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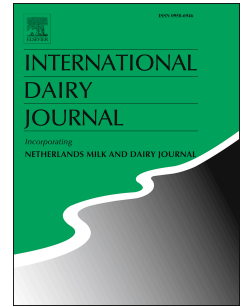
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Factors affecting the creaming of human milk

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1 **Factors affecting the creaming of human milk**

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24 ABSTRACT

25

26 The creaming properties of human milk have not been widely studied to date, and a  
27 mechanism for this phenomenon is not known. Here, the natural creaming of human  
28 milk, as affected by temperature and pre-treatments, was studied using dynamic light-  
29 scattering. The creaming rate of human milk increased with temperature in the range  
30 5 °C to 40 °C. Freezing human milk at -20 °C and thawing at room temperature had  
31 little influence on creaming. Compared with bovine milk, human milk showed a faster  
32 creaming rate at 40 °C, but a slower rate at 5 °C, suggesting a lack of cold  
33 agglutination; the mechanisms of creaming were also shown to differ in response to  
34 heat treatment. This study expands the current knowledge on milk creaming, and may  
35 have potential application to storage and handling of human milk in hospitals or  
36 homes, therefore supporting optimal nutrition of infants.

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## 38 1. Introduction

39

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

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

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

$$60 \quad v = \frac{g(\rho_p - \rho_f)d^2}{18\eta_p} \quad (1)$$

61 where  $g$  is acceleration due to gravity,  $d$  is the diameter of the fat globule,  $\rho_p$  and  $\rho_f$  are  
62 the densities of the plasma and fat, respectively, and  $\eta_p$  is the viscosity of the plasma.

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

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

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

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

95

## 96 **2. Materials and methods**

97

### 98 *2.1. Human milk samples*

99

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

108

### 109 *2.2. Compositional analysis*

110

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

118

### 119 2.3. *Size distribution of milk fat globules*

120

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

129

### 130 2.4. *Measurement of density of milk fat and viscosity of skimmed milk*

131

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



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

$$144 \quad \eta_{milk} = \frac{t_{milk} \rho_{milk}}{t_{H_2O} \rho_{H_2O}} \eta_{H_2O} \quad (2)$$

145 where  $t_{milk}$  and  $t_{H_2O}$  are the time (seconds) flow through the specific area of U-shape  
 146 viscometer of milk and water, respectively;  $\rho_{milk}$  is the density of skimmed human  
 147 ( $1.03 \times 10^3 \text{ kg m}^{-3}$ ; Neville et al., 1988) or skimmed bovine milk ( $1.04 \times 10^3 \text{ kg m}^{-3}$ ;  
 148 Ma & Barbano, 2000) at 5 °C;  $\rho_{H_2O}$  is the density of water at 5 °C, which is  $1.00 \times$   
 149  $10^3 \text{ kg m}^{-3}$  (Jones & Harris, 1992);  $\eta_{H_2O}$  is the viscosity of water at 5 °C, which is  
 150 1.52 mPa s (Kestin, Sokolov, & Wakeham, 1978). Fresh milk samples were measured  
 151 and each analysis was performed in triplicate.

152

### 153 2.5. *Sample preparation for creaming analysis*

154

155 For creaming analysis, five different temperatures, 5 [number of samples (n) =  
 156 11], 20 (n = 10), 37 (n = 13), 40 (n = 11) and 45 °C (n = 3) were selected in  
 157 individual experiments for simulation of refrigeration, room temperature (RT),  
 158 mammalian body temperature, and higher temperatures. Refrigerated milk samples  
 159 were incubated in a water bath for 20 min to achieve the target temperature, and each

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

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

174

## 175 2.6. *Creaming analysis*

176

177 The Turbiscan<sup>LAB™</sup> (Formulation, Ramonville St. Agne, France) with an 800  
178 nm near infrared (NIR) light source was used to study particle migration (creaming) in  
179 milk samples. The Turbiscan is based on multiple light scattering theory, where NIR  
180 photons are transmitted or back-scattered from the sample to transmission (T) or  
181 backscattering (BS) detectors (Carretero, Villepin, Brunel, & Carries, 2005).

