Postprandial metabolism of infant formulas containing different fat sources

Digestion, absorption, and metabolic responses

Jeske H.J. Hageman

Propositions

1. The fat source in infant formula plays a role in satiety.
   (this thesis)

2. Analysis of volatile organic compounds in exhaled air is a promising non-invasive method to use in nutrition intervention studies for insights in metabolism.
   (this thesis)

3. Less focus should be on p-values, and more to the clinical relevance of outcomes and the cohesion between parameters.

4. Since beekeeping can negatively affect biodiversity of wild pollinators (Valido *et al*. Scientific Reports, 2019, 9, 4711), the number of beehives kept by beekeepers should be restricted.

5. Alcohol breath tests should be implemented in physiological intervention studies to test the compliance of participants to the restriction of alcohol intake.

6. The existence of Netflix increases the feasibility of physiological intervention trials to study energy metabolism in a rested state.

7. PhD students should sing together, both in and outside the lab, to reduce stress.

8. When discussing the sustainability of diets the impact on health must be taken into account.

Propositions belonging to the thesis entitled

'Postprandial metabolism of infant formulas containing different fat sources

-Digestion, absorption, and metabolic responses-'

Jeske Hageman

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Chapter 1

General Introduction

Infant formula

Human milk is the best nutrition available for a newborn. The World Health Organisation recommends that infants should be exclusively breastfed for the first six months of life.[1] However, in some situations mothers are not able to breastfeed. In that case, infant formula (IF) is the best alternative. Most IFs are produced by using skimmed bovine milk to which proteins, fat, vitamins, and minerals are added to resemble the composition of human milk as close as possible. Besides lactose, protein, and oligosaccharides, fat is an important component of IF; about 50% of the energy in IF comes from fat.[2] Furthermore, the fat in IF contains the essential fatty acids linoleic acid and alpha-linolenic acid, needed for the synthesis of long-chain polyunsaturated fatty acids (LCPUFAs).[3] The fat blend also contains some additional components required for growth and development, such as cholesterol and fat-soluble vitamins. In IF, fat is mainly present in the form of triacylglycerols (TAGs), which consist of three fatty acids connected to a glycerol backbone (**Figure 1**).

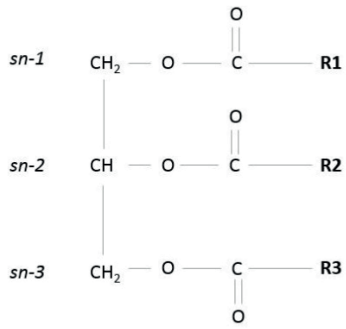

Figure 1: Composition of a triacylglycerol (TAG); three fatty acids attached at different stereospecific numbered (sn) locations of the glycerol backbone.

Fat digestion, absorption, and metabolism

Digestion of fat

After consumption of fat, digestion starts in the stomach. In the stomach emulsification takes place, in order to form smaller lipid droplets.[4] Gastric lipase starts the lipolysis, by releasing mainly the fatty acids at the sn-3 position, so that diacylglycerols (DAGs) are formed.[5,6] Thereafter, upon entering the duodenum the presence of TAGs, DAGs and fatty acids stimulates the production of gastrointestinal peptides.[7] Examples are cholecystokinin (CCK) and secretin, which stimulate pancreatic secretion and gall bladder emptying.[8–10] In this way pancreatic lipase and bile salts are secreted into the small intestine.[5] The bile salts emulsify the fat and thereby increase the surface area where lipase can act upon. Pancreatic lipase continues the lipolysis and cleaves the remaining fatty acids at the sn-3 position and fatty acids at the sn-1 position.[6] Most of the fatty acids at the sn-2 position remain attached to the glycerol backbone, as a monoacylglycerol (MAG) (**Figure 2**). Besides CCK and secretin, glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) are secreted upon presence of fat in the ileum. Some of these peptides enter the bloodstream and function as hormones.[11] Many of these hormones

act to delay gastric emptying, and thereby induce meal termination: satiation.[11] Another peptide which is involved in the regulation of appetite is ghrelin. This hormone stimulates appetite,[12] and is suppressed after digestion of fat.[7]

Absorption of fat

Short- and medium-chain fatty acids (SCFA and MCFA) are absorbed via passive diffusion, as unesterified fatty acids, or transported via anion exchange or monocarboxylate transporter 1 (MCT1) transporters into the enterocytes.[13,14] If the SCFA and MCFA are not stored or oxidized in the enterocytes, they are transported to the liver, directly via the portal vein.[14] Long-chain fatty acids (LCFA) and MAGs follow a different route of absorption. The apical side of enterocytes is covered by a gel-like layer, also known as the mucus layer.[15] Unesterified LCFA cannot easily cross this layer. However, the formation of micelles, together with bile salts and phospholipids, facilitates their transport over the mucus layer.[16,17] The uptake of fatty acids into enterocytes is believed to be mediated by membrane-associated fatty acid-binding proteins.[18] At the apical membrane, transport proteins, such as fatty acid transporter protein 4 (FATP4 (SLC27A4)), the scavenger receptor cluster determinant 36 (CD36), and scavenger receptor class B Type I (SCARB1) are expressed.[4,18,19] The fatty acids in the micelles can be transported into the enterocyte via those transport proteins (**Figure 2**). Once unesterified, fatty acids and MAGs cross the epithelial membrane, where they can have different fates.[20] A part of the fatty acids can be used for oxidation.[20] However, most of the fatty acids and MAGs will be re-esterified into TAGs, by monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT).[4] After a high-fat diet a part of these **TAGs** can be stored temporarily in lipid droplets in the enterocytes.[21] Formation of TAGs stimulates the production of microsomal triglyceride transfer protein (MTP). MTP transfers TAGs to a newly formed apolipoprotein B48 (apoB48), which rescues it from degradation, and forms it into a pre-chylomicron.[22,23] Then MTP transfers other TAGs, cholesterol, phospholipids, and fat-soluble vitamins into this pre-chylomicron.[4,19] Subsequently, apolipoprotein A-IV (apoA-IV) is added to the chylomicron membrane, which allows the particles to get larger and assimilate more lipids.[22] Thereafter, the chylomicron moves to the Golgi system, via fatty acid binding proteins (FABPs),[19] where apolipoprotein A-I (apoA-I) is added.[19] After final processing, the chylomicron is secreted via the basolateral membrane of the enterocytes.[22] Since chylomicrons are large in size, they cannot be secreted into the capillaries of the intestine. Instead, they are directed to the lymphatic system.

Postprandial metabolism of fat

Chylomicrons enter the bloodstream via the thoracic duct.[18,24] Once in the systemic circulation, chylomicrons can deliver fatty acids to skeletal muscle tissue, adipose tissue, and other peripheral tissues.[24,25] These tissues express lipoprotein lipase (LPL).[26] When chylomicrons pass peripheral tissues, especially skeletal muscle and adipose tissue, LPL hydrolyses TAGs in the chylomicrons.[20,27,28] In this way fatty acids are released and can be taken up by these tissues.[27,29]

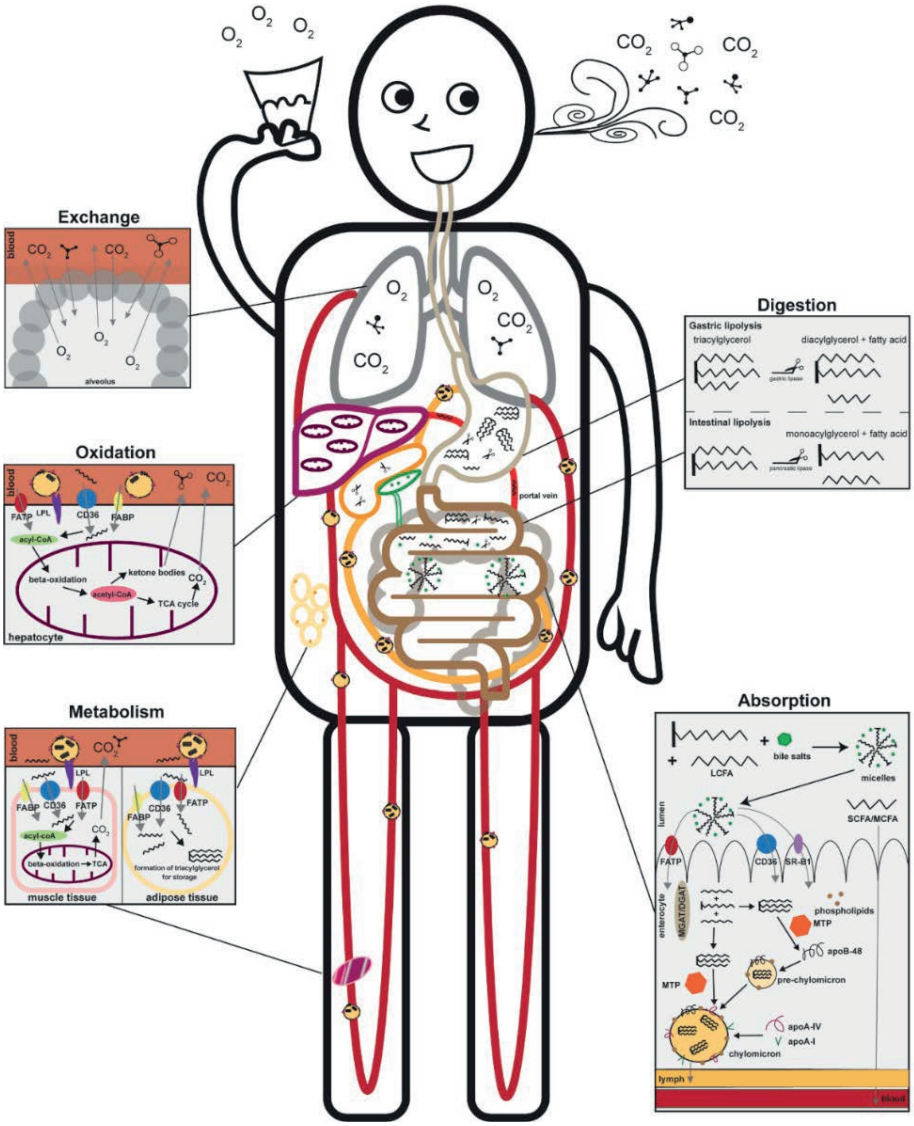

Figure 2: Schematic overview of postprandial digestion, absorption, and metabolism of triacylglycerols (TAGs). When TAGs are consumed, they are first digested in the stomach and small intestine, which results in the release of two free fatty acids (FFA) and one monoacylglycerol (MAG). Short- and medium-chain fatty acids (SCFA and MCFA) can directly transported from the lumen to the capillaries in the intestine, and are transported to the liver via the portal vein. Long-chain fatty acids (LCFA) and MAGs are, together with bile salts, formed into micelles. In the enterocyte, the unesterified LCFA and MAGs are esterified back into TAGs again. Chylomicrons are formed to transport the TAGs via the lymph system to peripheral tissues and the liver. In peripheral tissues, like muscle tissue and adipose tissue, lipoprotein lipase (LPL) is expressed, which releases FFA from the TAGs in chylomicrons. Those FFA will be absorbed into the tissues, and either be stored as TAGs or be used for beta-oxidation. Acetyl-coA, the product of beta-oxidation, can be used in the TCA cycle. The remaining chylomicrons, remnants, will be transported to the liver. There LPL is also expressed, and the remaining fatty acids are absorbed and are used for beta-oxidation. Acetyl-coA will either go into the TCA cycle or will be used to produce ketone bodies. CO_2, which is produced in the TCA cycle will transport via the blood to the lungs and will be exhaled.

Not all released fatty acids are taken up by muscle or adipose tissue, and will appear in the blood as non-esterified fatty acid (NEFA).[20,30] As such, they can be taken up by the liver or other tissues. Within adipose tissue fatty acids are re-esterified into TAGs and stored as such.[27,31] Skeletal muscle tissue takes up and stores fatty acids for oxidation.[27] This catabolism of TAGs in chylomicrons converts the chylomicrons in chylomicrons remnants.[20,27] These chylomicron remnants are transported to the liver, where fatty acids can be used for oxidation, and cholesterol is used for the formation of VLDL particles.[20,24]

Lipoprotein metabolism

In the liver, fatty acids can also be re-esterified into TAGs. Together with cholesterol esters, fatty acids are transported to synthesized apolipoprotein B100 (apoB100). Thereby, the degradation of apoB100 is prevented, and very large density lipoproteins (VLDL) are formed.[24] VLDL particles are then transported via the bloodstream to peripheral tissues, where LPL can act upon the TAGs and fatty acids will be released and used.[24] This process is similar to the fatty acid transport and release of chylomicrons.[24] When fatty acids are removed from VLDL, this particle becomes smaller, resulting in intermediate density lipoproteins (IDL) (**Figure 3**).[24] IDL particles can be cleared from circulation by the liver, and the remaining TAGs are hydrolysed resulting in the formation of low density lipoproteins (LDL).[24] LDL particles mainly contain cholesterol esters and apoB100. Another type of lipoprotein particles are the high density lipoproteins (HDL). The formation of HDL particles takes several steps. Each HDL particle contains apoA-I, which is synthesized in the liver, or can be acquired from chylomicrons.[24] To form a particle, cholesterol and phospholipids are obtained from the liver or intestine.[24] Furthermore, HDL particles acquire lipids and apolipoproteins from other tissues, and from other lipoproteins particles when those undergo lipolysis by LPL.[24] HDL functions as transport vehicle for transport of cholesterol from peripheral tissue to the liver for excretion and catabolism.[32]

Fat oxidation

In the muscle tissue and the liver fatty acids can be oxidized. Fatty acids are transported into the cell via transporters CD36 and FATP4, and to a lesser extent by FATP1 (SLC27A1) and FABPs.[33,34] Thereafter, fatty acids are converted to fatty acid-acyl coenzyme A (CoA) by acyl-CoA synthetases.[31] There are different acyl-CoA synthetases for the different fatty acid lengths. Long-chain acyl-CoA synthetases are located in the mitochondrial outer membrane and the endoplasmatic reticulum.[35] Fatty acyl-CoA are converted into fatty acyl carnitines by carnitine palmitoyltransferase 1 (CPT1),[36] transported over the mitochondrial inner membrane by CACT and reconverted into fatty acyl-CoAs by CPT2. Short- and medium-chain acyl-CoA synthetases are present in the mitochondrial matrix.[14] Shorter-chain unesterified fatty acids are able to pass the inner membrane, via carnitine acetyltransferase.[37] Inside the mitochondrial matrix the fatty acid-acyl CoAs enter the beta-oxidation cycle, which results in the formation of acetyl-CoA.[31] Acetyl-CoA can further be used in the tricarboxylic acid (TCA) cycle. The energy that is produced in these cycles, as NADH and FADH2, is used to generate ATP via oxidative phosphorylation.[31] Besides entering the TCA cycle, acetyl-CoA can also be used in the liver to form ketone bodies.[35,38] Ketone bodies can be used as energy substrates in other tissues, such as the brain.[39]

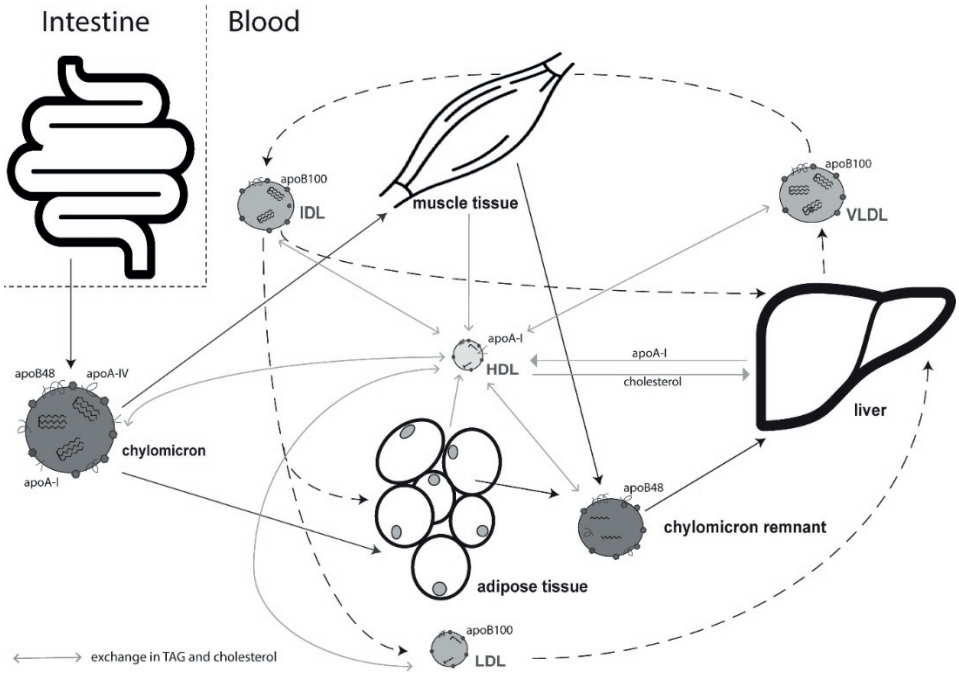

Figure 3: Schematic overview of lipoprotein metabolism. Chylomicrons are formed in the intestine, and are transported to peripheral tissues, like muscle and adipose tissue. There fatty acids are taken from chylomicrons, and chylomicron remnants are formed. Those are transported to the liver, where the remaining fatty acids are used for oxidation. In the liver very large density lipoproteins (VLDL) are formed. Those have a similar function as chylomicrons, transporting TAGs through the body. When fatty acids are taken intermediate density lipoproteins (IDL) and later even low density lipoproteins (LDL) are formed. High-density lipoproteins (HDL) are formed from apolipoprotein A-I (apoA-I) which is acquired from chylomicrons or the liver. HDL particles exchange TAGs and cholesterol with other lipoproteins, and transport cholesterol back to the liver.

