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Microbial growth, EPS concentration and textural properties of fermented milk supplemented with calcium and whey protein analysed using response surface methodology

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Abstract: The fermented milk gels were acidified by a ropy-EPS-producing strain of Streptococcus thermophilus ASCC 1275 at 42°C, stored at 4°C, 21 days. A central composite design containing two factors at five levels each: $CaCl_2$ (1-10 mM) and whey protein concentrate (WPC) or whey protein isolate (WPI) (1-5% w/w). The total solid content of yoghurt bases was 14% (w/w). All fermented milk was analyzed for the culture growth, EPS concentration, viscoelastic and flow behavior, firmness, and water holding capacity (WHC). Second order polynomial models estimated response variables with R^2 ranging from 0.704 - 0.943. WPI or WPC had no effect on the cell growth during storage, but increased the EPS concentration. Calcium reduced cell growth especially in WPI-supplemented yoghurt. Calcium disrupted gel structure by lowering G' and firmness, increasing flow behaviour and WHC. WPC addition increased G', consistency index, and firmness significantly ($P<0.05$). The interaction between calcium and either whey protein preparation weakened the structure further. These results showed that the texture of yoghurt was modulated by Ca and whey proteins supplementations and the extent and directions of the magnitude depended on the Ca concentration and type of whey protein preparation.

Keywords: Yoghurt, whey protein, calcium, rheology, water holding capacity

Introduction

Whey proteins, a by-product of cheese manufacturing, have been extensively used to improve functional, nutritional, therapeutic, and physiological properties of various food products. They influence several food textural properties mainly due to water- as well as protein- binding, emulsifying, and gelling characteristics (de Wit, 1998). Their functional properties have been used to improve colour, firmness, fracturability and reduce the oil content in deep-fried poultry products (Dogan *et al*., 2005), to prevent cook loss and improve texture profiles in meat batters (Barbut, 2006), to enhance homogeneity of the crystal size, coldness intensity, and creaminess in ice cream (Ruger *et al*., 2002), and to create more homogenous size of the air bubbles in whipped-frozen emulsions (Relkin and Sourdet, 2005). The nutritional and health related benefits of whey proteins include provision of essential amino acids in infant formula, biostatic and antibacterial activity (de Witt, 1998), weight control due to their calcium content (Pilvi *et al*., 2006), alleviation of stress related ailments (Schaafsma, 2006a) and enhanced satiation during weight loss program (Schaafsma, 2006b). Incorporation of calcium, vitamin D, and whey protein in the high sucrose and high fat diet, reduced fat mass, but increased lean mass in Winstar rat (Siddiqui *et al*., 2008). Therefore, the inclusion of these highly valuable dairy ingredients into various food products would certainly improve their healthy perception.

The effects of whey protein addition during yoghurt fermentation on the texture of yoghurt varies, apparently depending on factors such as whey protein type (Vasbinder *et al*., 2004), degree of whey protein denaturation (Sodini *et al*., 2006), point of whey protein addition such as before or after pasteurisation (Schorsch *et al*., 2001) and casein to whey protein ratio (Puvanenthiran *et al*., 2002). The interactions between the whey proteins in the form of native whey protein isolate (WPI) and caseins led to a weakening of the acid gel structure (Patocka *et al*., 2006). On the other hand, the WPC supplementation to yoghurt improved the yoghurt gel strength (Remeuf *et al*., 2003; Isleten and Karagul-Yuceer, 2006) especially after whey protein denaturation. The denaturation of whey proteins may also adversely (Sodini *et al*., 2006) or positively (Isleten and Karagul-Yuceer, 2006) affected water holding capacity, the expulsion of whey, upon prolonged storage.

Calcium is added to milk products not only for nutritional but also for functional purposes. Moreover, the calcium fortification in milk improved solubility, dialysis, transport and uptake rate of calcium, thus increasing its bioavalability (Perales *et al*., 2006), as well as enhancing heat stability of milk (Singh *et al*., 2007). Therefore, supplementation of both whey protein and calcium (Sanchez-Hidalgo *et al*., 2000) may synergistically improve nutritional status of

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certain food products. Our previous study showed a gel-weakening effect of calcium on Ca-supplemented fermented milk (Purwandari and Vasiljevic, 2009). Calcium addition may potentially affect textural characteristics through its interactions with yoghurt constituents such as casein, whey proteins, and various hydrocolloids including *in situ* produced bacterial exopolysaccharides (EPS). EPS produced by yoghurt cultures has been recognized to alter yoghurt texture. Either capsular or capsular-ropy type of EPS and added calcium showed synergistic effect on weakening final fermented milk texture, although elastic moduli of that with capsular-ropy EPS was less responsive towards changes in calcium concentration (Purwandari and Vasiljevic, 2009). Since sugar was not an effective agent to improve weak texture of Ca-supplemented fermented milk, it is important to find better alternatives. In this regard, whey protein may be a good candidate, since it showed synergy with $CaCl_2$ to form a strong gel (Lorenzen and Schrader, 2006). The information on the combined effects of the calcium addition and whey protein type on the textural and physico-chemical properties of yoghurt fermented with an EPS-producing cultures, is rather limited. Therefore, the aim was to assess the properties of calcium and whey proteins supplemented yoghurt bases produced with an EPS-producing strain of *Streptococcus thermophilus* using the response surface methodology.

Materials and Methods

Fermented milk cultures

Fermented milk batches were fermented by an EPS-producing strain of *Streptococcus thermophilus* ASCC 1275, which produces mixed EPS (capsular and ropy) (Zisu and Shah, 2003). The strain was obtained from the Australian Starter Culture Research Center (ASCRC, Werribee, Victoria, Australia). Frozen (-80oC) glycerol stock of the culture was activated by incubating it twice in 30 mL sterile 14% (w/v) skim milk at 42°C for 24 hours, before application in the fermented milk manufacturing.

