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Factors affecting the creaming of human milk

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ABSTRACT

The creaming properties of human milk have not been widely studied to date, and a mechanism for this phenomenon is not known. Here, the natural creaming of human milk, as affected by temperature and pre-treatments, was studied using dynamic light-scattering. The creaming rate of human milk increased with temperature in the range 5 °C to 40 °C. Freezing human milk at –20 °C and thawing at room temperature had little influence on creaming. Compared with bovine milk, human milk showed a faster creaming rate at 40 °C, but a slower rate at 5 °C, suggesting a lack of cold agglutination; the mechanisms of creaming were also shown to differ in response to heat treatment. This study expands the current knowledge on milk creaming, and may have potential application to storage and handling of human milk in hospitals or homes, therefore supporting optimal nutrition of infants.

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1. Introduction

Human milk is essential as our first natural food, providing both nutrients and immunity to infants. Infants, especially preterm infants, who receive no or limited milk from their own mothers, can also be fed by human milk from donors as a substitute. Those milk donations are screened, received, pasteurised and stored in human milk banks (Hartmann, Pang, Keil, Hartmann, & Simmer, 2007). Moreover, working mothers may store their milk in the fridge (4 °C) from 24 h to 8 d or in the freezer (–18 °C) for even longer times (Hands, 2003; Weiss, 2005).

Natural creaming of milk occurs during storage because of the lower density of milk fat globules compared with milk serum, which leads to fat rising to the top under the influence of gravity. The most well studied subject of creaming has been bovine milk, since at least the work of Babcock (1889). Creaming properties of caprine (El-Ghannam, Attia, & Zeidanr 1986), buffalo (Abo-Elnaga, 1966), carabao (Gonzales-Janolino, 1968) and camel (Farah & Rüegg, 1991) milk have also been studied, and all of these showed much slower creaming rates than bovine milk at refrigeration temperature. Human milk has been reported to cream more slowly than bovine milk at body temperature (Whittlestone & Perrin, 1954), but overall the creaming of human milk and the factors affecting this process have not been extensively studied to date.

The creaming rate, *v*, which is used to describe the creaming process in milk, is described by Stokes' Law (Walstra, 1995):

$$v = \frac{g(\rho_p - \rho_f)d^2}{18\eta_p} \quad (1)$$

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where g is acceleration due to gravity, d is the diameter of the fat globule, ρ_p and ρ_f are the densities of the plasma and fat, respectively, and η_p is the viscosity of the plasma.

However, applying Stokes' Law solely based on the size of individual milk fat globules cannot exactly predict the real creaming rate. For example, fresh bovine milk creams much faster than predicted by Stokes' Law at cold temperatures, which is explained by a phenomenon called cold agglutination, caused by the flocculation of fat globules (Sharp & Krukovsky, 1939). Cold agglutination is facilitated by agglutinins, which attach to the milk fat globule membrane (MFGM) and cause fat globules to aggregate; these agents have been identified as immunoglobulin M (IgM) (Euber & Brunner, 1984; Payens, Koops, & Mogot, 1965), and immunoglobulin A (IgA) (D'Incecco et al., 2018; Honkanen-Buzalski & Sandholm, 1981) in bovine milk. A homogenisation-labile component, termed the skim milk membrane but now sometimes referred to as exosomes or extracellular vesicles (Benmoussa et al., 2017), was also reported to be involved in cold agglutination (Euber & Brunner, 1984). Other factors that have been reported to influence the rate of creaming of bovine milk include the presence of bacteria (Jenness, Shipe, & Sherbon, 1974) and somatic cells (Geer & Barbano, 2014), heating (>70 °C), and high pressure treatment (≥400 MPa) (Huppertz, Fox, & Kelly, 2003).

Moreover, techniques for measuring creaming have improved. Traditionally, the creaming of milk was measured as the volume of cream produced from a specified volume of milk in a glass tube, of specific dimensions, at a stated temperature after certain time intervals, usually up to 24 h (Dunkley & Sommer, 1944; Euber & Brunner, 1984; Hammer, 1916; Kenyon, Jenness, & Anderson, 1966). More recently, the Turbiscan instrument has used to study the creaming behaviour of milk, based on multiple light-scattering principles; this gives information on creaming properties

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even when nothing is visible to the naked eye due to the opacity of milk samples, therefore removing the ambiguity of visual observation (Celia, Trapasso, Cosco, Paolino, & Fresta, 2009; Juliano et al., 2011).

In this study, the natural creaming properties of human milk under different temperature conditions were analysed using the Turbiscan stability analyser and compared with the creaming behaviour of bovine milk to understand the reasons behind any differences that might exist between them. In addition, the influence of different pre-treatments, such as freezing and pre-heating, which may be used in hospitals or milk banks to stabilise human milk, on the creaming were studied.

2. Materials and methods

2.1. Human milk samples

Ethical approval for this study was granted by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Cork, Ireland, and fresh human milk samples (collected after the infant was satisfied, and hence being largely mid- to hind-milk) were collected from Cork University Maternity Hospital, Cork, Ireland. Full-term individual fresh milk samples from healthy mothers who were one week after birth (i.e., first week of lactation) were collected and used within 24 h. Fresh raw bovine milk was obtained from a local market. Both human and bovine milk were stored at refrigeration temperatures until the creaming analysis.

2.2. Compositional analysis

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The fat, protein, lactose and total solids content of human milk was determined with a human milk analyser (MIRIS, Uppsala, Sweden) based on mid-infrared (mid-IR) transmission spectroscopy principles. Subsamples of fresh human milk (n = 12) were collected for compositional analysis were frozen at –80 °C, thawed at 4 °C, warmed to 40 °C and homogenised using a sonicator (MIRIS) before analysis. The fat, protein, lactose and total solids content of fresh bovine milk samples was measured using a Milkoscan FT 120 (Foss Electric, Hillerød, Denmark).

