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Free fatty acid release from vegetable and bovine milk fat-based infant formulas and human milk during two-phase in vitro digestion

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Abstract

23 Background: Bovine milk fat is increasingly used in infant formula (IF). The triacylglycerol
24 (TAG) structure of bovine milk fat might be beneficial for digestion and absorption. We
25 investigated the release of fatty acids (FAs) of IF containing different fat blends and
26 compared this to human milk.

27 Methods: Fresh human milk was sampled and two IFs were produced; one containing 100%
28 vegetable fat (IF1) and one with 67% bovine milk fat and 33% vegetable fat (IF2). Using a
29 static *in vitro* infant digestion model, consisting of a gastric and duodenal phase, the time
30 dependent release of individual free fatty acids (FFA) was studied, analysed using GC-MS,
31 and residual TAG levels were determined by GC-FID.

32 Results: Human milk and the IFs showed comparable total FA release. In the gastric phase, 5-
33 10% of lipolysis occurred, and mainly short (SCFA)- and medium chain fatty acids (MCFA)
34 were released. In the duodenal phase, lipolysis proceeded with release of SCFA but was
35 marked by a fast release of long-chain fatty acids (LCFA). The digestion of the IFs resulted in
36 different FFA profiles in the course and at the end of digestion. IF2 gave more SCFA and
37 MCFA release, which reflects the FA composition of bovine milk.

38 Conclusion: The addition of bovine milk fat to IF resulted in a total FA release comparable to
39 an IF with only vegetable fat and human milk. However, it did lead to a different time-
40 dependent release of individual FAs, which might result in differences in absorption and
41 other health effects *in vivo*.

42

43 **Introduction**

44 Human milk is the preferred nutrition for infants¹), and it is therefore recognized as the
45 golden standard for infant feeding. Fat is an important nutrient in human milk, delivering
46 about 50% of the energy to an infant.² Furthermore, it also delivers essential fatty acids
47 (FAs), fat-soluble vitamins and other components like cholesterol.³ Human milk fat is
48 composed for 98% of triacylglycerols (TAGs), formed by the esterification of fatty acids (FAs)
49 at the three stereospecific positions (sn-1, sn-2 and sn-3) of the glycerol backbone.

50 Digestion of TAGs starts in the stomach by the action of gastric lipase and is completed in the
51 small intestine by pancreatic lipase. Gastric lipase can potentially hydrolyse all three ester
52 bonds of TAGs but shows a stereo-preference for the sn-3 position of TAGs. Since in milk fat
53 the shorter FAs are placed here, this leads to the preferential release of the shorter FAs by
54 gastric lipase.⁴⁻⁶ Pancreatic lipase shows regioselectivity for the hydrolysis of the ester bonds
55 at the sn-1 and sn-3 positions, and seems to prefer medium-chain fatty acids (MCFA; C6:0-
56 C12:0) over long-chain fatty acids (LCFA; >C12:0).⁷ Therefore, hydrolysis of TAGs by
57 pancreatic lipase results in two free fatty acids (FFA) and a mono-acylglycerol (MAG) with a
58 FA esterified at the sn-2 position. Although the conversion of TAGs into FFA and MAG is
59 sufficient to ensure full intestinal absorption of fat, MAG can be further cleaved to some
60 extent by other enzymes showing high activity on MAG, namely bile salt-stimulated lipase
61 (BSSL)⁸ and pancreatic lipase-related protein 2.⁹⁻¹¹ Short-chain fatty acids (SCFA; C4:0-C5:0)
62 and MCFA can directly be absorbed in the stomach up to C12 [REF] and by enterocytes,
63 whereas LCFA, and MAGs require the presence of bile salts and incorporation into mixed
64 micelles. These micelles can be transported over the mucosal barrier into the enterocytes.

65 Fat digestion and absorption in adults is highly efficient, with 95-97% of the ingested lipids
66 being hydrolysed and absorbed.³ In infants, fat absorption is less efficient. Studies
67 investigating FA excretion in stool samples show that not all lipids are absorbed in infants; in
68 term infants about 10% of the consumed fat is not absorbed.¹² This amount of non-absorbed
69 FAs is found to be higher in formula fed infants compared to breast-fed infants.¹³ The fat
70 absorption seems to decrease with increasing FA chain length and increases with the
71 number of double bonds (unsaturated FA).³

72 Human milk fat has a specific TAG structure with most of the palmitic acid, about 70-88%,
73 attached at the sn-2 position¹⁴⁻¹⁷, whereas unsaturated FA are predominantly at the sn-1
74 and sn-3 positions.^{14,15} There are several hypotheses why this might be beneficial for an
75 infant.¹⁸ One of the explanations is that unesterified palmitic acid, like other long-chain
76 saturated fatty acids (LCSFA), is able to form indigestible complexes with calcium in the
77 lumen, called soap formation^{19,20}. These calcium soaps are not absorbed and are excreted in
78 faeces.¹⁹ With palmitic acid at the sn-2 position of the glycerol backbone, and thus not at the
79 sn-1 and sn-3 positions, both the MAG as well as the calcium will be absorbed, resulting in a
80 higher bioavailability of FAs and calcium and protection against stool hardening.

81 Sometimes human milk is not available, for whatever reason, and infant formula (IF) is used
82 to feed a baby. The fat blends that are used for IF are tailored to the fatty acid composition
83 of human milk, with C16:0 (palmitic acid) and C18:1 (oleic acid) being the most frequently
84 occurring FAs. Nowadays, IFs are mostly based on vegetable fats derived from palm
85 (kernel/olein) oil, sunflower oil, coconut oil, rapeseed oil and safflower oil.²¹ Bovine milk fat,
86 a rich source of palmitic acid, is also used, with increasing popularity.²² Whereas palm oil or
87 bovine milk fat based formulas can be designed to have a comparable FA composition, the

88 FA distribution on the TAGs differs. In bovine milk fat a higher percentage of palmitic acid is
89 positioned at the sn-2 position of the glycerol backbone, compared to a representative fat
90 blend containing palm oil (40-45% vs 10-20% respectively).^{14,23} In most of the vegetable fat
91 blends used in IF the LCSFA are positioned at the sn-1 and sn-3 positions.^{16,23} In bovine milk
92 fat, SCFA, MCFA, unsaturated fatty acids (UFA) and some LCSFA are positioned at sn-1 and
93 sn-3.¹⁷ The positioning of FA in bovine milk fat thus resembles human milk TAG to a higher
94 degree than a vegetable fat blend containing palm oil. Since bovine milk fat contains low
95 levels of linoleic acid, addition of vegetable fat is needed to reach the required amount of
96 linoleic acid.²⁴ Therefore, a maximum of 67% bovine milk fat can be used in IF. When bovine
97 milk fat is used as a source of palmitic acid, palm oil does not need to be added to reach
98 similar levels of palmitic acid as present in human milk.²⁴

99 Since gastric lipase is known to have an apparent preference for shorter FAs, due to its sn-3
100 stereo-specificity, and pancreatic lipase for unsaturated FAs, the usage of bovine milk fat
101 compared to palm oil may result in differences in lipolysis, which may affect lipid availability
102 to infants. Therefore, the aim of this study was to investigate the release of fatty acids of IF
103 containing either a vegetable fat blend containing palm oil or a mixture of bovine milk fat
104 and vegetable fat and to compare this to human milk. This was performed using a static two-
105 phase *in vitro* digestion model, mimicking both the gastric and the small intestinal
106 physiological conditions as occurring in an infant. Two different infant formulas and four
107 human milk samples were used. The release of different FAs, ranging from C4:0 to C18:3,
108 was analysed in both the gastric and the intestinal phase, and was compared for the IFs and
109 human milk samples. In addition, the residual TAG concentrations of human milk and the
110 different IFs were determined.

