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Similarity index and soap forming capacity in milk fat- and OPO-containing infant formulas

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ABSTRACT

Fat blends used in infant formulas (IFs) are designed according to the legally required total fatty acid (FA) composition. However, regional distribution of FAs on the *sn*-2 and *sn*-1/3 positions of the glycerol backbone in triglycerides is very important for the final functional (nutritional) properties of the fat. The regional distribution of FA in bovine milk fat- (MF) and OPO-containing IFs was compared in terms of similarity index, and "potential" soap forming capacity (SFC) as defined by the amount of long chain saturated FA on the *sn*-1/3 position that is linked to measured soaps in infant stools from an in vivo trial including IF with and without MF. Both MF- and OPO-type of ingredients can be used to improve similarity with human milk fat structure and to optimise potential soap formation: OPO is a more targeted approach on specific FAs; MF provides a wider range of FAs at the desired *sn*-positions.

1. Introduction

Human milk (HM) is the best source of nutrition for newborns, providing the optimal combination of nutrients such as protein, fat, carbohydrates, vitamins, and minerals. Fats are the main energy source for a newborn infant and provide the infant with essential fatty acids (FAs), mono- and diglycerides, vitamins, and cholesterol. The FAs in human milk fat (HMF) are mainly present in triglycerides and FA composition is important for the nutritional properties. In addition to the FA composition, the lipid structure, i.e., the distribution of the FAs over the *sn*-1, *sn*-2 and *sn*-3 position of the glycerol backbone, is also relevant.

The triglyceride (TAG) structure of HMF has been described and reviewed extensively (Delplanque, Gibson, Koletzko, Lapillonne, & Strandvik, 2015; Hageman, Danielsen, Nieuwenhuizen, Feitsma, & Dalsgaard, 2019; He, McClorry, Hernell, Lönnerdal, & Slupsky, 2020). The lipid structure in HMF is characterised by a strong prevalence of long-chain saturated FAs such as palmitic acid (C16:0) on the *sn*-2 position (70–88%), whereas long-chain unsaturated FA are mainly present on the *sn*-1/3 position (Haddad, Mozzon, & Frega, 2012; López-López, López-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargalló, 2002; Martin, Bougnoux, Antoine, Lanson, & Couet, 1993; Straarup, Lauritzen, Faerk, Høy, & Michaelsen, 2006). HMF is rich in OPO-type (1,3-dioleolyl-2-palmitate) of triglycerides. Human milk fat from Chinese origin is known to be richer in OPL-type of triglycerides in which one of the *sn*-1/3 positions is richer in linoleic acid (C18:2) compared with human milk fat from Western origin (Yuan et al., 2019).

When triglycerides are digested, the outer fatty acids (positioned at sn-1 and sn-3 of the glycerol backbone) are hydrolysed and released as free fatty acid. The free fatty acids and the monoglyceride can be absorbed although the efficacy of absorption is dependent on the length and saturation of the fatty acid. Short chain FAs (SCFAs: Cx with $2 \leq x \leq 4$) are primarily hydrolysed in the stomach and absorbed in the gut. The long chain FAs (LCFAs: Cx with $x > 11$) are primarily hydrolysed by pancreatic lipase and absorbed through passive transport of LCFA containing micelles. The fate of non-absorbed FAs depends on the part of the digestive tract where they are released and the physiological conditions in the intestine.

The released saturated LCFAs (LCSFA) from the *sn*-1 or *sn*-3 position may form insoluble complexes with cations such as calcium and magnesium in which calcium is the most abundant divalent cation in IFs. These so-called FA soaps prevent absorption of LCSFAs and decrease the uptake of calcium. It has been shown that soap formation is related to undesirable hard stools causing discomfort of the newborn (Lockton & Lucas, 1995). The lipid structure of infant formulas (IFs), and especially the *sn*-1 and *sn*-3 FA composition, is therefore of importance to control the extent of potential soap formation (Mehrotra, Sehgal, & Bangale, 2019).

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Bovine milk fat (MF) is a rich natural source of fatty acids, carotenes, flavor components, cholesterol and fat-soluble vitamins Their nature and their concentrations are reviewed by Mohan, O'Callaghan, Kelly, and Hogan (2020). Sources of MF applied in IFs are whole milk, cream, and anhydrous milk fat (AMF or butter oil). All these MF sources are obtained by applying solely physical processes to whole bovine milk. Besides the main fatty acids, MF contains also fatty acids present at lower concentrations such as branched chain fatty acids and cis/trans conjugated linolenic acid (CLAs). These minor fatty acids species also exist in human MF (Dingess et al., 2017). MF contains about 13 FAs that are present at weight percentages of 1% or higher. These FAs can form >1000 TAGs, differing in stereochemistry, making up about 95% of the total TAGs. The amount of palmitic acid at the sn-2 position is about 40–45% of the total amount of palmitic acid and MF also shows a high proportion of LCSFA at the *sn*-2 position (30–49%) (Hageman et al., 2019). The *sn*-1/3 position in MF is rich in SCFA and LCFA, both saturated and unsaturated. MF containing IFs have shown to reduce soap formation, reduce crying and improve stool characteristics in newborns (Manios et al., 2020; Sheng, Buthmanaban, Vonk, Feitsma, & Parikh, 2020).

Natural vegetable oils and fats do not have the optimal lipid composition and structure to mimic HMF. The overall composition and structure can be improved by blending different fat sources. A route to optimise the lipid structure is to engineer a triglyceride structure via inter- or intra-esterification. Chemical inter- or intra-esterification is not specific enough leading to too much randomisation of FAs over all *sn*-positions. Enzymatic restructuring of fats is more efficient but also more costly. A one step enzymatic synthesis of a structured lipid consists of an enzymatic inter-esterification of a fat source (e.g., a palm stearin) with another fat or FA source or via acidolysis of a fat source with non-esterified FA (e.g., obtained from high oleic sunflower oil). The first fat source is enzymatically hydrolysed with *sn*-1/3 specific enzymes and the hydrolysed fatty acids are replaced by the target FA (e.g., oleic acid) (Wei, Jin, & Wang, 2019). Examples of products obtained by this route are Betapol ® and Infat ®. This process can lead to about 65% of palmitic acid at the *sn*-2 position. A two-step approach yields much purer products (up to 95% of palmitic acid at the *sn*-2 position) but these methods are more expensive and may require the use of solvents and undesired reaction products need to be removed (Hasibuan, Sitanggang, Andarwulan, & Hariyadi, 2021). A recent development is the occurrence of ingredients enriched with OPL-type of triglycerides (Infat ® Plus). Fat blends containing these structured lipids have a TAG composition that is less diverse compared with human milk fat and bovine milk fat. IFs with these structured OPO-type of ingredients (also called β-palmitate, *sn*-2 palmitate) are known to reduce soap formation, reduce gut discomfort and reduce crying in newborns likely due to hard stools (Bar-Yoseph, Lifshitz, Cohen, Malard, & Xu, 2016; Litmanovitz et al., 2014; Yao et al., 2014).

The similarity index (*SI*) is a one-number result of a calculation method that compares the FA composition in IFs with that of HMF, an *SI* of 100 meaning total similarity to the reference composition. Wang et al. (2010) developed it for comparison of total FA and *sn*-2 FA composition. It is especially useful to compare compositions with a wide range of FAs. This approach was extended for the *sn*-1/3 FA composition (Kloek, Vonk, Feitsma, & Timmer, 2020). They compared the *SI* for MF formulas, palm oil (+PO) formulas and palm oil free (POF) formulas. It was shown that MF formulas were most similar to the HMF reference for total FA profile and for *sn*-2 profile, whereas the POF formulas were more similar for *sn*-1/3 profile. In addition, it showed that the type of vegetable fat source in the formula can be a discriminator with respect to similarity, independent on the presence of milk fat as an animal fat source. Recently another similarity method for total FA compositions was published leading to similar conclusions when comparing IFs with different oil sources (Hokkanen, Frey, Yang, & Linderborg, 2022).