Satiety induced by fat

With the stimulation of the release of gut peptides, fat intake can induce satiety. For example CCK has been found to induce satiety via stimulation of the nervus vagus.[40] Fat can also induce satiety via other routes, for example an increase in fat oxidation seems to be associated with satiety.[41,42] This might be explained by the increase in liver ATP, a product of the oxidative phosphorylation, which might influence food intake.[42] Another factor in fat-induced satiety is oleoylethanolamide (OEA), a lipid mediator. OEA, which is produced in the small intestine, has been found to increase after a meal.[43] OEA is thought to activate vagal sensory fibres in the small intestine to signal to the brain and inhibit food intake.[43] Furthermore, OEA increases the number of CD36 and FATP in the small intestine and adipocytes, which suggests a role in increased lipid absorption and oxidation.[43]

Methods to assess fat metabolism

Indirect calorimetry

When acetyl-CoA is used in the TCA cycle, carbon dioxide (CO_2) is produced as the final oxidation product. CO_2 is transported into the bloodstream and about 75% is taken up by red blood cells.[44] In the lungs, CO_2 is released from red blood cells and exhaled. In the production of ATP by oxidative phosphorylation, dioxygen (O_2, better known as oxygen) is used. By analysing exhaled air, O_2 and CO_2 can be measured.[45] O_2 consumption (VO_2) and CO_2 production (VCO_2) can be calculated, which provides a measure of energy expenditure; this method is referred to as indirect calorimetry.[46] The equations of Weir can be used to calculate energy expenditure from VO_2 and VCO_2.[47] When a subject is in rest, awake, healthy and at thermoneutral temperature, basal energy expenditure can be determined; the amount of energy that is needed to execute fundamental metabolic processes, such as breathing, beating of the heart, muscle tone, maintenance and turnover of cells, and ion homeostasis. The ingestion of food leads to an increase in energy expenditure, called diet-induced thermogenesis.[48] This energy is needed for the digestion and absorption of food, as well as for the metabolic effects of the consumed nutrients.[49] These processes lead to an increase in O_2 consumption, which can be measured via indirect calorimetry.

Metabolites in exhaled air

With the metabolism of fatty acids, not only CO_2 is produced, but also various other metabolites are formed. Some of these metabolites are also volatile, and can be transported from the bloodstream and the lungs into the air. [50] These volatile organic compounds (VOCs) can thus be detected in exhaled air. The analysis of VOCs has been studied as potential diagnostic tool in clinical settings.[51–53] Several VOCs have been identified as biomarkers for diseases such as chronic kidney disease,[54] cancer,[55,56] and asthma.[57] However, nutrition status also influences exhaled VOCs. An example is the ketone body acetone, which is formed by the oxidation of acetyl-coA. This is measured in the exhaled air of fasting subjects.[50] However, also other metabolites in exhaled air might be linked to lipid metabolism. Analysing VOCs in exhaled air could be a non-invasive method to analyse metabolic effects of nutritional intervention studies.

Different fat sources in IF

Fatty acids

IF can contain different fat sources, most IFs contain a mixture of vegetable fats. The most commonly used vegetable fats in IF are: palm (kernel) oil, soybean oil, rapeseed oil, (high-oleic) sunflower oil and coconut oil.[58] Some IFs contain a mixture of vegetable fat and bovine milk fat.[59] Bovine milk fat contains a wide variety of fatty acids. The major fatty acids in milk fat are palmitic acid (C16:0) and oleic acid (C18:1).[60] Besides those LCFA,(≥C12:0), bovine milk fat also contains SCFA (C4:0-C5:0) and MCFA (C6:0-C11:0).[60] The vegetable fat mixtures used in IF mostly

contain LCFA, with some MCFA.[61] The overall fatty acid composition of bovine milk fat and vegetable fat mixtures used in IF are given in **chapter 2, Table 1**.

Triacylglycerol structure

The positioning of the fatty acids at the different stereo-specific numbered (sn) locations of the glycerol backbone determines the structure of a TAG. In milk fat, mostly long-chain saturated fatty acids (LCSFA) are positioned at the sn-2 position.[62] Long-chain unsaturated fatty acids (LCUFA) are typically positioned at the sn-1 and sn-2 positions. SCFA and MCFA are mainly positioned at the sn-3 and sn-1 positions. In palm oil, the main vegetable oil used in most IFs, the sn-2 position of TAGs is mainly positioned by LCUFA and the sn-1 and sn-3 position mainly by LCSFA.[62,63] The TAG structure of bovine milk fat and the vegetable fats used in IF are described in more detail in **chapter 2**, and compared to human milk fat.

Physiological consequences

Physiological effects of TAG structure

In infants fat absorption is very efficient but not complete, about 10% of the fatty acids consumed are excreted via feces.[64] The amount of excreted fatty acids is found to be higher in formula-fed infants compared to breast-fed infants.[65] So, especially for formula-fed infants there is room for improvement. The TAG structure in IF has been found to affect absorption,[66,67] and postprandial lipid metabolism.[68,69] After consumption of IF, in which most LCSFA were present at the sn-2 position, a decreased fatty acid excretion has been found compared to an IF containing a commonly used vegetable fat, with most LCSFA at the sn-1 and -3 positions.[66,67] In a clinical trial the effect of LCSFA positioning on lipid metabolism was studied.[69] Positioning of LCSFA at the sn-2 position instead of the sn-1 and sn-3 positions, resulted in lower levels of HDL and higher levels of apolipoprotein B after 120 days of consumption.[69] As described above, vegetable fat and bovine milk fat have a different fatty acid composition and TAG structure. However, whether there are differences between a fat blend containing only vegetable fats and a fat blend containing bovine milk fat, used in IF, on postprandial lipid metabolism has not been studied before.

Physiological effects of fatty acid composition

Bovine milk fat contains about 10% of SCFA and MCFA, which are mainly present at the sn-1 and sn-3 positions. Since gastric and pancreatic lipase are thought to have a preference for shorter chain fatty acids,[6,70] the addition of bovine milk fat might enhance lipolysis. The digestion of an IF containing bovine milk fat compared to an IF containing vegetable fats only has not been studied before. Besides effects on lipolysis, the presences of SCFA and MCFA might also have other health effects. Consumption of medium-chain triglycerides increases energy metabolism and satiety compared to long-chain triglycerides.[71–76] Since SCFA and MCFA are easily absorbed, transported directly to the liver, and can be transported independent of the carnitine shuttle, they can be used quickly for oxidation. Therefore, addition of bovine milk

fat to IF might result in a higher postprandial diet-induced thermogenesis. Besides this, a faster switch from glucose to fat oxidation might spare some glucose from oxidation and might result in a prolonged satiety.[72] Furthermore, stimulation of satiety hormones, like CCK, also induces satiety via the nervus vagus.[40] Whether there are different satiety responses after consumption of an IF containing bovine milk fat or vegetable fat only is not known.

Aim and outline of this thesis

The aim of this thesis was to study the digestion, absorption, and metabolism of different fat sources used in infant formula; anhydrous bovine milk fat and vegetable fats. The second aim was to study whether the analysis of exhaled air can be used to assess metabolism of fat.

Our hypothesis was that the differences in fatty acid composition and TAG structure between anhydrous bovine milk fat and vegetable fat would result in a different digestion, absorption, and postprandial lipid metabolism, which may be relevant to health.

Since no detailed overview was available in which the different fat sources were described, in **chapter 2** we review the composition of human milk fat, bovine milk fat, and vegetable fat. Furthermore, the potential health effects of milk fat components are reviewed, and advantages and drawbacks of the different fat sources are discussed.

The digestion of an IF containing bovine milk fat compared to an IF containing only vegetable fat has not been studied before. **Chapter 3** focusses on the lipolysis of these IFs, which was studied in an *in vitro* infant digestion system. To study the effects of the fat sources on absorption and metabolism a human trial was executed. Since it was not possible to perform this study with infants, a proof-of-principle study was performed with young adults. The findings of this study are described in **chapter 4 and 5. Chapter 4** focuses on the effects of an IF with bovine milk fat compared to an IF containing only vegetable fats on lipid metabolism. **Chapter 5** focusses on the effects of these IFs on energy metabolism and satiety.

There is a need for non-invasive methods to study the effects of nutrition in vulnerable target groups, such as infants. To be able to investigate metabolism of lipids non-invasively, the analysis of VOCs in exhaled air was studied to investigate whether this would be an useful assessment for nutritional intervention studies. The results of the human studies performed to examine this are described in **chapter 6**.

In **chapter 7** the main findings of this thesis are discussed, along with strengths and weaknesses of the study designs, the translation from adults to infants, and the use of non-invasive methods.

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Chapter 2

Comparison of bovine milk fat and vegetable fat for infant formula: implications for infants health

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Abstract

Fat is an important component of human milk and infant formula (IF), delivering half of the energy a baby needs. Nowadays, mostly vegetable fats are used in IFs, however, the use of bovine milk fat in formulas is currently increasing. Bovine milk fat contains a different composition of fatty acids and lipid components than vegetable fats. We have compared the lipid profile of human and bovine milk to infant formulas with different fat sources. Furthermore, current knowledge of how infant digestion, absorption, metabolic responses, gut immunity, microbiota and/or cognition is affected by dietary fat is reviewed. The possible opportunities and drawbacks of the application of bovine milk fat in infant nutrition are described. Future perspectives for the development of IF containing bovine milk fat and future research directions are highlighted.

Introduction

Milk is essential for babies. For a newborn child breast milk is the preferred nutrition (EU Directive 2006/141). However, when breastfeeding is not an option, infant formula (IF) is the best alternative. About four percent of human milk consists of fat, which delivers approximately 50% of the total energy to infants.[1] Therefore, this is a major component to focus on in the development of optimal IF.

Currently, different fat sources are used for IF, of which most contain a mixture of vegetable fats. The most commonly used vegetable fats are coconut oil, corn oil, soybean oil, palm oil (palm olein, palm kernel oil), (high oleic) sunflower oil, high oleic safflower oil, and low erucic acid rapeseed oil.[2,3] Besides vegetable fats, the addition of bovine milk fat to IF is quite common. Sun *et al*. (2018) analyzed 180 infant formulas reflecting 75% of the market share in China, from which 66 products (37%) contained bovine milk fat. Bovine milk fat is added to IF in two different ways; either as anhydrous milk fat (containing triglycerides and other components like cholesterol and fat-soluble vitamins), or as full fat milk or cream (containing besides triglycerides and cholesterol all components of the fat globule membrane).

Until the 1970s, bovine milk fat was part of IF,[4,5] mainly through the use of whole milk in the recipes. However, as the formulas were further developed, animal fat was replaced by vegetable fats.[6] This was done for several reasons; to provide (higher levels of) mono- and poly-unsaturated fatty acids,[5] and due to the fear of contaminants, like dioxins. Also, it was believed that formulas similar to home-made evaporated milk formulas increased the level of constipation,[7] and the odor of regurgitated butterfat was found to be unpleasant.[8] In addition, the cost of using bovine milk fat was high, compared to the alternatives found in vegetable fats. Today, research focus is on adding complex lipids and milk fat globular membrane components to support infants' development.[9] Furthermore, EFSA states that "the obvious and previously used staple sources of fat for use in the production of IF and follow-on formula are cow's milk, to a certain extent goat's milk and different types of vegetable oils".[10] In this review, we compare the composition of human milk fat, bovine milk fat and vegetable fats and focus on their implications for infant health.

Table 1: Fatty acid composition (g/100 g fatty acids) of human milk, bovine milk and infant formulas (IF) containing different fat sources (mean+range).

Fatty acid		Milk			IF		
		Human milk– Europe [1, a]	Human milk – Asia [2, a]	Bovine milk [3]	IFs containing vegetable fat blends [4, b]	IFs containing milk fat [5, c]	IFs containing palm oil free vegetable fat blend [6, d]
SCFA							
C4:0	Butyric acid	ND	ND	3.50 (3.07-3.78)	ND	2.4	ND
MCFA							
C6:0	Caproic acid	0.39 [8]	0.07 [7]	2.29 (2.07 – 2.46)	ND	1.3	0.2
C8:0	Caprylic acid	0.19 (0.09-0.24)	0.17 (0.11-0.28)	1.38 (1.26-1.51)	1.2 (0.4-2.1)	1.7	2.5
C10:0	Capric acid	1.29 (0.83-1.63)	1.31 (0.52-2.48)	2.94 (2.60-3.23)	1.1 (0.1-1.7)	2.2	1.8
LCFA							
C12:0	Lauric acid	5.98 (4.15 – 8.33)	5.56 (2.97– 13.82)	3.87 (3.50-4.28)	5.4 (0.2-13.6)	6.3	13.4
C14:0	Myristic acid	6.44 (4.98 – 9.38)	5.70 (3.50 – 12.12)	11.29 (10.67 – 11.94)	4.6 (0.9-7.0)	7.2	5.2
C14:1	Myristoleic acid	0.18 [8]	0.26 (0.03-1.11) [9]	1.08 (1.01 – 1.19)	ND	0.8	ND
C15:0	Pentadecanoic acid	0.25 (0.16-0.32)	0.20 (0.08-0.50)	1.03 (0.97-1.10)	ND	0.6	ND
C16:0	Palmitic acid	21.93 (15.43-25.62)	21.78 (17.55-29.00)	30.20 (28.31 – 31.85)	26.3 (15.9-31.7)	18.9	7.7
C16:1 n-7	Palmitoleic acid	1.98 (1.65-2.31)	2.44 (1.29-4.59)	1.57 (1.45-1.68)	0.6 (0.2-1.1)	1.1	0.1
C17:0	Heptadecanoic acid	0.29 (0.22-0.33)	0.28 (0.19-0.41)	0.59 (0.53-0.72)	ND	0.3	ND
C18:0	Stearic acid	7.37 (5.58-9.52)	5.58 (3.90-6.79)	9.85 (8.75-11.39)	5.3 (3.2-7.7)	6.7	3.2
C18:1 n-9	Oleic acid	36.30 (28.93-41.69)	30.80 (21.85-36.96)	21.62 (19.37 – 24.25)	37.6 (31.6-42.3)	28.1	43.3
C18:2 n-6	Linoleic acid (LA)	13.99 (10.16-16.59)	16.90 (7.53-24.29)	1.44 (1.36 – 1.76)	14.0 (10.0-18.9)	16.7	20.5
C18:3 n-3	Alpha-linolenic acid (ALA)	0.76 (0.49-1.05)	1.47 (0.35-4.06)	0.49 (0.45-0.57)	1.6 (1.2-2.0)	1.5	1.8

C20:0	Arachidic acid	0.21 (0.14-0.31)	0.32 (0.03-2.97)	0.14 (0.12 – 0.17)	ND	0.3	0.3
C20:3 n-6	Dihomo-gamma-linolenic acid (DGLA)	0.38 (0.29-0.52)	0.42 (0.23-0.83)	0.07 (0.06-0.08)	ND	ND	ND
C20:5 n-3	Eicosapentaenoic acid (EPA)	0.09 (0.05-0.13)	0.31 (0.07-1.59)	0.07 (0.06-0.09)	ND	-	0.0
C22:0	Behenic acid	0.09 (0.05-0.13)	0.08 (0.05-0.14)	0.06 (0.05-0.07)	ND	0.1	0.4
C20:4 n-6	Arachidonic acid (ARA)	0.47 (0.37-0.64)	0.64 (0.30-2.57)	0.04 (0.03 - 0.05)	0.3 (0.1-0.4)	-	0.3
C24:0	Tetracosanoic acid	0.07 (0.03-0.16)	0.07 (0.01-0.14)	0.05 (0.04 – 0.07)	ND	ND	0.1
C22:6 n-3	Docosahexaenoic acid (DHA)	0.28 (0.18-0.42)	0.55 (0.19-1.13)	0.01 (0.00-0.04)	0.2	-	0.2
Total SC/MCFA		*1.86*	*2.14*	*10.11*	*2.3*	*7.6*	*4.5*
Total LCSFA		*42.62*	*39.59*	*57.08*	*41.6*	*40.4*	*30.3*
Total SFA		*44.48*	*41.73*	*67.19*	*43.9*	*48*	*34.8*
Total MUFA		*38.45*	*33.50*	*24.27*	*38.2*	*30.0*	*43.4*
Total PUFA		*15.97*	*20.27*	*2.12*	*16.1*	*18.2*	*22.8*
Total UFA		*54.42*	*53.77*	*26.39*	*54.3*	*48.2*	*66.2*

1: 11–19, *2:* 20,21,30,22–29, *3:* 31,32, *4:* 33, *5:* 2,34, *6:* 35–37, *7:* 25, *8:* 17, *9:* 28,29, [a] *studies from 2000-2018 are included, data about breast milk for infants <12 months of age,* [b] *IF contained palm oils, rapeseed oil, soybean oil and coconut oil as major fats,* [c] *IF contained bovine milk fat, corn oil, and other non specified vegetable fats,* [d] *IF contained high oleic sunflower oil, coconut oil, soy oil as major fats, ND: not determined, SCFA: short-chain fatty acid, MCFA: medium-chain fatty acid, LCFA: long-chain fatty acid, LCSFA: long-chain saturated fatty acid, MUFA: mono-unsaturated fatty acid, PUFA: poly-unsaturated fatty acid, SFA: saturated fatty acids, UFA: unsaturated fatty acids, note: The analytical methods for fatty acid analyses used in the various cited papers are subject to inaccuracies in quantitative measurements over the whole range of fatty acid lengths.*

Lipid composition in bovine milk, human milk and infant formula

Human as well as bovine milk contains approximately 4% fat in the form of globules.[38,39] During different stages of lactation the total fat content and fatty acid composition changes to a minor extent.[11,40–43] However, since this is not the focus of this review, and since the recommendations for the composition of IF is the same for newborns and up to 6 months, we chose to only include mature human milk as comparison for IF in this review. Fat globules are filled with triglycerides, which represent 98% of the total fat.[44] The so-called milk fat globular membrane (MFGM), which is composed of proteins and lipids, cover the milk fat globules (MFG). Proteins within the MFGM include glycoproteins and enzymes.[45,46] The structure of the MFGM was recently reviewed by Martini *et al.*[47] and nicely illustrated by Hernell *et al.*[48] The lipids within the MFGM include mainly polar lipids, but also some neutral lipids like triglycerides, diglycerides, monoglycerides, sterols (mainly cholesterol) and gangliosides. Furthermore, bovine milk fat contains trace amounts of ether lipids, hydrocarbons, fat-soluble vitamins, flavor compounds and other minor compounds.[39] The triglyceride composition and structure, polar lipids and cholesterol are described in more detail below.