Experimental design and statistical analysis

The experimental design consisted of twelve combinations according to a second order central composite design with two factors at five levels each, and $CaCl_2$, WPC or WPI concentrations as independent variables (Table 1). Every combination was at least replicated twice. The statistical analysis of the data was carried out using the SAS System (SAS, 1996). The full term second order polynomial response surface models were fitted to each of the response variables, according to the following equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 + \varepsilon,$$

Where β_0, β_1, …, β_{22} represented the estimated regression coefficients, with β_0 being the constant term; β_1, β_2 represented the linear effects, β_{11}, β_{22} the quadratic effects; β_{12}, β_{22} the interaction effects; ε was the random error; and X_1, X_2, were the independent coded variables (Myers and Montgomery, 2002). A simple correlational analysis was performed to reveal connection among parameters.

Table 1. Experimental design and levels of factors in natural and coded values

Factors	Coded factor				
	-2	-1	0	1	2
	Natural values				
Calcium concentration (mM)	0	2.5	5	7.5	10
WPC or WPI concentration (mM)	0	1.25	2.5	3.75	5

Run	Factors	
	Calcium concentration (mM)	WPC or WPI concentration (mM)
1	2.5	1.25
2	7.5	1.25
3	2.5	3.75
4	7.5	3.75
5	0	2.5
6	10	2.5
7	5	0
8	5	5
9	5	2.5
10	5	2.5
11	5	2.5
12	5	2.5

Preparation of fermented milk batches

Low heat-treated skim milk powder (Murray Goulburn Co-operative Co. Ltd., Brunswick, Victoria, Australia) was used alone or in conjugation with whey protein powders in preparation of the fermented milk mixes. A portion of skim milk solids was replaced with an appropriate amount of whey proteins maintaining the total solid content of the fermented milk mixes at 14 (% w/w). Proportions of skim milk solids, whey protein concentrate (WPC80 instantized, Wynpro, United Milk Tasmania, Australia) or whey protein isolate (WPI, Alacen 895, Fonterra, Melbourne, Victoria, Australia) and added calcium in samples were adjusted according to the central composite design as shown in Table 1. The powders were reconstituted in Milli-Q™ water to achieve 14% (w/w) total solid content and subsequently pasteurized at 90°C for 5 minutes holding time in a water bath with constant mixing. The appropriate amount of Ca was added prior to pasteurization in the form of $CaCl_2$. The fermented milk base was then cooled to 42°C and inoculated with 1% (v/v) of *S. thermophilus*. After inoculation, the pasteurized fermented milk base was poured aseptically into sterile 100 mL plastic containers, which were subsequently placed in an

incubator preset at 42°C. The process was terminated when pH reached 4.5 by immediate transfer into a cold room (4°C). Samples were examined for their rheological properties, after overnight cold storage (approximately 15 hrs) and after 21 days.

S. thermophilus *viable cell concentration*

The enumeration of *S. thermophilus* strains was according to an established procedure reported previously (Donkor *et al*., 2006) at day-1 (after overnight storage) and day-21. Briefly, 1 g of fermented milk sample weighed precisely was resuspended in 0.1% peptone water and serially diluted to required levels. Such diluted samples were then plated on M17 agar (Merck Pty Ltd., Kilsyth, Victoria, Australia) and incubated aerobically at 42°C for 48 h. The results obtained as means of 4 independent observations were expressed as a log of colony forming units per gram of fermented milk.

Concentration of crude EPS in fermented milk serum

The crude EPS concentration in fermented milk samples was determined following an established methodology (van Geel-Schutten *et al*., 1998) with some modifications, which was reported to be highly reliable (Rimada and Abraham, 2006). Approximately 30 g of fermented milk was first centrifuged (Model J2-HS, Beckman, Fullerton, CA, USA) at 11000xg at 4°C for 4 min. The supernatant was collected and combined with two volumes of chilled ethanol and stored at 4°C overnight. This was followed by centrifugation at 2000x g, at 4°C for 15 min (model RT7, Sorvall, DuPont, Newtown, CT, USA) to enhance the EPS precipitation. Collected EPS-containing precipitate was then dissolved in 10 mL of distilled water, followed by the addition of 250 µL 80% trichloroacetic acid for precipitation of the remaining proteins. After storing the mixture overnight at 4°C, it was centrifuged at 700 x g, 4°C for 15 min (Sorvall) to collect the EPS-containing supernatant. This procedure was repeated twice, the final precipitate was dried at 55 °C under vacuum and then weighed and expressed as the crude EPS.

Determination of water holding capacity

The level of water holding capacity during cold storage of the fermented milk batches was analysed by a centrifugation method previously reported (Jaros *et al*., 2002). For this test, the fermented milk batches were prepared by *in situ* fermentation in 50 mL centrifuge tubes (Falcon, Blue Max, Becton Dickinson and Company, Franklin Lakes, NJ., USA). Upon termination of fermentation, the tubes were stored in a cold room and centrifuged (Sorvall) at 700 x g at 8°C for 10 min at day-1 or at day-21. The weight of the drained liquid was recorded and related to the initial weight of fermented milk with the degree of water holding capacity expressed as a percentage.

$$WHC = 100\ \% - (\text{water expelled/initial weight} \times 100\%) \quad (1)$$

Rheological properties of fermented milk gels

The rheological properties of the fermented milks were measured using a controlled-stress rheometer (Physica MCR 301, Anton Paar, GmbH, Germany), equipped with a temperature and moisture regulating hood and a cone and plate geometry (CP50-1, 50 mm diameter, 1° angle and 0.49 mm gap). The temperature was regulated by a Viscotherm VT 2 circulating bath and controlled with a Peltier system (Anton Paar). The temperature during all determinations was maintained constant at 5+0.1°C. The data of all rheological measurements were analyzed with the supporting software Rheoplus/32 v2.81 (Anton Paar).