In this paper, the *SI* for OPO- and MF-containing commercial IFs is compared, also considering the other fat sources present in the IFs. Furthermore, the effect of the % of MF in the IF on the *SI* is studied. This is then related to the *sn*-1/3 FA composition and the *SI*-sn1/3. A newly defined soap forming capacity parameter (SFC), that is based on the *sn*-1/3 LCSFA distribution, is subsequently linked to the *SI* for *sn*-1/3 and discussed in terms of measured soap concentrations in stools from an in vivo infant trial comparing vegetable and MF containing IF.

2. Material and methods

2.1. Human milk data from literature

HMF reference compositions (total FAs, *sn*-1/3, *sn*-2) needed to calculate the *SI* were collected from publications ensuring a balance between ethnic origin (European and Chinese) and lactation stage (Haddad et al., 2012; López-López et al., 2002; Martin et al., 1993; Straarup et al., 2006; Sun et al., 2016; Wang et al., 2010; Wu et al., 2019; Zou et al., 2012). The geography, stage of lactation and number of human subjects per stage are given in Supplementary material Table S1. The average composition can be found elsewhere (Kloek et al., 2020).

2.2. Similarity index calculation

The *SI* of total FAs, *sn*-2 and *sn*-1/3 was calculated according to the basic procedure of Wang et al. (2010) taking into account the attention points with respect to data treatment by Kloek et al. (2020). The molar concentration of FA *i* (FA_i) in the sample B_i was compared with the concentration of that FA in the HMF reference, D_i. For the references, a relative standard deviation σ_{rel} of 20% was taken for each FA leading to a HMF FA concentration range of $A_{low,i}$ and $A_{up,i}$ given by respectively $D_i \cdot(1- \sigma_{rel})$ and $D_i \cdot(1+ \sigma_{rel})$. If the concentration in the sample fell inside the range of $A_{low,i}$ and $A_{up,i}$ there was no subtraction and constant C_i was 0. If the concentration fell outside that range, constant C_i became:

$$C_i = \frac{|B_i - A_{up,i}|}{A_{up,i}} \text{ if } B_i > D_i \quad (1)$$

$$C_i = \frac{|B_i - A_{low,i}|}{A_{low,i}} \text{ if } B_i < D_i \quad (2)$$

The subtraction E_i per FA is proportional to the relative concentration in the HMF composition D_i and is proportional to C_i.

$$E_i = 100 \cdot C_i \left(\frac{D_i}{\sum_i D_i} \right) \quad (3)$$

The *SI* was calculated by subtracting the summed subtraction over all FAs from 100:

$$\text{SI} = 100 - \sum_i E_i \quad (4)$$

The *SI* was calculated for the total FA profile (*SI*-FA), the FA profile at *sn*-2 position (*SI*-sn2) and the FA profile at the *sn*-1/3-position (*SI*-sn1/3).

2.3. Commercial infant formulas

A total of 29 commercial IFs were included in the analyses of which 20 were milk fat-containing (MF) and 9 were OPO-containing (OPO). The selection was based on presence of MF and OPO, not on other oil sources. The weight percentage of MF in the MF-containing IFs varied and was calculated based on the butyric acid (C4:0) content of the formula assuming 3.8% (w/w) C4:0 in bovine milk fat. Due to seasonal and source variation this may give an inaccuracy in the weight percentage of MF, but this is estimated to be no more than 10% of the calculated %MF. Most products were purchased in China and Hong Kong between 2016 and 2018 and originated from manufacturers in Western Europe (Denmark, France, Germany, Ireland, Switzerland, The Netherlands), China or Singapore. The fat blend composition in the IF was derived from the list of ingredients on the label. The MF IFs were categorised on being palm oil containing (+PO) or palm oil free (POF). The protein sources were of a bovine nature for all IFs. The IF data are compiled in Table 1.

2.4. Determination of stereo-chemical fatty acid composition

The fat structure in IFs was determined with a *sn*-1/3 specific pancreatic lipase-based hydrolysis of TAGs and subsequent FA-analysis of the 2-monoacylglycerols (Luddy, Barford, Herb, Magidman, & Riemenschneider, 1964). The 2-monoacylglycerols were isolated by thin layer chromatography, subsequently methylated for gas chromatographic analysis (GC) and quantified in weight concentrations, as FA methyl esters (FAME). The latter was analysed by standard ISO methods (ISO15884/IDF182 and ISO15885/IDF184: ISO, 2002a,b). Conversion to molar FA concentrations involved corrections for the FAME molecular weights. The *sn*-1/3 FA distribution per FA_i was calculated from the molar total FA composition ($FA_{tot,i}$) and the *sn*-2 FA composition ($FA_{sn2,i}$) using the following equation (Eq (5)):

$$FA_{sn13,i} = \frac{3 \cdot FA_{tot,i} - FA_{sn2,i}}{2} \quad (5)$$

Table 1
Overview of IF groups and formulas that were analysed for total FAs and sn-2 distribution, the composition of the specific IF in terms of oils and fats obtained from the ingredient declaration and the milk fat source used.

ID	IF group	% MF	MF source	Other fats	Oils
1	MF	10.5	WMP	PO	SBO, PO, PKO, SO
2	MF	11.2	whole milk	PO	PKO, SBO, HOSO, PO, CNO
3	MF	15.8	whole milk, AMF	POF	SO, SBO, CO, AMF
4	MF	18.4	WMP/AMF	POF	SO, SBO, CO, AMF
5	MF	19.9	whole milk	PO	PO, RO, PKO, SO
6	MF	19.9	whole milk	PO	PO, RO, PKO, SO
7	MF	20.0	whole milk	PO	PO, RO, PKO, SO
8	MF	20.1	whole milk	PO	PO, RO, PKO, SO
9	MF	20.2	whole milk	PO	PO, RO. PKO, SO
10	MF	21.1	whole milk	POF	RO, CO, CNO, SO
11	MF	23.7	whole milk, AMF	POF	CO, RO, CNO, SO, WNO, AMF
12	MF	26.3	whole milk	PO	RO, CO, PO, CNO
13	MF	39.5	whole milk, AMF	POF	CO, RO, SO, SBO, CNO, AMF
14	MF	50.5	cream	POF	SO, RO, CNO
15	MF	50.8	AMF	POF	AMF, CNO, SO, RO
16	MF	50.9	AMF	POF	AMF, CNO, SO, RO
17	MF	50.9	whole milk/cream	POF	SO, RO, CNO
18	MF	53.1	cream	POF	SO, RO, CNO
19	MF	53.3	AMF	POF	AMF, CNO, SO, RO
20	MF	65.8	cream	POF	SBO, SO, RO, CNO
21	OPO	<0.1	–		PKO, SO, RO, OPO
22	OPO	<0.1	–		PKO, SO, RO, OPO
23	OPO	<0.1	–		PKO, SO, RO, OPO
24	OPO	<0.1	–		PKO, SO, RO, OPO
25	OPO	<0.1	–		SBO, SO, CNO, OPO
26	OPO	<0.1	–		SBO, SO, CNO, OPO
27	OPO	<0.1	–		SBO, SO, CNO, OPO
28	OPO	<0.1	–		SBO, SO, CNO, OPO
29	OPO	<0.1	–	PO	PO, SO, RO, PKO, CO, CNO, OPO

Abbreviationsare: MF, bovine milk fat-containing formulas; OPO, OPO-containing formulas; WMP, whole milk powder; AMF, anhydrous milk fat; SBO, soybean; PO, palm oil; PKO, palm kernel oil; SO, sunflower oil; HOSO, high oleic sunflower oil; CO, corn oil; CNO, coconut oil; RO, rapeseed oil; WNO, walnut oil. The other fats column is used to categorise the IFs as palm oil containing (PO), palm oil free (POF) or OPO containing (OPO). For this classification OPO is not labelled palm oil containing. The %MF in MF containing IFs is calculated from the total FA composition assuming MF contains 3.8% (w/w) butyric acid.