182 Parameters quantified to describe creaming were the Turbiscan stability index  
183 (TSI), the mean value (of  $\Delta BS$ , %), the peak thickness ( $\Delta H$ ), and the migration rate.

184 The TSI was calculated by summing up the changes in BS at all measured positions,

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

196

## 197 2.7. *Statistical analysis of data*

198

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

206

207 **3. Results**

208

209 *3.1. Composition, size distribution and density of milk fat globules of human and*  
210 *bovine milk*

211

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

217 Differences in fat globule size between human and bovine milk were minor,  
218 with fat globules in human milk having a slightly lower average  $D[3,2]$  ( $2.3 \pm 0.3 \mu\text{m}$ )  
219 and higher average  $D[4,3]$  ( $4.1 \pm 0.9 \mu\text{m}$ ) than those in bovine milk ( $2.4 \pm 0.5 \mu\text{m}$  and  
220  $3.7 \pm 0.3 \mu\text{m}$ , respectively) (Table 2). In case of the size distribution of human MFGs,  
221 10% of particles were below  $1.1 \pm 0.3 \mu\text{m}$ , and 50% of particles below  $3.5 \pm 0.7 \mu\text{m}$ ,  
222 both of which are lower than the values presented for bovine milk; however, the  
223 average  $D_{v90}$  of human MFGs ( $7.1 \pm 1.6 \mu\text{m}$ ) was higher than that in bovine milk ( $6.6$   
224  $\pm 0.36 \mu\text{m}$ ). Overall, statistical analysis of human and bovine MFG size distribution  
225 parameters showed no significant difference ( $P > 0.05$ ) between the parameters  
226 measured for the two types of milk.

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

231

232 3.2. *Creaming profiles of human and bovine milk at different temperatures*

233

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

242 Bovine milk held at 5 °C had a lower  $TSI_{cream}$ , i.e., a higher stability, than that  
243 at 40 °C during the first hour; however, instability increased dramatically between 1  
244 and 4 h of measurement. The average  $TSI_{cream}$  values of human milk were lower at  
245 5 °C but higher at 40 °C, compared with those of bovine milk at each time point  
246 during 10 h. However, no significant difference ( $P > 0.05$ ) of  $TSI_{cream}$  was found  
247 between bovine and human milk at 10 h at either temperature.

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

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

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

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

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

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<sub>cream</sub> at each time point, whereas a 9-month frozen and thawed milk had  
290 a higher TSI<sub>cream</sub> 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<sub>cream</sub> 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  $\Delta BS$  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

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

313 After the addition of bovine WPI (0.83%, w/v) to human milk, both the peak  
314 thickness and the mean  $\Delta BS$  increased slightly. The initial creaming rates of human  
315 milk also increased after addition of both bovine WPI and bovine Igs, and the addition  
316 of bovine Igs had a higher impact on human milk creaming than the addition of WPI.  
317 However, the increases due to addition of bovine WPI and Igs were not significant ( $P >$   
318 0.05) in the case of  $TSI_{cream}$  (Supplementary material Fig. S4) at each time point.

319

#### 320 4. Discussion

321

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



332 post-partum), when milk contains a lower fat content and a higher protein content  
333 than in mature human milk (Gidrewicz & Fenton, 2014). A lactational effect was  
334 noticed and samples at 8 and 24 weeks post-partum appear to have higher values of  
335 peak thickness than samples at 1 week post-partum (data not shown). Mid- to hind-  
336 milk, which was collected after the babies have been satisfied in this study, may  
337 contain a higher level of fat than foremilk (Mizuno et al., 2009); however, the milk fat  
338 globule size is not expected to be significantly different (Mizuno et al., 2009). The  
339 content of macronutrients of human and bovine milk measured was, however,  
340 consistent with the literature values (Anderson, Atkinson, & Bryan, 1981; Bauer &  
341 Gerss, 2011; Gidrewicz & Fenton, 2014; Guo & Hendricks, 2008). The lower average  
342 fat content and a higher average solids-not-fat content in human milk than in bovine  
343 milk samples may slow fat separation in the milk (Webb & Hall, 1935), and may  
344 partly explain the observed slower creaming rate of human milk than bovine milk at  
345 5 °C.