111 **Material & Methods**

112 *Products*

113 Two IF base powders containing different fat blends were provided by FrieslandCampina. IF1
114 consisted of a mixture of vegetable fat (palm oil, palm kernel oil, rapeseed oil and sunflower
115 oil), IF2 contained 67% bovine milk fat and 33% of different vegetable fats (rapeseed oil,
116 sunflower oil and coconut oil). The IF products contained 30% fat (dry weight) and, besides
117 the different fat compositions, all other ingredients were the same. The IFs were dissolved in
118 40 °C demineralized water (0.134g/ml). The particle size distribution was determined using
119 laser light scattering using a Mastersizer 2000 (Malvern Instruments, Malvern, United
120 Kingdom) (**Figure 1**).

121 Human milk was obtained from four different Dutch women, who signed informed consent.
122 The milk was sampled right after pumping, and digestion experiments were started 45
123 minutes afterwards. Human milk samples 1-4 contained respectively 2.1%, 2.5%, 5.5%, and
124 3.2% fat, and the periods of collection were respectively 6 months, 6 months, 8 months, and
125 3 months. To determine the fatty acid composition of the IFs and human milk samples
126 methyl esters of the fatty acids were and analysed by capillary gas chromatography (**Table 1**)
127 Human and bovine milk fat contain a wide range of fatty acids²⁴, for these experiments we
128 have chosen to determine fatty acids ranging from C4:0-C18:3 since within those range the
129 most prevalent fatty acids are present.

130 *In vitro lipolysis experiments*

131 To simulate the digestive system of an infant, a two-phase static *in vitro* digestion model ,
132 including a gastric and duodenal phase, was used.^{25,26} The IFs or human milk, (40 ml), were

133 put in a temperature-controlled reaction vessel, kept at 37 °C. The products were
134 mechanically stirred and the pH was adjusted to 5.5 by addition of HCl. To simulate the
135 gastric phase of digestion at half gastric emptying, IFs or human milk (40 mL) were mixed
136 with simulated gastric fluid (SGF; 8.0 mL) at a 5 to 1 volume ratio [REF]. SGF was prepared
137 by dissolving rabbit gastric extract (RGE; CNRS, Marseille, France) at 1.8 mg/mL in 10 mM
138 MES buffer, 150 mM NaCl, pH 6.0. RGE contains rabbit gastric lipase and was chosen
139 because of similar properties to human gastric lipase, like regio- and stereo preference.²⁷
140 The lipase activity of the RGE powder was 68 U/mg, using tributyrin as substrate, which
141 corresponds to 57 µg/mg. A concentration of 1.8 mg/mL RGE in SGF allowed reaching a final
142 gastric lipase concentration of 17 µg/ml in the mixture of milk and SGF, which corresponds
143 to gastric lipase concentration in gastric contents at half gastric emptying [REF]. . During the
144 gastric phase the pH was kept constant at 5.5 by addition of HCL and NaOH. At -2, 15, and 29
145 minutes samples were taken for analysis. After 30 minutes, 25.2 ml of simulated intestinal
146 fluid was added to represent the ratio of meal to intestinal fluid observed *in vivo*.²⁹ Indeed, a
147 1.7 dilution factor is observed at half gastric emptying. To represent human pancreatic juice
148 and bile, porcine pancreatic extract (PPE or pancreatin; 8xUSP, Sigma-Aldrich, st Louis, USA)
149 and bovine bile salts (Sigma-Aldrich, st Louis, USA) were mixed and dissolved in 10 mM Tris
150 buffer, 150 mM NaCl, pH 6.0. The intestinal phase continued for 60 minutes, so the total
151 digestion experiment lasted 90 minutes. During the intestinal phase the pH was kept at 6.25
152 by addition of NaOH.. Samples were taken for analysis at 5, 10, 15, 30 and 60 minutes in the
153 intestinal phase. Enzymes in the samples were inactivated either by heat treatment (5
154 minutes at 72 °C) or acidification (addition of 200 µl 0.1M HCl), both well established
155 method to inactivate lipases. Samples were stored at -80 °C for further analysis. The
156 simulated gastric and intestinal fluids were made fresh before each experiment, and were

157 stored on ice until use. Digestion of the IFs was performed in triplicate. The four human milk
158 samples were digested in independent experiments.

159 *Release of individual fatty acids*

160 Release of individual FFAs was analysed by the ethyl chloroformate free fatty acid (ECF-FFA)
161 method of Amer *et al.*³⁰ Deuterated internal standards of all FFAs (C4-C18:1) were added to
162 samples and standards. This was followed by in solution derivatisation using a two-step
163 procedure. First 1000 µL of the digested samples were mixed with 200 µL of ECF. The pH was
164 adjusted to neutral pH with 100 µL 7 M NaOH, followed by a second addition of 200 µL of
165 ECF for neutral pH derivatisation. Subsequently, free fatty acids derivatives were extracted
166 with 1000 µL chloroform. 1 µL aliquots were injected in a splitless mode into an Agilent
167 Technologies 7890A gas chromatography system coupled to an Agilent Technologies 5975c
168 inert MSD quadrupole mass spectrometer (Agilent Technologies, Waghaeusel, Germany). An
169 HP-5MS capillary column coated with polyimide (60 m, 250 µm i.d., 0.25 µm film thickness;
170 Agilent Technologies) was used to separate the ECF-derivatised FFAs. The initial temperature
171 of the oven was held at 80 °C for 2 min, ramped to 140 °C at a rate of 10 °C/ min, to 240 °C at
172 a rate of 4 °C/ min, to 280 °C at a rate of 10 °C/ min, and then held at 280 °C for 3 min. A
173 constant flow rate of 1 ml/ min with helium as carrier was applied. The temperatures of the
174 ion source and injector were 230 and 260 °C, respectively. The mass spectral analysis was
175 performed in selected ion monitoring according to the ions determined by the use of a
176 standard for each compound with a quadrupole temperature of 150 °C and a fragmentation
177 voltage of 70 eV with a solvent delay of 6.50 min. Concentration of individual FFAs was
178 quantified using an external calibration curve including deuterated internal standards.
179 Deuterated C18:2 was used as internal standard for all unsaturated C18s.

180 *Analysis of residual triacylglycerol*

181 To analyse the residual TAG concentration upon digestion, lipids were extracted by mixing
182 0.5 ml of digestion sample with 125 μ L methanol, adding 1.25 ml ethyl acetate, mixing and
183 centrifuging for 10 minutes at 3000 rpm at 5°C (Hermile Labortechnik Z383K, Wehingen,
184 Germany). The ethyl acetate top layer was removed and stored. This procedure was
185 repeated by re-extracting the sample with 1.25 ml ethyl acetate and centrifugation, after
186 which the ethyl acetate layer was separated and pooled with the first one, and stored at 4 °C
187 until further analysis. Duplicate extractions were performed for all samples. Gas
188 chromatography coupled to flame ionization detection (GD-FID, Agilent 6990N, Amstelveen,
189 the Netherlands) was used to analyse the TAG concentrations. Using an injector (Gerstel CIS,
190 Mülheim an der Ruhr, Germany), the samples were injected at an initial temperature of
191 100°C and a split ratio of 20:1. A ZB-5HT capillary column (30m, 250 μ m i.d., 0.1 mm film
192 thickness; Phenomenex, Torrance, USA) was used to separate TAGs. The flame-ionization
193 detector (FID) was set to 400 °C. Chromatograms of each sample were obtained and
194 analysed using the Enhanced Data Analysis software from Agilent (Amstelveen, the
195 Netherlands). The values at t=0 were set to 100% to allow comparison of the different
196 samples. All used chemicals were of analytical standard.