Triglycerides

The fatty acids in human and bovine milk fat, as well as in vegetable fat, are mostly present in the form of triglycerides (~98%). A triglyceride consists of a glycerol backbone with three fatty acids attached to it. Both the fatty acids and the triglyceride structure of different fat sources are described in the sections below.

Fatty acids

Nearly 200 different fatty acids, ranging from C4:0-C26:0, are present in human milk fat.[38,39] For bovine milk fat this number is even higher, almost 400 fatty acids are present in bovine milk fat.[44] Only about 15% of those are present at 1% or higher, the others are only present in trace amounts. Since most vegetable fats (except coconut oil) do not contain fatty acids ranging from C4:0-C12:0, and no odd-chain fatty acids,[49] the variety of fatty acids in vegetable fats is lower compared to bovine and human milk fat **Table 1** shows the fatty acid composition of human milk, bovine milk and IF products with different fat blends. For clarity, very low abundant fatty acids were left out.

Fatty acids in human milk

Table 1 contains an average fatty acid composition of mature human milk (studies from 2000 until 2018 were included). Of all fatty acids in human milk, almost 98% are long-chain fatty acids (LCFA (>C10)), of which about 40% are saturated fatty acids (SFA). The remaining 2% of the fatty acids in human milk fat consist of medium-chain fatty acids

(MCFA (C6:0-C10:0)). Most studies are not able to detect the short-chain fatty acid (SCFA) butyrate (C4:0) in human milk; however, some studies do report the presence of butyric acid in low concentrations. For example, Wan *et al*. showed that human milk of Chinese mothers contained 0.6 g butyric acid per 100 g fatty acids.[25] The values represented in **Table 1** are an estimation of the true levels in human milk. Analytical factors influence the fatty acid compositions, including differences in extraction protocols and detection methods. Furthermore, there is a natural variation both between individual mothers and between geographical regions,[50] since the fatty acid composition of human milk is influenced by diet as well as genetics. To give an insight in these regional differences, data from human milk obtained in Asia and Europa is presented. Some regional differences are observed, as the level of PUFA is somewhat higher in Asia compared to Europe, and the level of SFA and MUFA is somewhat lower. Overall, the fatty acid composition between regions is quite similar.

Fatty acids in bovine milk

About 70% of bovine milk fat consists of SFA. Of all fatty acids, almost 90% are LCFA, 6-7% are MCFA, and butyrate presents about 3-4%. The most characteristic fatty acids for bovine milk fat are odd chain fatty acids, conjugated linoleic acid and butyrate. This latter fatty acid is not present in vegetable fats and only present in trace amounts in human milk.

Bovine milk fat contains higher levels of SFA compared to human milk fat, about 67% vs 43% respectively, and lower levels of MUFA's (24% vs 36%) and PUFAs (2% vs 18%). Even though low in human milk, docosahexaenoic acid (DHA) and arachidonic acid (ARA) are present in even lower amounts in bovine milk fat. Similar to human milk fat, the main fatty acids present in bovine milk fat are oleic acid and palmitic acid (C16:0). In human breast milk, palmitic acid alone accounts for approximately 10% of the infant's energy intake, making palmitic acid a key nutrient for infants.[51] In bovine milk fat, palmitic acid is present in higher levels compared to human milk fat (30% vs 22%), for oleic acid this is reverse (22% vs 34%). A major difference between human milk fat and bovine milk fat is the level of linoleic acid. Human milk fat contains around 15% linoleic acid, while in bovine milk fat this is only about 1.5%.

Fatty acids in vegetable fat

Different vegetable fats present in IF are blended in such a way that the fatty acid composition closely resembles that of human milk (**Table 1**). However, since different vegetable fats are used, there is also some variation between products. This is indicated by the ranges in **Table 1**, which shows examples of fat mixtures used in IF. Compared to an infant formula containing bovine milk fat, an infant formula that contains only vegetable fat contains lower levels of butyrate and MCFA and higher levels of MUFA.

When a mixture of only vegetable fats is used, a source of palm oil needs to be added to reach a similar level of palmitic acid as found in human milk. A vegetable source of palmitic acid is palm (kernel) oil. IFs without palm oil contain only 8% of palmitic acid, and higher levels of oleic acid, linoleic acid and lauric acid compared to human milk fat.

TAG structure

A triglyceride consists of a glycerol backbone with three positions for fatty acids to attach, the outer positions are called sn-1 and sn-3, and the center position is called sn-2. Specific fatty acids have their own favorable position at the glycerol backbone, which differ among species. With the current analytical methods available, only the percentage of fatty acids at the sn-2 position of the total fatty acids can be determined. The fatty acids present at sn-1 and sn-3 cannot be determined separately.

TAG structure in human milk fat

In human milk, the main fatty acid, palmitic acid, is mostly placed at the sn-2 position, representing about 70-88% of the total palmitic acid, see **Table 2.**[16,52,53] Of the other long-chain saturated fatty acids (LCSFA), 34-66% are also placed at the sn-2 position in human milk.[16,53] The only exception is stearic acid (C18:0), of which only 10% is placed at the sn-2 position.[16,53] The major TAG structures present in human milk are structures with palmitic acid at the sn-2 position, and oleic acid (18:1) attached to sn-1 or sn-3, like C18:1-C16:0-C18:2, C18:1-C16:0-C18:1, and C16:0-C16:0-C18:1.[54–56]

Table 2: Stereospecific distribution of C16:0 in human milk, bovine milk and vegetable fats

	% C16:0 at sn-2 position of total C16:0
Human milk	70-88% [1]
Bovine milk	40-45% [2]
Vegetable fats commonly used in IF	10-20% [3*]
Structured triglycerides	39-47% [3**]

1: [16,52,53], *2:* [52], *3:*[52,53] ** based on data of IFs containing vegetable fat without interesterified palm oil from figure 1 of Sun et al. 2018., ** based on data of IFs containing vegetable fat with interesterified palm oil from figure 1 of Sun et al. 2018*

TAG structure in bovine milk fat

In bovine milk fat, butyrate is mostly located at sn-3. MCFAs, as well as C12:0-C16:0, are preferably located at the sn-1 and sn-2 positions. Stearic acid (18:0) is selectively located at position sn-1, while oleic acid is mostly present at sn-1 or sn-3.[39] For bovine milk fat, the amount of palmitic acid at the sn-2 position is about 40-45% of the total amount of palmitic acid.[52] Sun *et al*. (2018) showed data for IFs containing bovine milk fat; however, the percentages of bovine milk fat used were not specified. Here, the percentage of LCSFA (excluding stearic acid) positioned at the sn-2, instead of sn-1 or sn-3, was between 30-49%.[53] Like human milk fat, bovine milk fat contains a wide variety of fatty acids, resulting in many different triglyceride structures. Just like human milk, the major TAG structures in bovine milk fat contain palmitic acid in the sn-2 position, and oleic acid attached to the sn-1 or sn-3 position.[57,58]

TAG structure in vegetable fat

The TAG structure of vegetable fats used in IF differ from human milk fat. For vegetable fat blends used in IF the amount of palmitic acid at the sn-2 position reaches levels of 10-20%.[52,53] Sun *et al*. reported that 19-59% of the LCSFA are positioned at the sn-2 position in IFs with vegetable fats, of which some contain interesterified palm oil.[53] Clearly, in vegetable fat-based IFs, high levels of triglyceride structures with saturated fatty acids at the sn-1 and/or sn-3 position are present, such as C18:1-C18:1-C16:0, C16:0-C18:1-C16:0, C18:2-C18:1-C16:0, and C16:0-C18:2-C16:0.[56] Since less different fatty acids are present in vegetable fat, also the pool of triglycerides is less diverse compared to human and bovine milk fat.

Structured TAGs

The distribution of fatty acids along the glycerol backbone at the sn-2 vs sn-1/sn-3 positions can be changed with inter-esterification.[2] Recently, TAGs generated through an enzymatic process from vegetable fats or combinations of vegetable and other fats e.g. from fish have become available.[59,60] The most common product is beta-palmitate, which is used in IF products currently on the market. Beta-palmitate is the resulting product of the enzymatic inter-esterification of palm oil and high oleic sunflower oil, where C16:0-C18:1n-9-C16:0 is transformed to C18:1n-9-C16:0-C18:1n-9.[61] These "structured TAGs" make it possible to produce IFs with TAG structures higher in sn-2 palmitate, often above 40% (ranging from 39-47%) of the total palmitic acid content (17-25%).[53,62]

Minor components

Polar lipids

Polar lipids encompasses amongst others phospholipids and sphingolipids. Those lipids contain a hydrophobic tail and a hydrophilic head.[45] Polar lipids have a fundamental role in milk; the emulsification of fat in water.[63] The concentration of total polar lipids is comparable between human milk fat and bovine milk fat. Human milk fat contains about 20.4± 2.8 mg of polar lipids per 100 ml compared to 19.2 ± 0.8 mg of polar lipids per 100 ml for bovine milk fat (calculated from Zou *et al.*, 2013).[64] The composition of the different polar lipids is slightly different between the two different fat sources. Furthermore, the exact phospholipid content of the bovine globule membrane is dependent on the cow breed, season, feed of the cow and size of the globule.[57,65] The main polar lipids present, in both the human and bovine fat globule membrane, are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM).[45,46] Human milk contains higher levels of sphingomyelin (40.2% ±1.1 vs 27.4 ±1.1) and phosphatidylserine (14.4 ± 2.0 vs 7.3 ± 1.0), while in bovine milk fat more phosphatidylethanolamine is present (12.5 ± 2.9 vs 30.2 ± 2.7),[64] see **Figure 1**. In IF, based on vegetable fat, the phospholipids are provided by lecithin, derived from either sunflower seeds or soybeans[4] and from residual bovine milk fat from skimmed milk powder.[2] The phospholipids from skimmed milk powder also account for the presence of sphingomyelin, which cannot be sourced via plant-based fat blends. The level of phospholipids varies among IFs, but IFs consist mostly of PC, SM, and PE with lower levels of PI and PS.[66,67]

Cholesterol

One of the minor components of human and bovine milk lipids are sterols, which make up 0.3% of total fat. Cholesterol constitutes about 95% of the total sterols. Human milk is a rich source of cholesterol, it contains about 90-150 mg/L of cholesterol.[2,9] Bovine milk fat contains higher levels, around 300 mg/L of cholesterol,[44] whereas IFs contain 0-4 mg/L of cholesterol.[9] A recent study investigating sterol contents of IFs showed that IFs based on vegetable fats contained on average 0.185 mg/L of cholesterol.[70] In line with the findings on phospholipids, the cholesterol present in IF based on vegetable fats also mostly originates from small amount of milk fat present in skimmed milk.[2] Newer types of IF, containing a blend of vegetable fats and bovine milk fat, contain higher levels of sterols, on average 0.927 mg/L,[70] which is still surprisingly low. However, the amount of milk fat in these IF products was not specified, so the fraction of bovine milk fat might have been low. Calculations based on literature values (NEVO online)

indicate that per addition of 10% bovine milk fat to a fat blend for infants formula 5.5 mg/L of cholesterol could be added.

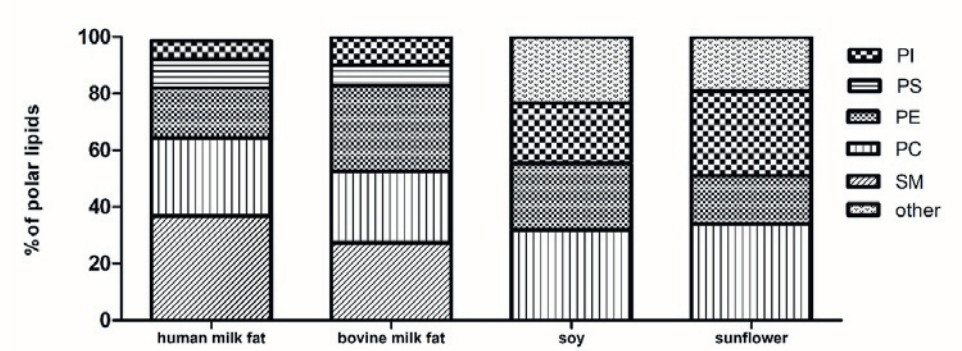

Figure 1: Relative proportion of polar lipids (% of polar lipids) from mature human milk and bovine milk [64,68], and from soybeans and sunflower kernels [69], (PE=phosphatidylethanolamine, PI=phosphatidylinositol, PS=phosphatidylserine, PC=phosphatidylcholine, SM=sphingomyelin).

Effects of milk fat related components on infant physiology and health

In recent years, the importance of dietary fats in infant nutrition has gained increasing scientific interest. Rather than merely a source of energy, it has become clear that the composition and structure of dietary fats in the infant diet could have profound influence on infant development, physiology and health. In this section, we will review how; 1) digestion/absorption, 2) metabolic responses, 3) gut immunity, 4) microbiota and 5) cognition could be affected by the composition and structure of milk fat related components. The main effects are illustrated in **Figure 2**. Since only very few studies have been performed to study the effects of these components in infants, other studies have been included to indicate possible interesting leads for infant health. These effects are indicated with a dotted line in **Figure 2.**

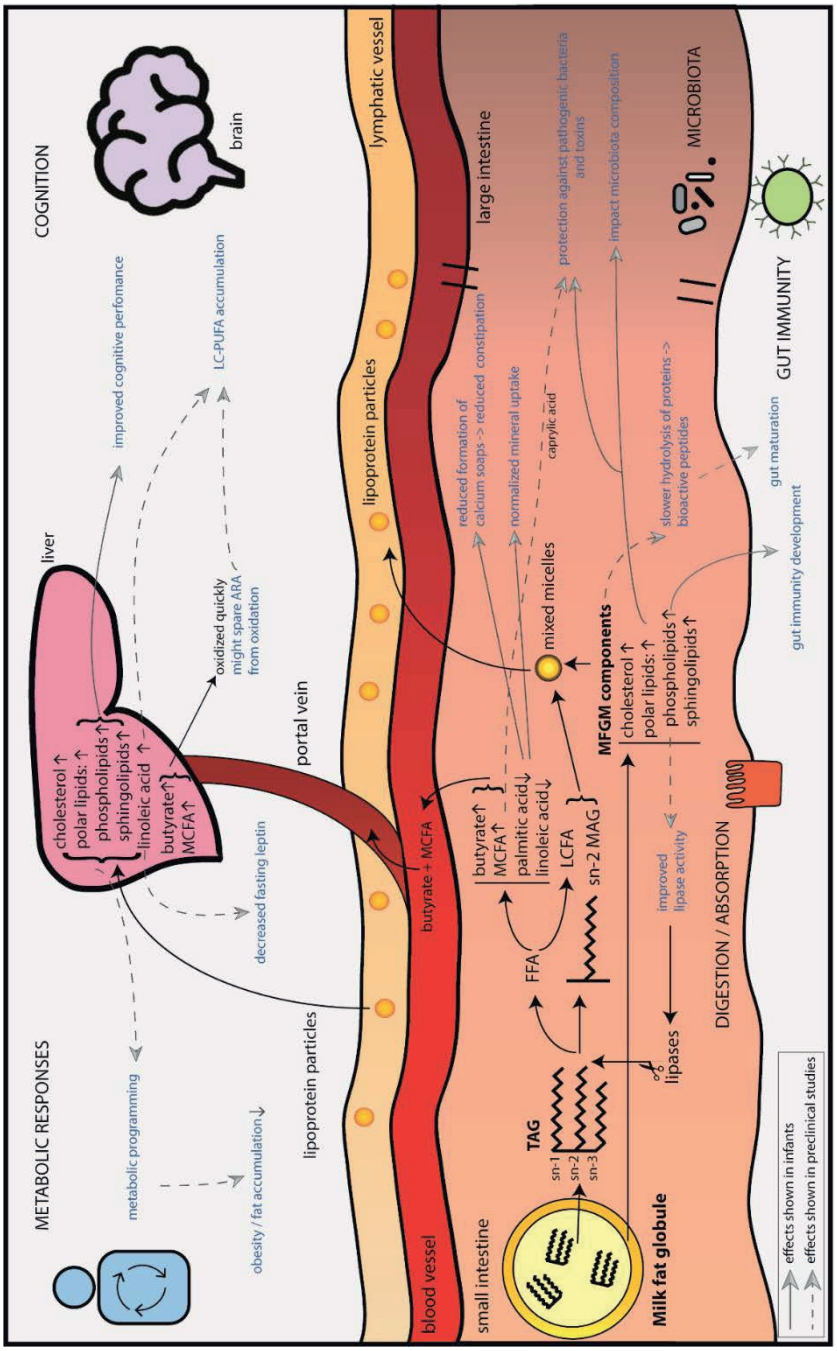

Figure 2: Schematic overview of the health effects of bovine milk fat (components) as described in this review, effects shown in infants are displayed with a solid arrow and effects shown in preclinical infants are displayed with a dotted arrow.