2.3. Size distribution of milk fat globules

The size distribution of milk fat globules (MFG) in human and bovine milk samples was determined with a Mastersizer 3000 laser particle size analyser (Malvern Instruments, Malvern, UK) equipped with a He–Ne laser (λ = 633 nm). The refractive indices of the fat globule and of the dispersant (water) at 25 °C were taken as 1.458 and 1.33, respectively, and the absorbance of fat globules was taken as 0.01 (Michalski, Briard, & Michel, 2001). The surface area mean diameter D[3,2], the volume-weighted mean diameter D[4,3], and the standard percentiles values, D_v10, D_v50 and D_v90 were measured in triplicate, as described earlier (Ménard et al., 2010).

2.4. Measurement of density of milk fat and viscosity of skimmed milk

Bovine milk fat was obtained by melting commercial butter and allowing to stand, followed by recovery of the upper fat layer. Human milk fat was obtained from a sample of donor milk that was agitated at high speed to destabilise the emulsion and recover a fat-rich phase, which was separated as for separation of bovine milk fat

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from butter to obtain a fat sample. The density of these human and bovine milk fat samples was determined using a pycnometer (SciLabware Ltd., Stoke-on-Trent, UK) at 20 °C by following standard method ASTM D3505 (ASTM, 2018). To obtain skim milk, milk was centrifuged at 3000 × g for 20 mins at 5 °C. Flow time of human skim milk, bovine skim milk, and pure water were measured by a U-shape glass viscometer (VWR International Ltd., Dublin, Ireland) at 5 °C following standard method ASTM D445 (ASTM, 1997), and viscosities of human and bovine skim milk were calculated by:

$$\eta_{milk} = \frac{t_{milk}\,\rho_{milk}}{t_{H_2O}\,\rho_{H_2O}}\,\eta_{H_2O} \qquad (2)$$

where t_{milk} and t_{H_2O} are the time (seconds) flow through the specific area of U-shape viscometer of milk and water, respectively; ρ_{milk} is the density of skimmed human (1.03×10^3 kg m^{-3}; Neville et al., 1988) or skimmed bovine milk (1.04×10^3 kg m^{-3}; Ma & Barbano, 2000) at 5 °C; ρ_{H_2O} is the density of water at 5 °C, which is 1.00×10^3 kg m^{-3} (Jones & Harris, 1992); η_{H_2O} is the viscosity of water at 5 °C, which is 1.52 mPa s (Kestin, Sokolov, & Wakeham, 1978). Fresh milk samples were measured and each analysis was performed in triplicate.

2.5. Sample preparation for creaming analysis

For creaming analysis, five different temperatures, 5 [number of samples (n) = 11)], 20 (n = 10), 37 (n = 13), 40 (n = 11) and 45 °C (n = 3) were selected in individual experiments for simulation of refrigeration, room temperature (RT), mammalian body temperature, and higher temperatures. Refrigerated milk samples were incubated in a water bath for 20 min to achieve the target temperature, and each

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sample (3 mL) was then inverted at least 10 times and placed in the Turbiscan immediately to make sure the back-scattering profile of the first scan was a flat line, and then scanned at each temperature for 10 h. That time was chosen as a compromise between the freshness and creaming duration of the milk, and because, after this time, the peak thickness in the cream layer of human milk reaches a plateau (as determined in preliminary work).

In the experiment with pre-heating, fresh milk samples were pre-heated in a water bath at 70 °C for 10 min. In a separate experiment, bovine whey protein isolate (WPI90, Carbery Group Ltd., Ballineen, Cork, Ireland) and immunoglobulins (Igs, isolated as described by McGrath, 2014), was added to human milk to achieve 1 g L^{-1} final Igs (average level of human and bovine Igs in milk). In a 'phase-reversal' experiment, human and bovine milk cream and serum were separated at 45 °C, which can concentrate agglutinins from the whole milk to the milk cream layer, and recombined with the opposite fraction as described by Jennes and Parkash (1971).

2.6. Creaming analysis

The Turbiscan$^{LAB™}$ (Formulaction, Ramonville St. Agne, France) with an 800 nm near infrared (NIR) light source was used to study particle migration (creaming) in milk samples. The Turbiscan is based on multiple light scattering theory, where NIR photons are transmitted or back-scattered from the sample to transmission (T) or backscattering (BS) detectors (Carrentero, Villepin, Brunel, & Carries, 2005).

Parameters quantified to describe creaming were the Turbiscan stability index (TSI), the mean value (of ΔBS, %), the peak thickness (ΔH), and the migration rate. The TSI was calculated by summing up the changes in BS at all measured positions,

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based on a scan-to-scan difference, over total sample height or a selected zone (Carrentero et al., 2005). In this study, the selected zone is the area where the cream layer forms; when the TSI value increases, the stability of the system decreases. The mean value represents changes in the concentration and size of particles, i.e., signal variation of ΔBS (%). It was calculated at the cream layer and for the bottom layer (lowest 20% of total height) of samples, and the ΔH measures the depth of the cream layer (represented by a peak in back-scatter data) that forms. A threshold value was set as 2% ΔBS. The slope of the curve of the ΔH as a function of time reflects the migration of the fat globules moving upwards within samples; the migration rate, calculated as the slope of the initial linear part of the ΔH plot, was taken to represent the creaming rate.

2.7. Statistical analysis of data

Data were analysed using Minitab® v18 (Minitab Inc., State College, PA, USA). Prior to analysis, data were tested for normality using the Anderson-Darling test. All TSI data, which were not normally distributed, were transformed according to a Box-Cox analysis. GLM ANOVA or one-way ANOVA followed by a paired multiple comparison test (Tukey's test) were used as appropriate. Human and bovine fat globule size distribution parameters were analysed using a 2-sample T-test. For all statistical analysis, the level of significance, α, was set at 0.05.

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3. Results

3.1. Composition, size distribution and density of milk fat globules of human and bovine milk

The averaged composition of fresh human milk samples and fresh bovine milk samples tested in this study is reported in Table 1 (details for the composition of milk samples from individual mothers are shown in Supplementary material Table S1). Compared with fresh bovine milk, human milk had lower levels of lipids and protein, but a higher level of carbohydrate and solids-not-fat content.