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

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

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

371         IgM and IgA are reported as major agglutinins to accelerate bovine milk  
372 creaming during cold storage (D'Incecco et al., 2018; Hansen, Larsen, & Wiking,  
373 2019), but remain in skim milk after separation at 45 °C (Jennes & Parkash, 1971).  
374 Although the protein content of human milk was lower than that of bovine milk,  
375 human milk is reported to contain a higher percentage of Igs (approximately 16% of  
376 total whey proteins) than bovine milk (approximately 10% of total whey proteins;  
377 Guo & Hendricks, 2008). Also, human milk has IgA-dominated Igs, whereas bovine  
378 milk Igs are IgG-dominated (Hurley & Theil, 2011). The level of IgA has been  
379 reported to be 0.13  $\text{kg m}^{-3}$  and 1  $\text{kg m}^{-3}$  for bovine and human milk, respectively,  
380 whereas the level of IgM is 0.04  $\text{kg m}^{-3}$  and 0.1  $\text{kg m}^{-3}$  for bovine and human milk,

381 respectively (Hurley, 2003), i.e., human milk contains higher levels of agglutinins  
382 (IgA and IgM). However, the cream volume and the creaming rate of human milk at  
383 5 °C were lower than that of bovine milk (Fig. 3, Table 4), which may result from a  
384 higher extent of agglutinins being associated with bovine milk fat globules than  
385 human milk. This is supported by the fact that the creaming ability of human milk was  
386 increased after mixing with bovine skim milk.

387         There are two possible reasons why human milk contains high levels of  
388 agglutinins, but has a lower creaming rate than bovine milk at 5 °C. Firstly, although  
389 hydrophobic interactions will be weakened at low temperatures, bovine milk has been  
390 reported to have the most hydrophobic secretory IgA among species, including  
391 humans, which can result in a higher level of bovine IgA associated with fat globules  
392 (Honkanen-Buzalski & Sandholm, 1981). Secondly, competition between soluble  
393 antigens and MFGM antigens to capture IgM may occur in human milk (Euber &  
394 Brunner, 1984). It is known that the interaction of IgM with MFG is specific and  
395 involves carbohydrate moieties (Euber & Brunner, 1984); therefore, milk  
396 oligosaccharides and  $\kappa$ -casein molecules containing carbohydrate moieties may be  
397 involved in the interaction of IgM. The oligosaccharides have not been considered as  
398 a factor in bovine milk due to their low concentration (Euber & Brunner, 1984), but  
399 should be considered in human milk due to their higher levels (Kunz & Rudloff,  
400 1993). Moreover, the content of sialic acid, which has been demonstrated to inhibit  
401 milk creaming (Euber & Brunner, 1984), is much higher in human milk than in  
402 bovine milk (Wang & Brand-Miller, 2003; Wang, Brand-Miller, McVeagh, & Petocz,  
403 2001). Further research on the reasons behind the difference of creaming between  
404 bovine and human milk appears warranted.