197 *Statistics*

198 For all parameters, the mean and SD of the three replicate experiments were calculated for
199 both IFs. The results of the individual four human milk digestion experiments were combined
200 and a mean and SD were calculated for all parameters. The data was compared via one-way
201 ANOVA analysis followed by a Bonferonni posthoc test, or Student's t-test when only data of

202 human milk and IF2 was available. GraphPad Prism (version 5.04 for Windows, GraphPad
 203 Software, San Diego California USA) was used.

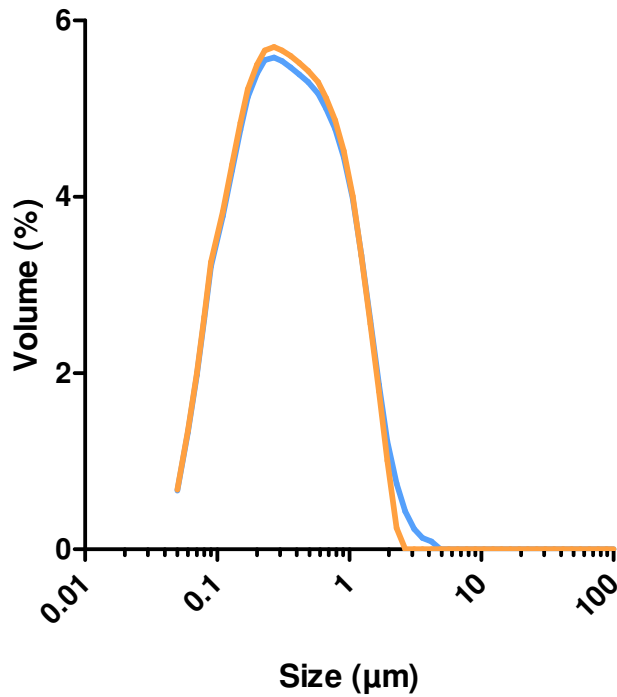
204 **Table 1: Initial fatty acid composition of the infant formula products and human milk samples (in % of total FAMES).**

				Human	Human	Human	Human
Fatty acid		IF1	IF2	milk-1	milk-2	milk-3	milk-4
SCFA	C4:0	<0.1	2.6	<0.1	ND	<0.1	<0.1
MCFA	C6:0	<0.1	1.6	<0.1	ND	<0.1	<0.1
	C8:0	0.6	1.2	<0.1	<0.1	0.2	0.3
	C10:0	0.5	2.1	0.9	0.7	1.6	1.6
	C11:0	<0.1	0.3	<0.1	<0.1	<0.1	<0.1
	C12:0	7.0	4.3	3.8	3.1	7.8	5.6
LCFA	C14:0	3.0	8.2	6.1	6.7	10.0	5.9
	C15:0	<0.1	0.7	0.5	0.6	0.4	0.3
	C16:0	24.7	22.8	30.0	28.3	21.0	18.4
	C18:0	3.1	7.1	8.1	9.4	6.6	4.7
	C18:1 n-9cis	25.4	25.9	30.3	29.8	32.2	30.8
	C18:2 n-6	12.9	12.0	10.5	10.0	10.3	20.6
	C18:3 n-3	1.8	1.4	0.8	0.8	0.8	1.6
	Total SCFA	<0.1	2.6	<0.1	ND	<0.1	<0.1
	Total MCFA	1.3	5.2	1.2	0.9	2.0	2.1

Total LCFA	78.0	82.4	90.1	88.7	89.1	87.9
Total SFA	39.3	50.9	49.8	49.0	47.9	37.1
Total MUFA	25.4	25.9	30.3	29.8	32.2	30.8
Total PUFA	14.7	13.4	11.3	10.8	11.1	22.2

205 FAME: fatty-acid methyl esters, IF: infant formula, LCFA: long-chain fatty acids MCFA: medium-chain fatty acids, MUFA:
 206 monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, SCFA: short-chain fatty acids, SFA: saturated fatty acids

207 **Results**

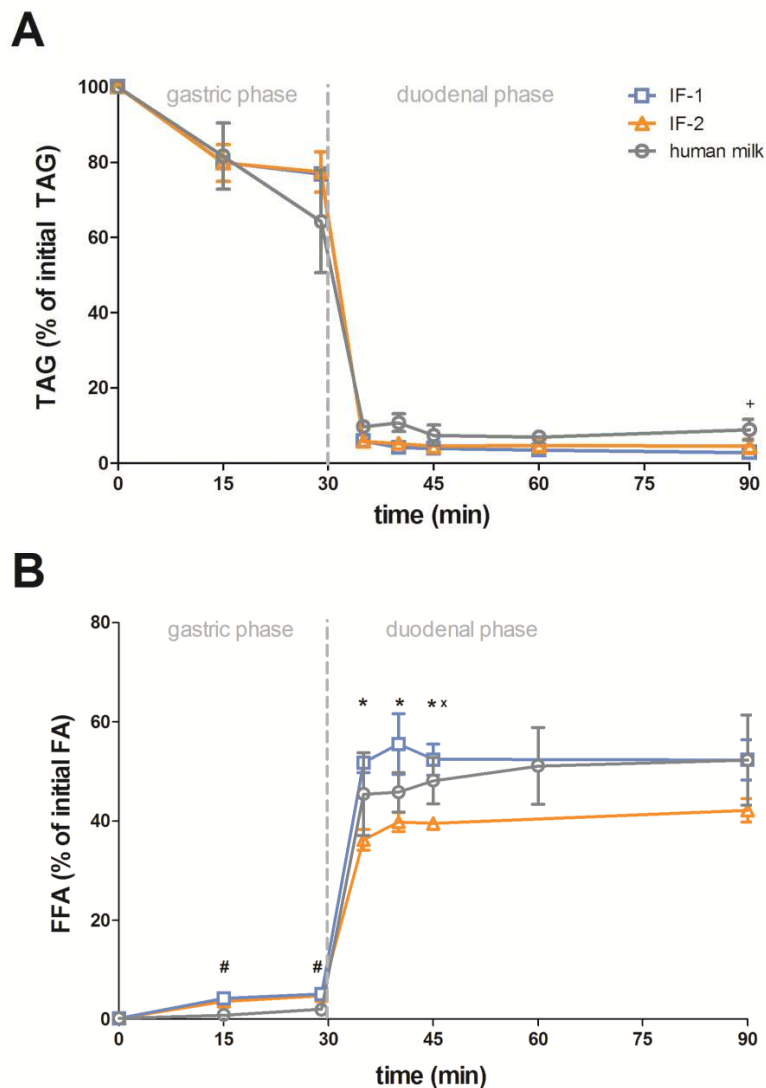


208
 209 **Figure 1:** The particle size distribution of the infant formulas (IF) tested in these digestion experiments. the blue line
 210 represents IF1, and the orange line represents IF2
 211

