units for NSTM. Based on these findings, all other data obtained were corrected for the actual DM content of each sample to eliminate the influence of DM differences on treatment effects.

During IIP, the pH of both SM and NSTM increased significantly ($P < 0.01$ and < 0.001, respectively), as illustrated in Figure 1. This was in contrast to HTST and HTLT with indirect heat transfer, where the pH decreased or remained similar to pH of the raw untreated milk (i.e. ∼pH 6.67). In SM, the pH increased linearly as the IIP temperature increased from 72 to 120°C, whereas the pH in NSTM increased mainly from 72 to 90°C, after which a steady state was reached ∼pH 6.75.

It has been known for years, that heating of milk results in a slight decrease in pH (Kaloyereas 1954; Bermudez-Aguirre *et al.* 2009), as also seen for the HTST and HTLT in Figure 1. This is ascribed to the establishment of a new equilibrium between monocalcium phosphate and dicalcium phosphate. Heating affects the buffer capacity of milk, colloidal phosphate increases and concentration of Ca^{++} decreases resulting in a pH decrease (Walstra *et al.* 1999). The change in pH is regarded as reversible with storage i.e. within 24 h (Walstra *et al.* 1999). The pH in milk has been observed to increase as a result of high-pressure induced solubilisation of micellar calcium phosphate (Huppertz *et al.* 2006). The present observation of pH increase in IIP-treated milk could be related to the steam pressure or degassing causing removal of CO_2 in the flash cooling after heating, but so far the underlying mechanism is unclear. Nevertheless, the pH was measured in samples stored > 24 h at 4°C, so the observed changes appear to be irreversible.

Inactivation of alkaline phosphatase

One of the most studied indigenous enzymes in milk is AP due to its heat lability resulting in inactivation. The effect of IIP of SM on the activity of AP is illustrated in Figure 2a with the results of experiments 1 and 4a as examples. A significant difference ($P < 0.001$) between the heat-treated samples and the raw SM was found, but the

Table 2 Milk dry matter content (%) as effect of heat treatment by instant infusion pasteurisation (IIP) and indirect heating (plate heat exchange) (HTST and HTLT) compared with untreated raw milk, $n = 2$

				IIP temperature (°C)							*HTST*	*HTLT*
Exp. no.	*Milk type*	*Holding time (ms)*	*Raw*	*72*	*80*	*85*	*90*	*100*	*110*	*120*	*72°C/15 s*	*85°C/30 s*
1	SM	100	8.85				8.25	8.45	8.31	8.19	8.56	
		200				8.64	8.59	8.57	8.48	8.41		
		700				8.28	8.43	8.24	8.40	6.61		
2	NSTM	100	8.65		8.86		9.06	9.07	9.14	9.17	8.21	
		700			8.65		8.89	8.88	9.23	9.26		
3	SM	200	8.63	8.16	7.89			7.78		7.54	8.45	
4a	SM	200	7.29	7.02	6.50		6.83	6.75	6.63	6.50	7.24	7.10
4b	NSTM	200	9.98	8.72	9.20		8.58	9.33	8.34	9.40	9.55	9.65

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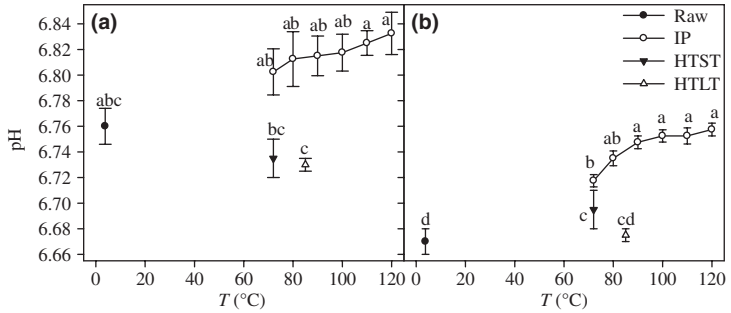

Figure 1 Experiment 4a and 4b. Mean ± SE of milk pH as function of treatment temperature *T* for (a) skim milk with points of different letters a–c being significantly different $P < 0.01$, and (b) nonstandardised milk with points of different letters a–d being significantly different $P < 0.001$. Symbols represent (black circle) Raw milk = unprocessed, (open circle) IIP = instant infusion pasteurisation, (black triangle) HTST = high temperature short time in plate heat exchanger (72°C/15 s) and (open triangle) HTLT = high temperature long time in plate heat exchanger (85°C/30 s), $n = 4$.