Prior to loading, all samples were stirred to eliminate thixotropy and heterogeneity of EPS distribution. A representative sample was loaded into the module and then pre-sheared at a high shear rate of 500/s for 15 s followed by 300 s rest to allow for structural rebuilding (Purwandari *et al*., 2007). The dynamic oscillatory measurements were carried out over a range of frequencies from 0.1 to 10 Hz to determine elastic properties (storage modulus G') of the samples. The strain was maintained constant at 0.5% and was inferred from the linear viscoelastic region determined previously by amplitude sweep at a constant frequency (1 Hz). The hysteresis loops were generated by measuring the shear stress upon increasing shear rate from 0.1 to 100/s in 300 s (upward curve in the rheogram), then holding at 100/s for 5 s and finally decelerated from 100 to 0.1/s in 300 s (downward curve in the rheogram). The data from the upward curve of the shear cycle were also fitted to Ostwald-de Waele power law model ($\tau = K \gamma^{n}$), where τ represents shear stress (Pa), γ shear rate (/s), while K and *n* are consistency factor (Pa s^n) and flow behaviour index, respectively.

The firmness of fermented milk gels was determined using a texture analyzer (TA-XT2plus, Stable Micro System Ltd., Surrey, UK), equipped with a 30 kg load cell and 20 mm aluminium cylinder probe (P/20, Stable Micro Systems). The cross-head speed during measurements was set at 1 mm/s with the 50% compression. Every combination was replicated twice with two sub samplings each.

Results and Discussion

Statistically, the cell growth was not significantly ($p > 0.05$) affected by either WPC or WPI concentrations (Tables 2, 3). Interestingly, the addition of calcium hindered the culture growth significantly ($p < 0.01$) in the WPI supplemented yoghurt only (Table 3). The concentration of the viable cells in all samples increased as expected during fermentations in both WPC- and WPI-supplemented fermented milk. During the cold storage, the culture continued to grow slowly in the WPC fermented milk with no apparent effect of the protein concentration ($p > 0.05$, Table 2). Conversely, the WPI-supplemented fermented milk supported the culture viability poorly, in which colony counts declined significantly ($p < 0.001$) as the WPI concentration was increased (Table 3). Similar to initial observations, the cell viability was significantly ($p < 0.01$) reduced in the presence of calcium, again, in the WPI-fermented milk only. Our findings were consistent with several other reports, which found that the supplementation of native whey protein preparations to growth media poorly supported growth of lactic acid bacteria (*Lb. delbrueckii spp. bulgaricus* RR), which was likely attributed to unavailability of lactose (Briczinski and Roberts, 2002) or lack of essential low molecular weight nitrogen compounds (Amrane and Prigent, 1993).

Table 2. Regression coefficients of the second-order polynomial model for the response variables (analysis has been performed using coded units) for WPC

Factors	Log G'[1] (mPa)	K[2] (mPa s^n)	n[3]	Firmness[4] g	Water holding capacity[5] (%, w/w)	EPS production[6] (mg/kg fermented milk)	Cell growth[7], (Log CFU/g fermented milk)
Day-1							
Constant	2.950*	4.600^{NS}	0.047*	11.567*	22.066***	520.305*	1.057*
Ca	-0.187*	8.272^{NS}	0.003**	-0.313^{NS}	5.879**	-188.230^{NS}	0.009^{NS}
WPC	0.593*	67.441*	0.017*	1.599***	0.175^{NS}	15.664*	-0.205^{NS}
$(Ca)^2$	-0.009**	-0.779***	-0.0003*	0.028^{NS}	-0.436**	15.664^{NS}	0.004^{NS}
$(WPC)^2$	-0.061*	-7.305*	-0.002*	-0.254**	5.218^{NS}	19.723**	0.067*
CaxWPC	-0.055*	-4.717**	-0.0007***	-0.129^{NS}	0.168^{NS}	8.346^{NS}	-0.016^{NS}
R^2	0.813	0.910	0.919	0.770	0.705	0.7842	0.4355
F	2.652	36.54	41.25	12.03	8.60	13.08	6.48
Probability of *F*	<0.0001	<0.0001	<0.0001	<0.0001	0.0003	<0.0001	0.0002
Day-21							
Constant	3.513*	0.653***	0.671*	8.652*	29.116*	308.718*	0.303^{NS}
Ca	0.092^{NS}	0.064^{NS}	0.029***	-0.375^{NS}	4.293**	-26.740*	-0.130**
WPC	0.484*	1.115*	-0.027^{NS}	1.244***	-0.881^{NS}	-141.112*	-0.037^{NS}
$(Ca)^2$	-0.005^{NS}	-0.001^{NS}	-0.002***	-0.025^{NS}	-0.333**	4.51*	0.0003^{NS}
$(WPC)^2$	-0.065*	-0.131*	-0.003^{NS}	-0.327*	-0.318^{NS}	24.222**	-0.046*
CaxWPC	-0.030^{NS}	-0.090*	0.004^{NS}	0.091^{NS}	0.358^{NS}	1.617^{NS}	0.047*
R^2	0.669	0.952	0.853	0.785	0.753	0.979	0.519
F	3.408	71.08	20.88	13.15	10.96	163.96	9.05
Probability of *F*	0.0007	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[1]Elastic moduli measured at 1Hz, 0.5% strain; [2]Consistency index derived from constant of power law function; [3]Flow behaviour index derived from power law function; [4]Firmness measured at 1 mm/s, 50% compression; [5]Measured as percentage of weight of serum after application of 70xg, 10 min, 8°C; [6]EPS collected as dry crude EPS; [7]Change of colony forming units during fermentation;*significant difference at P<0.001; **significant difference at P<0.01; ***significant different at P<0.05.