Digestion/absorption

Triglyceride digestion

The fat composition in the diet of infants affects the digestion and absorption of nutrients in infants. A well-studied example is the digestion and absorption of TAGs. During digestion, gastric and pancreatic lipases release the fatty acids positioned at the sn-1 and sn-3 positions of the TAG. As mentioned in paragraph 2.1.2, in human breast milk, these positions are predominantly occupied by MCFA, long-chain unsaturated fatty acids as well as low levels of butyrate. Butyrate and MCFA are, unlike LCFA, rapidly absorbed in the intestine as free fatty acids (FFA).[5] The sn-2 fatty acid remains on glycerol as sn2-monoglyceride (MAG). In human milk, the most abundant fatty acid in the sn-2 position is palmitic acid. Due to the more polar nature of the sn2-MAG, this fatty acid is more efficiently absorbed in the intestine in the form of sn2-MAG rather than as a FFA.[51] In contrast, IF based on vegetable fats mainly has palmitic acid in sn-1 and sn-3 position, that are released by the digestive lipases, resulting in large amounts of unesterified palmitic acid, as well as other low absorbable FA, freely present in the lumen.[5] These long-chain saturated FFA form complexes with calcium ions, generating non-absorbable soaps.[71,72] These calcium soaps are described to be associated with negative effects for infants, such as constipation, stool hardness,[73,74] and reduced bone mineralization.[75] As described in section 2.1.2 bovine milk and human milk contain respectively 40-45%[52] and 70-88%[16,52,53] of the palmitic acid at the sn-2 position and therefore less soap formation will most likely occur with IF containing bovine milk fat.

Cholesterol absorption

Cholesterol is a key component in cell membranes, it is important in brain maturation through myelination, and cholesterol is a precursor for bile acids and steroid hormones.[76] Furthermore, cholesterol is an important structural part of chylomicrons and lipoproteins, which are key factors for the absorption and transportation of LCFA in the body.

As mentioned in section 2.2.2, IFs contain much less cholesterol than human breast milk.[70,77] The low amounts of total cholesterol in IF, is most likely the reason for the lower serum levels of total cholesterol and LDL cholesterol found in formula fed infants compared to breast fed infants.[78] Furthermore, it could explain the three times higher cholesterol synthesis rate seen in formula fed infants,[79] as these infants would have to compensate for the lack of total cholesterol otherwise present in human breast-milk. Studies suggest that supplementing IF with cholesterol, does not entirely correct the lower plasma cholesterol levels found in formula fed neonates or piglets, respectively.[80,81] In contrast, Timby *et al*. showed that MFGM-enriched formula increased cholesterol levels, so at the age of 6 months, cholesterol levels were similar to breast-fed infants.[82] Although these studies are not directly comparable, these observations may indicate that cholesterol associated with the MFGM is more easily absorbed by the infant intestine than free cholesterol. Another factor which may influence cholesterol absorption in infants is the presence of plant sterols in IF, such as brassicasterol, campesterol, stigmasterol, β-sitisterol and sitostanol, which are absent in human breast milk.[70,77] Total plant sterol levels

exceeded the levels of total animal sterols in most formulas, except those with added anhydrous milk fat and/or MFGM, where total animal sterol levels were slightly higher than plant sterol levels.[70] Plant sterols have been described to reduce cholesterol intestinal absorption in adults.[83,84] However, the role of plant sterols in healthy term formula fed infants is unknown and needs to be investigated.

Effect of milk fat globular membrane on digestion and absorption

Bovine milk lipids in IF could also influence digestibility of proteins. *In vivo* and *in vitro* studies have shown that adding products including, but not exclusively containing MFGM and bovine milk fat to IF, leads to higher resistance of casein and β-lactoglobulin to digestion, as compared to formula based on vegetable fats. However, the exact composition and amount of the MFGM ingredients used in these studies are unknown and they may contain a variety of bioactive components. In a "minimally processed" model IF based on dairy fats with native MFG, casein and β-lactoglobulin were hydrolyzed slower, than the same formula after homogenization and pasteurization in an *in vitro* digestion system.[85] A similar reduction in protein digestion was reported in neonatal piglets receiving modified IF containing a mixture of milk and vegetable lipids and MFGM.[86] The resulting higher numbers of β-casein peptides in the gut, may exhibit bioactive functions that accelerates gut maturation.[86]

Lipolysis is also altered by lipid structure and components that are part of the MFGM, such as polar lipids. For example, the size and interfacial composition of MFG have shown to impact digestibility of lipids in simulated gastro-duodenal digestion.[87] Replacing polar lipids from soybean with milk polar lipids, changed the blood levels of lipids in mice after meals, with milk polar lipids resulting in a quicker elevation and clearance of plasma TAG.[88] Finally, Mathiassen *et al*. showed that exchanging soy lecithin with dairy phospholipids increased gastric lipase activity by 2.5-fold.[89] Human breast milk contains bile-salt stimulated lipase (BSSL), which accounts for 20-40% of lipase activity in infants.[90] Since this lipase is not present in IF, formula-fed infants lack this extra lipase activity. Thus, the increased gastric lipase activity, when replacing soy lecithin with bovine milk polar lipids, might possibly be beneficial for formula-fed infants. A review about the structure of the milk fat and the relation with digestibility has been published by Bourlieu and Michalski.[91]

Metabolic responses

Generally, the body compositions and growth curves differ between breastfed and formula-fed infants, as breastfed infants tend to have slower weight gain[92] and breastfeeding shows less association with childhood obesity.[93,94] These differences on infant growth performance have been linked to protein concentration (and thereby energy density)[95,96] and general feeding practices.[97] Nevertheless, there has recently been increasing focus in literature on how the lipid composition of the infant diet influence metabolism and metabolic programing in infants as well.

Milk fat globule membrane, cholesterol, polar lipids and metabolic responses

The dietary lipid structure is a focus area within neonatal lipid metabolism research. Both the lipid droplet size, as well as the components of the MFGM, may possibly contribute to the preventive effects of breastfeeding on childhood obesity. Studies in mice have shown, that consumption of pellets with phospholipid-coated large lipid droplets, reduced fat accumulation and improved the metabolic profiles in adult mice,[98] and protected against obesity in adult life during a Western-style diet (highly processed, high saturated fat and high carbohydrate content) challenge.[99] In a clinical study, where infants received a low-energy, low-protein, MFGM-enriched formula, cholesterol levels were normalized to the levels of breast-fed infants, most likely due to the cholesterol in MFGM.[82] However, there was no difference in growth performance between infants receiving standard or low-energy, low-protein, MFGM-enriched formula.[100]

Interestingly, mice fed a high-fat diet rich in polar lipids (phospholipids and sphingolipids) from soybeans, showed white adipose tissue hypertrophy and inflammation. White adipose tissue hypertrophy is indicative of an imbalance in fat metabolism that is associated with obesity mechanisms. This was not observed when the mice were fed a similar high-fat diet based on milk polar lipids.[101] In two other studies, feeding mice bovine milk sphingomyelin, compared to egg sphingomyelin, attenuated the consequences of high-fat-induced obesity in mice.[102,103] More long-term studies on infants are required to elucidate the relationship between MFGM, metabolism and metabolic programming. For a recent review on health-benefits of phospholipids in milk, see Verardo *et al.*[104]

Medium-chain fatty acids and metabolic responses

Since MCFA are not dependent on incorporation into the chylomicrons for absorption, MCFA are easily absorbed. Moreover, in contrast to LCFA, MCFA uptake in mitochondria occurs independent of the carnitine shuttling, contributing to a faster oxidation of MCFA.[105] Since the uptake of MCFA is easier, compared to LCFA, IFs for premature born children are enriched with MCFA, in the form of medium-chain triglyceride fats. Consumption of MCFA has been shown to increase diet-induced heat generation and fat oxidation in adults,[106–108] and in preterm infants the consumption of MCT was found to increase energy metabolism and improve thermoregulation.[109,110]

A few studies on rodents have investigated the impact of infant consumption of MCFA. In rats, high dietary intake of MCFA during pregnancy, prevented obesity in their offspring later in life.[111] In a study of both rats and mice, increased early-in-life intake of MCFA protected against the negative effects of a high-energy diet in adulthood, such as fat accumulation and insulin sensitivity.[112] In term infants, the role of MCFA on short- and long-term metabolism remains unclear.

Linoleic acid and metabolic responses

The essential fatty acid linoleic acid (LA) is needed by the body to synthesize arachidonic acid (ARA). Therefore, LA is added to IF in similar levels as found in human milk. The LA levels in commercially available IF are approximately around 16% of total FA (**Table 1**), which is similar to the LA levels in today's human milk. During the last 50-60 years the lipid composition in human breast milk has changed, so that today higher concentrations of LA are observed, from about 5% to 16% LA,[113] whereas levels of alpha-linolenic acid (ALA) have remained stable the past 40 years. This has brought up a lot of debate in the scientific field about the optimal level of LA and the optimal ratio with ALA.[114,115] In bovine milk, LA concentrations are approximately 10 times less than in the current human breast milk, 1.44% (**Table 1**). Bovine ALA levels are about half of the levels in human milk; 0.49% and 1.04%, respectively.

In recent studies on mice and rats, reducing LA (3.16 energy percentage (en%) vs 1.36 en%) in early life programmed towards relative metabolic resistance to a Western style diet (2.54 en%) in adult life. In mice, low LA diet (1.36 en% LA) decreased fat accumulation, reduced fasting TAG levels and lowered fasting leptin levels, whereas in rats a beneficial adipocyte composition was reported.[116] Furthermore, mice fed a Western-like diet high in LA and low in ALA (LA/ALA ratio 28), showed enhanced fat mass accumulation through four generations.[117] To elucidate the role of the ratio and levels of LA and ALA in infant nutrition more future research is required.

Gut immunity

The neonatal period is unique, in the sense that this is the time for maturation of the gut immune system and for the establishment of the gut microbiota. At birth, the gastrointestinal tract in humans is immature and adequate stimulation through diet and microbiota is essential for the gut to mature.[118,119] These processes are also influenced by the fat composition of the neonatal diet.

Dietary fats have been linked to host immune responses and have been associated with functions such as gut immune maturation, gut integrity and the establishment of gut immune homeostasis. Several studies have focused on the group of sphingolipids (including sphingomyelin, glycosphingolipids and gangliosides) and their potential protective functions against pathogenic bacteria and toxins, and their impact on gut immune maturation. The topic was recently reviewed by Nilsson.[120] In particular, sphingosine-1-phosphate (S1P), a metabolite from the degradation of sphingomyelin has gained much interest due to its intestinal immune modelling functions.[121] These include a role in intestinal epithelial cell barrier function, proliferation of IgA producing cells and lymphocyte trafficking, as demonstrated in cell lines.[122] Furthermore, imbalance of S1P may be involved in the development of diseases, which evolve due to inadequate regulation of the intestinal immune response, such as food allergies and intestinal inflammation, as reviewed recently by Kunisawa & Kiyonon.[123]

Besides the effect of sphingolipids, immunomodulatory effects of IF supplemented with bovine MFGM have been reported, in several animal and *in vitro* models, as well. The maturation of the mucosal immune system was accelerated in piglets receiving MFGM, based on the higher secretion of the immune system mediating cytokine interferon gamma from cells in the lymph nodes lining the small intestinal tissue (mesenteric lymph nodes). The authors indicate that these results might be related to the presence of sphingolipids in the MFGM fraction.[86] In some studies, gangliosides reduced proinflammatory signaling in the intestine in an *in vitro* gut model,[124] whereas others have not observed this effect in preterm piglets.[125]

Butyrate has been shown to have an important function in maintaining intestinal barrier function.[126] However, studies on Caco-2 cells have shown that in contrast to 2 mM butyrate, 8 mM butyrate has an adverse effect on a model for intestinal barrier function.[127] Furthermore, intestinal mucosal injury has been associated with administration of SCFA to young neonatal rats.[128] An effect, which ceases with intestinal maturation. These studies have led to the hypothesis that too much SCFA, as a result of microbial overproduction, may be a cause of necrotizing enterocolitis (a major condition of illness in newborn children) in premature infants.[129] However, when butyrate is digested (rather than produced by colonic microbes), butyrate is most likely rapidly absorbed in the upper gastrointestinal tract. The digestion and absorption of butyrate in premature and term infants is not well described in the literature, as this fatty acid is only present in human breast milk in very low levels (see **Table 1**). Therefore, further investigations are needed to elucidate the health effect of butyrate in bovine milk fat containing IF, since butyrate is digested and expected to be readily absorbed.

Clinical studies have shown that supplementing IF with bovine lipid components may potentially prevent some types of infection in infants as well. A fat blend containing bovine MFGM was shown to decrease episodes of bloody diarrhea in Peruvian infants/young children[130] and reduce the risk of acute otitis media (middle ear infection).[131] On the contrary, a study on rotavirus diarrhea did not show any effect of supplementing IF with a spray-dried ganglioside concentrate[132] and the study by Timby *et al*. did not show a reduction in other types of infections.[131] However, both studies were hampered by a low level of background infections. For reviews, see [48,133].

Microbiota

Distinct differences are observed in the microbiota between breast-fed and formula-fed infants[118,134,135] and it is wellknown that the gut microbiome plays a crucial role in the maturation of the gastrointestinal immune defense.[119,136,137] Key factors modulating the microbiota are the presence of human milk oligosaccharides[138,139] and maternal factors.[140] In addition, the lipid composition of the infant's diet could possibly alter the microbiota composition, as discussed below. SCFA and MCFA are described to exhibit antimicrobial effects against E. *coli*, Listeria *monocytogenes* and Staphylococcus *aureus in vitro* and *in vivo*.[141,142] In

particular, caprylic acid (C8:0) has shown inhibitory functions against pathogens, it both reduces bacterial growth in reconstituted IF[143] and weaning mortality in rabbits, fed a diet supplemented with caprylic acid-containing TAGs.[144] For a review on dietary fatty acids and food-borne bacterial infections, see Harrison *et al*.[145] This review mainly focuses on effects observed in chickens or cell cultures. Not much is known on the effect of milk fat on microbiota composition. In piglets, supplementing IF with bovine milk fat and MFGM increased Proteobacteria and Bacteroidetes while decreasing Firmicutes phyla, compared to piglets receiving formula exclusively based on vegetable lipids.[86] IF with structured vegetable TAGs increased Bifidobacteria and Lactobacillus strains compared to IF containing standard vegetable fats in two clinical intervention studies with a duration of respectively 6 and 8 weeks.[71,146] Furthermore, adding gangliosides to IF reduced the levels of fecal E. *coli* and increased fecal Bifidobacteria in pre-term newborn infants.[147] Although the lipid composition in the diet of neonates indeed does alter gut microbiota, the mechanisms, as well as the effects of milk fat based IF on the microbiota composition in the child needs to be further elucidated.

Cognition

Population studies have established that even after elimination of socioeconomic factors, breast-fed infants have an advantage over formula-fed infants when measuring cognitive functions.[148,149] Although IFs continuously are being improved, these data suggest that the nutritional components, composition and structure of IF still needs to be optimized, in order to achieve optimal infant neurodevelopment.