Differences in fat globule size between human and bovine milk were minor, with fat globules in human milk having a slightly lower average D[3,2] (2.3 ± 0.3 μm) and higher average D[4,3] (4.1 ± 0.9 μm) than those in bovine milk (2.4 ± 0.5 μm and 3.7 ± 0.3μm, respectively) (Table 2). In case of the size distribution of human MFGs, 10% of particles were below 1.1 ± 0.3 μm, and 50% of particles below 3.5 ± 0.7 μm, both of which are lower than the values presented for bovine milk; however, the average D_v90 of human MFGs (7.1 ± 1.6 μm) was higher than that in bovine milk (6.6 ± 0.36 μm). Overall, statistical analysis of human and bovine MFG size distribution parameters showed no significant difference ($P > 0.05$) between the parameters measured for the two types of milk.

The densities of human and bovine milk fat at 20 °C were $0.88 \times 10^3 \pm 0.46$ kg m^{-3} and $0.88 \times 10^3 \pm 0.61$ kg m^{-3}, respectively. The viscosity of skimmed human milk at 5 °C was measured as 2.53 ± 0.08 mPa s, which was lower than that of skimmed bovine milk at 5 °C (3.45 ± 0.02 mPa s).

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3.2. Creaming profiles of human and bovine milk at different temperatures

The formation of the layer of fat droplets (creaming) is illustrated in the back-scattering profiles of the samples (Supplementary material Fig. S1) as an increase in ΔBS at the top of samples over time, while a decrease of the back-scattering is indicative of clarification in the middle and lower phases of samples. As calculated from the backscattering profiles, the TSI values of cream layers (TSI_{cream}) in both types of milk increased with time (Fig. 1). In human milk, the TSI_{cream} increased with temperature from 5 to 40 °C, and there were significant differences ($P < 0.05$) between TSI_{cream} at 5 °C and TSI_{cream} at 40 °C at each time point.

Bovine milk held at 5 °C had a lower TSI_{cream}, i.e., a higher stability, than that at 40 °C during the first hour; however, instability increased dramatically between 1 and 4 h of measurement. The average TSI_{cream} values of human milk were lower at 5 °C but higher at 40 °C, compared with those of bovine milk at each time point during 10 h. However, no significant difference ($P > 0.05$) of TSI_{cream} was found between bovine and human milk at 10 h at either temperature.

The mean value of ΔBS in cream layer (MV_{cream}), which can be estimated from ΔBS profiles (Supplementary material Fig. S2), increased with temperature for human milk (Fig. 2A), i.e., the higher the temperature, the more rapidly the fat globules concentrated in the human milk cream layer. The averages of MV_{cream} for human milk ranged from 9.5% to 16.3% after 10 h from 5 °C to 40 °C. The average MV_{cream} for bovine milk after the same time at 5 °C and 40 °C were similar to each other (Fig. 2B). However, the time for bovine cream to reach the maximum ΔBS decreased from 6 h at 5 °C to 3 h at 40 °C. The rate of increase in ΔBS for milk from both species started to decrease after around 2 h creaming at all temperatures;

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however, this decrease in rate was more significant for bovine milk than for human milk.

The mean value in the bottom layers (MV_{bottom}) of the milk samples decreased from over time due to the fat globules moving upwards (Fig. 2C,D). In the bottom layer, human milk held at 20, 37, and 40 °C showed similar initial behaviour, while milk held at 5 °C had a lower initial rate of the MV_{bottom} curve. Interestingly, human milk held at 20 °C had a higher decrease of MV_{bottom}, i.e., a more complete clarification, compared with that at 5 °C. Moreover, in the bottom layer of both human and bovine milk, the clarification was more extensive at 5 °C than at 40 °C.

The peak thickness (ΔH) of human milk cream layer varies due to creaming temperature at different time points. As seen from Table 3, there were significant differences ($P < 0.05$) in ΔH among temperatures at 0.5 and 4 h, whereas there was no significant difference ($P > 0.05$) in ΔH among temperatures at 10 h creaming. Compared with bovine milk (Fig. 3), ΔH values of human milk cream increased steadily to a maximum at the end of 10 h, whereas it reached a plateau for bovine milk after 2 h at 5 °C. There was a lag phase for bovine milk cream formation at 5 °C, which was not seen 40 °C, while human milk started to cream earlier than bovine milk at both 5 °C and 40 °C. The final ΔH value of the human milk cream layer at 5 °C was lower than that at 40 °C, whereas it was notably higher for bovine milk at 5 °C than at 40 °C.

The average creaming rate, i.e., the migration rate of MFGs, or clusters thereof, of human milk increased with temperature (Table 4); there was a significant difference ($P < 0.05$) between rates at 5 °C and 40 °C. The rate was lower than that of bovine milk at 5 °C, whereas it was higher than that of bovine milk at 40 °C, suggesting different mechanisms of creaming in the milk of the two species. However,

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282 there was no significant difference ($P > 0.05$) in creaming rate between bovine and
283 human milk at 5 °C or at 40 °C.
284
285 *3.3. Influence of freezing-thawing and preheating on creaming*
286
287 The TSI cream values of individual human milk samples after different pre-
288 treatments as a function of time are shown in Fig. 4. Refrigeration for 1 week resulted
289 in a lower TSI_{cream} at each time point, whereas a 9-month frozen and thawed milk had
290 a higher TSI_{cream} at each time point. As seen in Fig. 4, short-term frozen storage (one
291 week) has less impact on the physical stability of human milk than refrigerated
292 storage and longer term frozen storage. However, no significant difference ($P > 0.05$)
293 was found among TSI_{cream} values for fresh milk, one-week refrigerated milk, one-
294 week frozen milk, and 9-month frozen milk at all time points.
295 The effect of preheating on human milk creaming was also studied. Compared
296 with the control, the TSI of human milk cream layer decreased significantly ($P < 0.05$)
297 after preheating (Supplementary material Fig. S3). The final peak thickness of the
298 cream layer was lower after preheating at 70 °C for 10 min; combined with the fact
299 that ΔBS also decreased significantly ($P < 0.05$) compared with the control, which
300 suggests that preheating human milk led to impaired creaming capacity.
301
302 *3.4. Mechanistic studies of creaming of human milk*
303
304 When human and bovine milk were mixed in a ratio of 1:1 (v/v), the mixture
305 behaved in a manner closer to bovine milk than to human milk (Fig. 5A), with a lag in
306 time before creaming commenced as observed above, but then the migration rate

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increased rapidly. When the cream from human milk was mixed with bovine skimmed milk, and vice versa, the recombined milk prepared by mixing human cream and bovine skim milk had higher TSI_{cream} (from 10 to 50 min, Fig. 5B), MV_{cream} (0 to 7 h, Fig. 5C), final ΔH of the cream layer, and initial creaming rate at each time point, compared with fresh bovine milk, fresh human milk, or milk recombined from skimmed human milk and bovine cream (Fig. 5D).