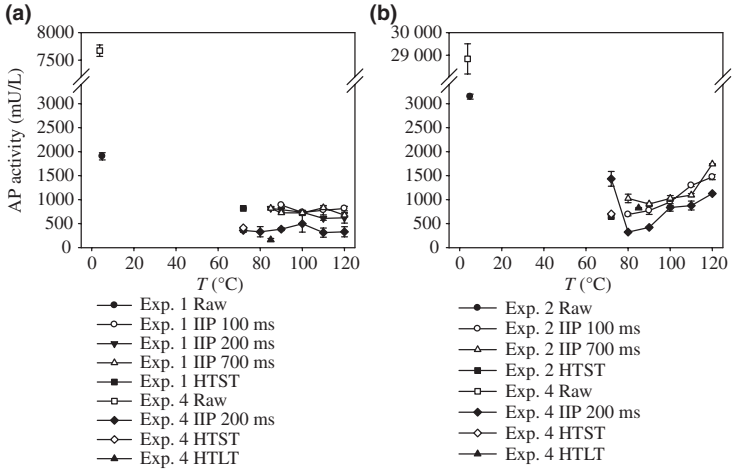

Figure 2 Alkaline phosphatase (AP) activity as function of treatment temperature *T* for (a) skim milk Experiments 1 and 4a, and (b) nonstandardised milk Experiments 2 and 4b, as Raw = unprocessed, IIP = instant infusion pasteurised at the given holding times from 100 to 700 ms, HTST = high temperature short time in plate heat exchange (72°C/15 s) and HTLT = high temperature long time in plate heat exchange (85°C/30 s), $n = 3$.

inactivation of AP was not influenced by the use of indirect (HTST and HTLT) or direct (IIP) heating. An effect of holding time ($P < 0.05$) was found for IIP-treated SM samples (experiment 1), i.e. a relatively lower inactivation at the shortest holding time (100 ms) compared with the longest (700 ms). Additionally, increasing IIP temperature resulted in relatively higher – although numerically small – inactivation of the enzyme ($P < 0.05$) at 120°C compared with 85 and 90°C. The AP activity in the raw NSTM samples (Figure 2b) was in general 2–3 times higher than in the SM (Figure 2a). In corroboration of this, an equal distribution of AP in SM and cream, but a higher activity of the enzyme in the cream, is reported (Fox and Kelly 2006).

As for SM, all heat treatments significantly ($P < 0.001$) reduced the activity of AP in the NSTM compared to the raw milk. In contrast to the effects on SM, there was a difference in AP activity of NSTM (experiment 2), where the longest holding time for IIP (700 ms) resulted in less inactivation than the HTST (Figure 2b). In experiment 4b, however, no significant differences on AP activity were found between the technologies of using indirect (HTST and HTLT) or direct (IIP) heat treatments. The response to IIP temperature was significantly different in experiment 4b where IIP treatment at 72°C resulted in higher AP activity than *T* of 80–90°C. For NSTM there was an unexpected increase in AP activity as temperature increased (Figure 2b). This might indicate that reactivation could occur at the higher IIP temperatures, a phenomena previously reported to occur in UHT (Fox and Kelly 2006) as well as high-pressure treated milk (Rademacher and Hinrichs 2006). Another explanation could be the influence of the smaller milk fat globules (MFG) created using IIP (Hougaard *et al.* 2009), which may have protected the AP against inactivation. The enzyme is bound to the MFG membrane via phosphatidyl inositol and can be released from the phospholipids of the MFG membrane by phospholipase (Bingham and Wand Malin 1992).