Table 3. Regression coefficients of the second-order polynomial model for the response variables (analysis has been performed using coded units) for WPI

Factors	Log G'[1] (mPa)	K[2] (mPa s^n)	n[3]	Firmness[4] g	Water holding capacity[5] (%, w/w)	EPS production[6] (mg/kg fermented milk)	Cell growth[7], (Log CFU/g fermented milk)
Day-1							
Constant	4.008*	0.765**	0.849*	8.906*	14.554^{NS}	211.478*	0.479^{NS}
Ca	-0.082***	-0.101^{NS}	-0.030^{NS}	-0.443^{NS}	9.511**	-15.480***	0.236**
WPI	-0.014^{NS}	0.356**	-0.142*	0.774^{NS}	9.112^{NS}	-76.600*	0.229^{NS}
$(Ca)^2$	-0.011*	0.002^{NS}	0.004*	-0.005^{NS}	-0.895*	2.463*	-0.019*
$(WPI)^2$	-0.029**	-0.061*	0.019*	-0.167**	-0.977^{NS}	8.761*	-0.040***
CaxWPI	0.048*	-0.006^{NS}	0.002^{NS}	0.0233^{NS}	-0.797^{NS}	4.072***	0.0006^{NS}
R^2	0.895	0.870	0.831	0.832	0.795	0.943	0.428
F	2.626	24.10	17.71	17.88	5.28	62.33	6.29
Probability of *F*	<0.0001	<0.0001	<0.0001	<0.0001	0.0037	<0.0001	0.0002
Day-21							
Constant	4.139*	0.104^{NS}	0.508*	7.014*	45.507*	152.796*	-0.445*
Ca	0.040^{NS}	0.104^{NS}	0.017^{NS}	0.461^{NS}	2.085^{NS}	-2.999^{NS}	0.144*
WPI	0.176^{NS}	0.511*	0.089**	2.24*	-11.031**	-54.484**	0.468*
$(Ca)^2$	-0.011*	-0.011**	-0.006*	-0.073*	0.057^{NS}	0.912^{NS}	-0.009*
$(WPI)^2$	-0.046*	-0.055**	-0.014*	-0.369*	1.663**	4.234***	-0.056*
CaxWPI	0.008^{NS}	-0.030^{NS}	0.005^{NS}	-0.034^{NS}	0.418^{NS}	4.416***	-0.029
R^2	0.758	0.813	0.935	0.830	0.901	0.896	0.730
F	2.636	15.67		5.28	32.56	31.12	22.6
Probability of *F*	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

1Elastic moduli measured at 1Hz, 0.5% strain; 2Consistency index derived from constant of power law function; 3Flow behaviour index derived from power law function; 4Firmness measured at 1 mm/s, 50% compression; 5Measured as percentage of weight of serum after application of 70xg, 10 min, 8oC; 6EPS collected as dry crude EPS; 7Change of colony forming units during fermentation *significant difference at P<0.001; **significant difference at P<0.01; ***significant different at P<0.05.

Similarly, growth of *S. thermophilus* was restricted in whey substrate despite its ability to utilize major whey proteins, α-lactalbumin and β-lactoglobulin (Bertrand-Harb *et al*., 2003). WPI may be depleted from important low molecular weight micronutrients as a result of the manufacturing process, which in turn limited the culture growth (Lorenzen and Schrader, 2006). The effect of calcium on the growth of lactic acid bacteria has not been extensively studied. However, calcium is reported to increase cell growth of a species of soil bacteria under acid environment (Maccio *et al*., 2002).

In fresh fermented milk, addition of WPC and WPI significantly ($p < 0.001$) affected the EPS production diametrically in different way (Tables 2, 3); whilst the WPC supplementation improved it, WPI addition decreased it. As the EPS production in most cases was growth-coupled (Ruas-Madiedo *et al*., 2005), the low EPS concentration in WPI fermented milk appeared to be in accordance with the limited cell growth. The production of EPS by a bacterial culture in a whey-based growth medium was only possible in the presence of hydrolized WPC (Briczinski and Roberts, 2002). In general, the estimated EPS production in the freshly prepared WPC-fermented milk (maximum of 542 mg/kg from combination of 0% WPC and 4.6 mM Ca) was higher than that produced in the WPI-fermented milk (maximum of 386.8 mg/kg from combination of ~1% WPI and ~8.8 mM Ca). Statistically, the calcium addition in both WPC and WPI fermented milks decreased the EPS production, but was significant ($p < 0.05$) only in WPI fermented milk (Tables 2, 3). However, the apparent trend according to the model showed that calcium increased EPS production in both types of the supplemented fermented milk (Figures 1A, 2A). In the WPC fermented milk, increasing the Ca concentration improved the EPS production at any level of WPC. Calcium is reported to improve cell growth as well as EPS production under low pH condition (Maccio *et al*., 2002). Interaction between whey protein and calcium positively affected EPS production in all fresh and storage fermented milk, but was only significant ($p < 0.001$) for WPI fermented milk (Tables 2, 3)

The storage time reduced the amount of the EPS in both WPC (estimated maximum of ~333 mg/kg from combination of 1.7% WPC and 9.7 mM Ca) and WPI fermented milk (estimated maximum of 202 mg/kg from combination of 0% WPI and 5.2 mM Ca) (data not shown). In this case, the decline in the EPS concentration in the WPI fermented milk was less