After the addition of bovine WPI (0.83%, w/v) to human milk, both the peak thickness and the mean ΔBS increased slightly. The initial creaming rates of human milk also increased after addition of both bovine WPI and bovine Igs, and the addition of bovine Igs had a higher impact on human milk creaming than the addition of WPI. However, the increases due to addition of bovine WPI and Igs were not significant ($P > 0.05$) in the case of TSI_{cream} (Supplementary material Fig. S4) at each time point.

4. Discussion

To our knowledge, this is the first detailed study on creaming behaviour of human milk, which contributes to knowledge of physico-chemical properties of human milk, specifically its creaming and cold agglutination behaviour. A similar or even higher creaming rate of human milk than that of bovine milk at warm temperature (40 °C) is inconsistent with the only report on human milk creaming, which suggested that the rate of creaming of human milk was lower than that in bovine milk at 37 °C (Whittlestone & Perrin, 1954). This inconsistency may be due to the high individual variation of human milk samples, more advanced analytical methodologies, and data coming from a larger sample size in our study. In this study, fresh human milk samples were obtained in the early stages of lactation (one week

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post-partum), when milk contains a lower fat content and a higher protein content than in mature human milk (Gidrewicz & Fenton, 2014). A lactational effect was noticed and samples at 8 and 24 weeks post-partum appear to have higher values of peak thickness than samples at 1 week post-partum (data not shown). Mid- to hind-milk, which was collected after the babies have been satisfied in this study, may contain a higher level of fat than foremilk (Mizuno et al., 2009); however, the milk fat globule size is not expected to be significantly different (Mizuno et al., 2009). The content of macronutrients of human and bovine milk measured was, however, consistent with the literature values (Anderson, Atkinson, & Bryan, 1981; Bauer & Gerss, 2011; Gidrewicz & Fenton, 2014; Guo & Hendricks, 2008). The lower average fat content and a higher average solids-not-fat content in human milk than in bovine milk samples may slow fat separation in the milk (Webb & Hall, 1935), and may partly explain the observed slower creaming rate of human milk than bovine milk at 5 °C.

The range of MFG size of human and bovine milk was similar to those reported by Rüegg and Blanc (1981), although some studies reported a higher MFG size of human milk than bovine milk (Lopez, Cauty & Guyomarc'h. 2015; Ma, Zhang, Wu, & Zhou, 2019). It has previously been noted that the human milk expressed from the fourth day to the first month of lactation, as for samples in this study, can have a lower average size than that of colostrum or mature milk from the first month until the end of lactation (Michalski, Briard, Michel, Tasson, & Poulain, 2005). According to Stokes' Law, the creaming rate rises when the diameter of MFG increases. A small extent of fat globule clustering, which increases the effective diameter of MFGs, was found in human milk at 5 °C, as the average MFG size of human milk cream layer

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decreased after dilution with 1% SDS (Supplementary material Table S2), which has also been reported in bovine cream (Boode, 1992; D'Incecco et al., 2018).

Considering the average diameter D[3,2] of human MFGs during the first week of lactation (2.27 μm) and the average of bovine MFG diameter (2.35 μm), the creaming rate for individual MFGs can be predicted by Stokes' Law [Equation (1)]. In this estimation, it is assumed that densities of human and bovine milk serum at 5 °C are 1.03×10^3 kg m^{-3} and 1.04×10^3 kg m^{-3}, respectively (Neville et al., 1988; Ma & Barbano, 2000), and, based on measurements in this study, densities of human and bovine milk fat are both 0.88×10^3 kg m^{-3}, and viscosities of human and bovine milk at 5 °C are 2.53 mPa s and 3.45 mPa s, respectively. On this basis, the calculated creaming rates of human and bovine milk are 0.60 mm h^{-1} and 0.51 mm h^{-1}, respectively. The predicted creaming rates for human and bovine milk are thus both lower than the measured averages (0.84 mm h^{-1} for human milk and 1.75 mm h^{-1} for bovine milk) at 5 °C, especially for bovine milk, which may reflect the occurrence of cold agglutination in bovine milk.

IgM and IgA are reported as major agglutinins to accelerate bovine milk creaming during cold storage (D'Incecco et al., 2018; Hansen, Larsen, & Wiking, 2019), but remain in skim milk after separation at 45 °C (Jennes & Parkash, 1971). Although the protein content of human milk was lower than that of bovine milk, human milk is reported to contain a higher percentage of Igs (approximately 16% of total whey proteins) than bovine milk (approximately 10% of total whey proteins; Guo & Hendricks, 2008). Also, human milk has IgA-dominated Igs, whereas bovine milk Igs are IgG-dominated (Hurley & Theil, 2011). The level of IgA has been reported to be 0.13 kg m^{-3} and 1 kg m^{-3} for bovine and human milk, respectively, whereas the level of IgM is 0.04 kg m^{-3} and 0.1 kg m^{-3} for bovine and human milk,

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respectively (Hurley, 2003), i.e., human milk contains higher levels of agglutinins (IgA and IgM). However, the cream volume and the creaming rate of human milk at 5 °C were lower than that of bovine milk (Fig. 3, Table 4), which may result from a higher extent of agglutinins being associated with bovine milk fat globules than human milk. This is supported by the fact that the creaming ability of human milk was increased after mixing with bovine skim milk.