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Based on these observations, the hypothesis that reactivation of AP can occur in IIP-treated SM and NSTM was tested on different samples originating from experiments 4a and 4b. The results are shown in Figure 3. Samples were stored cold (5°C) for up to 7 days, as this is a relevant temperature of milk storage, although previous studies have found 30°C, which is a relevant storage temperature for UHT products, to be the optimum temperature for reactivation of AP, which may take place only after 6 h (Fox and Kelly 2006). No samples exhibited reactivation of AP during the time studied (Figure 3) and the activity actually tended to decrease during storage. Furthermore, it was clear that IIP at 72°C was insufficient to achieve proper pasteurisation for NSTM.

Inactivation of lactoperoxidase

Another well-described indigenous milk enzyme is LPO, which has also been used for distinguishing between pasteurisations of HTST and HTLT. The LPO in milk is relatively heat stable, requiring between 5 s and 3.5 min at 80°C for complete inactivation depending on the heating method (Farkye and Imafidon 1995; Blel *et al.* 2002). The observed activity levels of LPO in raw samples of SM and NSTM were within the same range in the raw samples, which confirms other findings (Claeys *et al.* 2002). The overall effect of heat treatment was similar for the two milk types (Figure 4). A significant ($P < 0.001$) reduction in LPO activity was seen at 80°C. However, in experiments 4a and 4b, the relative inactivation of LPO was higher in SM (19.2%) vs NSTM (7.2%) at 72°C IIP, and a similar picture was seen for the indirect heat-treated samples. This may be explained by the fat in NSTM having a protective effect on LPO inactivation (Claeys *et al.* 2002). There were no significant differences between the technologies of heat transfer on LPO inactivation. Between IIP treatments, only at 80°C a holding time effect was found in experiment 2, i.e. shorter holding time (100 ms) resulted in a significant ($P < 0.01$) higher activity than 700 ms.

Inactivation of xanthine oxidase

The activity of XO as function of heat treatment (Figure 5) showed a general background level in all samples corresponding to ∼65–110 U with variations between the milk batches. The analytical method is based on the formation of urate, and milk may have a natural content of ∼2 mg/100 mL urate (Wolfschoon-Pombo and Klostermeyer 1981), explaining the observed background level. Generally, the NSTM had a higher initial level of XO activity than SM, which is expected as most of the XO is associated with the MFGM (Walstra *et al.* 1999). The HTST treatment did not affect XO activity, whereas HTLT significantly reduced it ($P < 0.001$). XO is activated by homogenisation caused by release of XO from the MFGM to the aqueous phase of milk at heating temperatures between 60 and 70°C (Griffiths 1986), whereas pasteurisation hereafter reduces XO activity by > 50% (Cerbulis and Farrell 1977).

For the SM, an IIP temperature of 80–85°C partly inactivated the XO, and at 90°C and higher the XO appeared to be completely inactivated regardless of holding time (Figure 5a).

The NSTM responded differently to the IIP treatments, as there was a significant ($P < 0.01$) holding time effect in experiment 2 (Figure 5b). The 100 ms at 80°C was insufficient to significantly decrease the XO activity, whereas the 700 ms at 80°C and, in experiment 4b, the 200 ms at 80°C significantly ($P < 0.001$) reduced the XO