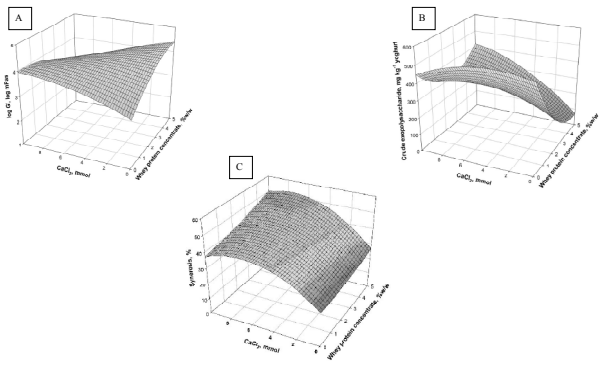

Figure 1. Typical estimated responses achieved by the response surface modeling showing the effects of the calcium chloride and whey protein concentrate additions on (A) the gel elastic modulus (G') (B) the EPS production by *Streptococcus thermophilus* and (C) the extent of the water holding capacity of the fermented milk batches after overnight cold storage

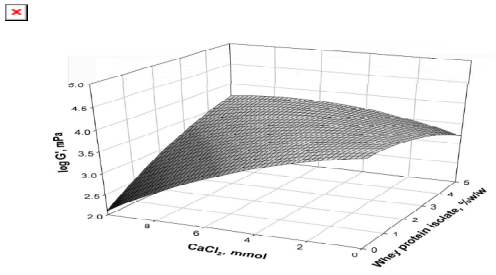

Figure 2. Typical estimated responses achieved by the response surface modeling showing the effects of the calcium chloride and whey protein isolate additions on (A) the gel elastic modulus (G') (B) the EPS production by *Streptoccocus thermophilus* and (C) the extent of the water holding capacity of the fermented milk batches after overnight cold storage

pronounced than that of the WPC fermented milk. The EPS reduction during fermented milk storage was also observed previously (Purwandari *et al*., 2007). The enzymatic degradation of the EPS beyond stationary phase was a common phenomenon observed (Degeest *et al*., 2002; Pham *et al*., 2000). The EPS may be incorporated in the culture metabolism when most of the nutrients in the medium were diminished (Tolstoguzov, 2003). Thus, the limited degradation of the EPS in WPI may be in conjunction with negative cell growth during storage. Trend in EPS concentration of storage WPC fermented milk was somewhat similar to that of the fresh fermented milk, in which WPC at medium concentrations adversely affected EPS concentration. During storage of WPI fermented milk, this reduction in the EPS concentration occurred in the region of high WPI-low Ca concentration.

Rheological and physical properties

The viscoelastic properties of fermented milk are commonly examined using small amplitude oscillatory measurement as elastic moduli (G') to assess its solid-like character. In general, the estimated log G' of the freshly prepared WPC fermented milk was higher (maximum of ~4.3 log mPa at 3.9% WPC and 0.91 mM $CaCl_2$) than that of the fresh WPI fermented milk (maximum of ~3.9 log mPa, at 1.6% WPI and 4.2 mM calcium) (Figures 1A, 2A). As the statistical analysis revealed, the elastic modulus of fermented milks upon the WPC supplementation was greatly ($p < 0.05$) increased by the WPC concentration (Table 2), which was in contrast to that of WPI fermented milk (Table 3). This effect in the fresh WPC fermented milk was diminished by high Ca concentration. The increasing effect of calcium concentration on G' of the fresh fermented milk was only significant ($p < 0.01$) in WPC fermented milk (Tables 2, 3) especially at its low concentration. In the WPI fermented milk, albeit statistically opposite effect of WPI on G', the G' was improved with the increase in the WPI concentration at high concentrations of Ca.

Native whey proteins usually disrupt the weak acid gel of fermented milk due to their 'inactive filler' nature (Lucey *et al*., 1999). However, our model describing WPC addition showed the opposite trend. Some whey denaturation may have likely taken place during heating of fermented milk-bases containing WPC at 90°C, enabling the formation of a complex between whey proteins and κ-casein, which altered the gel stiffness (Schorsch *et al*., 2001). On the other hand, Ca weakened the WPC gel (Lorenzen and Schrader, 2006), stabilized α-lactalbumin against unfolding and aggregation, thus prevented its denaturation in acidic environment (Pedersen *et al*., 2006). It also inhibited subsequent complexation between whey proteins with casein, leading to weak gel structure (Schorsch *et al*., 2001).

After 21 day of storage, the estimated log G' of both WPC (maximum of 4.4 mPa, from combination of 3.5% WPC and 0.4 mM Ca) and WPI (maximum of 4.38 mPa, from combination of 2.1% WPI and 2.6 mM Ca) fermented milks were higher than those of corresponding fresh fermented milks. Similar gel strengthening behaviour during storage was also previously noted in fermented milks produced with EPS producing cultures (Purwandari *et al*., 2007). Statistically, the WPC concentration substantially ($p < 0.001$) increased G' (Table 2), but the WPI concentration had no apparent effect ($p > 0.05$) (Table 3). The improvement of G' was more evident in WPI compared to that of WPC fermented milk. WPC contained more calcium, fat and phospholipid than WPI (Lorenzen and Schrader, 2006), that potentially hindered protein-protein interactions during cold storage in the acidic environment (Pedersen *et al*., 2006). Interaction between whey protein and Ca was only significant ($p < 0.05$) for fresh and storage WPI fermented milk (Table 3).

Firmness of the fermented milk gels was the textural characteristic analyzed in this work, while other parameters usually depicted by texture profile analysis were not assessed. Similar to G', fresh WPC fermented milk gel was harder (maximum firmness of 14 g, at 2.8% WPC and 0.04 mM calcium) than that of WPI fermented milk (maximum firmness of 9.8 g, at 2.4% WPI and 0.004 mM calcium). The firmness of WPC fermented milk was slightly but significantly altered by the types of mixture (Table 2). In WPI fermented milk, there was no apparent ($p > 0.05$) effect of supplementation on firmness of the gel. The calcium addition had no effect ($p > 0.05$) on firmness of both fresh WPC and WPI fermented milk (Tables 2, 3). In all fresh or stored fermented milk samples, there was no significant interaction ($p > 0.05$) of both whey protein and calcium.