405 Human milk was stable following short-term freezing, which is consistent with  
406 an unchanged size distribution of human MFG before and after freezing (data not  
407 shown). It has been reported that no significant change in concentration occurs for  
408 IgA, the major agglutinin in mature human milk, at  $-20\text{ }^{\circ}\text{C}$  for 1 month (Evans, Ryley,  
409 Neale, Dodge, & Lewarne, 1978) or 3 months (Reynolds et al., 1982). In contrast, a  
410 decrease in IgA level after 4 weeks at  $-20\text{ }^{\circ}\text{C}$  has also been reported (Akinbi et al.,  
411 2010), and Ramírez-Santana et al. (2012) reported a decrease in IgA concentration in  
412 human colostrum when the frozen storage period was increased to 12 months,  
413 indicating that IgA can be temperature labile. Moreover, although the MFG can be  
414 resistant to lipolysis as long as its structure remains intact, slow freezing or long-term  
415 freezing can lead to the destruction of MFGM and allow access of lipases to the core  
416 of MFG, i.e., to triglycerides (Berkow et al., 1984; Munkwitz, Berry, & Boyer, 1933).

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

426

## 427 5. Conclusion

428

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

440

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445

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447

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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

solid lines are averages of the  $TSI_{cream}$  before and after treatments;  $\blacklozenge$ ,  $\blacktriangle$ ,  $\bullet$  represent three individual samples in each treatment.

**Fig. 5.** The peak thickness profiles (A) of cream layer for fresh human milk ( $\blacklozenge$ ), fresh bovine milk ( $\triangle$ ), and bovine and mixed human milk (1:1, v/v;  $\bullet$ ) 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 ( $\circ$ ), and recombined milk of bovine cream and human serum ( $\bullet$ ) held at 5 °C, as a function of time. The profile is from an individual sample but is representative of data from others.

**Table 1**

Levels of macronutrients in human milk and bovine milk samples. <sup>a</sup>

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)

<sup>a</sup> Data (in g L<sup>-1</sup>) are means (human milk, n = 12; bovine milk, n = 4) with standard deviation in parenthesis.

**Table 2**Particle size distribution parameters of milk fat globules. <sup>a</sup>

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)

<sup>a</sup> Data (in  $\mu\text{m}$ ) are means (human milk, n = 7; bovine milk, n = 3) with standard deviation in parenthesis.



**Table 3**

Temperature (°C)	n	Time			Pea k thic kne ss of hum an mil
		0.5 h	4 h	10 h	
5	11	0.50 (0.37) <sup>a</sup>	1.73 (0.50) <sup>a</sup>	0.84 (0.82) <sup>a</sup>	
20	10	0.76 (0.63) <sup>b</sup>	2.22 (0.90) <sup>ab</sup>	1.65 (1.66) <sup>a</sup>	
37	13	0.94 (1.61) <sup>bc</sup>	2.36 (0.76) <sup>ab</sup>	1.78 (1.38) <sup>a</sup>	
40	11	1.17 (0.38) <sup>bc</sup>	2.57 (0.53) <sup>ab</sup>	1.97 (1.59) <sup>a</sup>	
45	3	2.38 (0.65) <sup>c</sup>	3.28 (1.10) <sup>b</sup>	3.29 (0.98) <sup>a</sup>	

k cream layer at different temperatures and time points. <sup>a</sup>

<sup>a</sup> Data ( $\Delta H$ ; 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
<b>Bovine milk</b>			
5	3	2.37 (1.45)	1.34 (0.58)
40	3	2.38 (0.52)	2.69 (0.27)
<b>Human milk</b>			
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)

<sup>a</sup> Data (in mm h<sup>-1</sup>) 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<sub>1</sub>; clarification, zone r<sub>2</sub>).