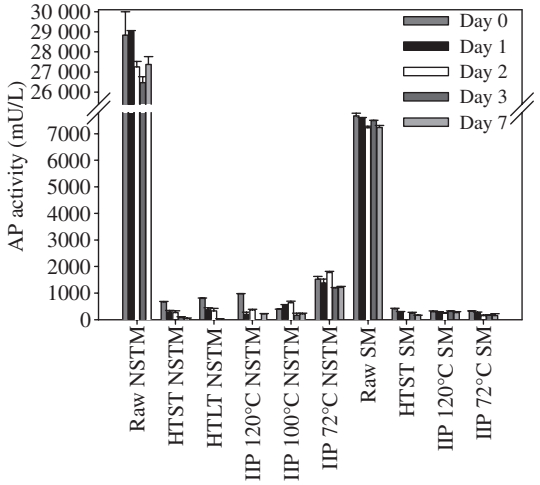

Figure 3 Experiments 4a and 4b. Effect of storage time of 0–7 days at 5°C of nonstandardised (NSTM) milk and skim milk (SM) samples on alkaline phosphatase (AP) activity for selected treatments of Raw = unprocessed, IIP = instant infusion pasteurised, HTST = high temperature short time in plate heat exchange (72°C/15 s) and HTLT = high temperature long time in plate heat exchange (85°C/30 s), $n = 3$.

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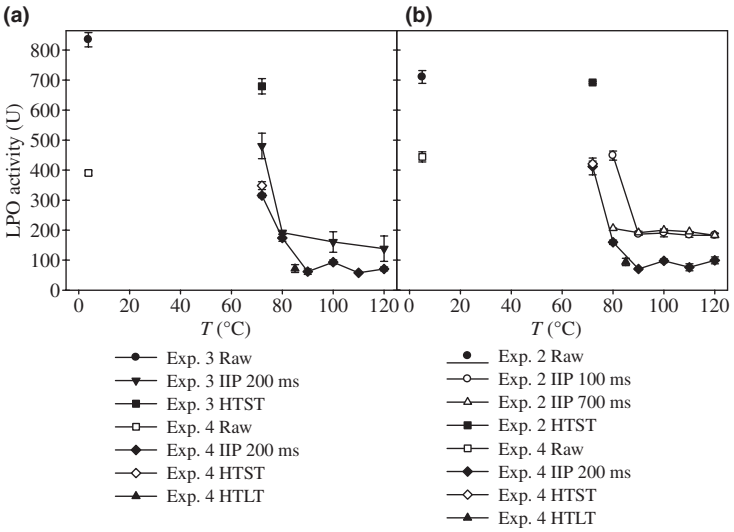

Figure 4 Lactoperoxidase (LPO) activity as function of treatment temperature *T* for (a) skim milk Experiments 3 and 4a and (b) NSTM Experiments 2 and 4b, as Raw = unprocessed, IIP = instant infusion pasteurised at the given holding times from 100 to 700 ms, HTST = high temperature short time in plate heat exchange (72°C/15 s) and HTLT = high temperature long time in plate heat exchange (85°C/30 s), $n = 3$.

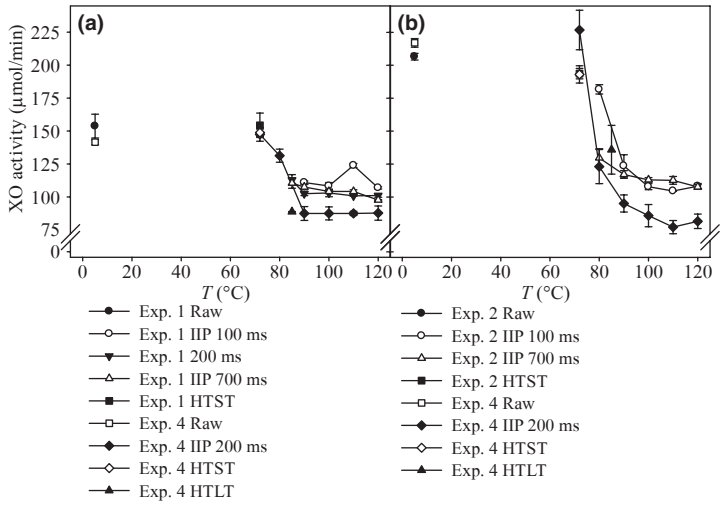

Figure 5 Xanthine oxidase (XO) activity as function of treatment temperature *T* for (a) skim milk Experiments 1 and 4a and (b) nonstandardised milk Experiments 2 and 4b, as Raw = unprocessed, IIP = instant infusion pasteurised at the given holding times from 100 to 700 ms, HTST = high temperature short time in plate heat exchange (72°C/15 s), and HTLT = high temperature long time in plate heat exchange (85°C/30 s), $n = 3$.

