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The Effects of Milk Components on Lactoperoxidase Activity

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Abstract

This study examined the effects of storage conditions (in particular temperature and media composition) and changes in composition of each milk fraction, and of individual components on lactoperoxidase (LP) activity. The enzyme demonstrated a pulsing characteristic especially in samples stored at 5 °C, however, temperature and media composition did not have great effects on the pulsing behaviour of the enzyme. The results also indicated small but statistically significant variations ($P \leq 0.05$) in LP activity between whole milk (control) and acid whey, rennet whey and cream and non-significant variations ($P > 0.05$) between the control and skimmed milk, and between skimmed milk and acid whey. The variations in activities were attributed to the presence or absence of casein. Though the results demonstrated that LP activity is a function of the interactive effects of all milk components, casein and Ca were the most influential milk components on LP activity. However, their net effects relied on the interactive effects of each other and other milk components. The promotive effect of Ca^{2+} on LP activity was shown to be indirect and reliant on pH changes and its influence on LP activity is within certain limits of its concentrations and under the control of casein.

Keywords: Lactoperoxidase, milk components, milk fractions, activity.

Introduction

Lactoperoxidase (LP) is one of the members of the large family of mammalian heme peroxidases that is found in exocrine secretions including milk (Sharma et al. 2013). LP is synthesised in the mammary gland and is a normal constituent of milk (Mullan 2003). The enzyme is one of the components of the lactoperoxidase system (LP-s). Lactoperoxidase system (LP-s) refers to an indigenous antibacterial system present in milk and other body secretions such tears, saliva, gastric juice, etc. The system consists of LP enzyme, and two substrates, SCN^- and H_2O_2. The main role of this enzyme is to oxidize thiocyanate ions (SCN^-) in the presence of hydrogen peroxide (H_2O_2) to products that exhibit antimicrobial activities (Magacz et al. 2019). Its antibacterial activities depend on the production of antibacterial compounds upon oxidation of SCN^- at the expense of H_2O_2 under the catalysis of LP, i.e. $(SCN^- + H_2O_2 \xrightarrow{LP} OSCN^- + H_2O)$. The concentration of LP necessary for the system to be active is only 1-2 µg/ml and is always present in bovine milk in sufficient concentration, 30 mg/l (Siva et al. 1991). In the presence of non-limiting activity of LP, the antimicrobial effects are related to SCN^- and H_2O_2 which occur naturally but in sub

optimal levels (Reiter and Härnulv 1984). Addition of H_2O_2 and SCN^- reactivates the system resulting in the production of antimicrobial agents. Raw milk preservation through activation of the LP-s has been demonstrated by results from trials already undertaken in different developing countries; however, limited information is available with regard to the effects of the different milk components on LP activity. Milk is known to be a complex mixture of different components, of which lacto-peroxidase (LP) enzyme is a small component. The compositions vary between species and within individual cows and also due to seasonal variations. The sum of these compositional changes affects the activity and functionality of LP enzyme. The present study examined the effects of changes in composition of each milk fraction, and of individual components on LP activity. Different reaction media were used (buffer, UHT, raw milk, and colostrum milk) to establish and broaden the understanding of the effect of milk composition on enzyme activity. The influence of storage conditions on LP activity was also investigated.

Materials and Methods

Sample collection and experiments were carried out during March to May 2006.

LP activity in milk fractions

Whole milk: Bulk raw fresh milk was from the Centre for Dairy Research (CEDAR, Reading, UK) unless stated otherwise. The milk was collected and transported at 4 °C to the laboratory for analysis.

Cream and skimmed milk: Cream and skimmed milk were prepared as described in subsequent section and the cream fraction concentrated by centrifugation at 604 x g for 30 min.

Rennet whey: Fresh milk warmed in a water bath (Type SB 15, Grant Instruments, Cambridge, England) maintained at 30 °C was skimmed by use of an electric cream separator (Elecrem, model 1, France). Commercial enzyme, Chy-Max chymosin (Chr Hansen, Pfizer, Inc. Wisconsin, USA) 1 ml was added to 1.5 L skimmed milk and incubated for 1 hour. The curd was cut using a stainless steel knife and further incubated for 15 min. This was then separated from whey using a muslin cloth, and clarified by centrifuging at 604 x g for 30 min and filtering through Whatman filter paper No. 4. Clarified rennet whey was dispensed into 250 ml sterile plastic bottles and frozen at –18 °C. When required, the whey was thawed at room temperature for about 4 hours and vacuum degassed through Whatman No. 4 filter paper (42.5 mm).

Acid whey: Skimmed milk was acidified using 0.5 M HCl to a pH value of 4.6; the resulting whey was separated from the curd by a muslin cloth, centrifuged at 604 x g for 30 min and then filtered through Whatman filter paper No. 4 .

Effects of storage time and temperature

To assess the effects of time and temperatures, different fractions of milk were stored at 5 °C, room temperature (22 °C), 25 °C and 30 °C and the enzyme activities determined at 0, 3, 6, 24, 48, 72, and 96 hours.

Effects of milk components on LP activities

Lactose (4.6%), casein (2.5%), NaCl (10 mM), $MgCl_2$ (10 mM), and $CaCl_2$ (10 mM) were prepared in phosphate buffer pH 6.7. The solutions were used to prepare 1 mM 2,2′-azino-bis-3-ethylbenz-thiazoline-6-sulphonic acid (ABTS) solution containing commercial LP enzyme. To further ascertain the effects of Ca^{2+} and casein on LP activities, the concentrations of Ca^{2+} in raw milk were manipulated as described below by using both cation exchange resin and $CaCl_2$ and casein model solutions prepared as described in the respective subsection below. Addition studies were carried in Ultra High Temperature (UHT) milk enriched with casein as described in the respective subsection below. However, due to difficulties in ascertaining the activities, an additional 0.1 ml of LP solution (5 mg/100 ml) was added in the assay volume

of UHT milk. The LP activities in all cases were then determined.

Manipulation of Ca^{2+} concentrations

Ca^{2+} reduction: Resins bind oppositely charged particles in exchange with the matrix charged groups. For the reduction of Ca^{2+} in raw milk, dry form cation exchange Na^{+} resin (Duolite C433 Resin, Permutit Co. Ltd., Isleworth, UK) in varying amounts (0.125% to 2.5%) was added in raw milk. The resin exchangeable ion, in this case Na^{+}, readily exchanged with milk Ca^{2+} leading to a reduction in milk Ca^{2+} concentrations.

Ca^{2+} increase: Addition of Ca-salts in milk leads into an increase in the concentrations of Ca^{2+}. In this experiment, different concentrations of $CaCl_2$ (0.063% to 2.5%) were added to raw milk to obtain samples with varying Ca^{2+} levels. Samples with both increased and reduced Ca^{2+} concentrations were immediately examined for LP activities.

Ca^{2+} determination: Ca^{2+} concentration was determined using Ciba Corning 634 Ca^{++}/pH analyser. The equipment is designed to measure pH value or potential difference displayed as mV, depending on whether it is connected to a pH electrode or ion selective electrode. In addition, the analyser contains the reference electrode. The Petterson 1030 Ca^{2+} ion selective electrode is designed for measuring the potential difference change related to the variation in the ionic calcium concentration in milk. Ca^{2+} standards were prepared and the corresponding mV measured on the machine. Similarly, the mV readings were taken for the samples and the corresponding Ca^{2+}concentrations determined from the standard curve.