Cognition and dairy fat components

Several individual lipid components present in human breast milk have been shown to be beneficial for brain development, including gangliosides, sphingomyelin and cholesterol. These lipids are all part of the MFGM and are present in lower concentration in IF, than in human breast milk, especially in formulas based entirely on vegetable fats.[70,150–152]

Clinical studies have demonstrated that supplementing IF with bovine lipid components, including MFGM fraction,[100] sphingomyelin,[153] and gangliosides,[154] improves the cognitive score of infants. Besides clinical trials on infants evaluated by cognitive tests, animal studies have given more insight in the influence of certain lipid components on brain development and cognitive function. In mice, the diet was supplemented with bovine phospholipids to obtain large phospholipids-coated lipid droplets, which improved cognitive performance.[155] Dietary cholesterol[76] and sphingomyelin[156] improved brain myelination in mice and rats, respectively, whereas sialic acid supplementation increased the levels of these gangliosides in rat brain.[157] Piglets received a diet supplemented with either MFGM, lactoferrin and prebiotics[158] or a combination of bovine phospholipids and gangliosides,[159] which in both cases induced physiological changes in the brain. Furthermore, mice fed diets supplemented

with dairy lipids, were protected against cognitive impairment due to LPS challenge in adulthood.[160]

Interplay between arachidonic acid, docosahexaenoic acid, linoleic acid and dairy lipids

Today, supplementing IF with ARA (from fungus Mortierella alpina) and DHA from either single cell oil (algae) or from fish (tuna) has become common, to ensure adequate levels for normal infant brain development. DHA is essential for normal growth and development of the infant brain, where DHA accumulates during the first years of life.[161] Like DHA, ARA is important for infant neurological development and together, DHA and ARA, account for approximately 25% of fatty acids in the brain.[162] When using human milk as a golden standard for IF, the ARA addition level should be higher than DHA levels.[9,163] Irrespective of the fat blend used, DHA and ARA are added as separate ingredients to IF.

Recently some studies have investigated whether differences in the dietary fat blends may affect the efficiency of DHA accumulation in the blood cells and ultimately in brain tissues. It has been proposed that a dairy fat matrix enriched in ALA might improve DHA accretion in rodents.[164] It has been suggested that lowering the LA/ARA ratio increase brain DHA, as both compounds compete in the same pathway to be converted from LA to ARA, and ALA through EDA to DHA, respectively. This has been reviewed by Astrup *et al*.[165] As mentioned before in paragraph 3.2.3, the levels of LA and the ratio with ALA in IF are under debate. In mice, reducing the LA in the maternal diet increased brain n-3 LC-PUFA (ALA, EPA, DPA (C22:5 n-3) and DHA) in the offspring,[166] whereas increasing ARA in sow diet increased DHA in piglet brains.[167] However, this topic is a matter of much debate. In one clinical trial, formulas with lower LA:ALA ratios increased DHA and ARA levels in plasma and erythrocyte phospholipids, but was insufficient to ensure DHA and ARA levels that match the levels of circulation of a breast-fed infant.[168] This study did not, however, include dairy fat.

It has been speculated that the high levels of butyric acid and MCFA in dairy fat may possibly spare ALA from oxidation, as energy is generated from the rapid absorption and oxidation of butyric acid and MCFA.[169,170] Therefore, bioconversion from ALA to DHA might be favored.

Further studies involving infant clinical trials are needed to elucidate the potential cogitative benefits of adding dairy fats to IF.

Advantages and drawbacks of different fat source for IF

In this review, we have discussed the different components of bovine milk fat, and compared those to human milk fat and vegetable fat. Furthermore, we have reviewed the existing evidence from both clinical trials and animal studies, on how bovine milk fat impacts (infant) physiology and health. Based on this, we would like to highlight some of the advantages and drawbacks of different fat sources for IF.

Bovine milk fat contains valuable lipids, such as cholesterol, phospholipids and sphingolipids. These lipids are present in human milk, but cannot be obtained from vegetable sources (see paragraph 2.2). Although more research is needed, these components seem to have several beneficial effects on infant physiology and health, as discussed in this review. Furthermore, bovine milk fat contains a high variety of TAGs, with a high percentage of palmitic acid positioned at the sn-2 position, which is also the case in human milk.[16,52,53] It has been shown that a high percentage of palmitic acid at sn-2 could positively affect TAG digestion and absorption in infants, as well as the comfort of infants.[72,73,171,172] So in contrast to that what was thought in the 1960s,[8] addition of bovine milk fat to IF might decrease constipation instead of causing it.

However, bovine milk fat cannot be used as a single source of lipids, as it contains higher levels of SFA compared to human milk fat and lower levels of LCFA (LA and ALA) and DHA and ARA (**Table 1**). Because of the low levels of LA in bovine milk fat, adding vegetable fat is necessary to reach the required level of LA. A maximum of 67% of bovine milk fat can currently be used in IF, when using todays preferred LA levels. These LA levels are based on current breast milk levels. However, LA levels can be lowered from an average of 16g/100g fatty acids to about 6 g/100g fatty acids without challenging current Codex Alimentarius legislation (FAO).[173] The minimum level LA required, reflects the levels of LA in human milk at the start of industrialization, and preclinical studies indicate that lowering the LA levels may possibly have a positive impact of infant health.[116,117]

In addition, bovine milk fat contains butyrate, which only is present in trace amounts in human milk, as well as elevated levels of MCFA (**Table 1**). Most likely, these components are rapidly absorbed and metabolized in infants. However, the nutritional needs of infants are complex matters, and although no adverse effects in infants have been reported on neither butyrate nor MCFA, the effect of elevated levels in IF on infant health and development remains unknown.

Vegetable fats can be blended in such a way, that they represent the fatty acid profile of human milk. This human milk profile includes some of the valuable LCFA (LA and ALA), which only can be obtained in low amounts from bovine milk fat. However, the structure of vegetable TAGs differ from that of human milk, which results in suboptimal digestion of specific triglycerides. To address this problem, vegetable fats can be re-structured by industrial processing. Thereby,

a TAG structure with more palmitic acid in the sn-2 position can be obtained. Still, the overall TAG composition is less diverse compared to human and bovine milk fat TAGs.

A commonly used vegetable fat is palm oil, although some commercial parties avoid the inclusion of palm oil in IF.[35–37] The latter is due to concerns related to digestion (discussed above), unsustainable production methods, and the presence of elevated levels of processing-induced contaminants in palm oil (i.e. glycidol esters and 3-monochloro-1,2-propanediol (3-MCPD-esters)) which are known to have adverse health effects.[174] However, when palm oil is avoided, the level of palmitic acid, one of the most abundant FA in human milk, is very low (**Table 1**). Another possible concern is the presence of plant sterols in vegetable fats, which are not present in human milk. Although this issue has gained little attention, it deserves further investigation

The use of fat blends containing both bovine milk fat and vegetable fats seems to be a good solution for making the best possible IF. This will provide infants with both the valuable bovine milk lipids, which cannot be obtained from vegetable fats, as well as the necessary LCFA profile by adding vegetable fats. Furthermore, combined bovine milk and vegetable fat blends allow the production of palm oil-free fat blends with the same palmitic acid level as observed in human milk (**Table 1**). Independent on the major fat source used for IF, DHA and ARA are always added separately to the chosen fat blend to accomplish their preferred fatty acid composition.

Although the levels of palmitic acid at the sn-2 position is higher in IFs containing either bovine milk fat or structured vegetable TAGs, the levels of palmitic acid at sn-2 of human milk is still not reached in the current IFs (see **Table 2**). Addition of structured vegetable TAGs to a blend with bovine milk fat and vegetable fat opens new possibilities to increase the sn-2 percentages, and to get closer to the TAG composition of human milk. Another possibility to improve IF is the generation of phospholipid coated droplets. A disadvantage of all current fat blends is that, due to processing, all fat droplets have the same globule size. This is unlike human milk fat, which contains larger globules in varying sizes. A new concept has emerged, in which larger phospholipid coated droplets are produced.[175] These artificial lipid droplets are closer to human MFG than regular produced infant formula, since they have a more comparable particle size with human milk fat, compared to normal IF lipid droplets, and they contain bovine MFGM components at their membrane.[175] However, these globules contain TAGs from vegetable fat, which are structurally different from human milk fat. Probably, it would be more optimal if both the membrane components, globule size and TAG composition and structure would more closely resemble the composition of human milk fat.

Future perspectives

In this review we have pointed out several health effects of bovine milk lipids. Still, the health impact of some bovine lipids have not been studied in infants yet. Although butyrate is well-known to be produced by the microbiota in the lower gastrointestinal tract, the health effects of butyrate in IF needs to be studied. Furthermore, MCFA, as MCT fats, are known to affect

metabolism. But more dedicated research is needed to elucidate how elevated MCFA levels in TAGs influence infant health. Clinical trials on MFGM do not always specify the dose and composition of the MFGM components used. Therefore, more research is needed to understand which specific MFGM components trigger the health effects that were found.

An alternative way to use bovine milk fat in IF in the future would be to use MFG with the milk fat globular membrane intact. Today, this is not possible due to the processing techniques used to produce IF powder, such as homogenization and spray drying. Recent work indicates that pasteurization after microfiltration may be a more gentle approach.[176] Mild processing seems to be a promising option to maintain bioactivity and structure of the milk components, but extensive research is required to identify technological options maintaining the nativity of the milk ingredients in a safe manner concerning microbiology. Technical possibilities include low heating, low or no homogenization, UV-C irradiation instead of pasteurization and alternative ways of (spray) drying. Current legislation does not allow the use of non-pasteurized milk for IF production, which makes collaboration between regulatory bodies and science a crucial part of any progress to take place in the future. However, recent investigations suggests that inactivation of bioactive components through donor human milk pasteurization is a key factor influencing growth performance in preterm infants.[177,178] Interestingly, UV-C treatment seems a promising alternative.[177]

In conclusion, inclusion of bovine milk fat in IF may bring additional health benefits to infant nutrition, as it delivers a variety of different components, which are present in human milk, but are lacking in vegetable fats. Hence, blending bovine milk fat with vegetable fat in combination with the development of more gentle processing techniques might be a future direction to improve IF.

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Chapter 3

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

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

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

Results: Human milk and the IFs showed comparable total FA release. In the gastric phase, 4-11% of lipolysis occurred, and mainly short (SCFA)- and medium chain fatty acids (MCFA) were released. In the duodenal phase, lipolysis proceeded with release of C4:0 but was marked by a fast release of long-chain fatty acids (LCFA). The digestion of the IFs resulted in different FFA profiles during and at the end of digestion. IF2 gave more release of C4:0-C11:0, which reflects the FA composition of bovine milk.

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

Introduction

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

Digestion of TAGs starts in the stomach by the action of gastric lipase and is completed in the small intestine by pancreatic lipase. Gastric lipase can potentially hydrolyse all three ester bonds of TAGs but shows a stereo-preference for the sn-3 position of TAGs. Since in milk fat short- and medium-chain FAs (SCFA, MCFA) (<C12:0) are placed here, this leads to the preferential release of the shorter FAs by gastric lipase.[4–6] Pancreatic lipase shows regioselectivity for the hydrolysis of the ester bonds at the sn-1 and sn-3 positions, and seems to prefer MCFA over long-chain fatty acids (LCFA; ≥C12:0).[7] Therefore, hydrolysis of TAGs by pancreatic lipase results in two free fatty acids (FFA) and a mono-acylglycerol (MAG) with a FA esterified at the sn-2 position. Although the conversion of TAGs into FFA and MAG is sufficient to ensure full intestinal absorption of fat, MAG can be further cleaved to some extent by other enzymes showing high activity on MAG, namely bile salt-stimulated lipase (BSSL)[8] and pancreatic lipase-related protein 2.[9–11] SCFA and MCFA can directly be absorbed in the stomach and by enterocytes, whereas LCFA, and MAGs require the presence of bile salts and incorporation into mixed micelles. These micelles can be transported over the mucosal barrier into the enterocytes. Fat digestion and absorption in adults is highly efficient, with 95-97% of the ingested lipids being hydrolysed and absorbed.[3] In infants, fat absorption is less efficient. Studies investigating FA excretion in stool samples show that not all lipids are absorbed in infants; in term infants about 10% of the consumed fat is not absorbed.[12] This amount of non-absorbed FAs is found to be higher in formula fed infants compared to breast-fed infants.[13] The fat absorption seems to decrease with increasing FA chain length and increases with the number of double bonds (unsaturated FA).[3]

Human milk fat has a specific TAG structure with most of the palmitic acid, about 70-88%, attached at the sn-2 position,[14–17] whereas unsaturated FA are predominantly at the sn-1 and sn-3 positions.[14,15] There are several hypotheses why this might be beneficial for an infant.[18] One of the explanations is that unesterified palmitic acid, like other long-chain saturated fatty acids (LCSFA; ≥C12:0), is able to form indigestible complexes with calcium in the lumen, called soap formation.[19,20] These calcium soaps are not absorbed and are excreted in faeces.[19] With palmitic acid at the sn-2 position of the glycerol backbone, and thus not at the sn-1 and sn-3 positions, both the MAG as well as the calcium will be absorbed, resulting in a higher bioavailability of FAs and calcium and protection against stool hardening.

Sometimes human milk is not available, for whatever reason, and infant formula (IF) is used to feed a baby. The fat blends that are used for IF are tailored to the fatty acid composition of human milk, with C16:0 (palmitic acid) and C18:1 (oleic acid) being the most frequently

occurring FAs. Nowadays, IFs are mostly based on vegetable fats derived from palm (kernel/olein) oil, sunflower oil, coconut oil, rapeseed oil and safflower oil.[21] Bovine milk fat, a rich source of palmitic acid, is also used, with increasing popularity.[22] Whereas palm oil or bovine milk fat based formulas can be designed to have a comparable FA composition, the FA distribution on the TAGs differs. In bovine milk fat a higher percentage of palmitic acid is positioned at the sn-2 position of the glycerol backbone, compared to a representative fat blend containing palm oil (40-45% vs 10-20% respectively).[14,17] In most of the vegetable fat blends used in IF the LCSFA are positioned at the sn-1 and sn-3 positions.[16,17] In bovine milk fat, SCFA, MCFA, unsaturated fatty acids (UFA) and some LCSFA are positioned at sn-1 and sn-3.[17] The positioning of FA in bovine milk fat thus resembles human milk TAG to a higher degree than a vegetable fat blend containing palm oil. Since bovine milk fat contains low levels of linoleic acid, addition of vegetable fat is needed to reach the required amount of linoleic acid.[23] Therefore, a maximum of 67% bovine milk fat can be used in IF. When bovine milk fat is used as a source of palmitic acid, palm oil does not need to be added to reach similar levels of palmitic acid as present in human milk.[23]

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

Material & Methods

Products

Two commercially available IF base powders containing different fat blends were provided by FrieslandCampina. IF1 consisted of a mixture of vegetable fat (palm oil, palm kernel oil, rapeseed oil and sunflower oil), IF2 contained 67% bovine milk fat and 33% of different vegetable fats (rapeseed oil, sunflower oil and coconut oil). The IF products contained 31% fat (dry weight) and, besides the different fat compositions, all other ingredients were the same. The composition of the IFs is provided in **Table 1**. The IFs were dissolved in 40 °C demineralized water (0.134g/ml). The particle size distribution was determined by laser light scattering using a Mastersizer 2000 (Malvern Instruments, Malvern, United Kingdom) (**Figure 1**).

Human milk was obtained from four different Dutch women, who signed informed consent. The milk was sampled right after pumping, and digestion experiments were started 45 minutes afterwards. Human milk samples 1-4 contained respectively 2.1%, 2.5%, 5.5%, and 3.2% fat, and

the periods of collection were respectively 6 months, 6 months, 8 months, and 3 months. To determine the fatty acid composition of the IFs and human milk samples methyl esters of the fatty acids were made and analysed by capillary gas chromatography **(Table 2).** Human and bovine milk fat contain a wide range of fatty acids,[23] for these experiments we have chosen to determine fatty acids ranging from C4:0-C18:3 since this range contains the most abundant fatty acids.

In vitro lipolysis experiments

To simulate the digestive system of an infant, a two-phase static *in vitro* digestion model, including a gastric and duodenal phase, was used.[24,25] The IFs or human milk (40 ml) were put in a temperature-controlled reaction vessel, kept at 37 °C. The products were mechanically stirred and the pH was adjusted to 5.5 by addition of 0.1M HCl. To simulate the gastric phase of digestion at half gastric emptying, IFs or human milk (40 ml) were mixed with simulated gastric fluid (SGF; 8.0 ml) at a 5 to 1 volume ratio.[26] SGF was prepared by dissolving rabbit gastric extract (RGE; CNRS, Marseille, France) at 1.8 mg/ml in 10 mM MES buffer, 150 mM NaCl, pH 6.0. RGE contains rabbit gastric lipase and was chosen because of similar properties to human gastric lipase, like regio- and stereo preference.[27] The lipase activity of the RGE powder was 68 U/mg, using tributyrin as substrate, which corresponds to 57 µg/mg. A concentration of 1.8 mg/ml RGE in SGF allowed reaching a final gastric lipase concentration of 17 µg/ml in the digestion mixture, which corresponds to the gastric lipase concentration in gastric contents at half gastric emptying.[26] During the gastric phase the pH was kept constant at pH 5.5 by addition of 0.1M HCl and/or 0.1 M NaOH. The gastric phase lasted for 30 minutes, starting with the addition of gastric fluid. At -2, 15, and 29 minutes samples were taken for analysis. After 30 minutes, 25.2 ml of simulated intestinal fluid was added to mimic the ratio of meal to intestinal fluid as observed *in vivo*, at half gastric emptying.[26,28] To represent human pancreatic juice and bile, porcine pancreatic extract (PPE or pancreatin; 8xUSP, Sigma-Aldrich, st Louis, USA) and bovine bile salts (Sigma-Aldrich, st Louis, USA) were mixed and dissolved in 10 mM Tris buffer, 150 mM NaCl, pH 6.0. The intestinal phase lasted for 60 minutes, from minute 30 to 90. The intestinal phase the pH was kept at pH 6.25 using 0.1M NaOH. Samples were taken for analysis at 5, 10, 15, 30 and 60 minutes after the start of the intestinal phase. Enzymes in the samples were inactivated either by heat treatment (5 minutes at 72 °C) or acidification (addition of 200 µl 0.1M HCl), both well-established methods to inactivate lipases.[29,30] Afterwards, samples were immediately frozen in liquid nitrogen and stored at -80°C for further analysis. The simulated gastric and intestinal fluids were made fresh before each experiment, and were stored on ice until use. Digestion of the IFs was performed in triplicate. The four human milk samples were digested in independent experiments.