activity compared with the raw NSTM. In contrast to SM, the NSTM responded to temperature for both the shortest holding times of 100 ms (experiment 2) and 200 ms (experiment 4b) by a further significant inactivation of XO at increasing temperatures up to 100 and 110°C respectively (Figure 5b).

Inactivation of γ-glutamyltranspeptidase

The results of heat treatments on γ-GTP activity are given with experiments 1–3 as examples in Table 3. The activity of the enzyme in the present study was at comparable levels for raw SM and NSTM samples in contrast to previous findings, where whole milk has higher activity (~4.3 mU/L) than SM (~3.1 mU/L) determined by the same method (McKellar and Emmons 1991). However, others report γ-GTP activity levels of 3.11 U/mL in a pool of whole milk (Zanker *et al.* 2001). The γ-GTP is a membrane bound enzyme located in MFGM, which points to a higher activity in NSTM, although apparently not the case in the present study. Actually, the enzyme appeared to be more sensitive towards heat treatment in the NSTM than in SM with relative mean inactivations by HTST of 54% and 28% respectively. By IIP treatments, some activity remained at 72°C (68%) and at 80°C (23%) dependent on holding time, whereas the γ-GTP was completely inactivated at IIP temperatures of 85°C and higher.

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Table 3 LS-means of γ-glutamyltranspeptidase activity (U/mL) in skim milk (SM) (Experiments 1 and 3) and nonstandardised milk (NSTM) (Experiment 2) samples heat treated by infusion pasteurisation (IIP) or plate heat exchanger (HTST) in comparison with raw untreated milk, $n = 3$

				IIP Temperature (°C)							
Exp. no.	*Milk type*	*Holding time (ms)*	*Raw*	*72*	*80*	*85*	*90*	*100*	*110*	*120*	*HTST 72°C/15s*
1	SM	100					0.05^{c}	0.04^{c}	0.04^{c}	0.04^{c}	2.64^{b}
		200	3.94^{a}			0.07^{c}	0.01^{c}	0.02^{c}	0.01^{c}	0.05^{c}	
		700				0.04^{c}	0.03^{c}	0.04^{c}	0.03^{c}	0.04^{c}	
2	NSTM	100	4.45^{a}		1.01^{c}		0.03^{d}	0.02^{d}	0.01^{d}	0.01^{d}	1.98^{b}
		700			0.02^{d}		0.04^{d}	0.02^{d}	0.01^{d}	0.04^{d}	
3	SM	200	4.30^{a}	2.92^{b}	0.21^{c}			0.01^{c}		0.03^{c}	3.33^{b}

Values within each experiment with different superscript letters are significantly different ($P < 0.001$).

When traditional indirect heating is used, the activity is completely lost after 15 s at 78–80°C (Zehetner *et al.* 1995). Whether the present remaining activity of ~23% after IIP at 80°C for 100 ms is caused solely by the very short holding time, or is a combinatory effect with the direct heating, is unknown. No other reports on inactivation of γ-GTP in infusion-treated milk have been found. The enzyme has been suggested as indicator for thermal treatment of milk (McKellar and Emmons 1991; Zehetner *et al.* 1995; Blel *et al.* 2002), as its thermostability is higher than that of AP but less than that of LPO, but so far determination of γ-GTP activity has not found application in the dairy industry.