Cleaning and calibration

Each week the electrodes were washed through with a deproteinizing solution containing active pepsin diluted in a solution containing NaCl, KCl, $CaCl_2$, LiCl and HCl, and a conditioning solution consisting of NaCl and NH_4FHF. The instrument was washed through and standardized every day automatically using a Ca^{2+} standard solution of 1.25 mM. Before each analysis, a calibration curve was prepared using 5 solutions of known Ca^{2+} concentration (1.0, 1.5, 2.0, 2.5 and 3.0 mM).

Total calcium determination

The EDTA (ethylenediamine tetraacetic acid) titration method was used to determine total calcium in milk using calgamite as an indicator. In this method, carboxylic acids containing tertiary amines form complexes with calcium ions. EDTA can form as many as six five-membered chelate rings and forms complexes with calcium.

Reagents for total calcium determination

Ammonia buffer solution: 7 g ammonium chloride and 25 g ammonia solution, specific gravity 0.88, were made up to 100 ml with distilled water.

Calgamite indicator: This was prepared by adding 0.2 g calgamite and 5 ml triethanolamine to 15 ml methylated spirit.

Procedures for total calcium determination

To 5 ml milk was added 1 ml ammonia buffer solution and 0.02 ml calgamite indicator. If calcium is present, a pink colour is produced. The mixture was titrated against 0.01 M EDTA solution until the colour of milk changed from pink to blue (the colour changes gradually from pink to purple then blue),

$$\%\ Calcium = \frac{\left(0.004 \times ml\ 0.01\,M\ EDTA\right)}{5} \times 100$$

$$mM\ Calcium = \frac{10}{4} \times \frac{\left(0.04 \times ml\ 0.01\,M\ \ EDTA\right)}{5} \times 100$$

$$mM\ \ Calcium = 2 \times ml\ 0.01\,M\ \ EDTA$$

Where: 0.004 is the calcium equivalent of 1ml of 0.01 M EDTA.

$CaCl_2$-model solution

1mM ABTS prepared in 10 mM $CaCl_2$ and 1 mM ABTS in phosphate buffer, pH 6.7 were used to prepare 2 mM - 10 mM $CaCl_2$ solutions. To each of the prepared solutions,

2 ml of commercial LP (5 mg/100 ml buffer) was added.

Casein- model solution

1 mM ABTS in 2.5% casein prepared in phosphate buffer, pH 6.7 and 1 mM ABTS in phosphate buffer, pH 6.7 was used to prepare 0.5% to 2.5% casein solutions. To each of the prepared solutions 2 ml of commercial LP (5 mg/100 ml buffer) was added.

Casein addition

Addition of casein in raw and UHT milk increases the concentration above the normal concentration. Powdered casein (0.063% to 2.5%) was added to both UHT and raw milks. The resulting samples were assayed for LP activity, Ca^{2+} and total Ca concentrations. To each of the prepared UHT milk samples, 2 ml of commercial LP (5 mg/100 ml buffer) was added.

Lactoperoxidase activity

The modified IDF method (Marks 1998) using ABTS (2, 2'-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid) as a chromogenic substrate was adopted in this study due to its higher sensitivity to peroxidase as compared to other substrates (Shindler and Bardsley 1975). All reagents in this experiment were prepared in phosphate buffer, pH 6.7 to assume the milk environment. The LP activity computational formula was slightly modified by changing the numerical constant from 93 to 96 to take care of the 0.1 ml increase in total assay volume (3.1 ml instead of 3.0 ml). The method is based on the oxidation of the chromogenic substrate (ABTS) in the presence of H_2O_2 producing a green coloured substance whose spectrophotometric reading is measured at 412 nm. The equation below summarises the reaction mechanism.

$$2ABTS + 2H_2O_2 \xrightarrow{LP} 2H_2O + 2ABTS^+$$

(*green coloured radical cation*)

Reagents and solutions for LP activity

0.1 M Phosphate buffer, pH 6.7 –was prepared using 0.1 M disodium hydrogen orthophosphate ($Na_2HPO_4.12H_2O$) and 0.1 M sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) in the ratio of 57: 43 ml, respectively. The pH was monitored with a pH meter standardized against standard solutions with pH 4.0 and 7.0, respectively.

1 mM ABTS solution – was prepared in 0.1 M phosphate buffer, pH 6.7.

0.3 mM Hydrogen peroxide – was prepared in 0.1 M phosphate buffer, pH 6.7 prepared from 30% hydrogen peroxide.

LP activity sample assay

To 0.1 ml of milk in phosphate buffer, pH 6.7 (dilution factor = 5) in a 4.5 cm^3 cuvette, 2 ml ABTS solution was added, mixed well and left for 5 minutes at room temperature to allow dispersion / solubilisation of the casein micelles. One ml of 0.3 mM H_2O_2 was added and mixed quickly. The first absorbance at 412 nm was recorded at exactly 15 seconds after addition of H_2O_2, and the second absorbance was taken after 1 minute and 15 seconds of the reaction time. The activity [E] was calculated using the equation below.

$$[E]_{milk} = \left\{ \frac{(R + R_o)(V_s + V_a)}{V_s} \right\} - 96$$

Where: $[E]_{milk}$ = LP activity (µM product/minute);

Vs = Sample volume (0.1 ml);

Va = Total volume of an assayed sample (3.1 ml);

Ro = 3 µM product/minute

R = Initial rate of generation of oxidized product which is given by this relation $(\Delta A/\Delta t)/ (32.4 \times 10^{-3})$ µM product/minute;

Where: 32.4×10^{-3} is the extinction coefficient of the ABTS oxidation product at 412 nm;

ΔA = change in absorbance;

Δt = change in time).

The numerical constant 93 was changed to 96 to take care of the 0.1 ml increase in assay volume. The total assay volume was 3.1 ml. All measurements were carried out at room temperature.

Zeta potential (ζ) and Micelle size

Zeta potential (mV) and micelle size (nm) were determined using Zetasizer Malvern System 5000 (Malvern Instruments Ltd., Worcestershire, UK). This operates by detecting light scattered from a suspension of particles (usually in the size range 5 nm - ≥ 5000 nm) and interprets the spectrum to extract a measurement of the velocity of the particles in the direction of an applied electric field. This velocity arises from the presence of the charge on the individual particles. The scattered light from the particles (casein micelles) is then mixed with the attenuated light from the laser and is directly focused on the detector. These parameters were determined using skimmed milk to avoid the interference of fat globules, which due to their large size (3 μm) can overlap with casein micelles. Samples were diluted (1:50) with deionised water and analysed at room temperature. The results include 10 sub-runs for casein micelle size and 3 replicates for zeta potential. For casein micelle size, the angle of the laser hitting the cuvette containing sample was always set at 90 °C.

pH effects on LP activity

Phosphate buffers (pH 5.7, 6.0, 6.3 and 6.7) were prepared according to Gomori's procedure (1957). Stock solutions of 0.1 M disodium hydrogen orthophosphate ($Na_2HPO_4.12H_2O$) and sodium dihydrogen orthophosphate ($Na\ H_2PO_4.2H_2O$) were mixed in pre-determined ratios to make solutions with the above pH values. Where necessary, solutions were titrated with 0.1 HCl to bring the pH to the correct pH value. These buffers were used to prepare 1 mM ABTS containing commercial LP enzyme and 0.3 mM H_2O_2 and used in activity assay.