212 The particle size distribution of the IFs is shown in **Figure 1**. The modal diameter of both IFs
 213 is 0.27 µm.

214 Lipolysis of the different milk products was studied by analysing the residual TAG and FFA
 215 concentrations of different time points of the *in vitro* digestion. The decrease in TAGs is
 216 shown in **Figure 1A**. In the gastric phase no significant difference between the IFs and human

217 milk was found ($p=0.18$). At the end of the gastric phase, 24 to 36% of initial TAG molecules
218 had already been hydrolysed. At the end of the duodenal phase more TAGs remained intact
219 in human milk (8.9%) compared to IF1 (2.9%) ($p=0.02$). The percentage of remaining TAGs
220 in IF2 (4.4%) was not different from either human milk or IF-1 ($p>0.05$). **Figure 1B** shows the
221 release of FFA. The human milk samples showed less release of FFAs during the gastric phase
222 compared to IF1 and IF2 ($2.0 \pm 0.2\%$ vs $5.1 \pm 0.2\%$ and $4.7 \pm 0.1\%$ respectively, $p<0.01$). As
223 percentage of FFA released after the total digestion, from human milk about 4% was
224 released during the gastric phase, for IF1 this was about 10% and for IF2 about 11% of FFA
225 were released in the gastric phase. Except for 45 minutes in the digestion ($p<0.01$), no
226 differences were found in FFA release between the IFs compared to the human milk samples
227 during the duodenal phase. However, the comparison of IF1 and IF2 did show some
228 differences. Compared to IF2, IF1 showed a higher release of FFAs at time points 35, 40 and
229 45 ($p=0.03$, $p<0.01$, and $p<0.01$ respectively) during the digestion, i.e. it was hydrolysed at a
230 faster rate. However, the total release of FAs at the end of the digestion, as percentage of
231 initial composition, was found to be similar for the different samples ($52.3 \pm 2.4\%$, $42.2 \pm$
232 1.4% , and $52.3 \pm 4.5\%$ for IF1, IF2 and human milk respectively, $p=0.14$).



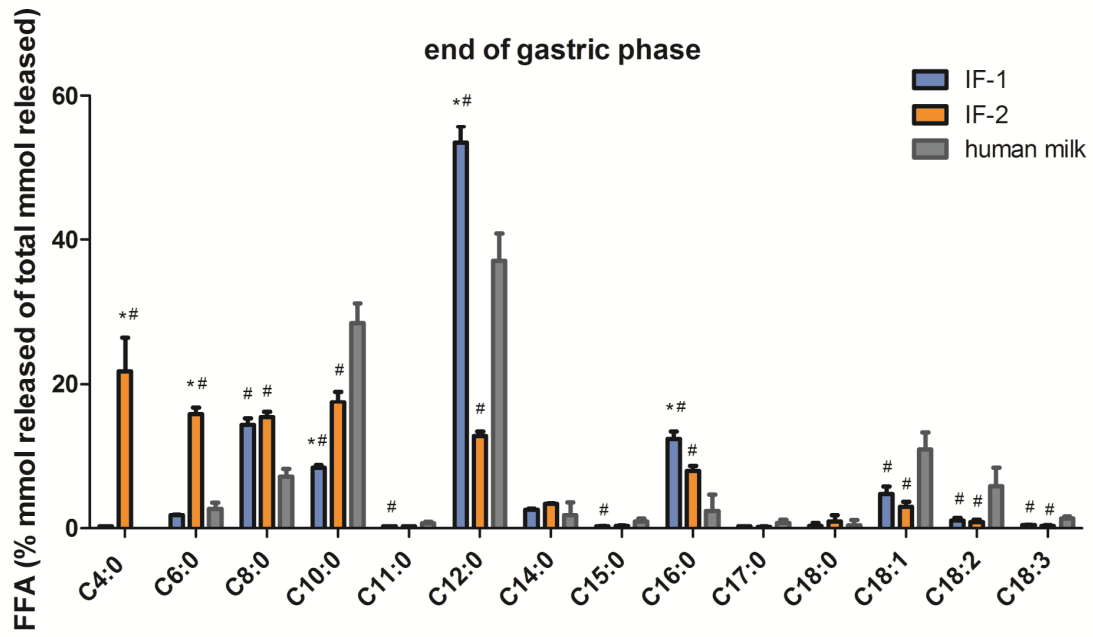
233

234 **Figure 1: The lipolysis of infant formulas and human milk (mean \pm SD). A: Decrease of triacylglycerols (TAGs) during *in***
 235 ***vitro* digestion in percentage of total TAGs initially present (mean \pm SD). B: Percentage of free fatty acids (FFA) in**
 236 **percentage of total esterified fatty acids (FA) in initial TAGs, during *in vitro* digestion (mean \pm SD). The vertical striped line**
 237 **indicates the start of the duodenal phase following the gastric phase. The grey line (○) represents the average of the**
 238 **human milk samples, the blue line (□) represents IF1, and the orange line (Δ) represents IF2. * Significant difference**
 239 **between infants formulas, # significant difference between both IFs and human milk, ⁺ significant difference between IF1**
 240 **and human milk, ^x significant difference between IF2 and human milk ($p < 0.05$), separate time points were compared with**
 241 **one-way ANOVA, followed by Bonferroni posthoc test.**

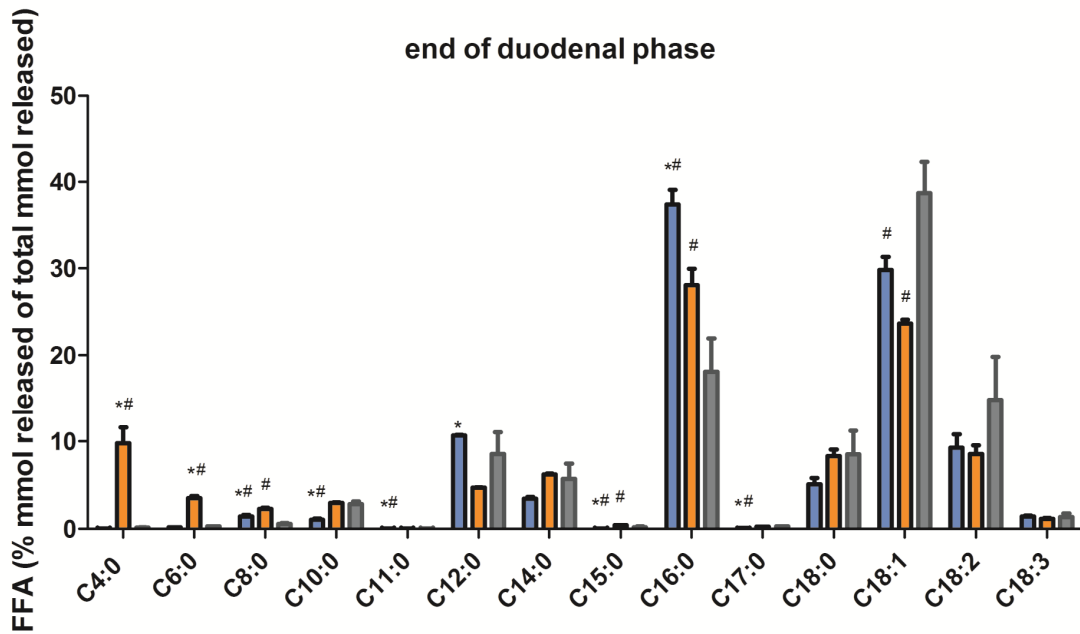
242 To obtain more insight in the digestion process, the release of individual FAs at the end of
 243 each digestion phase was analysed. **Figure 2** shows the distribution of individual FFAs