2.5. In-vivo data

The in-vivo data were derived from a double-blind randomised cross-over trial described in detail (Manios et al., 2020). A high MF IF (50%), a medium MF IF (20% MF) and a reference IF with only vegetable fat (VF) (also no OPO) were compared. The high MF IF is low in *sn*-1/3 LCSFA, whereas the medium MF IF is high in *sn*-1/3 LCSFA. The medium MF IF is slightly lower in *sn*-1/3 LCSFA than the VF IF. After a two-week run-in period, infants were allocated to one of the cross-over studies using block randomisation. In each of the studies infants were randomly assigned to receive either the VF formula or a MF-based formula: i) 50% MF + 50% VF (50MF) in cross-over study 1 (CS1) and ii) 20% MF + 80% VF (20MF) in cross-over study 2 (CS2). At the end of each two-week intervention period, stool samples were collected for FA, FA soaps and calcium analysis. The in-vivo data were used to compare stool composition with *SI* and stereochemistry of the IF fat source.

2.6. Statistical evaluation

Data analysis was performed in Excel using the add-in XL-Stat 2019.3.2 with the ANOVA function, followed by the Tukey HSD test using pairwise comparisons to test the differences between the FA compositions between the different IF groups for all FAs, FAs at *sn*-2 and FAs at *sn*-1/3 ($P < 0.05$).

3. Results and discussion

3.1. Comparison of regional chemistry and SI of MF- and OPO-containing IFs

Table 2 compiles the average FA composition of the average HMF reference and the MF- and OPO-containing IF groups for total FA, FA at sn-2 and FA at sn-1/3. Because of the wide range of % MF, the MF group is divided in a medium MF group (<25% MF) and a high MF group (>25%MF). Both groups enclosed 10 observations. Significant differences ($P < 0.05$) between the IF groups are indicated with letters and shortly summarised below.

For the overall FA composition, MF-containing IFs have higher concentrations C4:0, C6:0, C15:0, C17:0, C14:1 and C16:1 compared with OPO-containing IFs. The high MF-containing IFs have higher concentrations C4:0, C6:0, C8:0, C10:0, C14:0, C14; 1, C16:1 and C17:1 compared with the medium MF containing IFs. OPO-containing IFs have higher concentrations C12:0 and C18:1 compared with high MF containing IFs. There is no significant difference for C16:0.

For FA at *sn*-2, MF-containing IFs have higher concentrations C15:0, C17:0 and C16:1 compared with OPO-containing IFs. The high MF-containing IFs have higher concentrations C14:0, C16:0, C17:0, C14:1, C16:1 and C17:1 compared with medium MF-containing IFs. OPO-containing IFs have higher concentrations

Table 2
Molar distribution (expressed as % of total FA) of the main FAs in total fat, on sn-2 and on sn-1/3 in IF groups.[a]

FA	FA all					*sn*-2					*sn*-1/3				
	HMF	MF_med	MF_high	OPO	Pr > F(Model)	HMF	MF_med	MF_high	OPO	Pr > F(Model)	HMF	MF_med	MF_high	OPO	Pr > F(Model)
C4:0	0.000^{c}	1.760^{b}	4.528^{a}	0.000^{c}	<0.0001	0.000^{a}	0.000^{a}	0.150^{a}	0.000^{a}	0.388	0.000^{c}	2.640^{b}	6.718^{a}	0.000^{c}	<0.0001
C6:0	0.030^{c}	1.013^{b}	2.312^{a}	0.145^{c}	<0.0001	0.020^{a}	0.000^{a}	0.140^{a}	0.000^{a}	0.160	0.035^{c}	1.519^{b}	3.398^{a}	0.217^{c}	<0.0001
C8:0	0.205^{c}	1.443^{b}	2.234^{a}	1.748 ^{ab}	<0.0001	0.093^{b}	0.390 ^{ab}	0.650^{a}	0.300 ^{ab}	0.010	0.261^{c}	1.969^{b}	3.027^{a}	2.471 ^{ab}	<0.0001
C10:0	1.647^{b}	1.559^{b}	2.720^{a}	1.248^{b}	<0.0001	1.011^{b}	1.290^{b}	2.240^{a}	0.767^{b}	0.001	1.965^{b}	1.693^{b}	2.960^{a}	1.489^{b}	0.000
C12:0	6.286^{b}	8.193 ^{ab}	6.935^{b}	10.686^{a}	0.007	6.733^{c}	12.770^{b}	12.100 ^{bc}	20.889^{a}	<0.0001	6.062^{a}	5.905^{a}	4.352^{a}	5.584^{a}	0.676
C14:0	6.824^{a}	4.731^{b}	7.412^{a}	3.876^{b}	<0.0001	10.085 ^{ab}	7.880 ^{bc}	12.770^{a}	5.178^{c}	<0.0001	5.192^{a}	3.157^{b}	4.732 ^{ab}	3.225^{b}	0.003
C15:0	0.187^{b}	0.240^{b}	0.511^{a}	0.000^{c}	<0.0001	0.329^{a}	0.350^{a}	0.600^{a}	0.033^{b}	0.000	0.116^{b}	0.185^{b}	0.467^{a}	0.000^{b}	<0.0001
C16:0	24.078^{a}	20.445 ^{ab}	18.677^{b}	21.401 ^{ab}	0.029	51.821^{a}	14.930^{d}	21.390^{c}	27.367^{b}	<0.0001	10.179^{b}	23.202^{a}	17.321^{a}	18.418^{a}	<0.0001
C17:0	0.197^{a}	0.177^{a}	0.269^{a}	0.000^{b}	<0.0001	0.209^{a}	0.020^{b}	0.220^{a}	0.000^{b}	<0.0001	0.192^{a}	0.256^{a}	0.294^{a}	0.000^{b}	<0.0001
C18:0	5.847^{a}	4.294^{b}	5.208^{a}	3.475^{b}	<0.0001	1.459^{a}	2.830^{a}	2.860^{a}	1.544^{a}	0.152	8.046^{a}	5.025 ^{bc}	6.382^{b}	4.441^{c}	<0.0001
C20:0	0.140^{c}	0.249 ^{ab}	0.202^{b}	0.260^{a}	<0.0001	0.163^{a}	0.000^{b}	0.020^{b}	0.000^{b}	<0.0001	0.129^{c}	0.374^{a}	0.292^{b}	0.390^{a}	<0.0001
C22:0	0.040^{b}	0.215^{a}	0.193^{a}	0.195^{a}	<0.0001	0.043^{a}	0.000^{a}	0.020^{a}	0.000^{a}	0.172	0.038^{b}	0.323^{a}	0.280^{a}	0.292^{a}	<0.0001
C24:0	0.045^{a}	0.096^{a}	0.078^{a}	0.082^{a}	0.310	0.047^{a}	0.000^{a}	0.010^{a}	0.000^{a}	0.081	0.044^{a}	0.144^{a}	0.113^{a}	0.123^{a}	0.082
C14:1 ω5cis	0.152^{b}	0.163^{b}	0.535^{a}	0.000^{b}	<0.0001	0.092^{b}	0.120^{b}	0.470^{a}	0.000^{b}	0.004	0.182^{b}	0.184^{b}	0.568^{a}	0.000^{b}	<0.0001
C16:1 ω7cis	2.366^{a}	0.460^{c}	0.827^{b}	0.082^{d}	<0.0001	2.355^{a}	0.610^{c}	1.400^{b}	0.111^{d}	<0.0001	2.371^{a}	0.385 ^{bc}	0.540^{b}	0.106^{c}	<0.0001
C17:1 ω7cis	0.052 ^{ab}	0.010^{b}	0.087^{a}	0.000^{b}	0.003	0.011^{b}	0.010^{b}	0.100^{a}	0.000^{b}	0.013	0.072^{a}	0.015^{a}	0.086^{a}	0.000^{a}	0.036
C18:1 ω9 cis	32.395 ^{ab}	36.336^{a}	29.534^{b}	36.315^{a}	0.010	12.430^{c}	38.120^{a}	26.210^{b}	24.756^{b}	<0.0001	42.397^{a}	35.444^{b}	31.196^{b}	42.094^{a}	<0.0001
C20:1w9cis	0.469^{a}	0.276^{b}	0.271^{b}	0.281^{b}	0.010	0.298^{a}	0.000^{b}	0.030^{b}	0.000^{b}	<0.0001	0.556^{a}	0.415^{a}	0.391^{a}	0.421^{a}	0.197
C18:2 ω6cis (LA)	15.304^{a}	15.182^{a}	13.808^{a}	17.809^{a}	0.278	9.463^{b}	18.710^{a}	15.690^{a}	17.156^{a}	<0.0001	18.230^{a}	13.419^{a}	12.867^{a}	18.136^{a}	0.020
C18:3 ω3cis (ALA)	0.892^{b}	1.668^{a}	1.819^{a}	1.762^{a}	<0.0001	0.587^{b}	1.880^{a}	2.390^{a}	1.878^{a}	<0.0001	1.045^{b}	1.563 ^{ab}	1.533 ^{ab}	1.704^{a}	0.041
C18:3 ω6cis (GLA)	0.051^{a}	0.019^{a}	0.009^{a}	0.000^{a}	0.055	0.027^{a}	0.001^{b}	0.000^{b}	0.001^{b}	0.012	0.063^{a}	0.028^{a}	0.014^{a}	0.000^{a}	0.106
C20:5 ω3cis (EPA)	0.529^{a}	0.035^{b}	0.026^{b}	0.000^{b}	<0.0001	0.566^{a}	0.000^{b}	0.010^{b}	0.000^{b}	<0.0001	0.510^{a}	0.053^{b}	0.034^{b}	0.000^{b}	<0.0001
C20:4 ω6cis (AA)	0.079^{b}	0.368^{a}	0.322^{a}	0.302^{a}	<0.0001	0.037^{a}	0.010^{a}	0.050^{a}	0.000^{a}	0.431	0.099^{b}	0.546^{a}	0.458^{a}	0.453^{a}	<0.0001
C22:6 w6cis (DHA)	0.409^{a}	0.262 ^{ab}	0.194^{b}	0.157^{b}	0.001	0.656^{a}	0.000^{b}	0.050^{b}	0.022^{b}	<0.0001	0.285^{a}	0.392^{a}	0.266^{a}	0.224^{a}	0.169