There are two possible reasons why human milk contains high levels of agglutinins, but has a lower creaming rate than bovine milk at 5 °C. Firstly, although hydrophobic interactions will be weakened at low temperatures, bovine milk has been reported to have the most hydrophobic secretory IgA among species, including humans, which can result in a higher level of bovine IgA associated with fat globules (Honkanen-Buzalski & Sandholm, 1981). Secondly, competition between soluble antigens and MFGM antigens to capture IgM may occur in human milk (Euber & Brunner, 1984). It is known that the interaction of IgM with MFG is specific and involves carbohydrate moieties (Euber & Brunner, 1984); therefore, milk oligosaccharides and κ-casein molecules containing carbohydrate moieties may be involved in the interaction of IgM. The oligosaccharides have not been considered as a factor in bovine milk due to their low concentration (Euber & Brunner, 1984), but should be considered in human milk due to their higher levels (Kunz & Rudloff, 1993). Moreover, the content of sialic acid, which has been demonstrated to inhibit milk creaming (Euber & Brunner, 1984), is much higher in human milk than in bovine milk (Wang & Brand-Miller, 2003; Wang, Brand-Miller, McVeagh, & Petocz, 2001). Further research on the reasons behind the difference of creaming between bovine and human milk appears warranted.

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Human milk was stable following short-term freezing, which is consistent with an unchanged size distribution of human MFG before and after freezing (data not shown). It has been reported that no significant change in concentration occurs for IgA, the major agglutinin in mature human milk, at –20 °C for 1 month (Evans, Ryley, Neale, Dodge, & Lewarne, 1978) or 3 months (Reynolds et al., 1982). In contrast, a decrease in IgA level after 4 weeks at –20 °C has also been reported (Akinbi et al., 2010), and Ramírez-Santana et al. (2012) reported a decrese in IgA concentration in human colostrum when the frozen storage period was increased to 12 months, indicating that IgA can be temperature labile. Moreover, although the MFG can be resistant to lipolysis as long as its structure remains intact, slow freezing or long-term freezing can lead to the destruction of MFGM and allow access of lipases to the core of MFG, i.e., to triglycerides (Berkow et al., 1984; Munkwitz, Berry, & Boyer, 1933).

Pre-heating human milk at 70 °C for 10 min slowed cream separation and decreased the volume fraction of milk fat in cream layer, which has also been found for bovine milk (Caplan, Melilli, & Barbano, 2013). One possible reason could be that heat treatment increased the viscosity of the serum phase and thereby reduced the rate of creaming. Also, it has been hypothesised that heat-induced denaturation of proteins can result in interactions with milk fat globules and therefore increase their density (Caplan et al., 2013). A thinner cream layer can be thus formed after preheating (Supplementary material Fig. S3), shown as a lower ΔH of cream layer in preheated human milk than the untreated sample.

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5. Conclusion

The creaming behaviour of human milk was affected by temperature and pre-treatments, including freezing-thawing and preheating. The creaming capacity of human milk was correlated with the temperature in the range 5 to 40 °C. Short-term freezing-thawing had no effect on creaming while heating at 70 °C for 10 min slowed the creaming of human milk. Similarities and differences of human and bovine milk creaming behaviour at low temperature are discussed, and may be caused by the fat content, the properties of MFGM, the presence of agglutinins (IgA and IgM), and competition for attachment of agglutinins in milk serum. This study provides the information for both mothers and human milk banks in terms of handling and storing human milk and feeding infants with optimal methods in terms of ensuring proportionate levels of fat alongside other constituents.

Acknowledgement

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Figure legends

Fig. 1. Turbiscan stability index (TSI) distribution of the cream layer for fresh human milk (A) and fresh bovine milk (B) at 5 °C (□), 20 °C (▨), 37 °C (▥), and 40 °C (■) as a function of time. Horizontal lines inside boxes indicate the median value. Lines extending vertically from the boxes indicate variability outside the upper and lower quartiles. Mild outliers (○, values that lie between 1.5 and 3 times the interquartile range below the first quartile or above the third quartile) and extreme outliers (×, values that lie more than 3 times the interquartile range below the first quartile or above the third quartile) are indicated.

Fig. 2. Mean value of delta backscattering (%) of (A, B) cream layer for fresh human milk and the bottom layer (C, D) and cream layer for fresh human milk (A, C) and fresh bovine milk (B, D) at different temperatures as a function of time. Temperature are 5 °C (○), 20 °C (●), 37 °C (●), and 40 °C (●). Data points correspond to average mean value and error bars show the 95% confidence interval (CI).

Fig. 3. Peak thickness of the cream layer for fresh human milk (▲,▲) and fresh bovine milk (●,●) at 5 °C (▲,●) and 40 °C (▲,●), respectively. Peak thickness profiles are from individual samples but are representative of data from others.

Fig. 4. TSI profiles of human milk cream layer for milk stored at (A) 4 °C for 1 week, (B) milk frozen at –20 °C for 1 week (thawed at RT), and (C) milk frozen at –20 °C for 9 months (thawed at RT) compared with fresh human milk before treatments. The TSI_{cream} before treatments (black symbols) and the TSI_{cream} after treatments (grey symbols) are shown. The

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solid lines are averages of the TSI_{cream} before and after treatments; ◆, ▲, ● represent three individual samples in each treatment.

Fig. 5. The peak thickness profiles (A) of cream layer for fresh human milk (◆), fresh bovine milk (△), and bovine and mixed human milk (1:1, v/v; ●) and the TSI (B), mean value (C), and peak thickness (D) profiles of cream layer for fresh human milk, fresh bovine milk, recombined milk of human cream and bovine serum (○), and recombined milk of bovine cream and human serum (●) held at 5 °C, as a function of time. The profile is from an individual sample but is representative of data from others.

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Table 1

Levels of macronutrients in human milk and bovine milk samples. [a]

Type of milk	Fat	Crude protein	Carbohydrate	Total solids
Human milk	31 (1.04)	16 (0.18)	69 (0.25)	119 (1.16)
Bovine milk	40 (0.31)	35 (0.27)	45 (0.05)	130 (0.55)

[a] Data (in g L^{-1}) are means (human milk, n = 12; bovine milk, n = 4) with standard deviation in parenthesis.