Milk composition

Total protein, solid not fat (SNF) and lactose were measured using a Dairy Lab II analyser (Multispec Limited, York, UK) based on absorption of near infrared radiation at different wavelengths. Warm deionised water 40 °C was used to wash and zero the analyser. Samples were heated at 40 °C and shaken prior to analysis. Results were expressed as % w/v of the total milk composition and were the average of 3 replicates.

Statistical analysis

Data were analysed by analysis of variance (ANOVA) and the means compared using a multiple comparisons test of Least Significant Difference (LSD) at ($P \leq 0.05$).

Results and Discussion

LP activity in milk fractions

Fractionation is a preliminary stage during isolation and purification of LP. The present investigation aimed at examining the effects of fractionation and the contribution of each milk fraction on LP activity. Results (Figure 1) show small but statistically significant variations ($P \leq 0.05$) in LP activities between whole milk (control) and acid whey, rennet whey and cream, and non-significant variations ($P \leq 0.05$) between the control and skimmed milk, and between skimmed milk and acid whey. The variations in activities can be accounted for by the presence or absence of casein. The cream had a much lower activity as expected as LP enzyme is present only in aqueous phase. The higher activities recorded in whey samples is consistent with previous findings. Yoshida and Uyun (1991) reported higher yields of LP from acid whey than from rennet whey, but higher enzymatic activity in rennet whey than in acid whey similar to El-Baradei and Mahmoud (1997), while Fonteh (2001) recorded higher activity in acid whey than in rennet whey and higher activities in both sweet and acid whey than in other milk fractions.

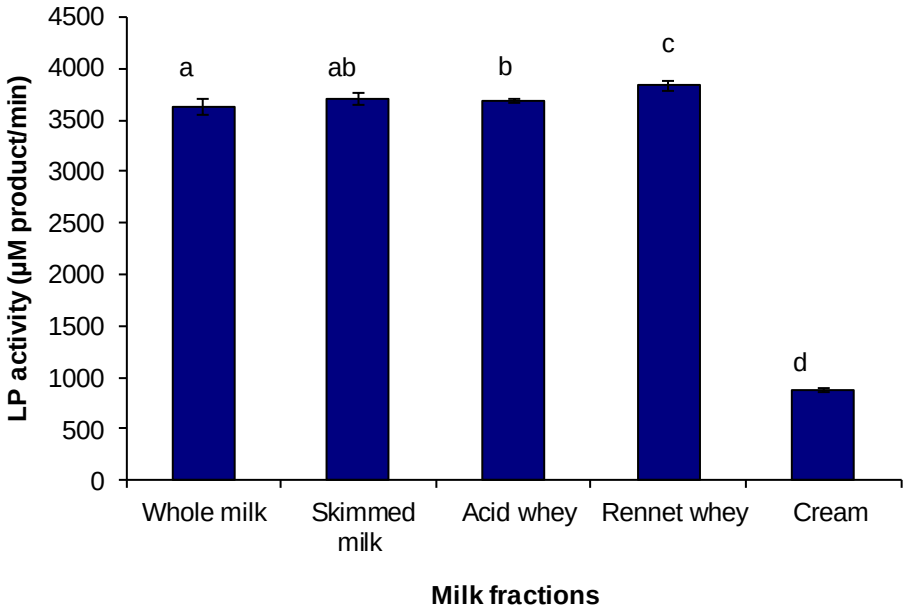

Figure 1: LP activity in various milk fractions. Bars carrying the same letter are not significantly different ($P \leq 0.05$) from each other ($n = 4$).

Effects of storage time and temperature

This study aimed at examining the effect of both storage conditions in particular temperature and media composition on LP activities. Results in Figure 2 show an interesting change in LP activity among milk fractions during storage. The enzyme pulsed as reported previously (Marks 1998, Fonteh 2001). The pulsing of enzyme was demonstrated especially in samples stored at 5 °C as samples stored at 22 °C and above spoiled after 24 h as depicted by the rapid decline in enzyme. However, temperature and media composition did not have great effect on the pulsing behaviour of the enzyme. The reason for pulsing is unclear, but previous work has linked it with the existence of isoenzymes (Fonteh 2001).

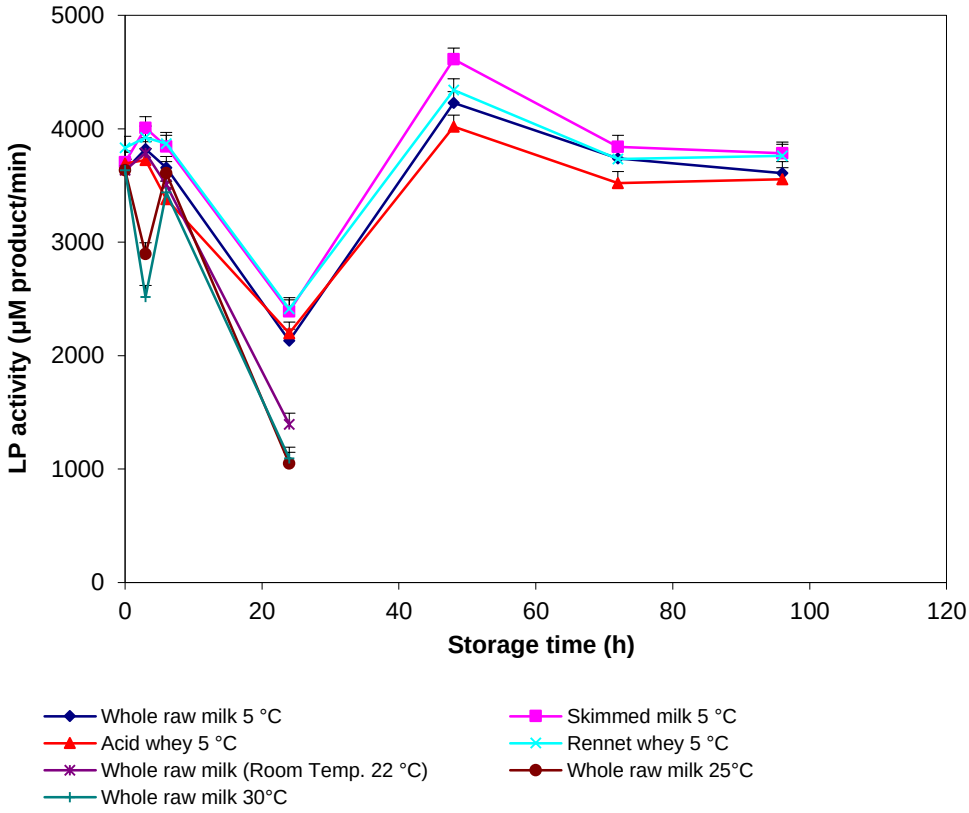

Figure 2: Effect of storage time and temperature on LP activity in milk fractions. Bars carrying the same letter are not significantly different ($P \leq 0.05$) from each other ($n = 4$).