[a] Abbreviations are: HMF, human milk fat reference; MF_med, milk fat containing IFs with MF<25%; MF_high, milk fat containing IFs with MF>25%; OPO, OPO containing. Difference in significance per FA is tested using the Tukey test ($P < 0.05$) and denoted with different superscript letters per FA group (all, sn-2 and sn-1/3).

Table 3
Similarity index (SI) per IF group calculated from the similarity indices per IF for total FA, sn-2 and sn-1/3 composition.[a]

IF group	OPO	MF_high	MF_med	Pr > F(Model)	Significant
SI-FA	89.2 ± 3.2[a]	89.4 ± 3.9[a]	82.8 ± 12[a]	0.134	No
SI-sn2	48.3 ± 3.8[a]	47.9 ± 9.8[a]	26.3 ± 13[b]	<0.0001	Yes
SI-sn1/3	84.2 ± 1.8[a]	78.0 ± 5.4 [ab]	72.0 ± 6.9[b]	0.0003	Yes
SFC (%)	21.6 ± 0.6[a]	22.8 ± 1.3[a]	25.7 ± 7.3[a]	0.157	No

[a] *SI* is calculated against an average HMF reference composition assuming a σ_{rel} of 20% per FA. Abbreviations are: OPO, OPO containing IFs; MF_med, bovine milk fat containing IFs with MF<25%; MF_high, bovine milk fat containing IFs with MF>25%; SFC, soap forming capacity. Difference in significance is tested using the Tuckey test ($P < 0.05$) and denoted with different superscript letters.

C12:0 and C16:0 compared with high MF-containing IFs. The medium MF-containing IFs have a higher concentration of C18:1 compared with the OPO and high MF-containing IFs.

For FA at *sn*-1/3, MF containing IFs have higher concentrations C4:0, C6:0 and C17:0 compared OPO-containing IFs. High MF-containing IFs have higher concentrations C4:0, C6:0, C8:0, C10:0, C15:0 and C14:1 compared with OPO-containing IFs. OPO-containing IFs are clearly higher in C18:1 compared with high MF-containing IFs. There is no significant difference for C16:0.

For the overall FA composition and the FA distributions at *sn*-2 and *sn*-1/3 the main significant differences between the IF groups are the FAs mainly present in MF.

The SIs were calculated to compare the total FA profile and their distribution on *sn*-2 and *sn*-1/3 position with those of the human reference (Table 3). This shows that the *SI*-FA is similar for the three IF groups and their values are high (82–90). The *SI*-sn2 is similar for OPO and high MF containing IFs and is significant lower for medium MF-containing IFs. The *SI*-sn2 values are low with values ranging from 26 to 48. The FAs causing the difference can be derived from the subtraction point *E* per FA. For the medium MF-containing IFs the FAs causing the largest subtraction for *SI*-sn2 are C16:0 (too low concentration) and C18:1 (too high concentration).

The *SI*-sn1/3 is similar for OPO and high MF containing IFs and have high values ranging from 78 to 84. The *SI*-sn1/3 of medium MF-containing IF is significantly lower compared with OPO-containing IFs but similar to that of high MF-containing IFs. The difference between OPO-containing IFs and medium-MF containing IFs is mainly caused by C16:0 (too high concentration in MF) and to a smaller extend by C18:2 (too low concentration in MF).

The high *SI* for all FA shows that FA distributions of the IF groups show much resemblance with human milk fat but are not significantly different for each other. The *SI*-sn2 and *SI*-sn1/3 shows that the regional chemistry of the FAs is more discriminating between the IF groups where difference in C16:0, C18:1 and C18:2 are the most discriminating FAs despite of the significant difference in concentrations of other FAs that contribute less to the *SI* due to their low concentrations.

3.2. Effect of percentage MF in MF containing IFs on SI

The MF-containing IFs have a wide range of percentage MF in the formula, varying from 10 to 66%. Furthermore, there is variation in other fat sources present in the blend. These IFs were classified in +PO- and POF-products since this classification affects the *SI* (Kloek et al., 2020). Generally, MF IFs comprising palm oil contain maximally ~25% MF, because a higher %MF will result in a too high saturated fatty acid content. Fig. 1 shows the *SI*-FA, *SI*-sn2 and *SI*-sn1/3 as function of the percentage MF in the IF. The average of the OPO-containing formula (0% MF) is plotted for reference. The variation in the *SI*s for the OPO-containing IFs is small. Since the percentage of OPO in the IFs was not known or could not be determined, it was not possible to determine the effect of percentage OPO on *SI*s.

Especially when using a POF, the *SI*-FA is influenced by the MF content. The most optimal *SI*-FA is obtained at a MF content of approximately 40–50% and the *SI*-FA of these products is similar to that of the OPO-products. At lower or even higher MF contents, the differences in C16:0 and unsaturated fatty acids between the IF and HMF become larger, resulting in a higher subtraction for these fatty acids. Using a PO-containing fat blend, the *SI*-FA can be increased at relatively low MF contents.