After storage, unlike G', firmness of both fermented milks changed to a lesser extent. While estimated firmness of WPC declined (maximum of 9.8 g from combination of 2.0% WPC and 0.1 mM Ca), that of WPI was slightly greater (maximum of 10.9 g resulted from combination of 3.3% and 0.3 mM Ca) (data not shown). The effect of both whey protein additions on gel firmness was significant ($p < 0.05$ and $p < 0.01$ for WPC and WPI fermented milk, respectively). In stored WPC fermented milk, addition of WPC improved firmness, but after ~4% WPC, it leveled off. In WPC-supplemented fermented milk, calcium addition decreased firmness. In WPI fermented milk, high values of firmness were achieved by combination of WPI at all concentration and Ca at low concentration (up to ~4 mM). Increasing WPI concentration improved firmness. However, at high WPI concentration, addition of high concentration of Ca decreased it. Although not significant ($p > 0.05$), addition of Ca tended to increase firmness but gradually reduced firmness at high (~3 %) WPI concentration.

A close relation between G' and firmness was noted previously (Kealy, 2006). Moreover, a harder gel of WPC fermented milk maybe due to higher fat content in WPC. However, the firmness of fermented milk may be better correlated to a yield stress rather than to G'. Both firmness and yield stress are a measure of force to start breaking of gel, while G' was a measurement of gel strength upon oscillation. Nevertheless, in our work, firmness appeared to be

less affected by storage in comparison to G'.

Consistency index K is an indicator of the material resistance to deformation (Rao, 1999). Therefore, similar to G' and firmness, it also indicates the strength of gel, but more specifically related to shear. In our work, similar to G' and firmness, estimated maximum K values of fresh WPC fermented milk were greater (around ten fold) than those of WPI fermented milk. Whey protein concentration was the only factor significantly ($p < 0.001$ to $p < 0.05$ for WPC and WPI, respectively) contributing to the increase in K for both types of fermented milk (Tables 2, 3). In WPC fermented milk, addition of WPC increased K at any Ca concentration. However, increasing WPC concentration as Ca was continuously added gradually reduced K values considerably to reach very low value (close to 0 mPa s^n) at highest concentration of both Ca and WPC (data not shown). Similarly, addition of WPI up to ~4 % also increased K value at any level of Ca, after which K decreased slightly in about similar extent at all Ca concentrations. The effect of Ca was not significant ($p > 0.05$) on either WPC or WPI fermented milk (Tables 2, 3), but its decreasing effect on K of WPI fermented milk was apparent. In WPC fermented milk, Ca addition up to ~6 mM only very slightly increased K, but decreased it afterwards.

During storage, estimated K values of WPC fermented milk were substantially ($p < 0.001$) reduced around 20 times without considerable change in trend, while those of WPI fermented milk were not altered greatly ($p > 0.05$) (Table 2,3). This difference in the magnitude of reduction of K values during storage between WPC and WPI fermented milk was somewhat similar to that of firmness. Ca addition had no apparent influence ($p > 0.05$). While the trend in storage WPC did not differ from that of fresh one, Ca addition up to ~6 mM in storage WPI fermented milk increased K, but reduced it afterwards. WPI addition also increased K values. However, at high Ca concentration, K was reduced. Interaction between whey protein and Ca was only significant for fresh and storage WPC fermented milk ($p < 0.01$ and $p < 0.001$, respectively).

Syneresis or the whey expulsion can result from a weak gel incapable of retaining serum or gel compaction that leads to water expulsion from protein network (Lucey, 2001). The degree of syneresis of the two fresh fermented milks (Figures 1C, 2C) varied slightly, around 30-50% and 20-40% for WPC and WPI fermented milk, respectively. In fresh fermented milk, addition of calcium played a significant ($p < 0.01$) role in enhancing syneresis in both WPC and WPI fermented milk (Table 2, 3). In WPC fermented milk, addition of either WPC or Ca induced syneresis, although the effect of WPC was lesser than that of Ca. In the WPI fermented milk, the addition of WPI increased syneresis, followed by a steady decline at high concentration of both WPI (~4 %) and Ca (from ~5 mM).

After the storage, the syneresis of the WPC fermented milk was reduced slightly (around 20-50%), while that of the WPI fermented milk increased (up to ~80%), especially at high Ca concentrations. Although not statistically significant ($p > 0.05$), WPC intensified syneresis of storage fermented milk at any Ca concentrations with similar trend as that of fresh fermented milk. In the WPI fermented milk, however, increasing both WPI and Ca concentration intensified ($p < 0.01$) syneresis steadily. The influence of interaction between the two factors examined was not significant ($p > 0.05$) in all samples. Relating syneresis to parameters of the gel strength, especially G' and firmness, revealed negative correlations which became more significant (r ranged from -0.483 to –0.8816) after storage. This may indicate that syneresis in both fermented milks was induced by gel-weakening effect, which became more intense during storage. This is in contrast to normal fermented milk, which tended to reduce syneresis upon storage (Purwandari *et al*., 2007).