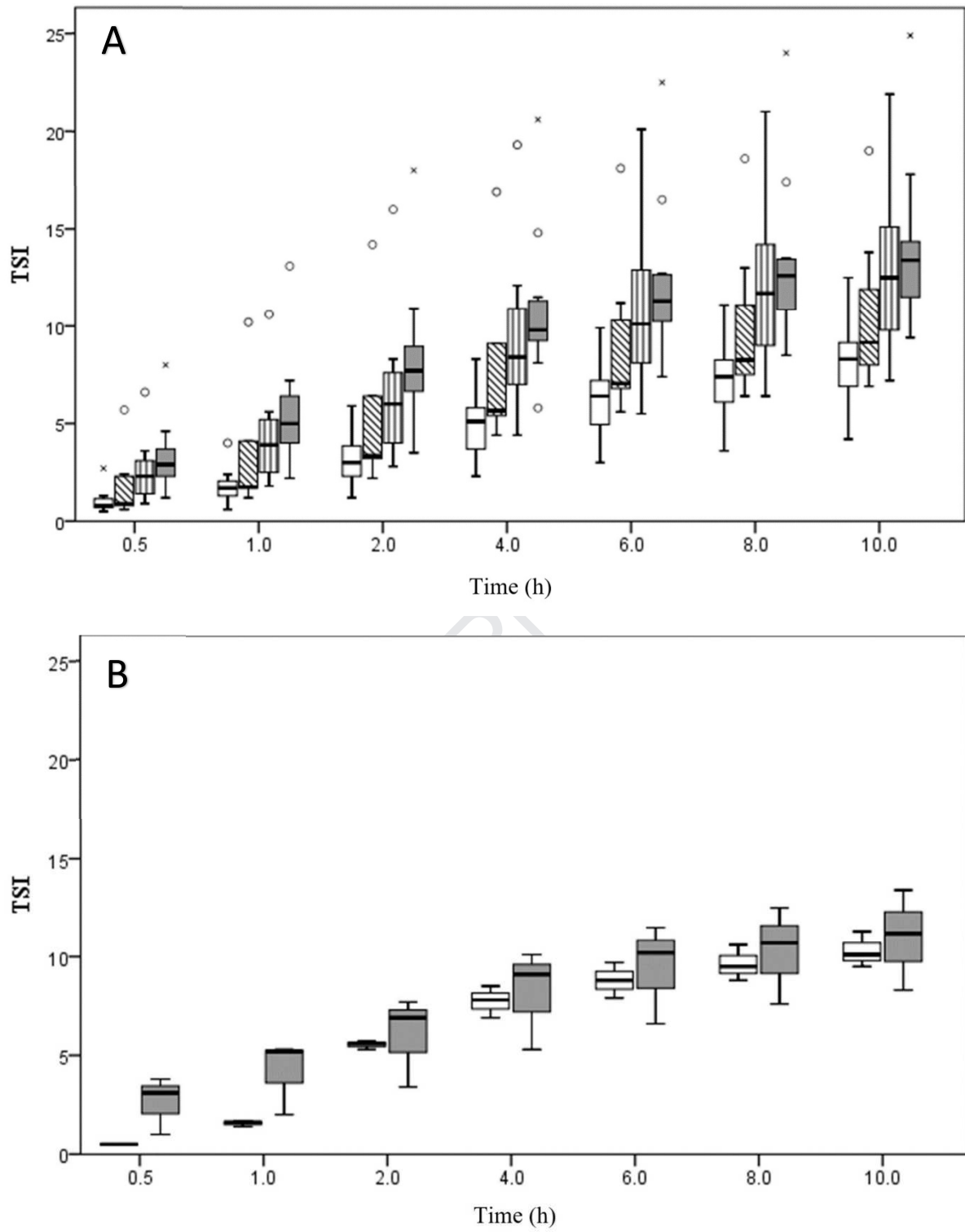


Figure 1