The *SI*-sn2 is most affected by the milk fat content. It increases for both POF-IFs and PO-containing IFs with increasing amount of MF in the IF. The increase is most clear for POF-IFs. PO-containing IFs having slightly higher *SI* values at similar milk fat contents. The IF with soybean oil as the first fat source on the ingredient declaration has the highest *SI*-2n for PO-containing IF with low MF. Products with 50% MF have a slightly higher *SI* than the OPO-containing IFs.

SI-sn1/3 for POF products is hardly affected by the MF content and is almost as high as the *SI*-sn1/3 of OPO-containing IFs, except for the product with 66% MF that has a clearly lower *SI*-sn1/3. This is an IF with soybean oil as the first fat source on the ingredient declaration. PO-containing fat blend have a lower *SI*-sn1,3 at low MF content. Since exact oil ratios in the IF formula are not known, these effects cannot be linked specifically to oil sources.

Thus, increasing the percentage MF in IFs will increase the *SI*-sn2 over a broad percentage MF range independent on other fat sources present. More MF can improve the *SI* for all FA for palm oil free IFs up to 30% MF, while the *SI*-sn1/3 for palm oil-containing IFs can be improved by addition of MF up to 30%. This shows that MF can improve the similarity between the fat stereochemistry of IFs and HMF, although the degree of improvement depends on the composition of the fat blend used.

3.3. Soap forming capacity

During digestion the *sn*-2 MAGs are absorbed and the hydrolysed FA from the sn-1/3 position can form soaps provided calcium is present and pH is sufficiently high. Therefore, theoretical soap formation is expected to be linked to the FA composition at the *sn*-1/3 position and especially to the concentration of LCSFA at this position. We define the so-called soap forming capacity (SFC) as the mole % of LCSFA present at the *sn*-1/3 position on the total mole % FA present where $FA_{sn13,I}$ is the mole % FA at *sn*-1/3 summed over all LCSFA *i*:

$$SFC = \frac{2}{3} \cdot \sum_{i} FA_{sn13,i} \quad (6)$$

Using this definition, the HMF reference has a SFC of 19%. This value should be seen as a relative value to compare with other fat sources or blends.

The calculated SFCs for the IFs are compiled in Table 3 and show no significant differences in SFC between OPO- and medium or high MF-containing IFs despite of a significant difference in SI-sn1/3. This can be explained by the SFC being only determined by the LCSFA at the sn-1/3 position, whereas the SI-sn1/3 is determined by all FAs on these positions. MF contains low concentrations C18:1 and C18:2 and high concentrations of SCFA and MCFA at this sn-1/3 position.

Fig. 2 shows the SFC as function of the percentage MF in the IF for PO-containing and POF IFs. PO-containing IFs have a higher SFC compared with POF IFs except for the IF with 26%MF that is high in rapeseed oil and corn oil. This is also the IF with high *SI*-sn1/3 of 81.

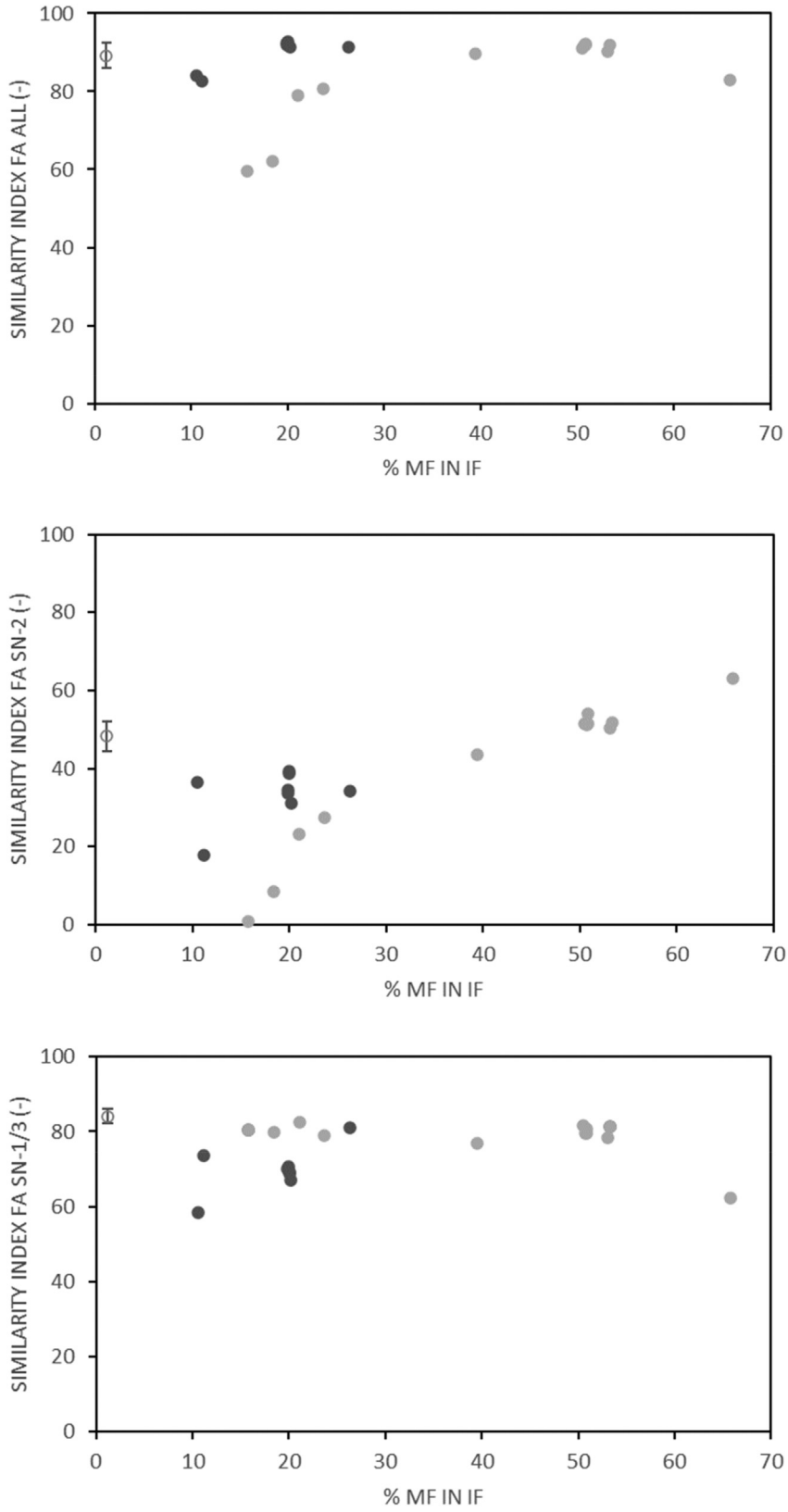

Fig. 1. Similarity index (*SI*) for FA all, FA at *sn*-2 and FA at *sn*-1/3 of MF containing IFs as function of the percentage MF in the IF. Symbols indicate whether the IF contains palm oil (●) or is palm oil free (●). The open symbol indicates the *SI* for the OPO containing IF without MF.