244 released as percentages of total FFAs after the gastric phase (A) and after the duodenal
245 phase (B). During gastric lipolysis MCFA were the main FAs released, followed by palmitic
246 acid (C16:0) and oleic acid (C18:1) (**Figure 2A**). The digestion of IF2 was distinctly marked by
247 the release of C4:0 originating from bovine milk. The long-chain unsaturated fatty acids
248 (LCUFA) C18:1 and C18:2 from human milk were released at a higher level, whereas for the
249 IFs the percentage released of these fatty acids was much lower. During duodenal lipolysis,
250 the main FAs released were LCFA (**Figure 2B**). Lipolysis of human milk resulted in higher
251 levels of unsaturated fatty acids (C18:1 and C18:2) compared to the IFs. The profile of FFAs
252 released from IF2 was still marked by a significant level (9.8%) of C4:0, although this was not
253 found for IF1 and human milk. Furthermore, a higher percentage of all MCFA, except C12:0,
254 was found after lipolysis of IF2.

A



B



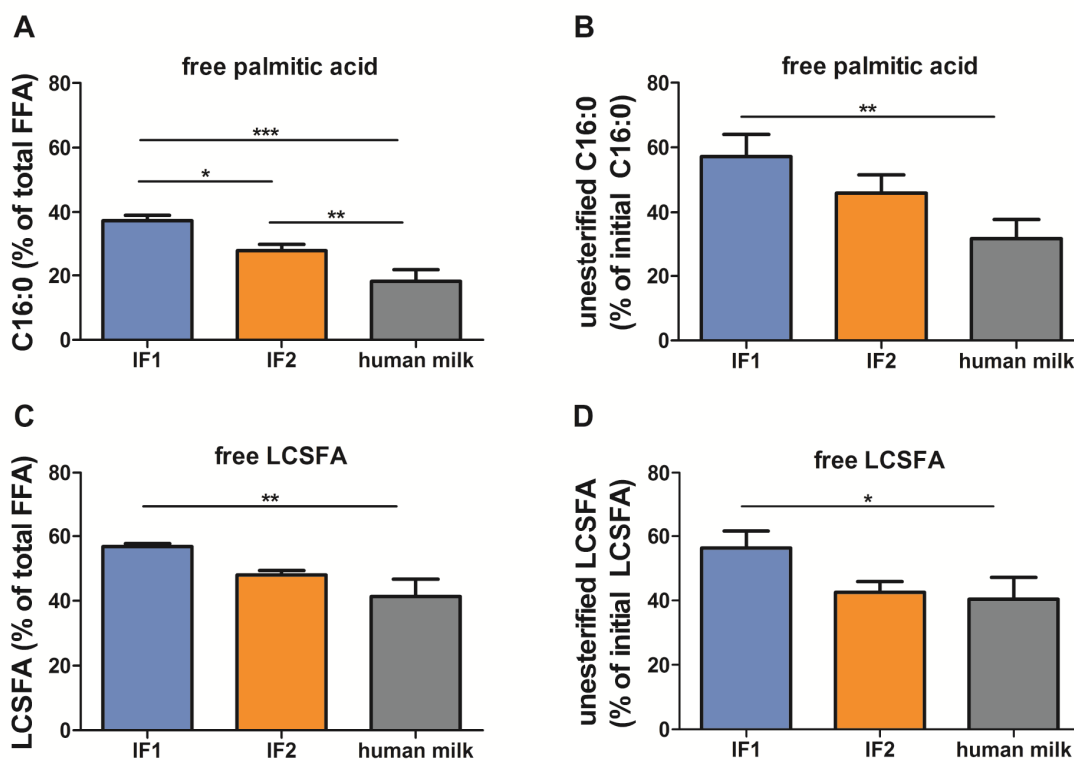
255

256 **Figure 2: Free fatty acid profile at the end of *in vitro* gastric digestion (A) and duodenal digestion (B) of IF1 (blue bar), IF2**

257 **(orange bar), and human milk (grey bar) (mean ±SD). * significant difference between IFs, # significant difference**

258 **between IF and human milk (p<0.05), as compared with one-way ANOVA.**

259 Since LCSFA are able to form complexes with calcium, the release of the sum of LCSFA and
 260 the most prominent LCSFA, palmitic acid, were compared between the IFs and human milk
 261 after digestion (end of duodenal phase). Free palmitic acid was less represented after
 262 digestion of human milk ($18.2 \pm 3.8\%$ of the FFAs) compared to digestion of IF1 ($37.4 \pm 1.6\%$;
 263 $p < 0.001$) and IF2 ($28.0 \pm 1.9\%$; $p < 0.001$) (**Figure 3A**). Of the initial palmitic acid present in
 264 TAGS from IFs and human milk, the lowest percentage of palmitic acid ($31.8 \pm 6.0\%$) was
 265 released from human milk (**Figure 3B**), while $57.2 \pm 6.9\%$ and $46.0 \pm 5.6\%$ respectively was
 266 released from IF1 and IF2 ($p < 0.01$). The total release of LCSFA, as percentage of FFA released
 267 (**Figure 3C**) or as percentage of LCSFA initially present in the IF and human milk TAGs (**Figure**
 268 **3D**), was lower in the human milk samples compared to IF1. IF2 did not significantly differ
 269 from human milk or from IF1.