Effects of milk components on LP activity

Figure 3 shows the effects of addition of milk components on LP activity in buffer. The three metal chlorides all resulted in small but significant increases ($P \leq 0.05$) in activity compared to the control in the order; Ca (36%) > Mg (26%) > Na (9%), while casein resulted in a massive significant decrease ($P \leq 0.05$) in activity (90%).There was no effect due to lactose. The results are consistent to those reported by Fonteh et al. (2005) except for lactose which also demonstrated an increase in LP activity. The reason for this surprisingly high reduction in activity by casein is not known, but we speculate that it may be due to either adsorption of the enzyme to casein or due to interaction of LP with casein making it unavailable for the reaction. This high reduction in activity by casein (>50%) was also reported in previous studies (Fonteh 2001). This reinforces the assumption that the variations in LP activity between milk fractions are greatly due to the presence or absence of casein fraction (Figure 1).

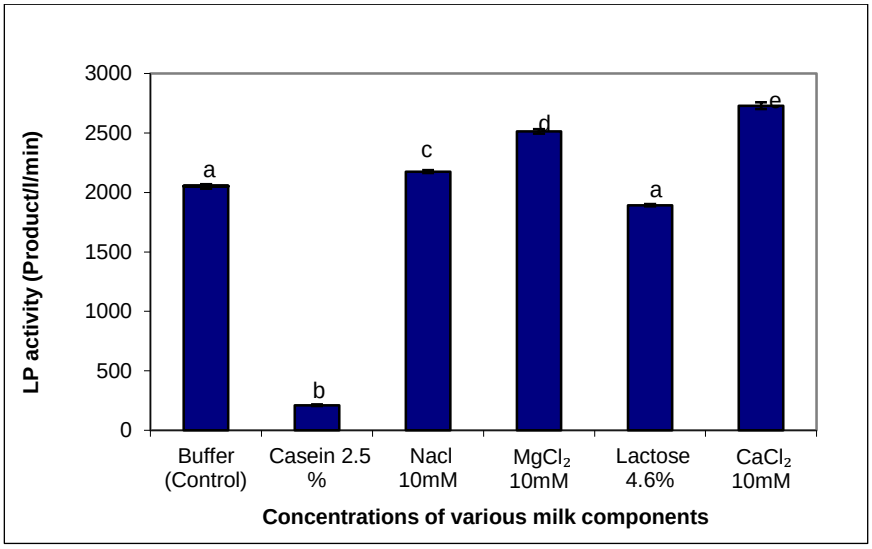

Figure 3: Effect of milk components (in phosphate buffer, pH 6.7) on LP activity. Bars carrying the same letter are not significantly different ($P \leq 0.05$) from each other (n = 4).

Effects of casein on LP activity

Additional experiments were conducted to further examine the influence of casein on LP activity. Varying levels of casein were used in both buffer (Figure 4 a) and UHT milk (Figure 4 b) enriched with commercial LP. The higher LP activity recorded in UHT milk is due to the amount of enzyme applied. It was not possible to assay the LP activity in UHT milk when the same amount of enzyme as that used in buffer was used hence additional amount of enzyme was added to the assay sample to be able to ascertain the LP activity at different casein levels in UHT milk.

A consistent significant decrease ($P \leq 0.05$) in enzyme activity with increasing casein concentrations was clearly observed in these media especially in phosphate buffer (Figure 4 a). This implies that the inhibitory effect of casein was much greater in buffer than in UHT. The decrease in LP activity was between 13% and 95% in buffer, whereas in UHT milk the decrease ranged from 7 to 14 % (Figure 4 b). The dependence of the inhibitory effect of casein on the reaction media suggests the role of other milk components in neutralising the effects due to casein. The mineral components, NaCl, $MgCl_2$ and $CaCl_2$ have been shown to exhibit a promotive effect on LP activity (Figure 3). Gastaldi et al. (1994) described the possibility of added Ca binding to casein either directly as Ca^{2+} or indirectly through such salts as phosphates on ester phosphate groups. Tercinier et al. (2014) also reported interaction between the casein micelles in milk and insoluble calcium phosphate particles, such as hydroxyapatite (HA). The net effect of casein on LP activity is thus a function of the interactive effects of all milk components. The mechanism by which casein affects LP activity is unclear; however, it is speculated that the net negative charges of casein due to phosphate, carboxyl and sulfhydryl groups could be a contributing factor. This may result in an interaction between casein and LP enzyme, reducing the reactive groups of the enzyme and hence its overall reactivity.

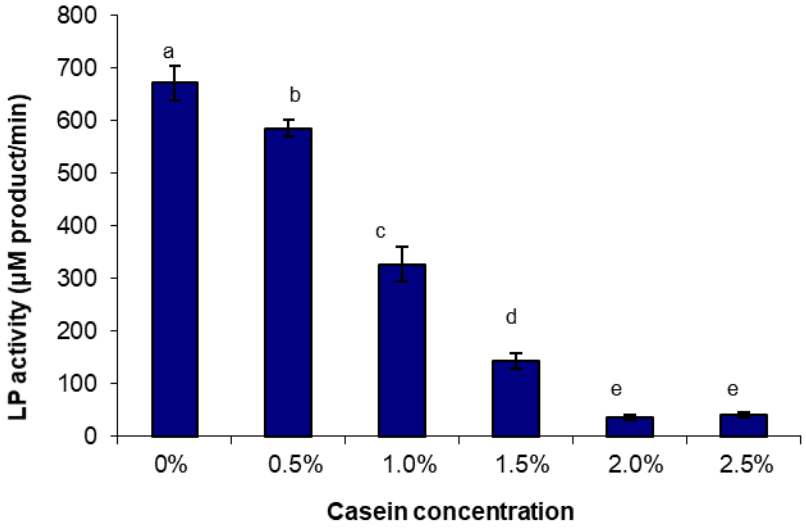

Figure 4 (a): Variation of casein concentration with LP activity in buffer solution. Error bars are standard deviations (n = 4).

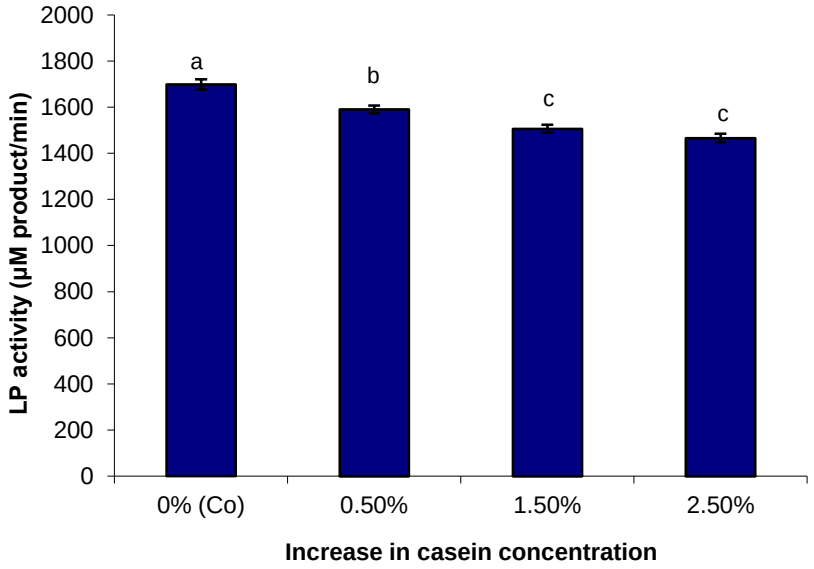

Figure 4 (b): Variation of casein concentration (wider range) with LP activity in UHT milk. Error bars are standard deviations (n = 4). Co = control.