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Table 2

Particle size distribution parameters of milk fat globules. [a]

Size distribution parameters	Human milk	Raw bovine milk
D [3,2]	2.27 (0.33)	2.35 (0.47)
D [4,3]	4.11 (0.92)	3.69 (0.32)
Dv10	1.09 (0.32)	0.97 (0.12)
Dv50	3.53 (0.74)	3.63 (0.32)
Dv90	7.10 (1.56)	6.64 (0.36)

[a] Data (in μm) are means (human milk, n = 7; bovine milk, n = 3) with standard deviation in parenthesis.

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Table 3

Peak thickness of human milk cream layer at different temperatures and time points. [a]

Temperature (°C)	n	Time		
		0.5 h	4 h	10 h
5	11	0.50 (0.37)[a]	1.73 (0.50)[a]	0.84 (0.82) [a]
20	10	0.76 (0.63)[b]	2.22 (0.90)[ab]	1.65 (1.66) [a]
37	13	0.94 (1.61)[bc]	2.36 (0.76)[ab]	1.78 (1.38) [a]
40	11	1.17 (0.38)[bc]	2.57 (0.53)[ab]	1.97 (1.59) [a]
45	3	2.38 (0.65)[c]	3.28 (1.10)[b]	3.29 (0.98) [a]

[a] Data (ΔH; in mm) are means with standard deviation in parenthesis; n is the number of samples at each temperature. Different superscript letters indicate statistically significant differences at $P < 0.05$ between temperatures within each column.

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Table 4.

Creaming rates measured in the cream layer and the clarification layer of human and bovine milk.

Temperature (°C)	n	Migration rate	
		Creaming zone	Clarification zone
Bovine milk			
5	3	2.37 (1.45)	1.34 (0.58)
40	3	2.38 (0.52)	2.69 (0.27)
Human milk			
5	11	1.25 (0.95)	0.61 (0.14)
20	10	1.81 (1.85)	1.46 (1.80)
37	13	2.27 (1.61)	2.18 (1.31)
40	11	3.34 (2.11)	3.33 (3.00)
45	3	6.06 (1.69)	6.88 (3.82)

[a] Data (in mm h^{-1}) are means with standard deviation in parenthesis; n is the number of samples at each temperature. Zones are as defined in Supplementary material Fig. S1 (creaming, zone r_1; clarification, zone r_2).

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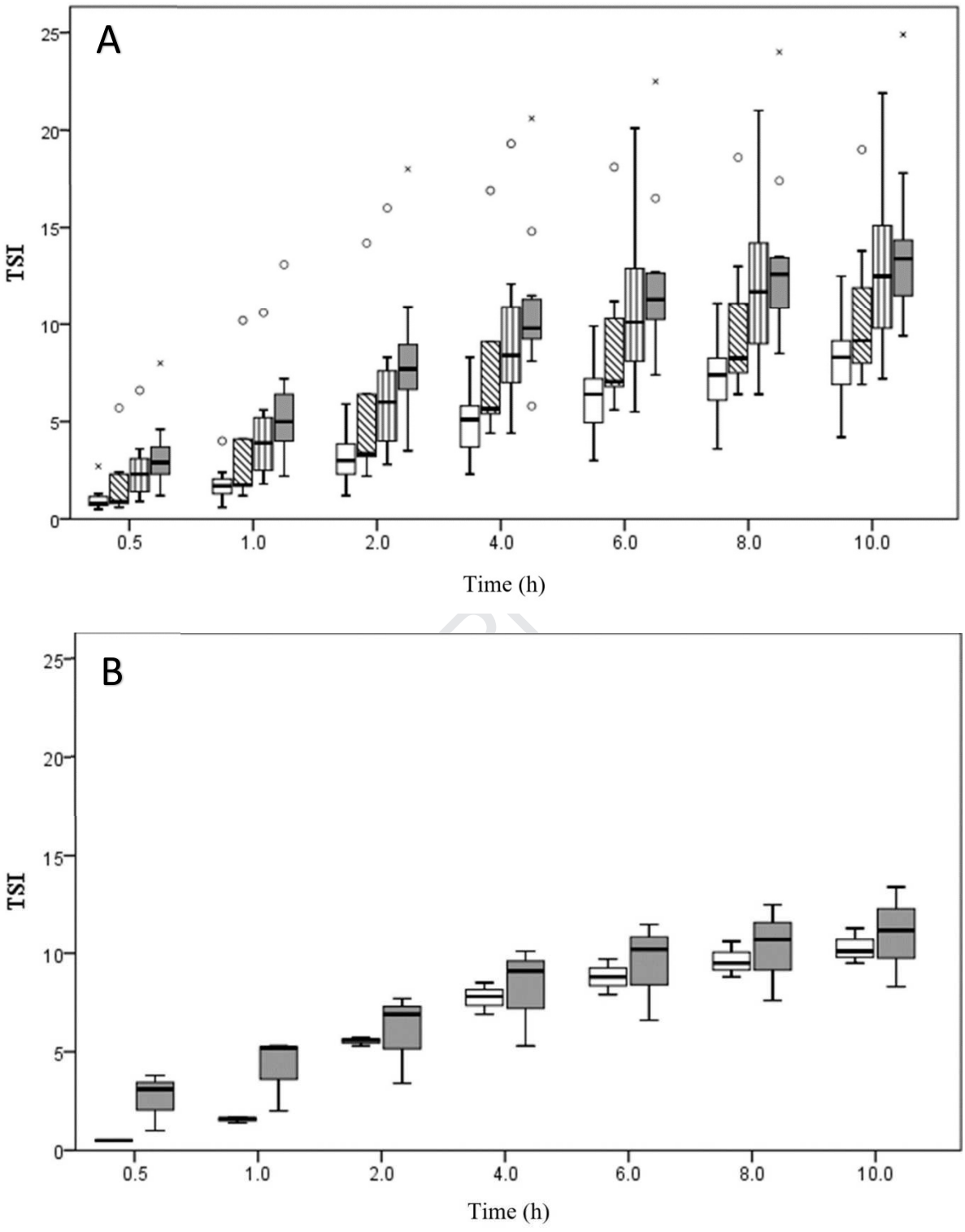

A
TSI
25
20
15
10
5
0
0.5
1.0
2.0
4.0
6.0
8.0
10.0
Time (h)
B
TSI
25
20
15
10
5
0
0.5
1.0
2.0
4.0
6.0
8.0
10.0
Time (h)