Denaturation of whey protein

Figure 6 presents the FPLC chromatograms of the whey fractions obtained for raw SM and selected indirect and direct heating treatments. The peaks of both immunoglobulins, BSA, α-lactalbumin and β-lactoglobulin have previously been used for evaluation of whey protein denaturation by heating (Resmini *et al.* 1989; Law *et al.* 1994; Law and Leaver 2000). The immunoglobulins and BSA are the most heat sensitive, α-lactalbumin is the most heat resistant, and the heat lability of β-lactoglobulin lies inbetween (Resmini *et al.* 1989; Law *et al.* 1994), which also appears from Figure 6a (raw vs HTST) and Figure 6b (raw vs HTLT). We chose to use β-lactoglobulin to evaluate the effect of IIP on whey protein denaturation in relation to the raw unprocessed milk samples (Table 4).

In SM, the applied HTST treatment resulted in a significantly lower DD than the IIP except for experiment 4a, where 200 ms holding time at 72–80°C resulted in a DD of β-lactoglobulin comparable to HTST. The β-lactoglobulin in the NSTM samples generally exhibited higher heat sensitivity than in SM (Table 4, Figure 7).

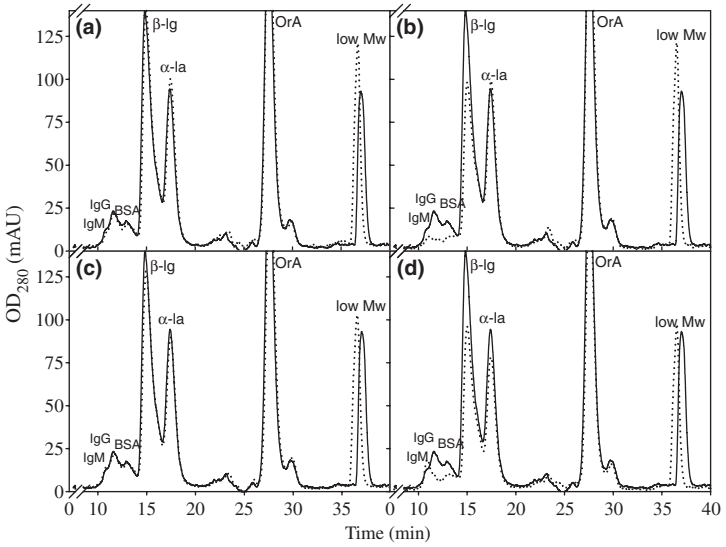

Figure 6 Fast protein liquid chromatography separation of whey proteins in the supernatant sample after acid precipitation on a Superdex 75 HR 10/30 column by gel permeation chromatography in 0.1 M TRIS–HCl/0.5 M NaCl buffer. Raw SM (——) and (a) high-temperature short-time-treated SM (·····), (b) high-temperature long-time-treated SM (·····), (c) instant infusion pasteurised (IIP) treated SM at72°C (·····) and (d) IIP treated SM at 120°C (·····). Ig = immunoglobulin, BSA = bovine serum albumin, β-lg = β-lactoglobulin, α-la = α-lactalbumin, OrA = orotic acid and low Mw = low molecular weight fraction of salts, vitamins, etc.

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Table 4 Denaturation degree (DD, %) of β-lactoglobulin in milk samples heat treated by instant infusion pasteurisation (IIP) or plate heat exchanger (HTST and HTLT) relative to raw untreated milk (=0%), $n = 3$