Effects of Ca^{2+} concentrations on LP activity

Further experiments with different levels of Ca^{2+} in Ca^{2+} model solutions similarly showed a positive correlation between Ca^{2+} concentration and LP activity (Figures 5 a) and confirmed the promotive role of Ca^{2+} recorded in the previous experiment (Figure 3). Small but significant increases in LP activities ($P \leq 0.05$) recorded at almost all Ca^{2+} levels were associated with the increase in Ca^{2+} concentrations which varied with pH from pH 6.7 at 0 mM to pH 6.52 at 10 mM. The variations in activities were however not

significant between 4 mM, 6 mM and 8 mM samples, but were significant between this group and 2 mM and 10 mM samples group. However, manipulations of Ca^{2+} in raw milk by either addition of Ca^{2+} or reduction of the actual Ca^{2+} concentration by cation exchange Na^{+} resin did not cause any significant changes in LP activity in raw milk with an initial concentration of 2.45 mM (Figure 5 b) but resulted in small significant changes in raw milk with an initial Ca^{2+} concentration of 2.01 mM (Figure 5 c). These results show the dependency of the effects of Ca^{2+} on media and probably suggest the reliance of the net promotive effect of Ca^{2+} on the net interactive effect of Ca^{2+} with other milk components. Casein has been shown in earlier experiments to have an inhibitory effect on LP activity which is about three times the promotive effects of Ca^{2+}. The interaction between Ca^{2+} and casein described in previous studies (Gastaldi et al. 1994) could possibly describe the effects due to these components on LP activity.

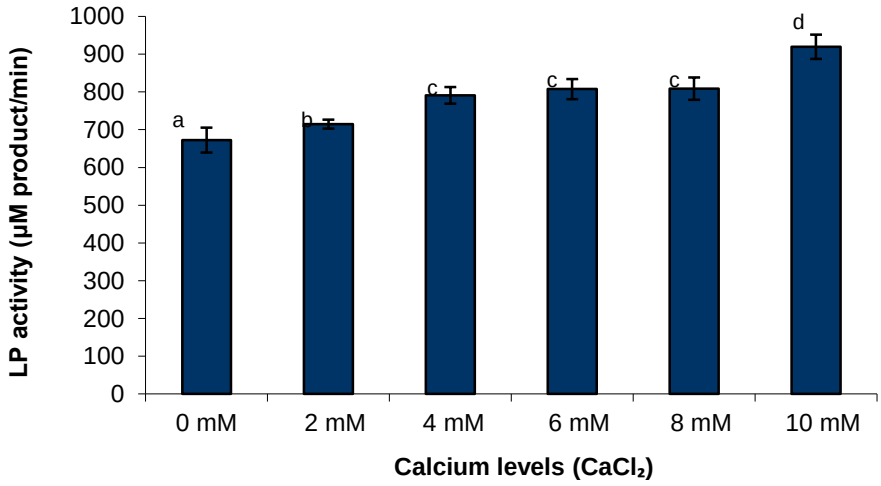

Figure 5(a): Variation of LP activity with Ca^{2+} concentration prepared in phosphate buffer, pH 6.7 without adjusting the final pH. Bars carrying the same letter are not significantly different ($P \leq 0.05$) from each other ($n = 4$).

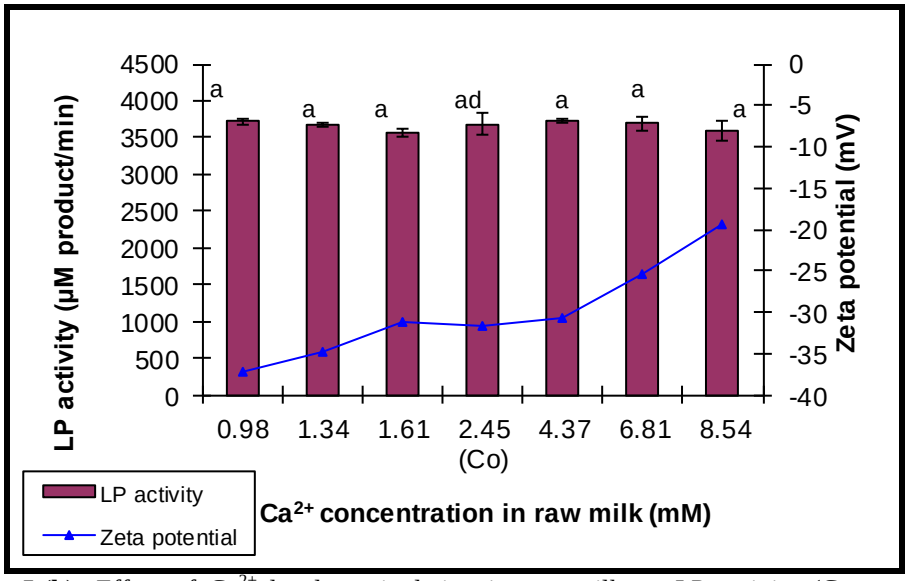

Figure 5 (b): Effect of Ca^{2+} level manipulation in raw milk on LP activity (Co = control sample). Bars carrying the same letter are not significantly different ($P \leq 0.05$) from each other (n = 3).

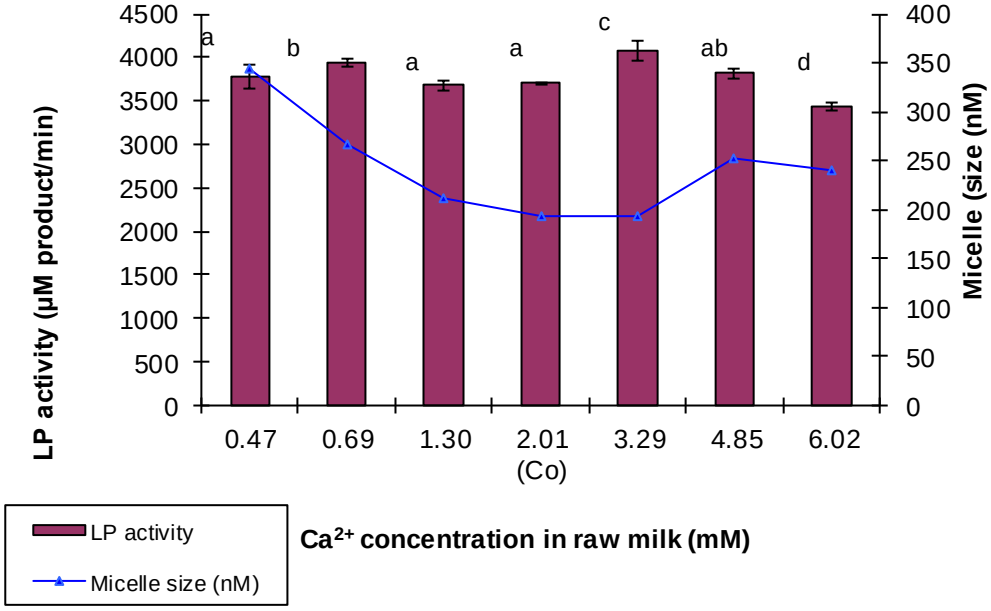

Figure 5 (c): Effect of Ca^{2+} level manipulation in raw milk on LP activity (Co = control sample). Bars carrying the same letter are not significantly different ($P \leq 0.05$) from each other (n = 3).