The correlation between parameters in this study revealed a significant negative correlation between the EPS concentration and all parameters of the gel strength: firmness, G', and K in both types of supplemented fermented milk. In most cases, the storage intensified the extent of these correlations. For example, coefficient of correlation between firmness and EPS concentration in fresh WPC fermented milk was –0.4370, it was then increased to –0.9263 after storage. Similarly, in the case of G', the coefficient was increased from –0.6059 in fresh fermented milk to -0.8852 after storage. Moreover, there was a shift of correlation between EPS concentration and syneresis of both fermented milks, from less significant negative (r of –0.3512 and –0.3087 in WPC and WPI fermented milks, respectively) in fresh fermented milk, became more significant positive (r of 0.4178 and 0.9426 in WPC and WPI fermented milks, respectively) after storage. Probably, although EPS was able to support water holding before storage, in the later stage as it was reduced in quantity, it contributed to the weakening structure leading to the increase in syneresis, as well as reduce in firmness, G' and K of some samples. EPS had been considered to be capable of hindering the development of protein-protein network (Tuinier *et al*., 2000).

Conclusion

The supplementation of non-fat EPS-containing fermented milk with calcium and whey protein affected microbial, physical and rheological characteristics. Data derived from small deformation measurement showed trends better than those of large deformation method. The calcium addition tended to weaken the structure, resulted in induced syneresis and lowered storage moduli as well as firmness. The addition of whey protein, on the contrary, tended to strengthen the gel structure as shown by the increase in storage moduli, consistency index K, and firmness. The effect of whey protein addition was only significant in WPC fermented milk. The interaction of calcium and whey proteins substantially reduced fermented milk gel strength. Therefore, in WPC fermented milk, low concentration of Ca and high concentration of WPC appeared to result in better fermented milk texture. In WPI supplemented fermented milk, both low concentration of Ca and WPI was apparently more supportive to texture.

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References

Amrane, A. and Prigent, Y. 1993. Influence of media composition on lactic acid production rate from whey by *Lactobacillus helveticus*. Biotechnology Letters 15(3): 239-244.

Barbut, S. 2006. Effects of caseinate, whey and milk powders on the texture and microstructure of emulsified chicken meat batters. LWT-Food Science and Technology 39(6): 660-664.

Bertrand-Harb, C., Ivanova, I.V., Dalgalarrondo, M. and Haertlle, T. 2003. Evolution of beta-lactoglobulin and α-lactalbumin content during yoghurt fermentation. International Dairy Journal 13(1): 39-45.

Briczinski, E.P. and Roberts, R.F. 2002. Production of an exopolysaccharide-containing whey protein concentrate by fermentation of whey. Journal of Dairy Science 85(12): 3189-3197.

De Witt, J.N. 1998. Nutritional and functional characteristics of whey proteins in food products. Journal of Dairy Science 81: 597-608.

Degeest, B., Mozzi, F. and De Vuyst, L. 2002. Effect of medium composition and temperature and pH changes on exopolysaccharide yields and stability during *Streptococcus thermophilus* LY03 fermentations. International Journal of Food Microbiology 79(3): 161-174.

Dogan, S.F., Sahin, S. and Summu, G. 2005. Effects of batters containing different protein types on the quality of deep-fat-fried chicken nuggets. European Food Research Technology 220: 502-508.

Donkor, O.N., Henriksson, A., Vasiljevic, T. and Shah, N.P. 2006. Effect of acidification on the activity of probiotics in yoghurt during cold storage. International Dairy Journal 16(10): 1181-1189.

Isleten, M. and Karagul-Yuceer, Y. 2006. Effects of dried dairy ingredients on physical and sensory properties of nonfat yogurt. Journal of Dairy Science 89(8): 2865-2872.

Jaros, D., Rohm, H., Haque, A., Bonaparte, C. and Kneifel, W. 2002. Influence of the starter culture on the relationship between dry matter content and physical properties of set-style yogurt. Milchwissenschaft-Milk Science International 57(6): 325-328.

Kealy, T. 2006. Application of liquid and solid rheological technologies to the textural characterisation of semi-solid foods. Food Research International 39(3):265-276.

Lorenzen, P.C. and Schrader, K. 2006. A comparative study of the gelation properties of whey protein concentrate and whey protein isolate. Lait 86(4): 259-271.

Lucey, J.A. 2001. The relationship between rheological parameters and whey separation in milk gels. Food Hydrocolloids 15(4-6): 603-608.

Lucey, J.A, Munro, P.A. and Singh, H. 1999. Effects of heat treatment and whey protein addition on the rheological properties and structure of acid skim milk gels. International Dairy Journal 9(3-6): 275-279.

Maccio, D, Fabra, A. and Castro, S. 2002. Acidity and calcium interaction affect the growth of *Bradyrhizobium* sp and the attachment to peanut roots. Soil Biology and Biochemistry 34(2): 201-208.

Mendoza, C., Peerson, J.M., Brown, K.H. and Lonnerdal, B. 2004. Effect of a micronutrient fortificant mixture and 2 amounts of calcium on iron and zinc absorption from a processed food supplement. American Journal of Clinical Nutrition 79(2): 244-250.

Myers, R.H. and Montgomery, D.C. 2002. Response Surface Methodolgy: process and product optimization using design experiments. New York: J. Wiley.

Patocka, G., Cervenkova, R., Narine, S. and Jelen, P. 2006. Rheological behaviour of dairy products as affected by soluble whey protein isolate. International Dairy Journal 16(5): 399-405.

Pedersen, J.B., Fojan, P., Sorensen, J. and Petersen, S.B. 2006. Towards control of aggregational behaviour of α-lactalbumin at acidic pH. Journal of Fluorescence 16(4): 611-621.

Perales, S., Barbera, R., Lagarda, M.J. and Farre, R. 2006. Fortification of milk with calcium: Effect on calcium bioavailability and interactions with iron and zinc. Journal of Agricultural and Food Chemistry 54(13): 4901-4906.

Pham, P.L., Dupont, I., Roy, D., Lapointe, G. and Cerning, J. 2000. Production of exopolysaccharide by *Lactobacillus rhamnosus* R and analysis of its enzymatic degradation during prolonged fermentation. Applied and Environmental Microbiology 66(6):

2302-2310.