Release of individual fatty acids

Release of individual FFA was analysed by the ethyl chloroformate free fatty acid (ECF-FFA) method of Amer *et al* .[31] Deuterated internal standards of all FFA (C4-C18:1) were added to samples and standards. This was followed by in solution derivatisation using a two-step procedure. First 1000 µL of the digested samples, were mixed with 200 µL of ECF. The pH was adjusted to neutral pH with 100 µL 7 M NaOH, followed by a second addition of 200 µL of ECF for neutral pH derivatisation. Next, FFA derivatives were extracted with 1000 µL chloroform. 1 µL aliquots were injected in a splitless mode into an Agilent Technologies 7890A gas chromatography system coupled to an Agilent Technologies 5975c inert MSD quadrupole mass spectrometer (Agilent Technologies, Waghaeusel, Germany). An HP-5MS capillary column coated with polyimide (60 m, 250 mm i.d., 0.25 mm film thickness; Agilent Technologies) was used to separate the ECF-derivatised FFA. The initial temperature 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 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 constant flow rate of 1 ml/ min with helium as carrier was applied. The temperatures of the ion source and injector were 230 and 260 °C, respectively. The mass spectral analysis was performed in selected ion monitoring according to the ions determined by the use of a standard for each compound with a quadruple temperature of 150 °C and a fragmentation voltage of 70 eV with a solvent delay of 6.50 min. Concentration of individual FFA was quantified using an external calibration curve including deuterated internal standards. Deuterated C18:2 was used as internal standard for all unsaturated C18s.

Analysis of residual triacylglycerol

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

Statistics

For all parameters, the mean and SD of the three replicate experiments were calculated for both IFs. The results of the individual four human milk digestion experiments were combined and a mean and SD were calculated for all parameters. The data was compared via one-way ANOVA analysis followed by a Bonferonni posthoc test, or Student's t-test when only data of human milk and IF2 was available. GraphPad Prism (version 5.04 for Windows, GraphPad Software, San Diego California USA) was used for the statistical analyses.

Table 1: Composition of infant formulas (IFs) (per 100 gram dry weight)

Per 100 g	IF1	IF2
Energy (kcal)	535	525
Protein (Nx6.25) (g)	11.3	10.6
Fat (g)	31.2	30.5
Carbohydrates (g)	52.8	54.3

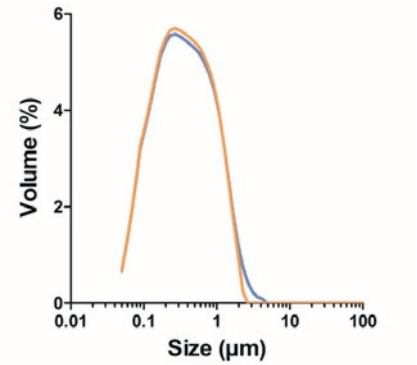

Figure 1: The particle size distribution of the infant formulas (IF) basepowders tested in these digestion experiments. the blue line represents IF1, and the orange line represents IF2.

Table 2: Initial fatty acid composition of the infant formula products and human milk samples (in % of total FAMEs).

Fatty acid	IF1	IF2	Human milk-1	Human milk-2	Human milk-3	Human milk-4
C4:0	<0.1	2.6	<0.1	ND	<0.1	<0.1
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
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	42.3	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 SC/MCFA	1.4	7.8	1.3	0.9	2.1	2.2
Total LCFA	94.9	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	42.3	25.9	30.3	29.8	32.2	30.8
Total PUFA	14.7	13.4	11.3	10.8	11.1	22.2

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

Results

The particle size distribution of the IFs is shown in **Figure 1**. The modal diameter of both IFs is 0.27 µm. Lipolysis of the different milk products was studied by analysing the residual TAG and FFA concentrations of different time points of the *in vitro* digestion. The decrease in TAGs is shown in **Figure 2A**. In the gastric phase no significant difference between the IFs and human milk was found (p=0.18). At the end of the gastric phase, 24 to 36% of initial TAG molecules had already been hydrolysed. At the end of the duodenal phase more TAGs remained intact in human milk (8.9%) compared to IF1 (2.9%) (p=0.02). The percentage of remaining TAGs in IF2 (4.4%) was not different from either human milk or IF-1 (p>0.05). **Figure 2B** shows the release of FFA. The human milk samples showed less release of FFA during the gastric phase compared to IF1 and IF2 (2.0 ± 0.2% vs. 4.3 ± 0.2% and 4.7 ± 0.1% respectively, $p < 0.01$). Compared to the amount of FFA released after the digestion, during the gastric phase 4% of FFA were released from human milk, about 10% from IF1, and about 11% from IF2. Except for 45 minutes ($p = 0.04$), i.e. 15 minutes after the start of the duodenal phase, no differences were found in FFA release between the IFs compared to the human milk samples during this phase. The total release of FAs at the end of the digestion, as percentage of initial composition, was found to be similar for the different samples (43.9 ± 2.0%, 42.2 ± 1.4%, and 52.3 ± 4.5% for IF1, IF2 and human milk respectively, $p = 0.14$).

To obtain more insight in the digestion process, the release of individual FAs at the end of each digestion phase was analysed. **Figure 3** shows the distribution of individual FFA released as percentages of total FFA after the gastric phase (A) and after the duodenal phase (B). During gastric lipolysis fatty acids up to and including C12:0 were the main FAs released, followed by palmitic acid (C16:0) and oleic acid (C18:1) **(Figure 3A**). The digestion of IF2 was distinctly marked by the release of C4:0 originating from bovine milk. The long-chain unsaturated fatty acids (LCUFA) C18:1 and C18:2 from human milk were released at a higher level, whereas for the IFs the percentage released of these fatty acids was much lower. During duodenal lipolysis, the main FAs released were LCFA (**Figure 3B**). Lipolysis of human milk resulted in higher levels of unsaturated fatty acids (C18:1 and C18:2) compared to the IFs. The profile of FFA released from IF2 was still marked by a significant level (9.8%) of C4:0, although this was not found for IF1 and human milk. Furthermore, a higher percentage of C6:0-C11:0 was found after lipolysis of IF2.

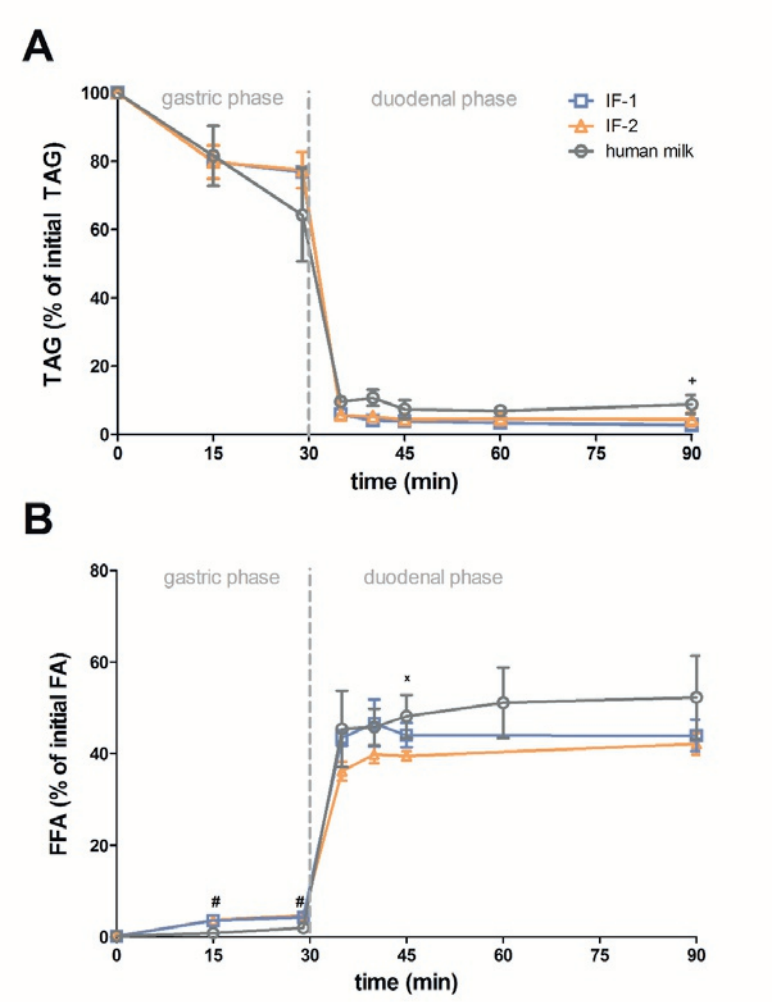

Figure 2: The lipolysis of infant formulas and human milk (mean ±SD). A: Decrease of triacylglycerols (TAGs) during *in vitro* digestion in percentage of total TAGs initially present (mean ±SD). B: Percentage of free fatty acids (FFA) in percentage of total esterified fatty acids (FA) in initial TAGs, during *in vitro* digestion (mean ±SD). At time point 0 the FFA percentages were 0.15%, 0.20%, and 0.22% for IF-1, IF-2 and human milk respectively. The vertical striped line indicates the start of the duodenal phase following the gastric phase. The grey line (○) represents the average of the human milk samples, the blue line (□) represents IF1, and the orange line (Δ) represents IF2. # significant difference between both IFs and human milk, + significant difference between IF1 and human milk, x significant difference between IF2 and human milk ($p<0.05$), separate time points were compared with one-way ANOVA, followed by Bonferroni posthoc test.

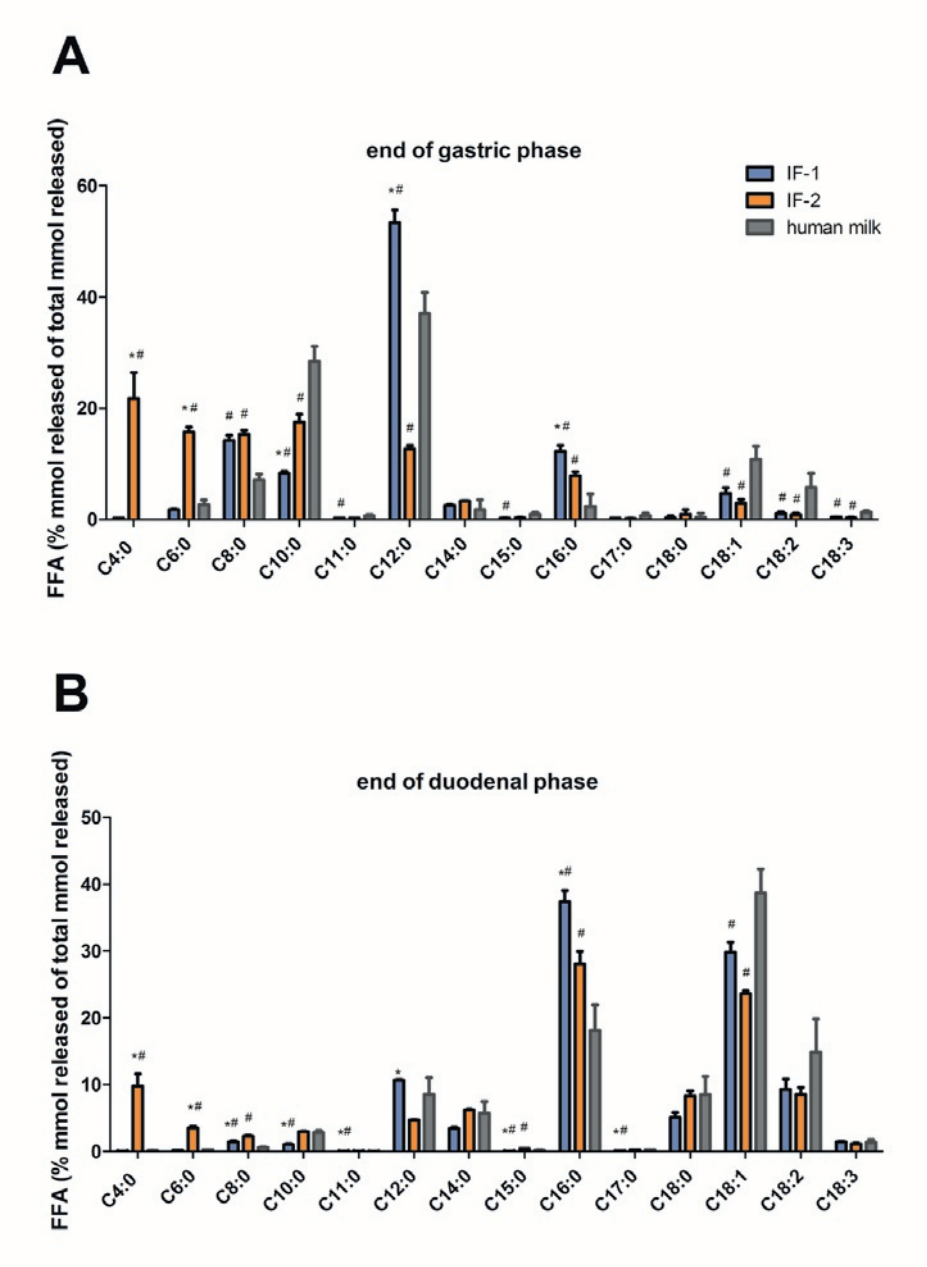

Figure 3: Free fatty acid profile at the end of *in vitro* gastric digestion (A) and duodenal digestion (B) of IF1 (blue bar), IF2 (orange bar), and human milk (grey bar) (mean ±SD). * significant difference between IFs, # significant difference between IF and human milk ($p<0.05$), as compared with one-way ANOVA.

Since LCSFA are able to form complexes with calcium, the release of the sum of LCSFA and the most prominent LCSFA, palmitic acid, were compared between the IFs and human milk after digestion (end of duodenal phase). Free palmitic acid was less represented after digestion of human milk (18.2 ± 3.8% of the FFA) compared to digestion of IF1 (37.4 ± 1.6%; p<0.001) and IF2 (28.0 ± 1.9%; p<0.001) (**Figure 4A**). Of the initial palmitic acid present in TAGS from IFs and human milk, the lowest percentage of palmitic acid (31.8 ± 6.0%) was released from human milk (**Figure 4B**), while 57.2 ± 6.9% and 46.0 ± 5.6% respectively was released from IF1 and IF2 (p<0.01). The total release of LCSFA, as percentage of FFA released (**Figure 4C**) or as percentage of LCSFA initially present in the IF and human milk TAGs (**Figure 4D**), was lower in the human milk samples compared to IF1. IF2 did not significantly differ from human milk or from IF1.

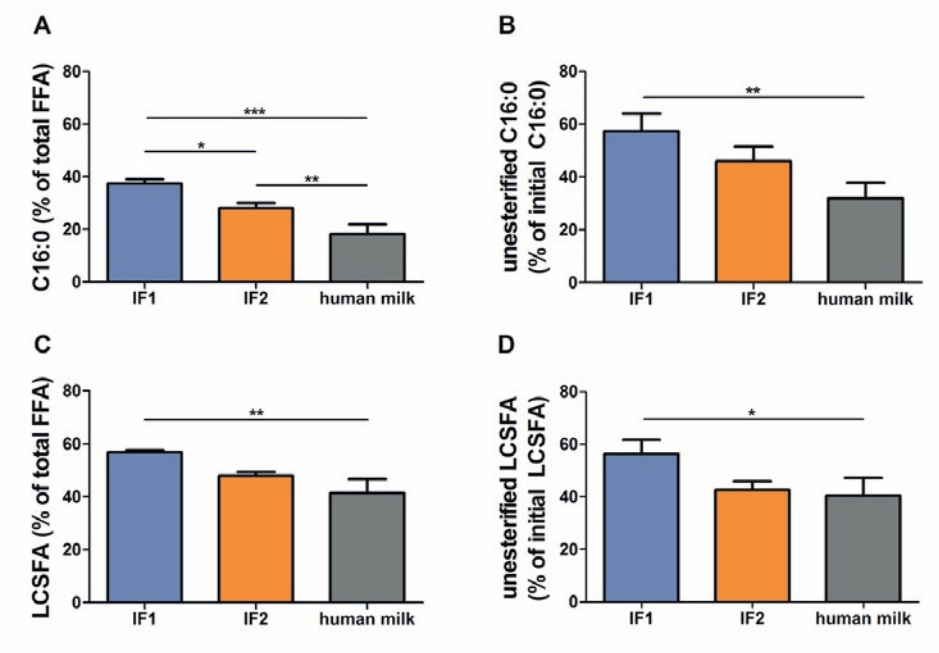

Figure 4: Free palmitic acid (C16:0) and total free long-chain saturated fatty acids (LCSFA) released from IFs and human milk at the end of duodenal digestion. A) free palmitic acid expressed as % of total free fatty acids (FFA) released upon digestion B) free palmitic acid expressed as % of initial amount of C16:0 in milk products. C) total free LCSFA expressed as % of total FFA released upon digestion; D) total free LCSFA expressed as % of initial amount of total LCSFA in IFs and human milk. IF1, blue bar; IF2, orange bar; human milk, grey bar. Data are (mean ±SD), and were analysed using one-way ANOVA, and Bonferroni posthoc test, * p<0.05, **p<0.01, ***p<0.001.