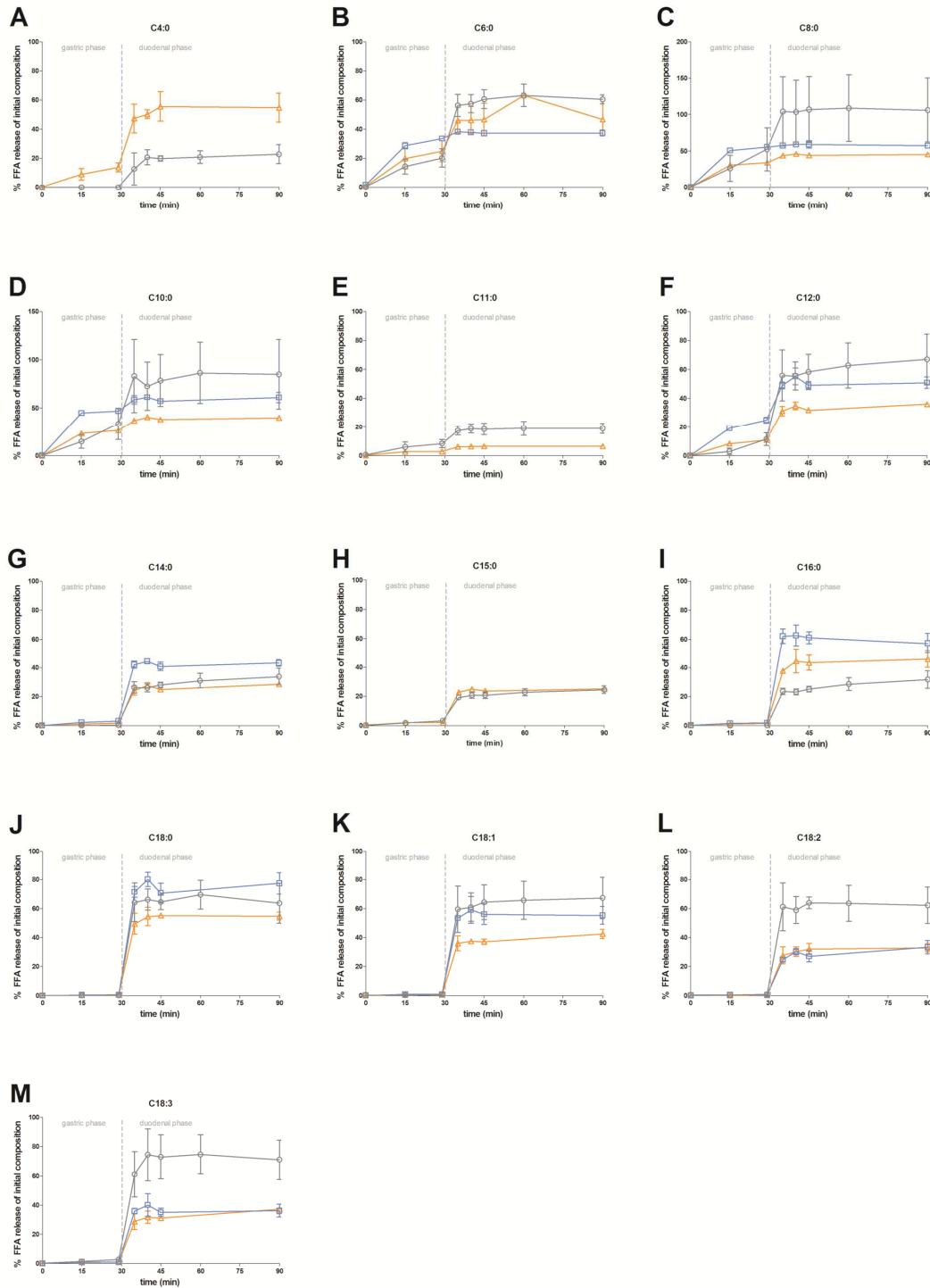


270
 271 **Figure 3: Free palmitic acid (C16:0) and total free long-chain saturated fatty acids (LCSFA) released from IFs and human**
 272 **milk at the end of duodenal digestion. A) free palmitic acid expressed as % of total free fatty acids (FFA) released upon**
 273 **digestion B) free palmitic acid expressed as % of initial amount of C16:0 in milk products. C) total free LCSFA expressed as**

274 % of total FFA released upon digestion; D) total free LCSFA expressed as % of initial amount of total LCSFA in IFs and
275 human milk. IF1, blue bar; IF2, orange bar; human milk, grey bar. Data are (mean \pm SD), and were analysed using one-way
276 ANOVA, and Bonferroni posthoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

277 Next, the release of individual FAs in time was examined. **Figure 4** shows the release of FAs
278 as a percentage of their initial concentration in the IFs and human milk. **Table 2** shows the
279 corresponding mean percentages (\pm SEM) of the individual fatty acids released at the end of
280 digestion (90 min) as well as during gastric phase (0-30 min) and duodenal phase (30-90 min)
281 separately. The SCFA, C4:0, which is present in the IF with bovine milk fat (IF2) and found at
282 very low levels in human milk, was partly released in the gastric phase for IF2 (14%), whereas
283 for human milk no free butyric acid could be detected (**Figure 4A, Table 2**). The release of
284 C4:0 mainly took place in the duodenal phase, and total release was higher for IF2 compared
285 to human milk (respectively 55% and 23%, $p = 0.01$). The release of C6:0 was highest from
286 human milk ($p = 0.02$), and showed different patterns between the IFs and human milk
287 (**Figure 4B, Table 2**). For IF1 C6:0 was primarily released in the gastric phase. For IF2, the
288 release in the gastric and duodenal phase was comparable (respectively 25% and 21%). For
289 human milk the release of C6:0 in the duodenal phase was higher compared to the gastric
290 phase (respectively 40% vs 20%). The release of the MCFA C8:0 and C10:0 was similar in the
291 gastric phase and after total digestion for the IFs and human milk ($p = 0.38$, $p = 0.13$ and,
292 $p = 0.06$, $p = 0.11$ respectively). However, in the duodenal phase the release of C8:0 and C10:0
293 were higher compared to the IFs ($p < 0.01$ and $p < 0.01$ respectively). (**Figure 4C-D, Table 2**). All
294 of the LCFA, both saturated and unsaturated, were mainly released in the duodenal phase
295 (**Figure 4G-M, Table 2**). An exception is C12:0, lauric acid, which was also partly released in
296 the gastric phase (**Figure 4F, Table 2**). The release of C12:0 after total digestion was similar
297 for both IFs and human milk ($p = 0.33$). However, during the gastric phase the release was

298 highest for IF1 ($p < 0.01$), while during the duodenal phase the release was higher for human
299 milk compared to the IFs ($p < 0.01$). From the other LCSFA, except for C15:0, the release from
300 human milk is or tends to be lower compared to IF1 (Table 2). The release of LCUFA after
301 total digestion was higher from human milk compared to the IFs (Table 2)



302

303

Figure 4: Release of individual fatty acids during *in vitro* digestion, expressed as percentage of their initial FA content

304

present in IFs and human milk (mean \pm SD); A) C4:0, B) C6:0, C) C8:0, D) C10:0, E) C11:0, F) C12:0, G) C14:0, H) C15:0, I)

305

C16:0, J) C18:0, K) C18:1, L) C18:2, M) C18:3. The vertical striped line indicates the start of the duodenal phase following

306

the gastric phase. The grey line (\circ) represents human milk, the blue line (\square) represents IF1, and the orange line (Δ)

307

represents IF2.

308

309 **Table 2: Release of the individual fatty acids, as percentage of their initial FA content present in IFs and human milk (mean ± SEM) upon digestion of IF1, IF2 and human milk. P-values**310 **represent the results of one-way ANOVA, or in case of C4:0, C11:0 and C15:0 the results of a Student's t-test, difference between products is indicated with letters (Bonferroni posthoc**