			Temperature (°C)							*HTST*	*HTLT*
Exp. no.	*Milk type*	*Holding time (ms)*	*72*	*80*	*85*	*90*	*100*	*110*	*120*	*72°C/15 s*	*85°C/30 s*
1	SM	100				3.62^{de}	8.73^{bc}	10.03^{ab}	11.78^{a}	2.08^{e}	
		200			9.20^{cd}	6.66^{de}	12.74^{bc}	15.58^{ab}	21.38^{a}		
		700			5.04^{cd}	6.73^{de}	12.80^{bc}	14.83^{ab}	20.36^{a}		
2	NSTM	100		2.07^{e}		9.23^{d}	23.97^{b}	48.51^{a}	49.52^{a}	3.30^{e}	
		700		13.24^{cd}		13.13^{cd}	14.51^{c}	22.78^{b}	24.43^{b}		
3	SM	200	5.80^{c}	8.49^{c}			15.13^{b}		18.63^{a}	0.11^{d}	
4a	SM	200	8.80^{de}	7.72^{de}		11.89^{cde}	14.63^{bcd}	20.39^{b}	18.96^{bc}	6.08^{e}	29.41^{a}
4b	NSTM	200	10.29^{e}	13.11^{e}		18.49^{d}	24.69^{c}	29.67^{b}	35.40^{a}	9.55^{e}	35.24^{a}

Values within each experiment with different superscript letters are significantly different ($P < 0.001$).

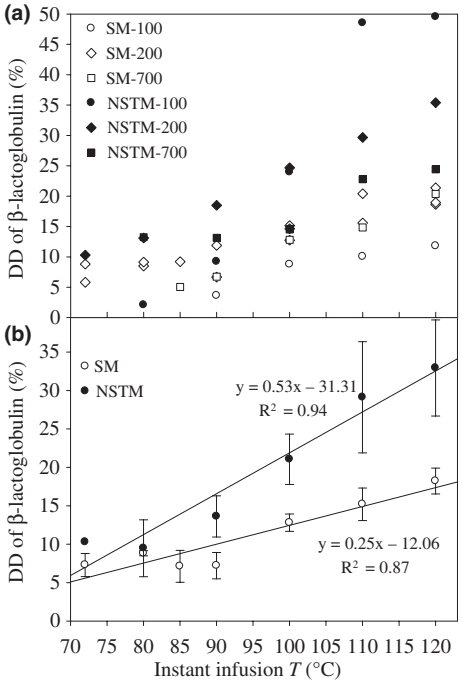

Figure 7 Denaturation degree (DD, %) of β-lactoglobulin in instant infusion treated milk samples as function of infusion temperature T (°C). (a) All data means of Experiments 1–4 of nonstandardised milk (NSTM) closed symbols and skimmed milk (SM) open symbols at holding times of 100, 200 and 700 ms and (b) linear correlations of NSTM and SM, respectively, with each data point representing the mean ± SE of all experimental holding times applied at the respective temperature.

The 100 ms at 80°C in experiment 2 resulted in a DD of β-lactoglobulin that was comparable to the HTST treatment. HTLT heat treatment (85°C for 30 s, used in experiments 4a and 4b) resulted in a degree of thermal damage to the whey proteins (Table 4) of a magnitude that required IIP temperatures of 120°C for NSTM or > 120°C for SM to match.

Increasing pH in the interval pH 6–8 (Paulsson and Visser 1992) or pH 5.2–8.8 (Law and Leaver 2000) has been shown to result in reduced denaturation temperature of β-lactoglobulin and increasing denaturation rates under isothermal conditions. Therefore, the pH increase observed with increasing IIP temperatures (Figure 1) can be expected to contribute to the increased denaturation of β-lactoglobulin.

There were highly significant ($P < 0.001$) effects of IIP temperature and holding time on the β-lactoglobulin denaturation. The shortest holding time of 100 ms (experiment 1) resulted in a significantly lower denaturation of the protein in SM compared with 200 and 700 ms. In contrast to this, the shortest holding time at the highest IIP temperatures of 100–120°C resulted in a significantly higher denaturation in the NSTM (experiment 2).