Figure 1

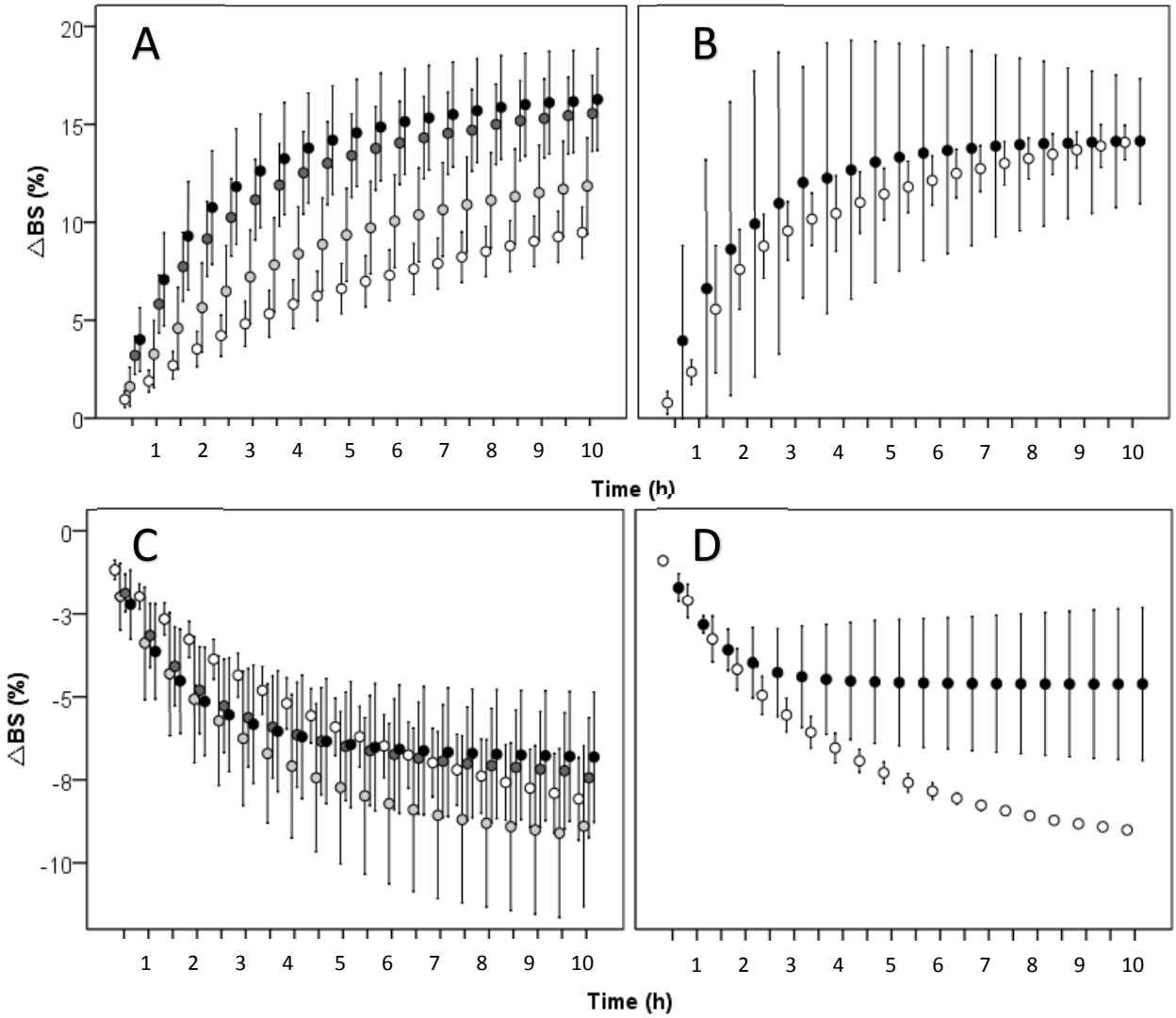


Figure 2

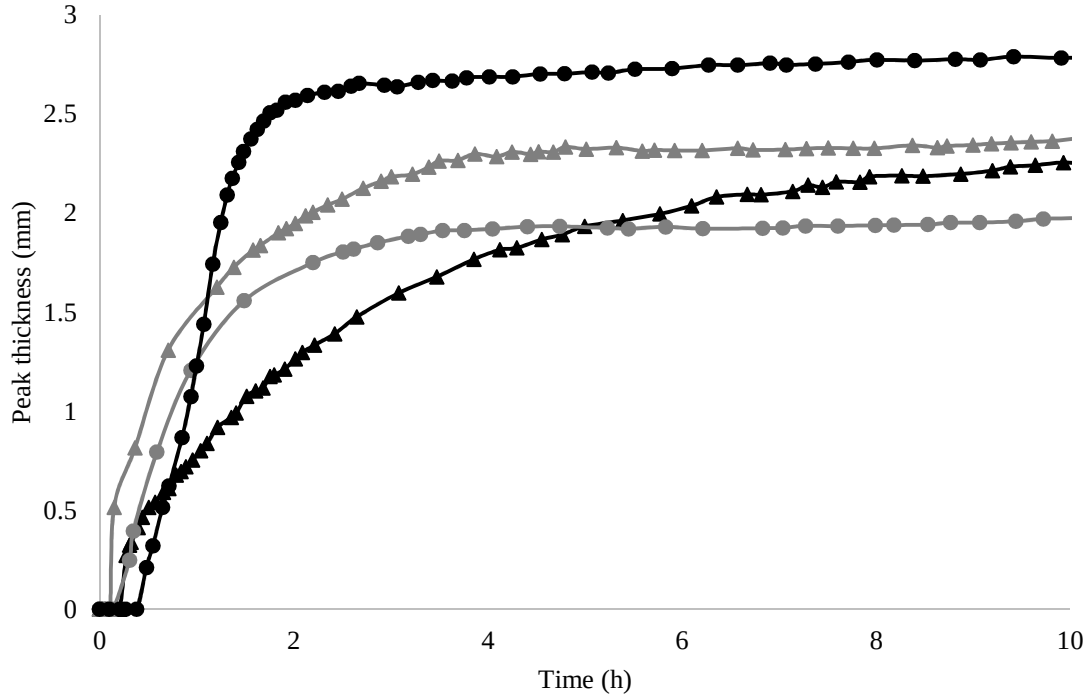
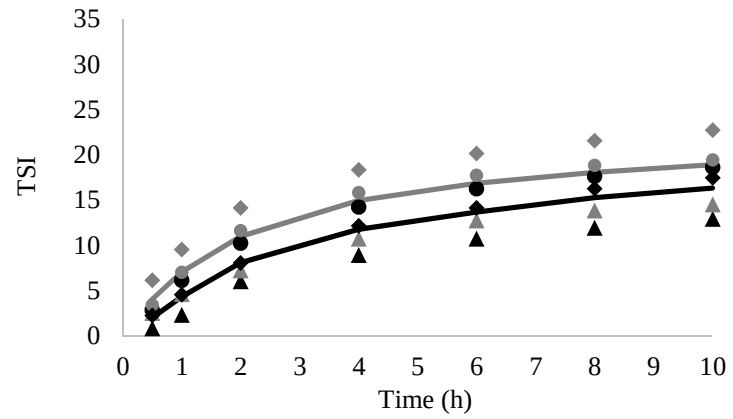