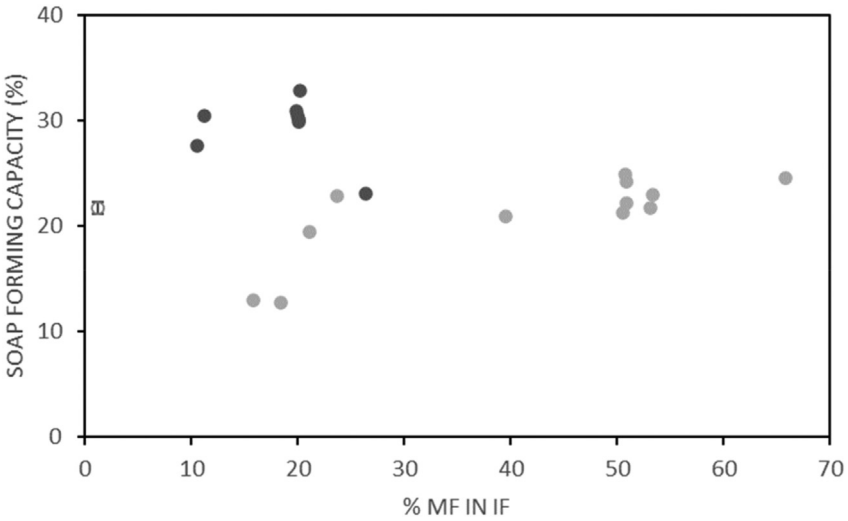

Fig. 2. Soap forming capacity of MF containing IFs as function of the percentage MF in the IF. Symbols indicate whether the IF contains palm oil (●) or is palm oil free (●). The open symbol indicates the *SI* for the OPO containing IF without MF.

For POF IFs the SFC increases with an increase of percentage MF in the IF. The effect on the SFC is small if the composition comprises more than 40% MF. The large effect at low percentage MF can be ascribed to the fats and oils other than the MF. The SFC of PO is about 41%, whereas the SFC of MF is 32%. Partial replacement of PO by MF will lead to a reduction of the SFC. If the vegetable fat blend is POF and rich in oils such as rapeseed oil, (high oleic) sunflower oil or soybean oil, addition of MF will lead to an increase in SFC.

Fig. 3 shows an overview of calculated SFCs of typical fats and oils based on internal data. From the semi-solid fats, MF has the lowest SFC and is therefore the best option to keep the SFC low in an IF blend with typical oils. One should keep in mind that human MF also has a certain SFC (~20%) indicating that optimising rather than minimising SFC should be performed when designing fat blends for IFs with respect to soap formation.

Fig. 4 shows that the *SI* for FA at *sn*-1/3 gives an indication of the SFC but does not fully predict it. Up to a SFC of 19–20%, the *SI* for FA at *sn*-1/3 is independent on SFC. For higher SFC value, the *SI*-sn1/3 decreases. Two groups can be distinguished that seem to be affected by the composition of the vegetable fat blend. The group represented by the lowest line contains IFs that have soybean oil as one of the first oils in the fat source declaration, indicating it is present at a high proportion. Soybean oil is rich in C18:2 of which large part is present at the *sn*-1/3 position. This gives a high subtraction for C18:2 at *sn*-1/3 and therefore a lower *SI*. The POF IFs have a lower SFC compared with PO-containing IFs but the POF IFs are also the ones that mainly consist of high MF-containing IFs. The PO-containing IFs have higher SFC but are also the one that consist of medium MF-containing IFs.

3.4. Similarity index and soap forming capacity linked to in vivo soap data in stools

The stool data are compiled in Supplementary material Table S2 and the similarity indexes, soap forming capacity number and regional FA distributions are compiled in Supplementary material Table S3 and Supplementary material Table S4.

The SI-FA is similar for the high and medium MF IF and is clearly lower for the IF without MF (Fig. 5). The IF without MF has higher SI subtractions for C12:0. The *SI*-sn2 is highest for high MF (about 50)

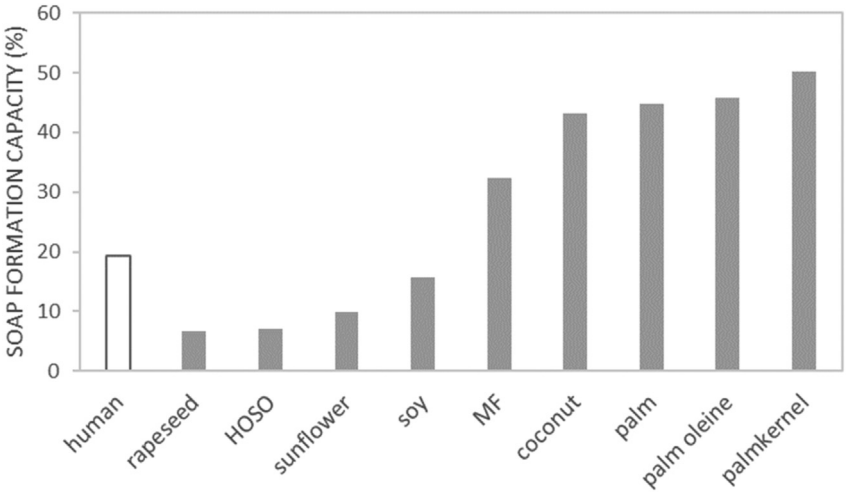

Fig. 3. Theoretical soap forming capacity (SFC) of fat sources used in IFs based on regional FA composition data (own data). Human is calculated from literature data. Oils and fats are ranked on increasing SFC.

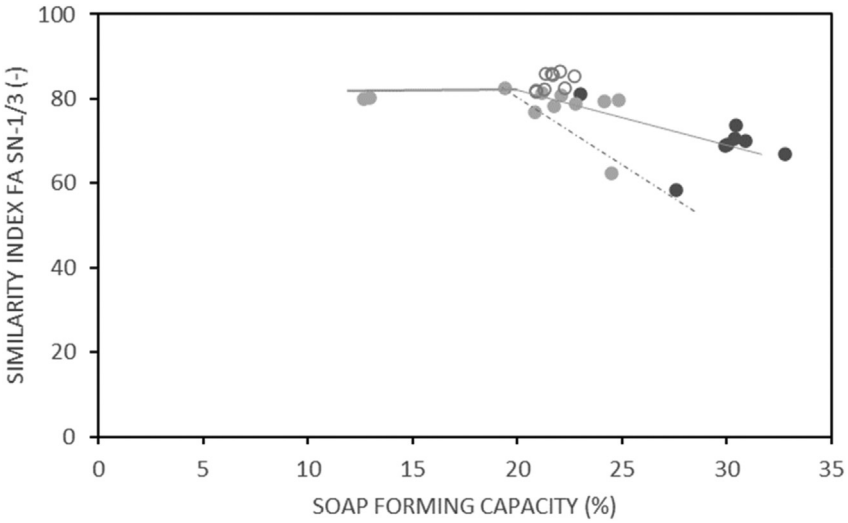

Fig. 4. Similarity index (*SI*) for FA at *sn*-1/3 of MF containing IFs as function of the soap forming capacity (SFC) of the IF. Symbols indicate whether the IF contains palm oil (●) or is palm oil free (●). The open symbol indicates the *SI* and SFC for the OPO containing IF without MF. The lines are a guide to the eye. The dashed grey line represents MF containing IFs rich in soybean oil.

and decreases in the order of medium MF IF and VF IF. The main subtractions are for C16:0 and C18:1, where both decrease with increasing amount of MF in the IF. The *SI*-sn1/3 is highest for high MF (about 80) and is clearly lower for the medium MF IF and the VF IF. Subtractions for the high MF are mainly on C16:0 and C18:2. There is no difference between the *SI*-sn1/3 between the medium MF IF and the VF IF. The subtractions for the latter two are mainly for C16:0.

The FA soap concentration in the stools decreases in the order C16:0, C18:0, C14:0, C18:1, C12:0 and C18:2. This order is independent on the IF type. Compared with the non-soaped FA in the stools, C16:0, C18:0, C14:0 and C12:0 are the dominant FAs to form soaps having a ratio soaped FA to non-soaped FA of respectively 20, 19, 7 and 4 (Manios et al., 2020). This ratio for C18:1 and C18:2 is respectively 1.7 and 0.2. Therefore, we will only consider the LCSFA soaps. The total soaped LCSFA concentration is significantly lower for the high MF compared with the other IFs. There is no significant difference between the medium MF IF and the VF IF (Manios et al., 2020). The total soaped LCSFA concentration shows a similar trend when plotted as function of the SFC (Fig. 6a). A plot of the individual soaped FA concentrations as function of the *sn*-1/3 concentration of the individual FAs for all three IFs (Fig. 6b) also shows a positive correlation. It is dominated by the C16:0 FA soaps and to a lesser extent the C18:0 soaps.