Next, the release of individual FAs in time was examined. **Figure 5** shows the release of FAs as a percentage of their initial concentration in the IFs and human milk. **Table 3** shows the corresponding mean percentages (±SEM) of the individual fatty acids released at the end of digestion (90 min) as well as during gastric phase (0-30 min) and duodenal phase (30-90 min) separately. The SCFA, C4:0, which is present in the IF with bovine milk fat (IF2) and found at very low levels in human milk, was partly released in the gastric phase for IF2 (14%), whereas for human milk no free butyric acid could be detected (**Figure 5A, Table 3**). The release of C4:0 mainly took place in the duodenal phase, and total release was higher for IF2 compared to human milk (55% and 23% respectively, $p=0.01$). The release of C6:0 was highest from human milk ($p=0.02$), and showed different patterns between the IFs and human milk (**Figure 5B, Table 3**). For IF1 C6:0 was primarily released in the gastric phase. For IF2, the release in the gastric and duodenal phase was comparable (25% and 21% respectively). For human milk the release of C6:0 in the duodenal phase was higher than in the gastric phase (40% vs 20% respectively). The release of C8:0 and C10:0 was similar in the gastric phase and after total digestion for the IFs and human milk ($p=0.38$, $p=0.13$ and, $p=0.06$, $p=0.11$ respectively). However, in the duodenal phase the release of C8:0 and C10:9 was higher compared to the IFs ($p<0.01$ and $p<0.01$ respectively). (**Figure 5C-D, Table 3**). All of the LCFA, both saturated and unsaturated, were mainly released in the duodenal phase (**Figure 5G-M, Table 3**). An exception is C12:0, lauric acid, which was also partly released in the gastric phase (**Figure 5F, Table 3**). The release of C12:0 after total digestion was similar for both IFs and human milk ($p=0.33$). However, during the gastric phase the release was highest for IF1 ($p<0.01$), while during the duodenal phase the release was higher for human milk compared to the IFs ($p<0.01$). From the other LCSFA, except for C15:0, the release from human milk was or tended to be lower compared to IF1 (**Table 3**). The release of LCUFA after total digestion was higher for human milk compared to the IFs (**Table 3**).

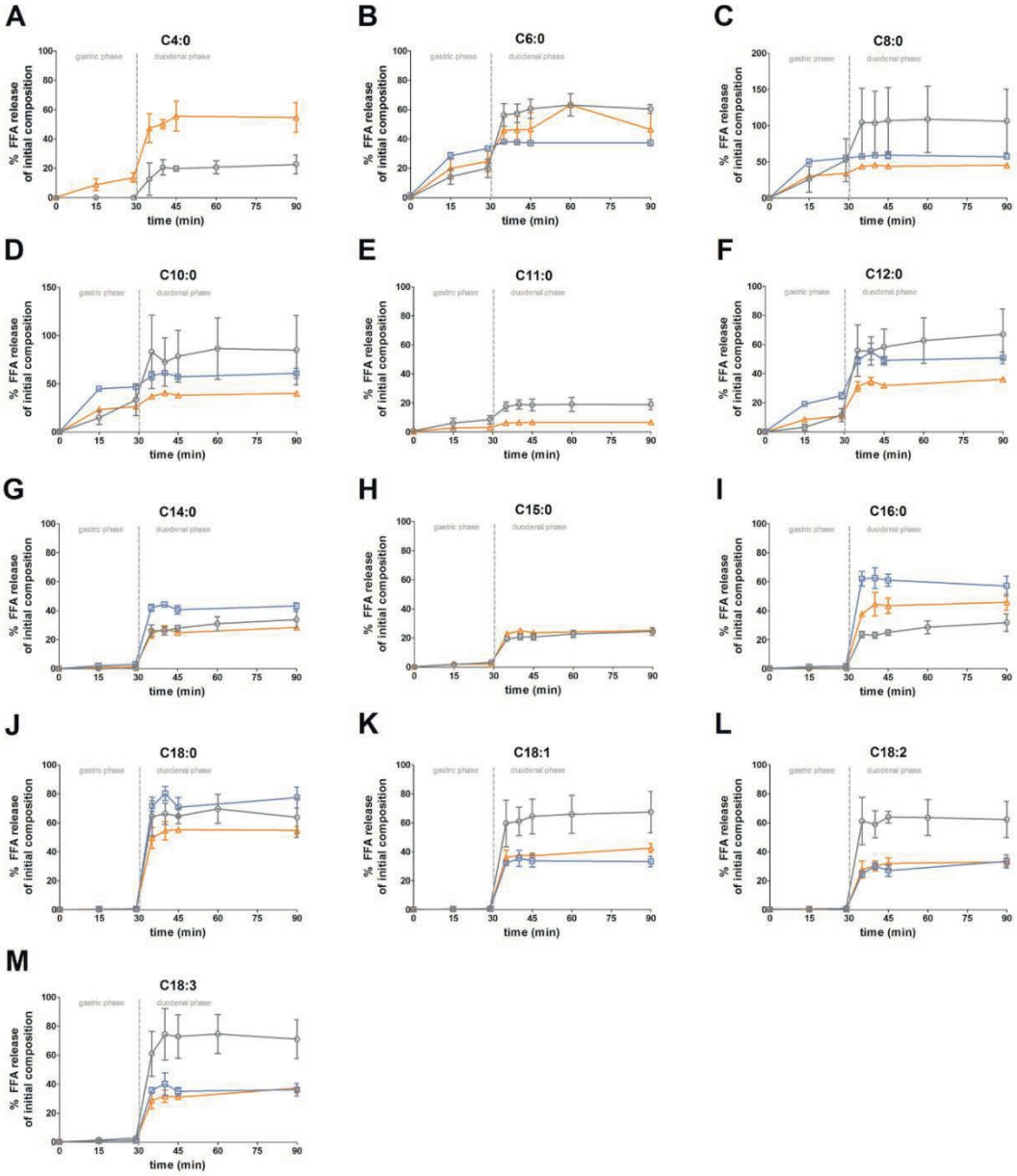

Figure 5: Release of individual fatty acids during *in vitro* digestion, expressed as percentage of their initial FA content present in IFs and human milk (mean ±SD); A) C4:0, B) C6:0, C) C8:0, D) C10:0, E) C11:0, F) C12:0, G) C14:0, H) C15:0, I) C16:0, J) C18:0, K) C18:1, L) C18:2, M) C18:3. The vertical striped line indicates the start of the duodenal phase following the gastric phase. The grey line (○) represents human milk, the blue line (□) represents IF1, and the orange line (Δ) represents IF2.

Table 3: 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 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 test). nd, not detected

	Gastric phase				Duodenal phase				Total digestion			
Fatty acid (%)	**IF1**	**IF2**	**Human milk**	***p-value***	**IF1**	**IF2**	**Human milk**	***p-value***	**IF1**	**IF2**	**Human milk**	***p-value***
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 ± 5.8 ^{a}	22.7 ± 3.7 ^{b}	*0.01*
C6:0	33.5 ± 0.7 ^{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 ± 0.8	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 ± 0.6	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 ± 1.2 ^{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 ± 0.3 ^{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 ± 0.1 ^{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 ± 0.3	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	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	*0.40*	32.7 ± 3.5 ^{a}	41.9 ± 3.2 ^{a}	66.6 ± 14.0 ^{b}	*0.04*	33.2 ± 2.1 ^{a}	42.5 ± 1.8 ^{a}	67.4 ± 7.1 ^{b}	*0.04*
C18:2	0.4 ± 0.1 ^{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 ± 0.2 ^{a}	1.2 ± 0.2 ^{a}	2.9 ± 0.5 ^{b}	*0.02*	35.2 ± 4.1 ^{a}	36.1 ± 3.1 ^{a}	68.2 ± 12.6 ^{b}	*<0.01*	36.2 ± 2.5 ^{a}	37.4 ± 1.9 ^{a}	71.1 ± 6.7 ^{b}	*<0.01*

Discussion

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

In vitro lipolysis

In this study we tried to mimic the digestion as occurring in infants. Therefore, the gastric pH was set to infant levels, which is higher compared to that of adults.[32] Gastric and duodenal pH values were chosen to correspond to conditions existing in the stomach and duodenum at half gastric emptying time of a meal.[26] The gastric lipase that is used has characteristics similar to human gastric lipase, including stereo-preference and pH-activity level.[27] In addition, the duration of digestion that was used in this model was quite short compared to that of other *in vitro* digestion models. Whereas some other models use a gastric phase of several hours,[33–35] the gastric phase of the current model lasted only 30 minutes. However, this is in line with the findings of Roman *et al.*, who showed that the gastric emptying time of an infant is approximately 30 minutes.[6] Moreover, the gastric lipolysis levels reached after 30 min under these conditions of pH and gastric lipase concentration are equivalent to those recorded *in vivo* during the whole gastric digestion period.[26,36] Further improvement of the model could involve stepwise transfer of product fractions to the duodenal phase, mimicking the process of gastric emptying, and gradual addition of digestive enzymes. These two effects potentially negate each other. In view of the rapid lipolysis that is observed, these alterations are unlikely to affect the currently observed outcomes.

FFA analysis

Previous studies investigating the *in vitro* lipolysis of bovine milk, human milk and IFs only determined the release of FA with a carbon number equal to or higher than 6.[37–42] The extraction of the SCFA C4:0 and its quantification are notoriously difficult due to its partial solubility in water, volatility and poor detection with various analytical techniques. Therefore, the level of C4:0 released from milk TAGs are often not determined during both *in vitro* and *in vivo* digestion studies. However, since C4:0 is present in relatively high levels in bovine milk fat, we determined the release of this FA. Therefore, an in-solution derivatization approach was used, in which all FFA were converted to ethyl esters in the investigated matrix before extraction. This method thus prevents loss of in particular shorter chain FAs and allows quantitative measurements.[31] Therefore, the total FFA profile, including C4:0, could be studied, which normally fails to be investigated due to their water solubility. The in-solution derivatization approach allowed us to show a full picture of the lipolysis, concerning all more abundant FAs in the investigated matrices.

Gastric lipolysis

The present *in vitro* digestion experiments showed that during the gastric phase, only a small percentage of total FAs were released. For the IFs about 10% of total FFA were released in the gastric phase, which is in line with previous findings.[24,32,35] The gastric lipolysis of human milk samples was around twofold lower, with about 4% of total FFA being released. This may be due to the presence of the human milk fat globule membrane in fresh human milk, which is less accessible to lipases than the globules in IF.[43,44] Another possible explanation might be the larger lipid-water interface available for the lipases[33], since the IFs used in these experiments have a modal diameter of 0.3 µm, while human milk is known to have a larger modal diameter, of around 4 µm.[45] Since no significant difference was found for the clearance of TAGs between human milk and IFs in the gastric phase, the differences in FFA release and lipolysis levels can easily be explained by further hydrolysis of DAGs generated from TAGs of IFs. However, this was not studied here and thus remains speculative.

Since samples were taken at different time points during the *in vitro* digestion it was possible to study the time-dependent release of the individual fatty acids. During gastric lipolysis mainly C6:0-C11:0 and part of C4:0 (for IF2 only) were released, with only some LCSFA. Most of the released LCSFA was C12:0. In this study we defined C12:0 as a LCFA since it has the ability to form calcium soaps like other LCSFA.[20] However, lauric acid is more water soluble than FA ≥ C14,[46] and can partly be directly absorbed into the portal vein.[47] Therefore it is often considered as being a MCFA.[48] This study shows that the release of C12:0 in time is in line with MCFA, and therefore the results support the categorization of C12:0 as a MCFA when using digestion kinetics as basis for classification. The preferential release of fatty acids <C12 is according to expectation, since it corresponds to the specificity of gastric lipase.[4,5] For human milk no release of C4:0 was found in the gastric phase, however, the levels of C4:0 in the human milk samples was very low (**Table 2**), therefore, the amount of C4:0 released might have been below detection.

Duodenal lipolysis

In the duodenal phase, lipolysis occurred at a faster rate and reached higher levels. After 10 minutes almost maximum lipolysis was reached for all samples. Thus, the pancreatic extract, containing pancreatic lipase, in combination with bile salt, acted very efficiently. This high efficiency may have precluded identification of subtle differences in digestion kinetics. Such differences may possibly have been observed using lower concentrations of lipases. However, lower lipase concentrations would not have been physiologically relevant for term infants, as the conditions we used were based on *in vivo* data obtained from term infants.[36] The release of FA from initial TAGs found in these experiments after the duodenal phase is between 42 and 52%. These values have to be compared with a lipolysis level of 67% (TAGs totally converted in MAGs and FFA) that allows full intestinal absorption of lipolysis products. The model used here has been shown to well reproduce the lipolysis levels observed *in vivo* at the Angle of Treitz, i.e. the end of the duodenum.[49] It does not allow to observe full lipolysis, but it appears suitable for comparing various type of meals,[26] emulsions[25,50] and, as shown here, IFs versus milk.

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

Not only LCFA were released in the duodenal phase in the course of human milk digestion. Unexpectedly, also part of C6:0-C11:0 from human milk and part of C4:0, from human milk and IF containing bovine milk fat, were released. Since fatty acids <C12 are mostly positioned at the sn-1 and sn-3 position of the glycerol backbone in human and bovine milk fat, they are thought to be preferentially released by gastric lipase in the stomach. However, gastric lipolysis is rapidly inhibited by FFA,[51] and therefore some of those fatty acids may have reached the duodenal phase still esterified in TAGs (or DAGs). Gastric lipase can still work in the duodenal phase where its inhibition by lipolysis products, which is observed in gastric conditions,[52] is abolished by bile salts. This has been shown in chronic pancreatitis patients with no pancreatic lipase.[53] In addition, pancreatic lipase is a 1,3-regioselective lipase that can also preferentially release the FAs esterified to the sn-3 position of the glycerol backbone. The difference in time points of release of C6:0-C11:0 between human milk and IFs may also be explained by differences in TAG composition. It has been shown that the percentage of C8:0 at the sn-2 position is higher in the milk of Chinese mothers compared to IF.[17] Such positioning of those FAs at the glycerol backbone could have partly protected them from lipolysis in the gastric phase of digestion.

Possible health effects

The preferential release of C4:0 and C6:0-C11:0 in the gastric phase of digestion is potentially linked with the fact that these FAs can already be absorbed by the gastric cells.[54,55] When they reach the intestinal lumen they could possibly exert effects on epithelial cells. MCFA are hypothesized to have beneficial effects, like antimicrobial effects.[56,57] The same holds true for C4:0, butyric acid, which is present in bovine milk fat and in trace amounts in human milk. Butyrate, and other SCFA, are largely produced by microbial fermentation in the intestine and are generally considered as important mediators of the beneficial health effect of intestinal microbiota, contribution to epithelial maturation and barrier function.[58,59] Since microbial fermentation occurs predominantly in the colon, it is of interest that milk and IF have the potential to deliver C4:0 to the small intestine. A study performed in piglets showed that

consumption of butyrate improved development of the jejunum and ileum crypt depth, villi length and mucosa thickness were increased.[60] Whether the levels of butyric acid present in human milk and IF containing bovine milk fat contributes to small intestinal maturation and functioning still needs to be determined.

Conclusion

The addition of bovine milk fat to IF, and thereby changing the triglyceride structure, did not influence the total amount of release of fatty acids in time compared to an IF with vegetable fat only or human milk. However, the profile of the fatty acids that are released was found to be different. More SCFA and MCFA, and less LCSFA, especially palmitic acid, were released from an IF containing a combination of bovine milk fat and vegetable fat compared to an IF containing only vegetable fat. The FFA profile of human milk distinguishes from the IF products by a higher release of C6:0-C11:0 and an even lower release of palmitic acid in the duodenal phase.

Conflict of interest

Jeske Hageman and Anouk Feitsma are employees of FrieslandCampina

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Chapter 4

The effect of partly replacing vegetable fat with bovine milk fat in infant formula on postprandial lipid metabolism: a proof-of-principle study in healthy young male adults

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Abstract

In infant formula (IF) bovine milk fat can be used in addition to vegetable fats. These fat sources differ in fatty acid (FA) composition and triacylglycerol (TAG) structure. The type of FAs and TAG structure affect the postprandial lipemic response. A maximum of 67% bovine milk fat can be used in IF. Whether bovine milk fat influences the postprandial lipemic response compared to an IF containing only vegetable fats is unknown. Here, this is examined in a proof-of-principle study with 20 healthy male adults. Using a randomized controlled double-blind cross-over design, subjects consumed test drinks with either 100% vegetable fat (VEG) or with 67% bovine milk fat and 33% vegetable fat (BOV) on two separate days. Before and every 30 minutes after consumption venous blood samples were taken, until five hours postprandially. Indirect calorimetry was performed continuously. No differences in concentrations of serum lipids, lipoprotein metabolism or substrate oxidation were observed between test drinks. Particle size of chylomicrons was slightly different, but this was not due to differences in lipid content. The differences in FA profile of chylomicrons observed between the two test drinks was comparable to the differences in the initial FA profile of the test drinks. Concentrations of serum ketone bodies were increased following consumption of the BOV-test drink, which may because of the higher percentage of short- and medium-chain FA. In conclusion, the use of bovine milk fat in IF does not affect postprandial lipemic response in healthy adults. Whether the exact same effects occur in infants requires experimental verification.