Pilvi, T.K., Korpela, R., Vapaatalo, H. and Mervaala, E. 2006. High calcium diet with whey protein inhibits weight gain in a model of diet induced obesity. Atherosclerosis Supplements 7(3): 431-431.

Purwandari, U., Shah, N.P. and Vasiljevic, T. 2007. Effects of exopolysaccharide-producing strains of *Streptococcus thermophilus* on technological and rheological properties of set-type yoghurt. International Dairy Journal 17(11): 1344-1352.

Purwandari, U. and Vasiljevic, T. 2009. Rheological properties of fermented milk produced by a single exopolysaccharide producing *Streptococcus thermophilus* strain in the presence of added calcium and sucrose. International Journal of Dairy Technology 62(3): 411-421.

Puvanenthiran, A., Williams, R.P.W. and Augustin, M.A. 2002. Structure and visco-elastic properties of set yoghurt with altered casein to whey protein ratios. International Dairy Journal 12(4): 383-391.

Rao, M.A. 1999. Rheology of Fluid and Semisolid Foods, Principles and Applications. Maryland: Aspen Publisher Inc.

Relkin, P. and Sourdet, S. 2005. Factors affecting fat droplet aggregation in whipped frozen protein-stabilized emulsions. Food Hydrocolloids 19(3): 503-511.

Remeuf, F., Mohammed, S., Sodini, I. and Tissier, J.P. 2003. Preliminary observations on the effects of milk fortification and heating on microstructure and physical properties of stirred yogurt. International Dairy Journal 13(9): 773-782.

Resch, J.J., Daubert, C.R. and Foegeding, E.A. 2005. The effects of acidulant type on the rheological properties of beta-lactoglobulin gels and powders derived from these gels. Food Hydrocolloids 19(5): 851-860.

Rimada, P.S. and Abraham, A.G. 2006. Kefiran improves rheological properties of glucono-δ-lactone induced skim milk gels. International Dairy Journal 16(1): 33-39.

Ruas-Madiedo, P., Alting, A.C. and Zoon, P. 2005. Effect of exopolysaccharides and proteolytic activity of *Lactococcus lactis* subsp. *cremoris* strains on the viscosity and structure of fermented milks. International Dairy Journal 15(2): 155-164.

Ruger, R.P., Baer, R.J. and Kasperson, K.M. 2002. Effect of double homogenization and whey protein concentrate on the texture of ice cream. Journal of Dairy Science 85(7): 1684-1692.

Sanchez-Hidalgo, V.M., Flores-Huerta, S., Matute-Gonzalez, G., Urquieta-Aguila, B., Bernabe-Garcia, M. and Cisneros-Silva, I.E. 2000. A fortifier comprising protein, vitamins, and calcium-glycerophosphate for preterm human milk. Archives of Medical Research 31(6): 564-570.

SAS. 1996. SAS/STAT Softwares: Changes and enhancements through release 6.11. Cary, NC: SAS Inst Inc.

Schaafsma, G. 2006a. Health issues of whey proteins: 1. Protection of lean body mass. Current Topics in Nutraceutical Research 4(2): 113-121.

Schaafsma, G. 2006b. Health issues of whey proteins: 2. Weight management. Current Topics in Nutraceutical Research 4(2): 123-126.

Schorsch, C., Wilkins, D.K., Jones, M.G. and Norton, I.T. 2001. Gelation of casein-whey mixtures: effects of heating whey proteins alone or in the presence of casein micelles. Journal of Dairy Research 68(3): 471-481.

Siddiqui, S.M.K., Chang, E., Li, J., Burlage, C., Zou, M., Buhman, K.K., Koser, S., Donkin, S.S., Teegarden, D. 2008. Dietary intervention with vitamin D, calcium, and whey protein reduced fat mass and increased lean mass in rats. Nutrition Research 28 (11): 783-790.

Singh, G., Arora, S., Sharma, G.S., Sindhu, J.S., Kansal, V.K. and Sangwan, R.B. 2007. Heat stability and calcium bioavailability of calcium-fortified milk. LWT-Food Science and Technology 40(4): 625-631.

Sodini, I., Mattas, J. and Tong, P.S. 2006. Influence of pH and heat treatment of whey on the functional properties of whey protein concentrates in yoghurt. International Dairy Journal 16(12): 1464-1469.

Tolstoguzov, V. 2003. Thermodynamic considerations on polysaccharide functions. Polysaccharides came first. Carbohydrate Polymers 54(3): 371-380.

Tuinier, R., Dhont, J.K.G. and De Kruif, C.G. 2000. Depletion-induced phase separation of aggregated whey protein colloids by an exocellular polysaccharide. Langmuir 16(4): 1497-1507.

Tuinier, R., Rieger, J. and de Kruif, C.G. 2003. Depletion-induced phase separation in colloid-polymer mixtures. Advances in Colloid and Interface Science 103(1): 1-31.

van Geel-Schutten, G.H., Flesch, F., ten Brink, B., Smith, M.R. and Dijkhuizen, L. 1998. Screening and characterization of *Lactobacillus* strains producing large amounts of exopolysaccharides. Applied Microbiology and Biotechnology 50(6): 697-703.

Vasbinder, A.J., de Velde, F.V. and de Kruif, C.G. 2004. Gelation of casein-whey protein mixtures. Journal of Dairy Science 87(5): 1167-1176.

Zisu, B. and Shah, N.P. 2003. Effects of pH, temperature, supplementation with whey protein concentrate, and adjunct cultures on the production of exopolysaccharides by *Streptococcus thermophilus* 1275. Journal of Dairy Science 86(11): 3405-3415.