The concentrations C12:0 and C14:0 soaps are lower than expected if a linear relation between soap concentration and *sn*-1/3 FA concentration is assumed, but this can be well explained by the fact that the solubility index of calcium soaps is higher if the fatty acids are shorter (Graham & Sackman, 1983). This indicates that the individual FA composition at the *sn*-1/3 position, and the soap forming capacity derived from this, can be a good indicator for formation of soaps in stools. However, one should keep in mind that the amount of soap depends on many more factors than only *sn*-1/3 FA composition. It also depends on the location in the gastrointestinal tract where the fatty acids are released during digestion and the amount of dissolved calcium or other soap-forming cations at the time the fatty acids are released. The FA

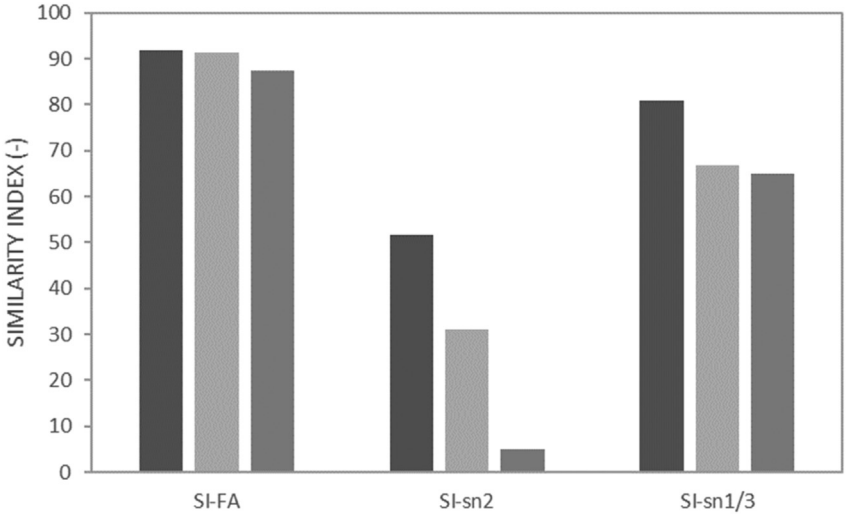

Fig. 5. Similarity index for total FA (SI-FA), FA at sn-2 location (SI-sn2) and FA at sn-1/3 location (SI-sn1/3) for the three different IFs tested in the in-vivo study. High MF IF(■), Medium MF IF (■) and VF IF (■).

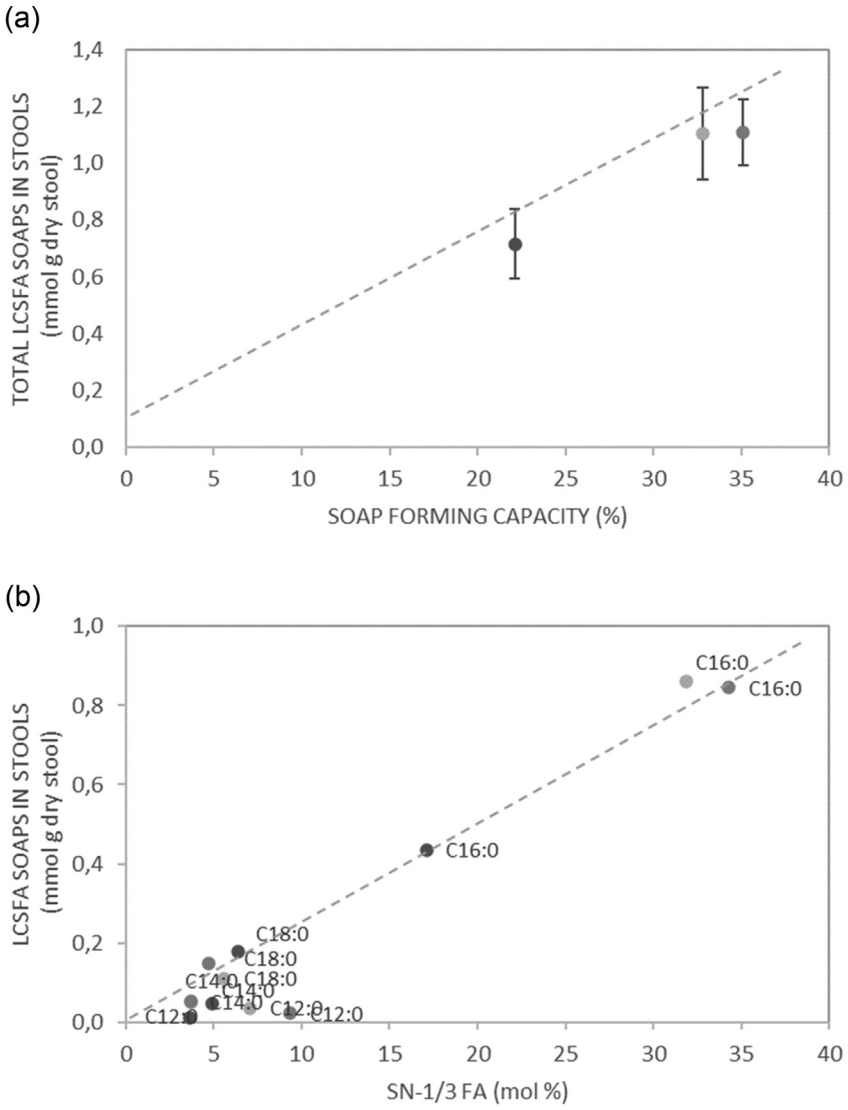

Fig. 6. Soap composition in stools for in vivo trial for high MF IF (●), medium MF IF (●) and VF IF (●), expressed as total LCSFA concentration as function of soap forming capacity (upper chart) and expressed as the individual soaped FA concentration as function of the FA concentration *sn*-1/3 position (lower chart). FAs are indicated as labels. Lines are guides to the eye.

release during digestion strongly depends on the how the fat is stabilised in the IF, the size of the fat droplets and the type of calcium salts that are present.

Furthermore, soap solubility will depend on the extent of water resorption that occurs in the last part of the digestive system. In the in vivo study, the molar ratio LCFA soaps/calcium increased with decreasing amount of MF in the IF, which was mainly due to an increase in the amount of soaped FA rather than a decrease in the amount of calcium in the stools). The molar ratio varied from 0.62 for high MF IF to 0.96 for IFs without MF which in all cases is much smaller than the stoichiometric ratio of 2. The lower value might be due to an excess of calcium not associated with FAs or due to a wide range of FAs in the stools disturbing the packing into soap structures. The wider range of FAs present in MF might be a reason that the ratio decreases with increasing amount of MF in the IF. All these factors will affect the SFC. Quinlan estimated molar ratios of soaps to calcium of 1.2 and explained this by mixed soap complexes (Quinlan, Lockton, Irwin, & Lucas, 1995).

Since LCSFA FAs at the *sn*-1/3 position are the main FAs to form soaps, their concentration is expected to be correlated with the *SI* subtraction points for *sn*-1/3 for LCSFAs. The correlation coefficients for the regression between concentration LCSFA soaps and concentration FAs at *sn*-1/3 (Fig. 6b) and between concentration LCSFA soaps and subtraction points for sn-1/3 FAs (Fig. 7) are, respectively, 0.951 and 0.938 indicating these parameters are highly associated to each other.