Relationship between LP activity and either Zeta potential or micelle size

The addition of Ca^{2+} resulted in a steady increase in zeta potential (ζ) from –31.6 mv (control, 2.45 mM) to –19 mV at 8.54 mM Ca^{2+} and its partial reduction caused a steady decrease in ζ from –31.6 to –37 mV at 0.98 mM Ca^{2+} (Figure 5 b). The increase in ζ with increasing Ca^{2+} concentrations corresponds to the reduction in net negative charge, while the decrease in ζ with decreasing Ca^{2+} concentrations corresponds to the increase in net negative charge. This suggests a partly neutralisation of the negative charge through interaction of $Ca^{2+}$with casein as reported previously (Dalgleish 1984).

On the other hand, both addition of excess Ca^{2+} or partial removal of the actual Ca^{2+} in raw milk was associated with increases in micelle size, MS (Figure 5 c). The average MS increased from 193 nm at 2.01 mM Ca^{2+} (control - Co) to 252 nm at 4.85 mM Ca^{2+}. However, further addition of Ca^{2+} reduced the MS to 240 nM at 6.02 mM Ca^{2+} concentration. Negligible changes in MS were observed between 1.30 mM and 3.29 mM as reported previously (Holt et al. 1986). The MS expanded much more on Ca^{2+} removal than on Ca^{2+} addition. The largest MS (344 nm) was recorded at the lowest Ca^{2+} concentration, 0.47 mM. These changes are related to either aggregation of micellar components or disintegration of micelles. Removal of Ca^{2+} in small amounts leads to dissociation of the relatively weak bonds holding β- and κ-caseins without micellar disintegration (Lin et al. 1972). Excessive removal however, dissociates the CCP (colloidal calcium phosphate) and Ca-α-caseinate bond. The reduction of Ca^{2+} also increases the negative charge and repulsive forces and prevents flocculation (Horne 1984). However, in both cases there is no correlation of LP activity with either ζ (R^2=-0.512) or MS (R^2 = –0.028). It might have been expected that if changes in LP activities were related to adsorption or binding of the enzyme to the micelle surface as postulated earlier, then activity would be related either to total surface area or surface charge on the micelle.

Effects of changes in Ca^{2+} on pH and their effects on LP activity

To examine the link between changes in milk Ca^{2+} concentrations and changes in pH and the effects of these pH changes on LP activity, the following experiment was carried out. Calcium ion was reduced in raw milk by use of dry form cation exchange Na^{+} resin and increased by addition of $CaCl_2$. The reduction of the actual milk Ca^{2+} resulted in an increase in pH, from 6.73 to 7.26, whereas addition of Ca^{2+} led to a decrease in pH, from 6.73 to 5.46 (Figure 6 a). Udabage et al. (2000) related the decrease in pH to increased Ca^{2+} activity and serum Ca^{2+} as demonstrated; $3Ca^{2+} + 2HPO_4^{2-} \rightarrow Ca_3(PO_4)_2 + 2H$. To examine how these changes in pH could affect LP activity, buffers with different pH values within the range of changes observed for raw milk were prepared and the activity determined. The LP activity varied significantly ($P \leq 0.05$) at different pH values: with pH being inversely related to LP activity (Figure 6 b). That is, the lower the pH the higher the activity and vice-versa. Significant variations ($P \leq 0.05$) in LP activities observed at all four pH values justify the great influence of pH on enzyme activity. It is thus logical to suggest that the effects of Ca^{2+} on LP activity could be indirect and reliant on changes in pH. The negative correlation between pH and LP activity agrees with previous findings (Wolfson and Sumner 1993). Other researchers (Deveau and Ramet 1995, Kussendrager and van Hooijdonk 2000) reported the optimum LP activity to lie between pH 4.5 and 6.0 but with yet significant activity at pH 7 (Marshall et al. 1986).

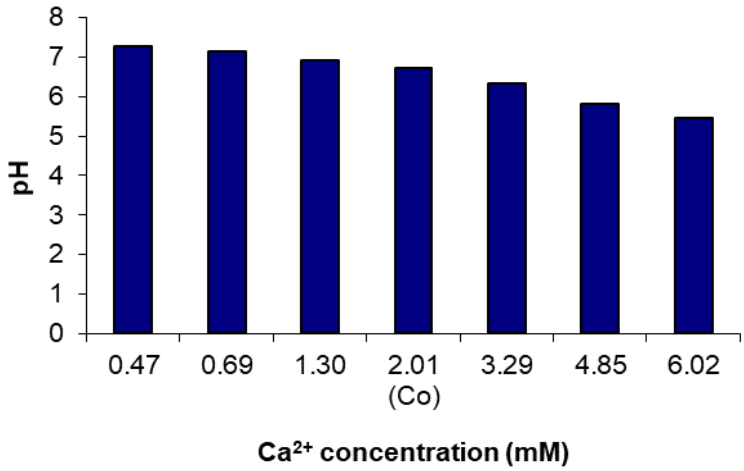

Figure 6 (a): The effect of Ca^{2+} level manipulation in raw milk on changes in pH. Error bars are not seen due to very small standard deviations (n = 3).

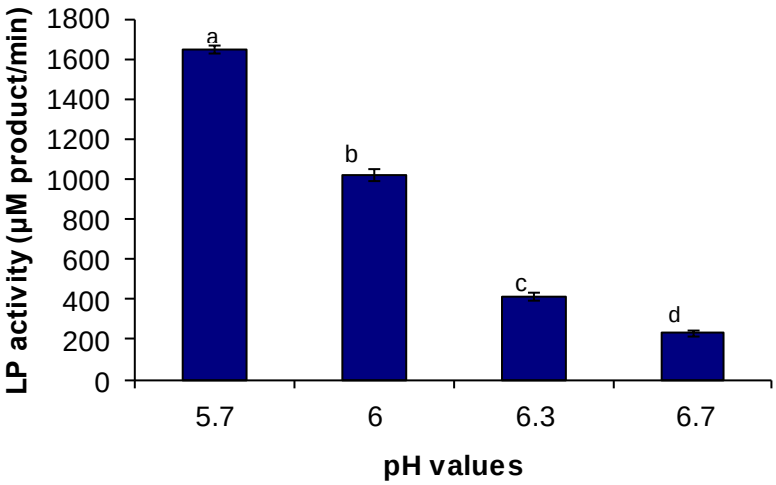

Figure 6 (b): pH effects on LP activity (commercial enzyme-in buffer). Error bars are standard deviations (n = 4).