Fatty acid (%)	Gastric phase				Duodenal phase				Total digestion			
	IF1	IF2	human milk	<i>p-value</i>	IF1	IF-2	Human milk	<i>p-value</i>	IF1	IF2	human milk	<i>p-value</i>
C4:0	nd	13.7 ± 1.8 ^a	0.0 ± 0.0 ^b	<0.01	nd	41.1 ± 7.6	22.7 ± 3.7	0.10	nd	54.7 ± 5.78 ^a	22.7 ± 3.7 ^b	0.01
C6:0	33.5 ± 0.71 ^b	24.9 ± 2.4 ^a	20.1 ± 3.7 ^a	0.07	3.9 ± 0.9 ^a	20.7 ± 1.5 ^b	40.9 ± 3.1 ^c	<0.01	37.4 ± 1.1 ^a	46.6 ± 3.6 ^{a,b}	60.5 ± 1.8 ^b	0.02
C8:0	55.5 ± 0.76	34.1 ± 0.4	52.4 ± 14.8	0.38	1.8 ± 0.8 ^a	11.3 ± 0.7 ^a	53.8 ± 15.8 ^b	<0.01	57.3 ± 1.1	45.4 ± 0.0	106.2 ± 22.0	0.06
C10:0	46.8 ± 0.59	26.5 ± 1.0	33.4 ± 8.2	0.13	14.1 ± 4.5 ^a	13.5 ± 2.7 ^a	51.6 ± 19.6 ^b	<0.01	60.9 ± 3.0	40.0 ± 0.9	85.0 ± 18.0	0.11
C11:0	nd	3.0 ± 0.1 ^a	8.5 ± 1.4 ^b	0.02	nd	3.6 ± 0.3 ^a	10.4 ± 2.1 ^b	<0.01	nd	6.6 ± 0.2 ^a	19.0 ± 1.8 ^b	<0.01
C12:0	24.9 ± 1.20 ^a	10.9 ± 0.2 ^b	11.6 ± 2.2 ^b	<0.01	26.0 ± 3.9 ^a	25.1 ± 1.5 ^a	55.5 ± 12.9 ^b	<0.01	50.9 ± 2.3	36.1 ± 0.9	67.1 ± 8.7	0.33
C14:0	3.1 ± 0.25 ^a	1.71 ± 0.0 ^b	0.4 ± 0.1 ^c	<0.01	40.2 ± 2.0 ^a	26.9 ± 1.4 ^b	33.5 ± 5.7 ^{a,b}	0.01	43.3 ± 1.3 ^a	28.6 ± 0.8 ^b	33.9 ± 2.9 ^{a,b}	<0.01
C15:0	nd	2.1 ± 0.2	3.3 ± 0.9	0.35	nd	23.1 ± 1.0	18.7 ± 4.5	0.16	nd	25.2 ± 0.6	24.6 ± 2.6	0.83
C16:0	1.8 ± 0.13 ^a	1.5 ± 0.1 ^a	0.2 ± 0.1 ^b	<0.01	55.4 ± 6.7 ^a	44.5 ± 5.7 ^{a,b}	31.7 ± 6.0 ^b	<0.01	57.2 ± 4.0 ^a	46.0 ± 3.2 ^{a,b}	31.8 ± 3.0 ^b	<0.01
C18:0	0.5 ± 0.33	0.7 ± 0.4	0.2 ± 0.2	0.51	76.9 ± 7.7	54.1 ± 2.7	63.5 ± 13.6	0.07	77.4 ± 4.2	54.8 ± 1.6	63.7 ± 6.9	0.07
C18:1	0.9 ± 0.11	0.6 ± 0.1	0.7 ± 0.1	0.40	54.4 ± 5.8 ^{a,b}	41.9 ± 3.2 ^a	66.6 ± 14.0 ^b	0.04	55.3 ± 3.5 ^{a,b}	42.5 ± 1.8 ^b	67.4 ± 7.1 ^a	0.04
C18:2	0.4 ± 0.07 ^a	0.4 ± 0.1 ^a	1.0 ± 0.2 ^b	0.02	33.0 ± 4.6 ^a	32.6 ± 3.0 ^a	61.1 ± 12.4 ^b	<0.01	33.4 ± 2.6 ^a	32.9 ± 1.7 ^a	62.3 ± 6.3 ^b	<0.01
C18:3	1.0 ± 0.16 ^a	1.2 ± 0.2 ^a	2.9 ± 0.5 ^b	0.02	35.2 ± 4.1 ^a	36.1 ± 3.1 ^a	68.16 ± 12.6 ^b	<0.01	36.2 ± 2.5 ^a	37.4 ± 1.9 ^a	71.1 ± 6.7 ^b	<0.01

311 **test). nd, not detected**

312

313

314 **Discussion**

315 This is the first *in vitro* study to investigate the difference in lipolysis between an IF
316 containing bovine milk fat and an IF with vegetable fats only, and to compare this with the
317 lipolysis of human milk. Using a two-step *in vitro* infant digestion model, which consisted of a
318 gastric and a duodenal phase, no differences in total FA release at the end of digestion
319 between the different IFs and human milk were found in these experiments. Therefore, this
320 study showed that addition of bovine milk fat to IF did not results in a different total extent
321 of lipolysis compared to an IF containing vegetable fats only or human milk.

322 *In vitro lipolysis*

323 In this study we tried to mimic the digestion as occurring in infants. Therefore, the gastric pH
324 was set to infant levels, which is higher compared to that of adults.³¹ Gastric and duodenal
325 pH values were chosen to correspond to conditions existing in the stomach and duodenum
326 at half gastric emptying time of a meal.²⁹ The gastric lipase that is used has characteristics
327 similar to human gastric lipase, including stereo-preference and pH-activity level.²⁷ In
328 addition, the duration of digestion that was used in this model was quite short compared to
329 that of other *in vitro* digestion models. Whereas some other models use a gastric phase of
330 several hours³²⁻³⁴, the gastric phase of the current model lasted only 30 minutes. However,
331 this is in line with the findings of Roman *et al*, who showed that the gastric emptying time of
332 an infant is approximately 30 minutes.⁶ Moreover, the gastric lipolysis levels reached after
333 30 min under these conditions of pH and gastric lipase concentration are equivalent to those
334 recorded *in vivo* during the whole gastric digestion period.^{29,35} Further improvement of the
335 model could involve stepwise transfer of product fractions to the duodenal phase, mimicking
336 the process of gastric emptying, and gradual addition of digestive enzymes. These two

337 effects potentially negate each other. In view of the rapid lipolysis that is observed, these
338 alterations are unlikely to affect the currently observed outcomes.

339 *SCFA analysis*

340 Previous studies investigating the *in vitro* lipolysis of bovine milk, human milk and IFs only
341 determined the release of FA with a carbon number equal to or higher than six.³⁶⁻⁴¹ The
342 extraction of C4:0 and its quantification are notoriously difficult due to its partial solubility in
343 water, volatility and poor detection with various analytical techniques. Therefore, the level
344 of C4:0 released from milk TAGs are often not determined during both *in vitro* and *in vivo*
345 digestion studies. However, since C4:0 is present in relatively high levels in bovine milk fat, in
346 this study it was essential to determine the release of this FA as well. Therefore, an in-
347 solution derivatization approach was used allowing all FFA to be extracted after an
348 conversion to ethyl esters in the investigated matrix. This method thus prevents loss and
349 allows for quantitative measurements.³⁰ Therefore, the total FFA profile, including C4:0,
350 could be studied, which normally fail to be investigated due to their water solubility. The in-
351 solution derivatization approach allowed us to show a full picture of the specificity of gastric
352 lipase and gastric lipolysis, concerning all FAs in the investigated matrices.

353 *Gastric lipolysis*

354 The present *in vitro* digestion experiments showed that during the gastric phase, only a small
355 percentage of total FAs were released. For the IFs about 10% of total FFAs were released in
356 the gastric phase, which is in line with previous findings.^{25,31,34} The gastric lipolysis of human
357 milk samples was around twofold lower, with about 4% of total FFA release. This may be due
358 to the presence of the human milk fat globule membrane in fresh human milk, which is less
359 accessible to lipases than the globules in IF.^{42,43} Another explanation might be the larger

360 lipid-water interface exposed to the lipases⁴⁴, since the IFs used in these experiments have a
361 modal diameter of 0.3 μm , while human milk is known to have a larger modal diameter,
362 around 4 μm .⁴⁵ . Since no significant difference was found for the clearance of TAGs
363 between human milk and IFs in the gastric phase, the differences in FFA release and lipolysis
364 levels can easily be explained by further hydrolysis of DAGs generated from TAGs of IFs.
365 However, this was not studied here and thus remains speculative.