This effect can possibly be explained by the effect of IIP on the MFGs (Hougaard *et al.* 2009). Heating will induce interactions of β-lactoglobulin and κ-casein with MFGM components (Houlihan

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et al. 1992; Ye *et al.* 2004), either by direct adsorption to the fat or by interaction with the MFGM (Corredig and Dalgleish 1996), which in the present FPLC analysis would be measured as 'denatured'. Conditions of increasing heating time (0–40 min) and temperatures (65–95°C) increase the concentration of β-lactoglobulin associated with the MFGM surface (Ye *et al.* 2004). However, a maximum quantity of ∼1% of the total β-lactoglobulin appear to be able to associate with the MFGM upon heating (Corredig and Dalgleish 1996; Ye *et al.* 2004). The relative quantity of β-lactoglobulin that associate to the MFGM is rather small and cannot in itself explain the high DD levels observed in treatments at elevated IIP temperature, but it may partly explain the observed differences between SM and NSTM.

The DD of β-lactoglobulin correlated linearly with the IIP temperatures studied (Figure 7b). In the range of 72–120°C, the rate of denaturation of β-lactoglobulin was more than twice as high for NSTM as for SM, 0.53%/°C vs 0. 25%/°C. At $T = 68.8$°C the two regression lines intersect, meaning that at lower temperatures the presence of fat during IIP has little influence on β-lactoglobulin denaturation, which support previous results (Claeys *et al.* 2002).

The disulfide bonding occurring between denatured β-lactoglobulin (as well as α-lactalbumin) and κ-casein (Dalgleish 1990; Corredig and Dalgleish 1996; Claeys *et al.* 2002) decreases β-lactoglobulin solubility and induces co-precipitation with caseins at low pH. This is quantitatively more important than the association to the MFG, as the open structure of the micelle provides a large surface available for interaction (Corredig and Dalgleish 1996).

Fredsted *et al.* (1996) report a DD of β-lactoglobulin of 22% when using IIP at 130–145°C for < 1 s, and Rysstad and Kolstad (2006) report 13.6% DD for IIP at 135°C for half a second. Steam injection, another direct heating technology, is reported to cause disruption of the MFG (Corredig and Dalgleish 1996) similar to our previous observations (Hougaard *et al.* 2009). Whey proteins bind to the MFG, which also contains some casein that was probably adsorbed to the smaller fat globules during their formation (Corredig and Dalgleish 1996). Homogenisation is known to cause adsorption of caseins to the MFGM (Lee and Sherbon 2002), which might also occur during the IIP treatment, thereby increasing the possibility for β-lactoglobulin to associate with MFGM via adsorbed caseins. The total amount of whey protein associated to the MFG was ∼3 times higher in milk heated by direct steam injection than by indirect heating (Corredig and Dalgleish 1996). However, the surface load of whey proteins on the MFGs was incomparable between the two heating systems because of changes in MFG size and membrane composition caused by the direct steam injection (Corredig and Dalgleish 1996). At the same time, the interaction between whey proteins and casein micelles was reduced compared with milk treated by indirect heating, but still accounted for the quantitatively most important pathway for denatured β-lactoglobulin association.

Although the steam injection and instant infusion technologies are different technologies, there appear to be similarities with regard to the way they affect milk constituents. We thus hypothesise that the behaviour of β-lactoglobulin in terms of denaturation and association during heating is different for the studied IIP when compared with the HTST and HTLT treatments.

CONCLUSIONS

The DM content of milk subjected to instant IIP decreased significantly and this was taken into consideration when comparisons were made with indirect heating. The pH increased significantly with increasing temperature applied during IIP in contrast to indirect heating. This may contribute to the increase in DD seen for β-lactoglobulin. The whey protein denaturation increased linearly with IIP temperature of 72–120°C, but with higher rates for nonstandardised milk than for skimmed milk. The homogenisation effect of the IIP treatments at increasing temperature appeared to play an important role in association of whey protein to the MFG surface.

All the indigenous milk enzymes studied were inactivated at high IIP temperatures, but their different inactivation profiles can possibly be used in evaluating the impact of different heat processing on milk. Specifically, there appears to be no reactivation of AP as result of IIP.

In conclusion, the IIP technology appears less gentle than the applied HTST (72°C/15 s) but gentler than the HTLT (85°C/30 s) treatment with regard to whey protein denaturation and the studied enzymatic activities.

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