The effects of casein on LP activity at different pH values

The promotive role of Ca^{2+} on LP activity in previous experiments was related to its effects on pH (Figure 6 a). To examine the extent to which the inhibitory effect of casein on LP activity would be affected by Ca^{2+} induced changes in pH, the following experiment was carried out. Buffers with 4 different pH values were prepared and each divided into two halves, 100 ml each. To one half, casein was added to achieve a 2.5 % casein concentration and no casein was added in the other half. The enzyme activity was determined in both samples (2.5% casein and casein free) at each pH value and the changes in activity compared.

The results showed significant variations in LP activity at all pH values

(Figure 7). The maximum LP activity was recorded at pH 5.7 and the minimum activity at pH 6.7 in both casein free and 2.5% casein samples. The reduction in activity in the 2.5% casein samples was 7–fold at pH 6.7, 4–fold at pH 6.3 and about 2–fold at pH 6.0. For, the casein free samples the decrease in LP activity was 4–fold at pH 6.7, 2–fold at pH 6.3, and by less than 2–fold at pH 6.0. The higher reduction in LP activity in 2.5% casein samples than in casein free samples further confirms the higher inhibitory effects of casein compared to the promotive effect of Ca^{2+} which relies on pH drop. The reduction in LP activity in casein free samples was purely due to the increase in pH, while in the 2.5% casein samples the extra reduction in LP activity was due to the presence of casein. These results suggest that the influence of pH is under the control of casein. The extra reduction in LP activity recorded in the 2.5% casein sample at each pH value was almost twice as much as the reduction in activity recorded in casein free samples, which reflects the overshadowing effects of casein over pH effects.

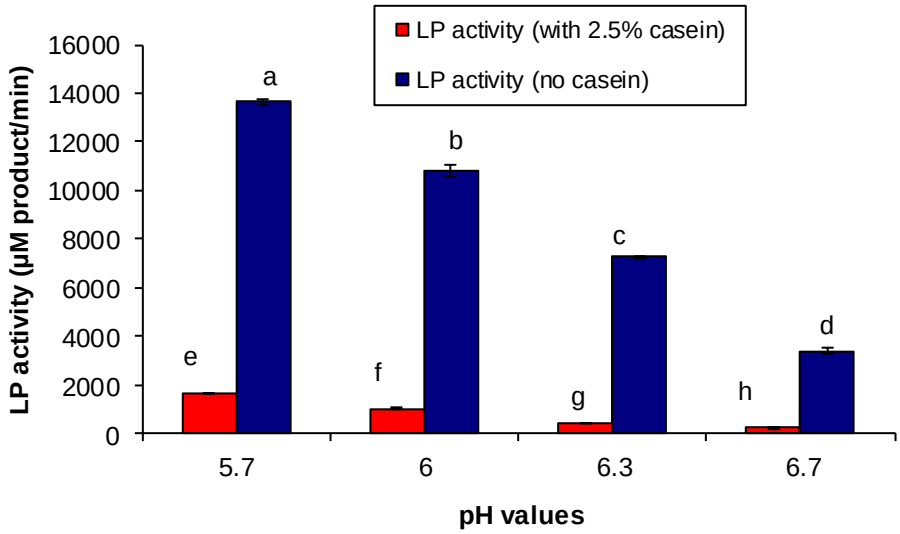

Figure 4: Effect of casein on LP activity at different pH values (in phosphate buffer). Error bars are standard deviations (n = 4).

LP activity in colostrum milk

Ca^{2+} is present in colostrum milk in a much higher concentrations and its concentrations vary with post parturition days. Previous experiments demonstrated the promotive role of Ca^{2+} on LP activity. To broaden the understanding of the role of Ca^{2+} on LP activity this additional investigation was carried out on colostrum milk collected from twelve individual cows for the first five days after parturition. Samples were frozen and thawed when needed for experimentation.

Table 1 summarises the compositional variations of colostrums, changes in pH, titratable acidity (TA), zeta potential and micelle size and the corresponding LP activity. Total Ca and Ca^{2+} were highest in day 1 colostrum and their concentrations decreased with normalisation of colostrum. The changes were concurrent with increases in pH and decreases in TA. The LP activity was extremely low in all day 1 samples, whereas maximum values were registered in days 2, 3, 4 and 5 colostrums. The maximum values in day 2 colostrum were recorded for cows H & J, day 3 for cows C,

D, F, & I, day 4 for cows A, G, K & L and in day 5 colostrum for cows B & E. The LP activity ranged from 21 µmole product/min for day 1 colostrum of cow L to 6179 µmole product/min/ml for day 3 colostrum of cow I. Korhonen (1977) recorded the maximum LP content during the third and fourth milkings based on twice milkings a day, which corresponds with day 2. While Reiter (1985) reported maximum LP levels on 4 – 5 days postpartum.

The results generally show a poor non-significant ($P < 0.05$) negative correlation between LP activity and Ca^{2+}, but the correlation was however highly significant between LP activity and total calcium ($P < 0.01$). It is possible that the positive effect of Ca^{2+} is within certain limits of its concentration or is counteracted by the excessively higher amounts of other components in colostrum. Earlier results suggest the dependency of LP activity on the interactive effects of all individual milk components. Manners and Craven (2003) documented twice as much the level of casein (5 g/100 ml) in colostrum milk as compared to that in normal milk produced 10 days (2.6 g/100 ml) later during lactation. This is consistent with the high protein concentrations recorded in the current study. Casein constitutes 80% of the total proteins. The higher inhibitory effect of casein on LP activity than the promotive role of Ca^{2+} has been demonstrated. This offers the possible explanation for the low LP activities recorded in colostrums, especially in day 1 colostrum. This is consistent with a highly significant negative correlation between LP activity and both protein and TA ($P < 0.01$) and a positive significant correlation with Ca/protein ratio ($P < 0.05$). Among the 10 cows, whose Ca/protein ratios were computed, the maximum LP activities in 5 cows were recorded for days having the highest Ca/protein ratio, while for the other 4 colostrums samples with the maximum activity their TA values were higher than the TA values of their counterparts with higher Ca/casein ratios. The results further suggest the interactive effects of milk components on LP activity. Lactose and fat however, have no impact on LP activity as demonstrated by a non-significant correlation with LP activity.

There was no significant correlation between LP activity and MS but a significant correlation ($P < 0.05$) was observed between LP activity and ζ. This is likely to be due to the compositional effects on LP activity as observed earlier other than its reliance on ζ.

Table 1: The relationships between LP activity and compositional changes of day 1 to day 5 colostrums of 12 individual cows. *shows the maximum LP activity values and maximum Ca/protein ratios in each cow's colostrum. nd = not determined.