3.5. General discussion

SI depends on the selected HMF reference, which in this paper is an average of European and Chinese HMF sources. Since FA composition and regional distribution depends of FA depend on geography and food habits, a specific reference can be selected that is of most interest. This will affect the outcome of the *SI* comparisons.

Comparison of *SI* for MF- and OPO-containing IF groups do not show significant differences on *SI*-FA. *SI*-sn2 shows that OPO-containing IFs and high-MF containing IFs are equally similar to HMF with respect to sn-2 whereas medium-MF containing IFs are less similar to HMF. MF at sufficient high concentration can deliver

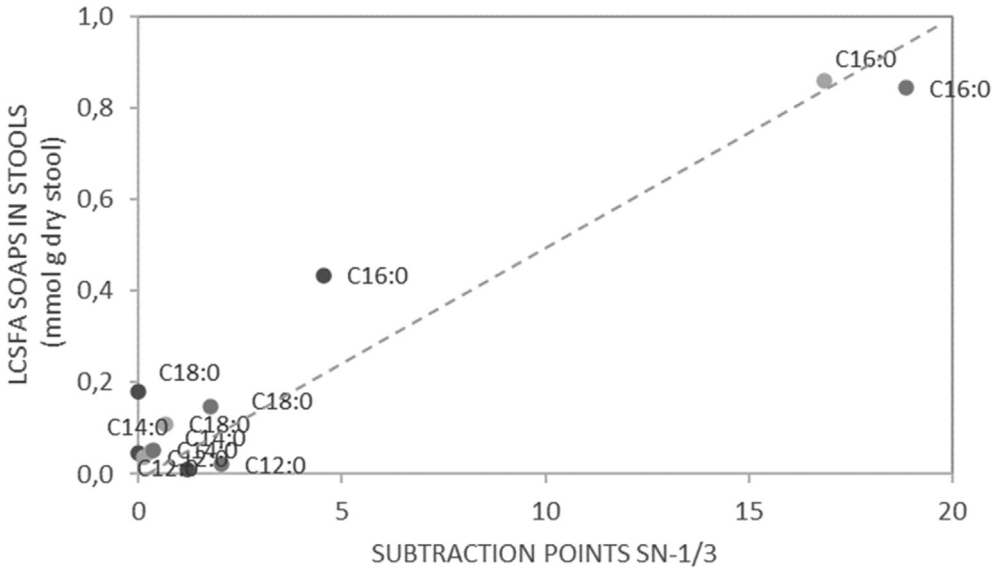

Fig. 7. Soap composition in stools for in vivo trial for high MF IF (●), medium MF IF (●) and VF IF (●), expressed as individual LCSFA concentration as function of as *sn*-1/3 subtraction points for the individual FAs. FAs are indicated as data labels.

similarity close to OPO-containing IFs. The *SI*-sn1/3 of POF MF-containing IFs are almost the same as that of OPO-containing IFs and is mainly a high MF effect. Presence of PO lowers the *SI*-sn1/3 but is indirectly also a low MF effect. Addition of OPO-type of ingredients leads to a higher *SI*-sn1/3 even if OPO is not the main ingredient in the IF. This is due to the high amount of the synthetic TAGs being rich in palmitate on *sn*-2 and oleic acid on *sn*-1/3. This makes the IF less sensitive for the presence of other fat sources.

The above effects strongly depend on the type of oils and fats used in the fat blend and in what ratios they are used. An important discriminator is whether the fat blend is PO-containing or is POF. The use of PO gives a high *SI*-FA whereas POF gives a high *SI*-sn1/3. A similar effect was observed when comparing non-MF PO-containing IFs and non-MF POF IFs (Kloek et al., 2020). The current paper also suggests that soybean oil is a discriminating oil source: high soybean oil concentrations lead to lower *SI*-sn1/3 which is due to high concentrations of C18:2 at *sn*-1/3. Therefore, it is important to consider all the fat sources used when comparing IFs in terms of presence or absence of certain fat sources like MF, PO, POF or OPO.

When designing fat blends for IFs, both MF and OPO type of ingredients can be used to improve *SI*-sn2 or *SI*-sn1/3. Use of MF will give similarity on a wider range of FAs and delivers specific FAs unique for MF and present in HMF, but present at low concentration and therefore not contributing substantially to the *SI*s. Use of OPO-type of ingredients is a more targeted approach on increasing *sn*-2 palmitate and *sn*-1/3 oleate using a triglyceride designed for this but lacking the wide range of FAs like MF.

A specific *sn*-2 palmitate and/or *sn*-1/3 oleate functionality to be delivered by the OPO ingredient is reduced soap formation. We defined the soap forming capacity as the concentrations of LCSFA on the *sn*-1/3 position to predict soap formation. These concentrations correlate well with the specific FA soaps formed in stools obtained from an in-vivo study in which IFs with different amounts of MF were tested and of which the FA distribution on *sn*-2 and *sn*-1/3 were known. Unfortunately, no similar detailed data are available for OPO-containing Ifs, so no comparison with our data can be made.

Optimising fat blends to reduce calcium soaps in stools can be done by optimising the total *sn*-1/3 composition (*SI*-sn1/3) or optimising the LCSFAs on the *sn*-1/3 position. Optimising on *SI*-sn1/3 will focus on being closer to the total FA pattern at *sn*-1/3 of HMF, whereas optimising on LCSFAs on the *sn*-1/3 position is a more targeted approach on soap forming capacity. This paper has shown that there is a relation between the sn-1/3 similarity and the SFC, although the SFC is not fully correlated to the sn-1/3 similarity but oil-type specific. At the end, it is the choice of formulating an IF with an OPO-ingredient that is designed to have a specific function or with MF, to obtain a wide FA range and broader fat-related beneficial nutrients for multiple functionalities. Less black and white, IFs containing both OPO and MF can result in low soap formation capacity and a wide range of FA present.

It should be kept in mind that the distribution of FA over the sn-2 and sn-1/3 position, and therefore *SI*, does not indicate the concentration of TAGs. The TAG profile indicates what are the neighbouring FAs at position *sn*-2 and *sn*-1/3. This is not a random distribution but designed by nature with a certain purpose. The TAG profile might be a next step in designing fat blends for IF formula.

4. Conclusions

MF- and OPO-containing IF groups are similar with respect to *SI*-FA. The OPO-containing IFs and high-MF containing IFs are equally similar to HMF with respect to sn-2 whereas medium-MF containing IFs are less similar to HMF. The OPO-containing IF group and high-MF containing IFs are similar with respect to *SI*-sn1/3 whereas the medium MF group is like the high MF-group but lower compared with OPO-containing IFs. Using stool data from an *in*-vivo infant trial, a positive correlation between LCSFA concentration at the *sn*-1/3 position and SFC was identified. Both the SFC and the *SI*-*sn*1/3 can be used to optimise the composition of the fat part of an infant formula. The *SI* and the SFC are not only affected by the presence of OPO or MF in the fat blend, but also by the choice of other oil/fat types: palm oil, palm oil free and soybean oil are discriminating. The effect of the other fats in the blend is larger when MF or OPO are present at lower concentrations. Use of OPO type of ingredients will provide a more targeted approach on supplying specific FAs on the foreseen *sn*-positions. Use of MF in IFs may increase similarity compared with human milk fat structure and provide a wide range of FAs and other dissolved components. *SI*-sn1/3 and SFC are important calculation tools to design fat

blends where the choice depends on either wanting to design a targeted composition (SFC) or wanting to design a blend with a wide range of FAs similar to HMF structure (*SI*-sn13).

Author contribution

William Kloek: Conceptualization, methodology, formal analysis, writing-original draft, writing-review. **Christel Timmer:** resources, writing-review. **Nanda de Groot:** conceptualization, writing-review. **Anouk Feitsma:** writing-review.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2023.105619.

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