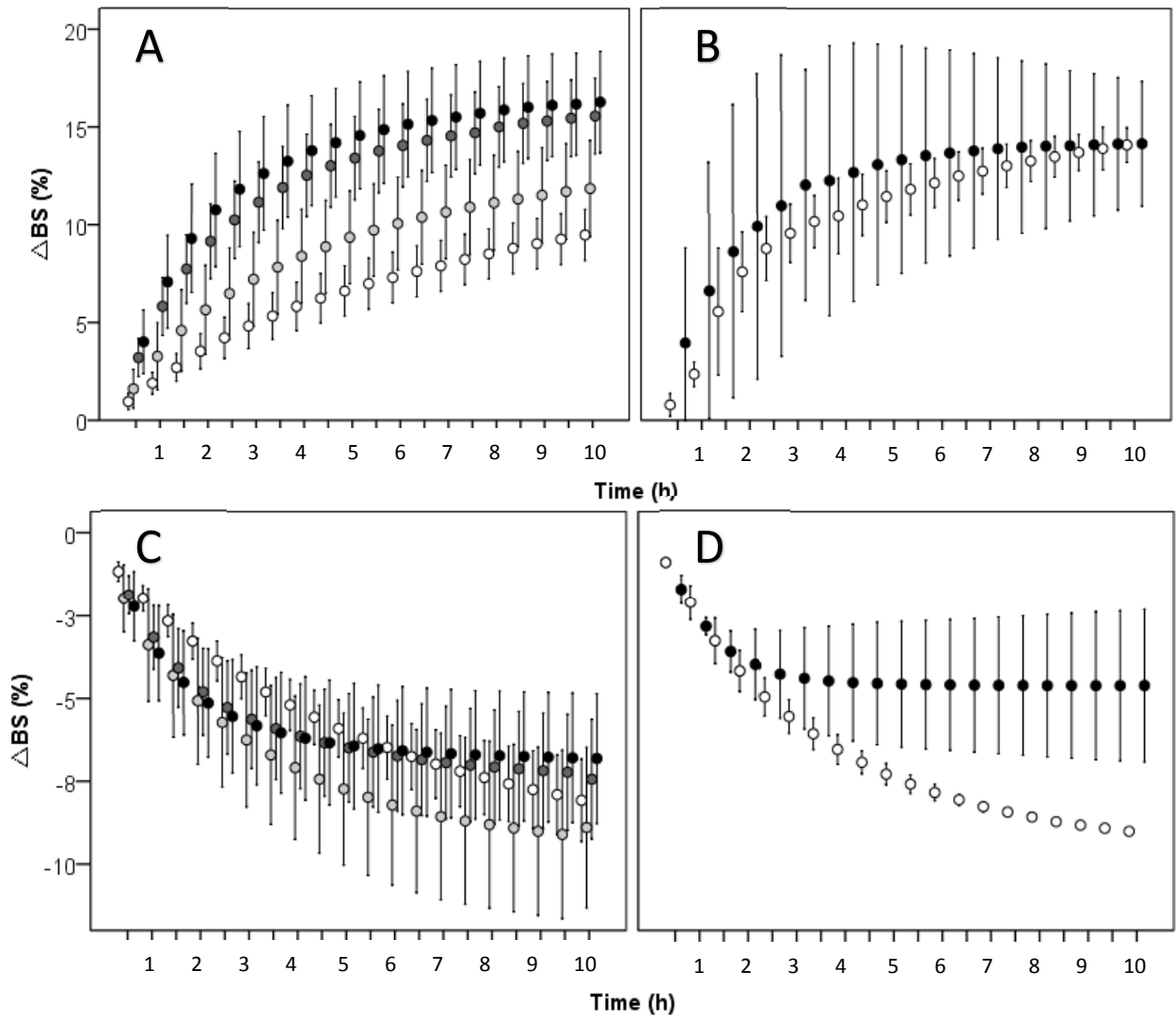


Figure 2

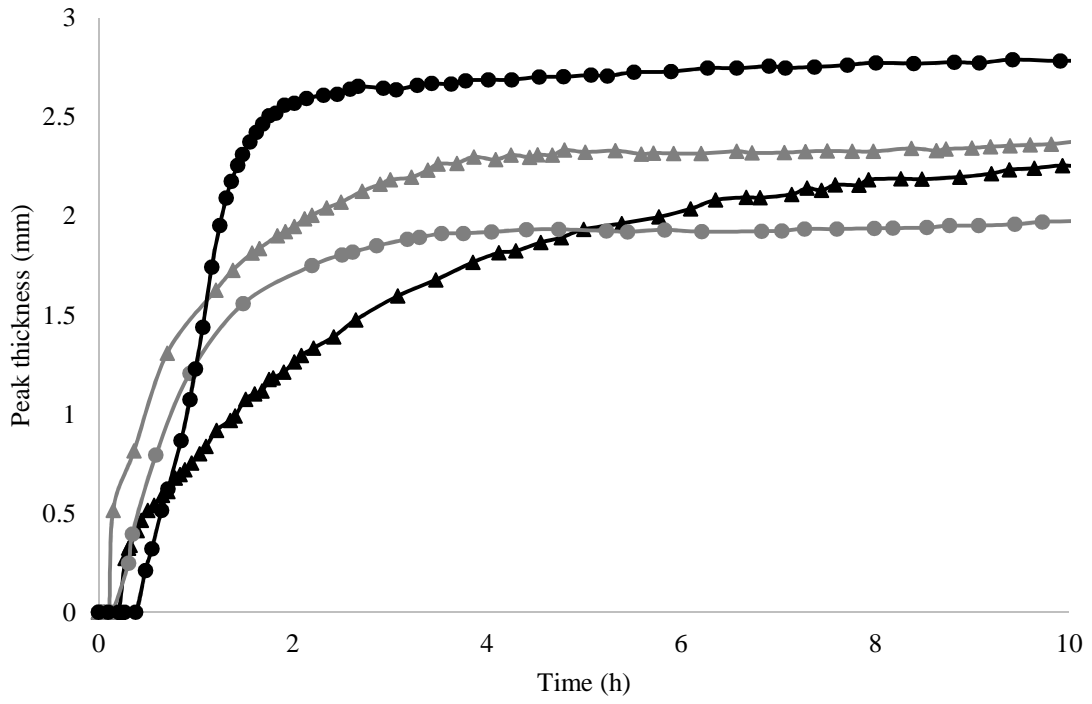
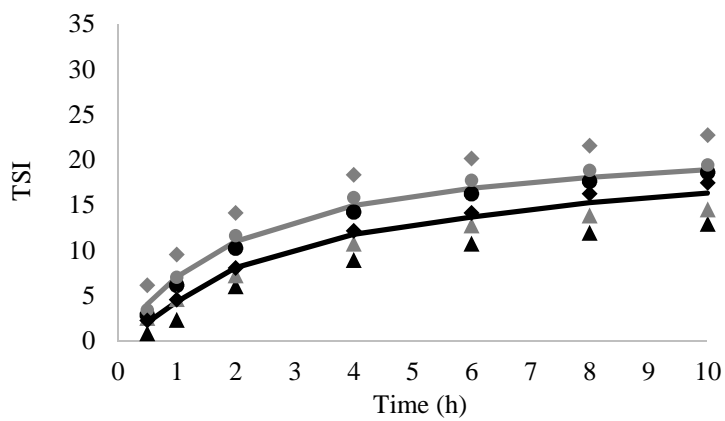