Cow	Day	LP activity (μM product/min)	Ca^{2+}(mM)	Ca (mM)	Size (mM)	Zeta (mV)	Fat	Protein	Lactose	SNF	pH	TA	Ca/Prot. ratio
A	1	47	3.19	60.0	241	–22.0	3.51	17.20	1.65	19.52	6.27	ND	3.49
	2	65	3.00	56.8	238	–19.7	4.27	17.30	1.61	19.66	6.25	0.360	3.28
	3	184	2.99	49.6	203	–23.6	2.46	6.50	1.58	8.84	6.37	0.300	7.63
	4	467*	2.75	42.0	192	–23.6	2.73	6.57	3.12	10.41	6.49	0.270	**6.39**
	5	390	2.70	41.8	169	–26.3	2.03	5.09	3.64	9.43	6.64	0.225	8.21*
B	1	37	3.62	79.8	241	–23.4	0.84	21.72	2.46	24.92	6.24	**nd**	3.67
	2	46	3.34	68.2	221	–24.6	1.4	12.78	3.24	16.74	6.28	0.410	5.34
	3	106	3.27	53.4	195	–23.6	3.69	8.07	3.68	12.45	6.29	0.330	6.62
	4	237	3.12	45.3	177	–25.9	2.46	5.21	4.16	10.00	6.29	0.280	8.69
	5	**316 ***	3.11	42.4	172	–27.3	2.13	4.44	4.05	9.19	6.32	0.250	**9.55***
C	1	205	3.21	62.0	192	–23.8	4.02	8.61	3.77	13.08	6.31	**0.300**	7.20
	2	317	2.92	49.0	194	–26.1	4.79	5.93	4.04	10.66	6.34	0.286	8.26
	3	**344 ***	2.88	45.0	188	–26.2	4.19	4.96	4.13	9.78	6.35	**0.285**	**9.07***
	4	328	2.70	41.6	188	–27.4	4.23	4.60	4.22	9.51	6.35	0.270	9.04
	5	295	2.18	41.0	188	–27.3	5.14	4.63	4.29	9.61	6.37	0.255	8.86
D	1	38.3	3.48	69.8	240	–24.6	4.29	14.48	2.66	17.87	6.21	0.360	4.82
	2	242	3.16	63.6	227	–25.8	2.89	10.20	3.55	14.46	6.21	0.360	6.24
	3	**491 ***	2.72	53.4	232	–29.2	8.07	6.50	3.45	10.65	6.21	0.300	**8.22**
	4	473	1.83	45.6	236	–28.2	7.22	5.24	3.45	9.40	6.28	0.260	8.70
	5	477	1.41	44.8	241	–28.4	4.71	4.72	3.99	9.40	6.36	0.250	9.49*
E	1	217	2.93	70.2	290	–27.5	10.53	20.54	1.74	23.03	6.35	**0.435**	3.42
	2	224	2.84	71.4	230	–27.2	7.24	17.97	2.78	21.47	6.32	0.440	3.97
	3	364	2.82	62.8	195	–25.0	2.19	8.76	3.55	13.01	6.37	0.330	7.17
	4	488	2.80	59.0	191	–28.0	3.54	7.30	3.68	11.67	6.41	0.320	8.08
	5	**604 ***	1.94	52.6	189	–27.2	8.87	5.44	3.79	9.92	6.64	0.265	**9.67***
F	1	558	3.38	60.3	292	–30.3	5.6	25.15	1.42	27.32	6.28	**nd**	2.40
	2	751	3.25	54.8	260	–26.3	2.75	19.54	1.93	22.22	6.21	0.360	2.80
	3	**1025***	3.07	52.0	220	–23.8	2.28	12.21	2.97	15.90	6.27	0.360	**4.26**
	4	990	2.57	38.8	224	–26.7	3.94	6.91	3.65	11.26	6.3	0.275	5.62
	5	990	2.17	36.2	224	–27.6	3.01	5.38	3.79	9.88	6.32	0.245	6.73*
G	1	22	3.90	61.4	210	–17.1	2.50	15.75	2.68	19.16	6.23	0.380	3.90
	2	1436	3.39	47.7	179	–20.9	4.32	5.52	3.75	9.97	6.36	0.245	8.64
	3	2648	3.25	46.4	192	–20.2	2.96	4.55	3.61	8.87	6.38	0.220	10.19
	4	**3106***	3.12	45.2	195	–24.2	2.38	4.36	3.83	8.90	6.42	0.200	**10.37***
	5	2512	2.82	42.0	196	–22.6	2.75	4.12	4.10	8.92	6.47	0.185	10.19
H	1	1103	3.29	72.1	210	–15.2	5.46	13.70	3.17	17.58	6.26	0.440	5.26
	2	**3302***	2.99	46.8	201	–20.6	5.44	5.28	3.94	9.92	6.29	0.290	**8.86**

Cow	Day	LP activity (μM product/min)	Ca^{2+}(mM)	Ca (mM)	Size (mM)	Zeta (mV)	Fat	Protein	Lactose	SNF	pH	TA	Ca/Prot. ratio
	3	2778	2.74	41.4	202	–22.6	6.25	4.42	4.05	9.16	6.34	0.250	9.37
	4	2510	2.55	40.6	214	–22.9	6.88	4.37	4.13	9.18	6.41	0.230	9.29
	5	1864	2.37	40.0	223	–23.2	5.34	4.22	4.26	9.17	6.50	0.200	9.48*
I	2	4371	2.40	29.3	202	–20.3	2.15	5.20	1.25	7.22	6.39	0.210	5.63
	3	**6179***	3.90	41.9	212	–21.9	3.48	5.08	3.00	8.79	6.15	0.290	**8.25***
	4	3165	2.60	36.7	215	–22.1	3.97	4.65	3.62	8.98	6.27	0.265	7.89
	5	889	1.50	34.8	168	–23.7	4.45	4.29	3.98	8.97	6.47	0.215	8.11
	1	145	7.10	46.3	243	–16.7	0.96	17.98	1.93	20.65	5.97	0.450	2.58
J	**2**	**921***	4.90	40.9	190	–20.8	2.04	5.72	3.22	9.66	6.10	0.320	**7.15**
	3	884	3.70	36.0	194	–21.9	5.30	4.11	3.40	8.22	6.23	0.245	8.76
	4	648	3.20	36.1	170	–23.2	1.96	3.94	3.77	8.40	6.31	0.215	9.16
	5	766	3.20	37.2	176	–22.4	3.11	3.93	3.90	8.52	6.39	0.200	9.47
	3	2461	3.00	40.5	190	–25.0	nd	nd	nd	nd	6.32	0.260	nd
K	**4**	**2740***	3.10	42.9	188	–24.5	nd	nd	nd	nd	6.39	0.260	nd
	5	1960	2.70	39.3	179	–25.3	nd	nd	nd	nd	6.41	0.240	nd
	1	21	5.80	80.0	203	–18.0	nd	nd	nd	nd	6.10	0.460	nd
L	2	1515	3.60	58.0	175	–21.6	nd	nd	nd	nd	6.27	0.330	nd
	3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	4	**2054***	2.60	44.0	172	–22.8	nd	nd	nd	nd	6.43	0.220	nd
	5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Conclusions

The different fractions and treatments of milk have varying effects on LP activity. The presence or absence of casein greatly influences the changes in LP activity. The significant variation in LP activity of aqueous fractions of sweet (rennet) and acid whey reflects the effects of the treatments involved on LP activity. Casein and Ca are the most influential milk components on LP activity; however, their net effects rely on the interactive effects of each other and other milk components. The promotive effect of Ca^{2+} on LP activity is indirect and reliant on pH changes and its influence on LP activity is within certain limits of its concentrations and under the control of casein. It might be worthwhile to further examine the effects Ca on LP activity by inducing very small changes in the actual Ca concentration of raw milk and also relate the Ca/casein ratio with LP activities of different milk samples.

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