366 Since samples were taken at different time points during the *in vitro* digestion it was possible
367 to study the time-dependent release of the individual fatty acids. During gastric lipolysis
368 mainly MCFA and part of C4:0 (for IF2 only) were released, with only some LCSFA. Most of
369 the released LCSFA was C12:0. In this study we defined C12:0 as a LCFA since it has the
370 ability to form calcium soaps like other LCSFA.²⁰ However, lauric acid is more water soluble
371 than FA \geq C14⁴⁶, and can partly be directly absorbed into the portal vein.⁴⁷ Therefore it is
372 sometimes considered as being a MCFA.⁴⁸ This study shows that the C12:0 release in time
373 are in line with MCFA, and therefore the results support the categorization of C12:0 as a
374 MCFA. The preferential release of the short and medium chain FA is according to
375 expectation, since it corresponds to the specificity of gastric lipase.^{4,5} For human milk no
376 release of C4:0 was found in the gastric phase, however, the levels of C4:0 in the human milk
377 samples was very low (**Table 1**), therefore, the amount of C4:0 released might have been
378 below detection.

379 *Duodenal lipolysis*

380 In the duodenal phase, lipolysis occurred at a faster rate and reached higher levels. After 10
381 minutes almost maximum lipolysis was reached for all samples. Thus, the pancreatic extract,
382 containing pancreatic lipase, in combination with bile salt, acted very efficiently. This high

383 efficiency may have precluded identification of subtle differences in digestion kinetics. Such
384 differences may possibly have been observed using lower concentrations of lipases.
385 However, lower lipase concentrations would not have been physiologically relevant for term
386 infants, as the conditions we used were based on *in vivo* data obtained from term infants.³⁵
387 The release of FA from initial TAGs found in these experiments after the duodenal phase is
388 between 42 and 52%. These values have to be compared with a lipolysis level of 67% (TAGs
389 totally converted in MAGs and FFAs) that allows full intestinal absorption of lipolysis
390 products. The model used here has been shown to well reproduce the lipolysis levels
391 observed *in vivo* at the Angle of Treitz, i.e. the end of the duodenum.⁴⁹ It does not allow to
392 observe full lipolysis, but it appears suitable for comparing various type of meals²⁹,
393 emulsions^{50,51} and, as shown here, IFs versus milk.

394 As expected, the FFA profile in the duodenal phase was somewhat different between the
395 different fat blends. In the course of human milk lipolysis, higher levels of unsaturated fatty
396 acids (C18:1 and C18:2) were released, and lower levels of saturated fatty acids, compared
397 to the IFs. This is in line with the starting hypothesis that human milk has its LCUFA mostly
398 placed at the sn-1 and sn-3 positions and LCSFA at the sn-2 position. Whereas both IFs
399 contain similar levels of palmitic acid, less was released in the bovine milk fat containing
400 blend, which is likely due to the higher level of palmitic acid at the sn-2 position in this blend.
401 Palmitic acid, and also the other LCSFA, are known to form insoluble calcium soaps in the
402 intestinal lumen.^{19,20} The formation of calcium soaps is linked to digestive problems, such as
403 constipation and therefore discomfort.¹⁹ The release of LCSFA from the IF containing bovine
404 milk fat was not different from human milk. Thus, after digestion of IF with bovine milk fat
405 less unesterified palmitic acid is present and the total LCSFA is not different from human

406 milk.. Therefore, it is expected that addition of bovine milk fat to IF leads to less calcium
407 soap formation and thereby less gut discomfort.

408 Not only LCFA were released in the duodenal phase in the course of human milk digestion.
409 Unexpectedly, also part of the MCFA from human milk and part of C4:0, from human milk
410 and IF containing bovine milk fat, were released. Since C4:0 and MCFA are mostly positioned
411 at the sn-1 and sn-3 position of the glycerol backbone, they are thought to be preferentially
412 released by gastric lipase in the stomach. However, gastric lipolysis is rapidly inhibited by
413 FFA⁵², and therefore some C4:0 and MCFA may have reached the duodenal phase still
414 esterified in TAGs (or DAGs). Gastric lipase can still work in the duodenal phase where its
415 inhibition by lipolysis products, which is observed in gastric conditions⁵³, is abolished by bile
416 salts. This has been shown in chronic pancreatitis patients with no pancreatic lipase.⁵⁴ In
417 addition, pancreatic lipase is a 1,3-regioselective lipase that can also preferentially release
418 the FAs esterified to the sn-3 position of the glycerol backbone. The difference in time points
419 of release of MCFA between human milk and IFs may also be explained by differences in TAG
420 composition. It has been shown that the percentage of C8:0 at the sn-2 position is higher in
421 the milk of Chinese mothers compared to IF.²³ Such positioning of those FAs at the glycerol
422 backbone could have partly protected them from lipolysis in the gastric phase of digestion.

423 *Possible health effects*

424 The preferential release of C4:0 and mainly MCFA in the gastric phase of digestion is
425 potentially linked with the fact that these FAs can already be absorbed by the gastric
426 cells.^{55,56} When they reach the intestinal lumen they could possibly exert effects on epithelial
427 cells. MCFA are hypothesized to have beneficial effects, like antimicrobial effects.^{57,58} The
428 same holds true for C4:0, butyric acid, which is present in bovine milk fat and in trace

429 amounts in human milk. Butyrate, and other SCFA, are largely produced by microbial
430 fermentation in the intestine and are generally considered as important mediators of the
431 beneficial health effect of intestinal microbiota, contribution to epithelial maturation and
432 barrier function.^{59,60} Since microbial fermentation occurs predominantly in the colon, it is of
433 interest that milk and IF have the potential to deliver SCFA to the small intestine. A study
434 performed in piglets showed that consumption of butyrate improved development of the
435 jejunum and ileum crypt depth, villi length and mucosa thickness were increased.⁶¹ Whether
436 the levels of butyric acid present in human milk and IF containing bovine milk fat contributes
437 to small intestinal maturation and functioning still needs to be determined.

438 **Conclusion**

439 The addition of bovine milk fat to IF, and thereby changing the triglyceride structure, did not
440 influence the total amount of release of fatty acids in time compared to an IF with vegetable
441 fat only or human milk. However, the profile of the fatty acids that are released was found to
442 be different. More SCFA and MCFA, and less LCSFA, especially palmitic acid, were released
443 from an IF containing a combination of bovine milk fat and vegetable fat compared to an IF
444 containing only vegetable fat. The FFA profile of human milk distinguishes from the IF
445 products by a higher release of MCFA and an even lower release of palmitic acid in the
446 duodenal phase.

447 **Abbreviation (optional)**

448 FA: fatty acid

449 FFA: free fatty acid

450 LCFA: long-chain fatty acid (C12:0>higher)

451 LCSFA: long-chain saturated fatty acid

452 LCUFA: long-chain unsaturated fatty acid

453 MCFA: medium-chain fatty acid, (C6:0-C11:0)

454 SCFA: short-chain fatty acid, (C4:0)

455 SFA: saturated fatty acid

456 TAG: triacylglycerol

457 UFA: unsaturated fatty acid

458 **Conflict of interest**

459 Jeske Hageman and Anouk Feitsma are employees of FrieslandCampina

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