Figure 3



B

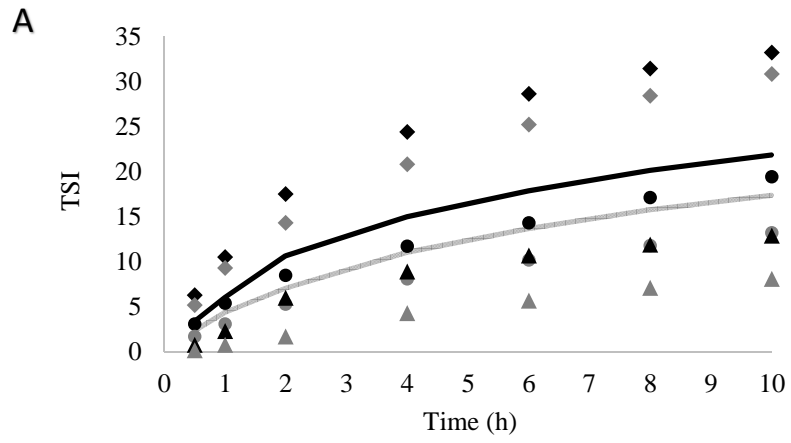


Figure 4

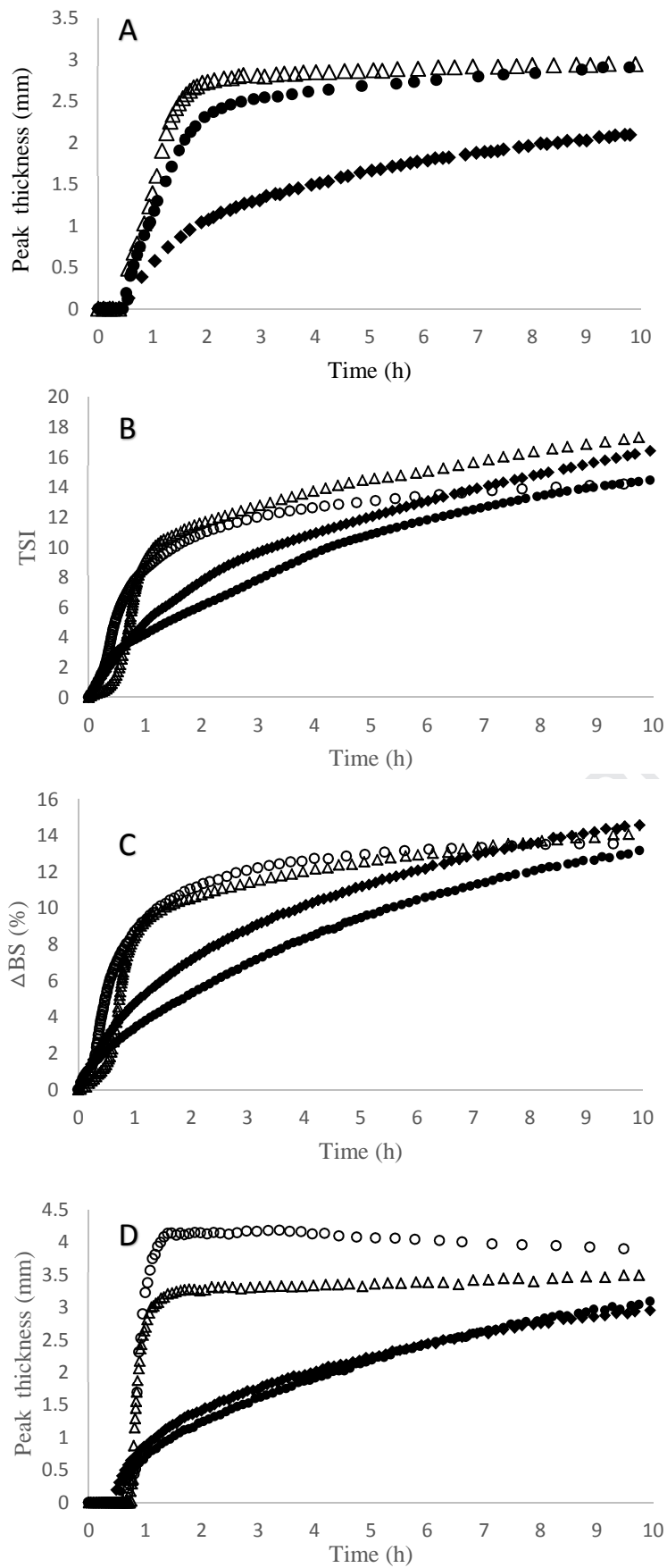


Figure 5