Peak thickness (mm)
Time (h)
0
0.5
1
1.5
2
2.5
3
0
2
4
6
8
10

Figure 3


TSI
Time (h)
0
5
10
15
20
25
30
35
0
1
2
3
4
5
6
7
8
9
10

B

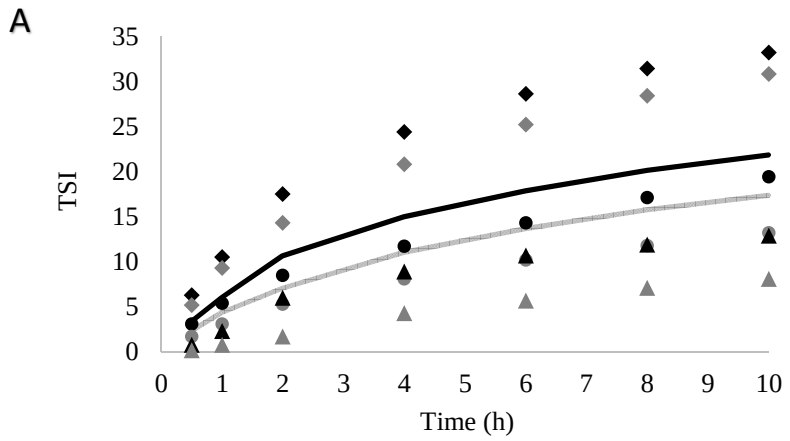
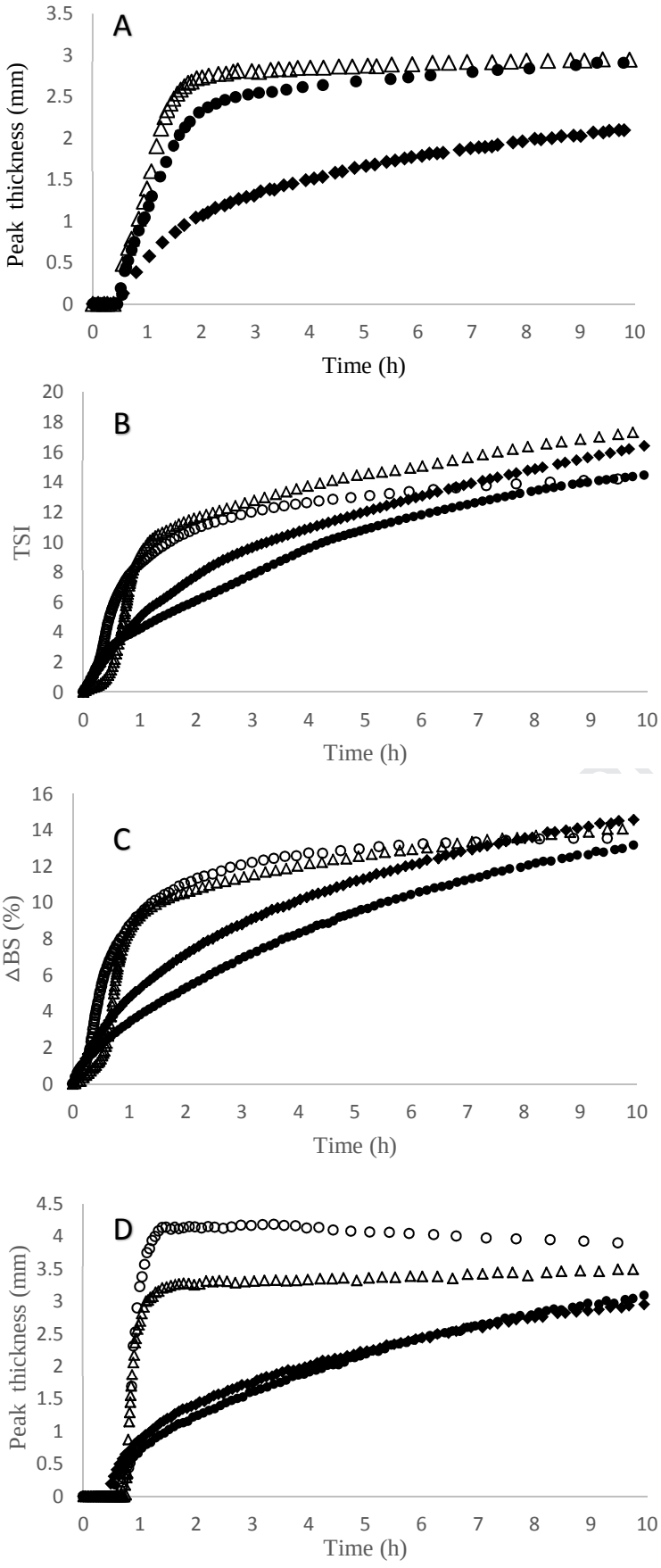


Figure 4

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Figure 5