Keywords: lipid metabolism, bovine milk fat, infant formula, lipidomics, chylomicrons

Introduction

Breast milk is the best available nutrition for infants. In case this is not available, infant formula (IF) is the best alternative. Fat is an important component of IF, it delivers about 50% of the energy to an infant.[1] Fat blends for IF are designed to mimic the FA composition of human milk fat, they can contain fat from various sources, mostly a mixture of vegetable fats is used, e.g. palm oil, (high-oleic) sunflower oil, and coconut oil.[2] Animal sources are also used, such as bovine milk fat.[3] Vegetable fat and bovine milk fat differ in fatty acid (FA) composition.[2] With the use of bovine milk fat, a wider variety of FAs is introduced into IF, compared to the use of vegetable fats only. The level of saturated FA (SFA), including short- and medium-chain FA (SCFA and MCFA), increases and the level of monounsaturated FA (MUFA) slightly decreases with addition of bovine milk fat to IF.[2] Besides FA composition, the two fat sources also differ in positioning of FAs on the glycerol backbone, i.e. the triacylglycerol (TAG) structure, since in TAGs of bovine milk fat more long-chain saturated FAs (LCSFA) are present on the sn-2 position compared to vegetable fat.[2]

Both the type of FA and their positioning on the glycerol backbone affect the postprandial rise in circulating TAG-containing lipoproteins, the lipemic response.[4–6] Postprandial lipemic responses have gained a strong interest since a prolonged or elevated postprandial lipemic response, hypertriglyceridemia, is associated with increased metabolic disease risk, such as risk for cardiovascular diseases (CVD).[7,8] This lipemic response is largely caused by chylomicron production.[4] Chylomicron production is influenced by endogenous factors, including genetic variation, as well as exogenous factors, such as food components.[9] Despite conflicting results in literature, the general believe is that lipemic responses are highest for SFA, followed by MUFA, n-6 PUFA and lowest for n-3 PUFA.[4] In a clinical trial it was shown that positioning of LCSFA at the sn-2 position, instead of the sn-1 and sn-3 positions of a TAG, resulted in higher levels of apolipoprotein B in infants, indicating higher concentrations of chylomicrons and lipoproteins.[6] Thus, the different lipid composition and structure of bovine milk fat and vegetable fat may affect its postprandial lipemic response.

A maximum of 67% of bovine milk can be introduced in IF to still have the minimum preferred level of linoleic acid. It is not known whether an IF containing 67% bovine milk fat and 33% vegetable fats differs in the postprandial lipemic response compared to a fat blend containing 100% vegetable fats. This is what we have investigated in this study. Since invasive blood sampling is needed to study the lipemic response, we conducted a proof-of-principle study with healthy male adults, rather than with infants. Blood samples were taken before and every 30 minutes after consumption of the test drinks until five hours postprandially. Comprehensive analyses of serum samples and chylomicron-rich fraction of plasma samples were performed to obtain a detailed view of the postprandial lipemic response.

Material&Methods

Study ethics

This intervention study, with the acronym MELC, was performed at the Human Research Unit at the Wageningen University. It was approved by the Medical Ethics Committee of Wageningen University (METC-WU), and was conducted according to the declaration of Helsinki. The trial was registered at the Dutch Trial Register (NTR7083). Written and oral information was provided to the volunteers. All subjects gave an informed written consent before enrolment.

Subjects

Twenty healthy Caucasian males, with an average age of 21.4 years, a healthy body weight (75.9 ± 8.3 kg) and BMI (22.5 ± 1.6 kg/m^2) were included in the study. Exclusion criteria that were used were: claustrophobia, (symptoms of) lactose intolerance or cow's milk allergy, known metabolic diseases, autoimmune diseases, gastro-intestinal diseases, cardiovascular diseases, smoking, excessive alcohol consumption (>21 glasses per week), and blood donation during the two months before the study.

Study design

The study design has been described and diet-induced thermogenesis, satiety, blood glucose levels, and gastrointestinal peptide concentrations were reported (**chapter 5**). Briefly, the MELC study was a double-blind randomized cross-over trial, in which two test drinks were compared on separate test days with at least 1 week wash-out. Subjects were restricted from exercise, alcohol consumption, and use of drugs on the day prior to a test day. A standardized dinner was consumed (2443 kJ, 15 g fat, 64 g carbohydrates, 43 g protein), followed by an overnight fast of 12 hours. After being transported by car to the research unit, an indwelling venous catheter was placed, and participants lied down on a bed for at least 30 minutes. Exhaled air was sampled continuously using a ventilated hood system. After blood samples were taken to determine baseline concentrations of outcome parameters, subjects consumed one of the two test drinks (time point 0 min). The order of the test drinks was randomized by a random sequence generator. After consumption participants were placed under the ventilated hood again and exhaled air measurements continued for five hours. Every 30 minutes blood samples were taken. At 150 minutes after consumption of the test drinks subjects received a glass of water and were allowed to have a toilet break.

Test drinks

The test drinks were isoenergetic and equal in nutrient composition, with about 50 en% from fat, 42 en% from carbohydrates, and 8 en% from protein (**Supplemental Table 2**). The fat source differed between the test drinks, one contained a fat blend of vegetable fats only (VEG), the other one contained a fat blend of 67% anhydrous bovine milk fat and 33% vegetable fats (BOV). The FA profile was determined by conversion of FAs into methyl esters followed by

capillary gas chromatography (NEN-ISO 15884 and 15885) (**Table 1**). Participants received a total amount of test drinks similar to 30% of their estimated total energy expenditure, calculated using the Harris-Benedict equation[10] and a physical activity level of 1.75.

Indirect calorimetry

Concentrations of O_2 and CO_2 were determined in exhaled air via the indirect calorimetry system MAX-II Metabolic System (AEI technologies Inc, USA). Volume of O_2 consumption (VO_2) and volume of CO_2 production (VCO_2) were calculated. Non-protein fat and glucose oxidation rates were calculated via the equations of Péronnet & Massicotte.[11] Fat and glucose oxidation rates were averaged per 30 minutes.

At time points -2, 60, 120, 180, 240, and 300 minutes exhaled air was sampled into Tedlar sample bags, via a sampling device coupled to the MAX-II Metabolic system. Concentrations of m/z 59 (acetone) were determined via PTR-MS (Ionicon Analytik, Austria).

Table 1. Fatty acid composition of the two test drinks (in % of total FAMEs).

	VEG- test drink	BOV-test drink
C4:0	<0.1	2.6
C6:0	<0.1	1.6
C8:0	0.6	1.2
C10:0	0.5	2.1
C11:0	<0.1	0.3
C12:0	7.0	4.3
C14:0	3.0	8.2
C14:1n-5	<0.1	0.7
C15:0	<0.1	0.7
C16:0	24.7	22.8
C16:1n-7	0.2	1.1
C18:0	3.1	7.1
C18:1 trans	<0.1	1.1
C18:1n-9	42.3	25.9
C18:1n-7	1.6	1.1
C18:2 n-6	12.9	12.0
C18:3 n-3	1.8	1.4
C18:3n-6	0.1	<0.1
C20:0	0.3	0.2
C20:1n-9	0.5	0.2
Total SFA	39.6	51.1
Total MUFA	44.8	30.1
Total PUFA	14.8	13.5

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids

Blood analysis

Blood was sampled into sterile vacutainers, containing coagulation activators (serum), or EDTA (plasma). After inverting the tubes ten times, the serum samples were left at room temperature for 60 minutes to clot. Afterwards, serum samples were centrifuged (1200 g, 10 min, RT). Plasma samples were centrifuged directly after inverting (1200 g, 10 min, 4°C). Aliquots of serum and plasma were stored at -80°C until analysis. Concentrations and compositions of different lipoprotein subclasses, apolipoproteins, and ketone bodies were analysed in serum samples of all time points by a metabolomics platform using nuclear magnetic resonance spectroscopy.[12]

Isolation and analysis of chylomicron-rich fraction of plasma

Plasma was used for the isolation of the chylomicron-rich fraction (CMRF). Four ml of plasma was carefully overlaid with 8 ml of demineralized water and centrifuged for 180 minutes at 22.500 rpm, at 21°C (Beckmann Avanti J-25, rotor JA 25.50). The upper layer, the CMRF, was separated, snap frozen and stored at -80°C until analysis. Analysis of TAG and FAs in the CMRF were performed as described by Tvrzická *et al.*[13] Briefly, total lipid was extracted from 0.1 ml of CMRF by the method of Folch *et al.*[14] using dichloromethane instead of chloroform.[15] Nonadecanoic acid ethyl ester (NuCheck Prep, USA) was added to each sample before extraction as an internal standard. Samples were transmethylated to FA methyl esters (FAME). Chromatographic analyses were performed with a Trace-GC gas chromatograph combined with AS 2000 autosampler (Thermo-Finnigan, USA), equipped with a capillary split/splitless injector and a flame ionization detector. Analyses of FAME were performed on a fused-silica capillary column coated with chemically bond stationary phase DB-23 (60 m, 0.25 mm I.D., film thickness 0.25 um) (Agilent Technologies, USA). The oven temperature was programmed as follows: 100°C isothermal for 2 min, then to 180°C at 10°C /min, isothermal for 25 min, then to 250°C at 8°C /min, where it was maintained for 15 min. The injector and detector temperatures were set at 250°C and 270°C, respectively. Hydrogen carrier gas was maintained at a head pressure of 80 kPa and total flow of 30.6 ml/min, with a split flow 20 ml/min and splitless time 0.25 min. Integration software Clarity version 2.4.1.57 (Data Apex Ltd. Prague, Czech Rep.) was used for data acquisition and handling. TAG concentrations were determined with an analyzer UNICEL DxC 880i (Beckman Coulter, USA), using reaction set for TAGs (Cat. Nr. 445850, Beckman Coulter, USA).

To measure the particle size of particles in the CMRF, samples were thawed in a waterbath at 40°C, and diluted 1:1 with 0.01M PBS. Dynamic light scattering at 40°C with a Zetasizer Nano ZS (Malvern Pananalytics, United Kingdom) (refractive index of 1.33) was used to determine the particle size distribution of the CMRF samples.

Statistics

One participant was excluded from analyses due to non-compliance. CMRF samples of one participant were lost during isolation. Averages of fat and glucose oxidation were calculated per 30 minutes. Area under the curve (AUC) was calculated for all outcome parameters and

compared with either a paired t-test or a Wilcoxon rank test, if data was not normally distributed. GraphPad Prism (version 5.04, GraphPad Software Inc, USA) was used to perform statistical tests. Differences were considered to be significant when p-values were below 0.05.

Results

Serum lipids

After consumption of the test drinks an increase in serum concentrations of total TAG and FA is observed, as shown in **Figure 1**. Esterified cholesterol slightly increased after consumption of the drinks, with a maximum at 60 minutes postprandially, after which levels gradually returned to baseline. Free cholesterol appeared to decrease from 60 minutes postprandial onwards. No differences in AUC between the VEG- and BOV-test drinks were found.

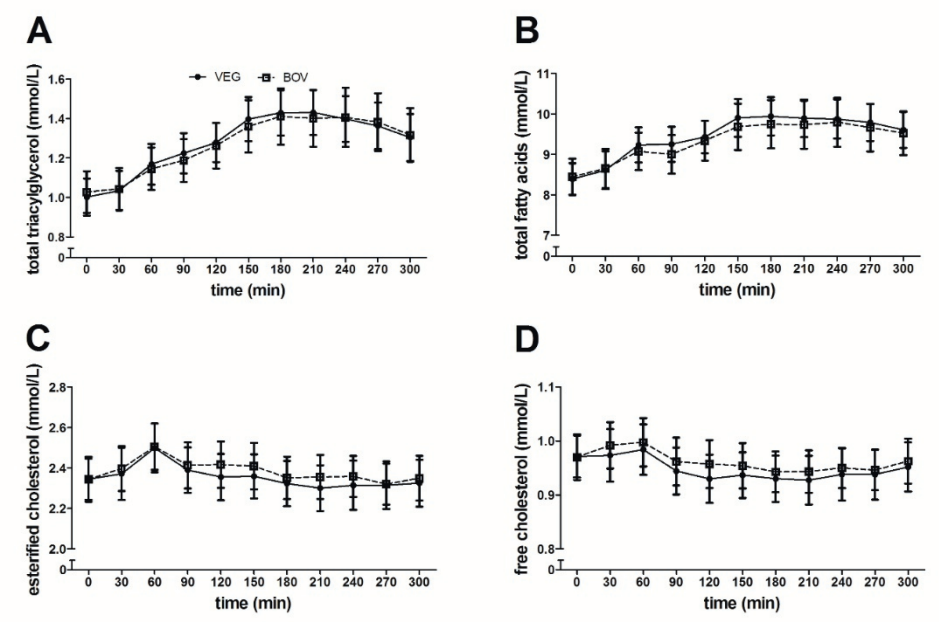

Figure 1: Serum concentrations of total triacylglycerol (TAG) (A), total fatty acids (B), esterified cholesterol (C), and free cholesterol (D). The black circles (●) represent the VEG-test drink, the open squares (□) represent the BOV-test drink. AUC was compared with either a paired t-test or Wilcoxon signed rank test if data was not normally distributed. No differences were observed in AUC of total TAG (p=0.86), total FA (p=0.41), esterified cholesterol (p=0.51), and free cholesterol (p=0.48).

Chylomicrons

After consumption of the VEG- and BOV-test drinks an increase in chylomicron concentration and size was found, as well as an increase in TAG and FA concentrations in the CMRF, and an increase in total cholesterol and phospholipids in chylomicrons (**Figure 2**). No significant differences were seen between the test drinks, except that the AUC of the chylomicron particle size was slightly more increased after consumption of the BOV-test drink (p=0.03).

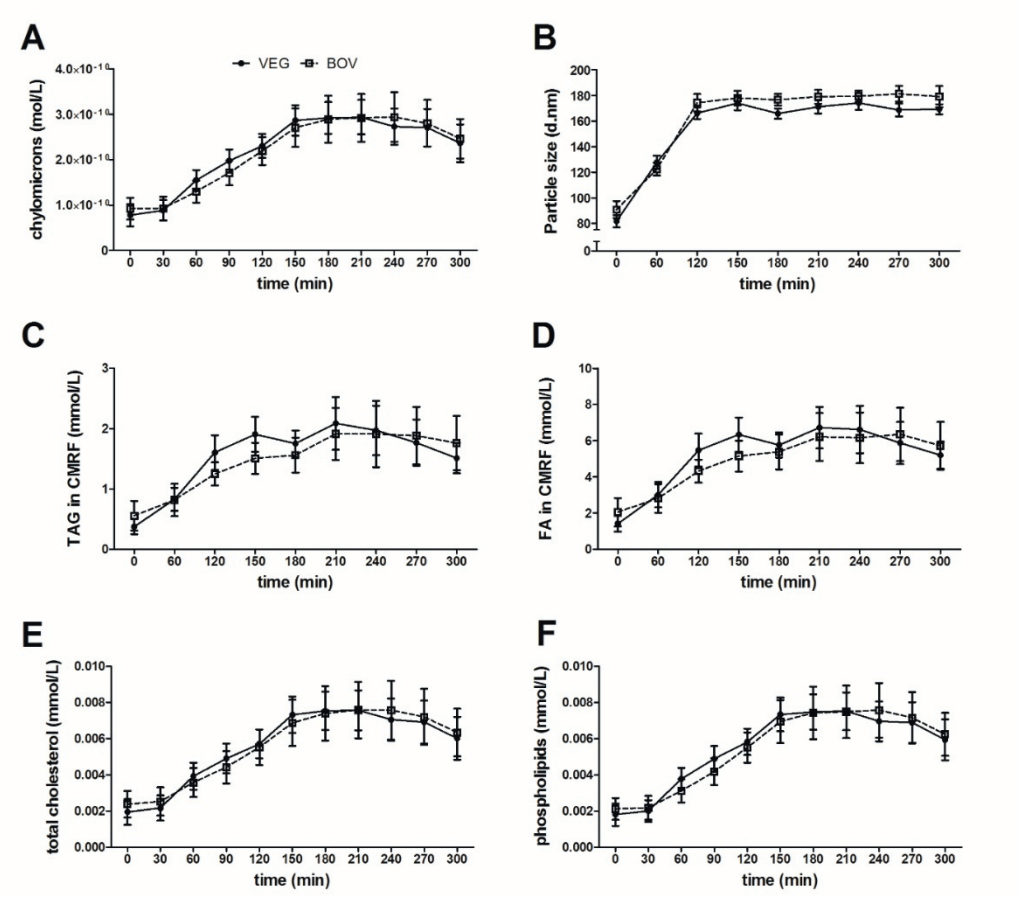

Figure 2: Chylomicron concentration (A), size (B), and content (C-F) after consumption of a milk drink containing either 100% vegetable fat (VEG-test drink) or a mixture of 67% bovine milk fat with 33% vegetable fat (BOV-test drink) (mean ± SEM, n=19 for A, E, and F, n=18 for B,C and D). No significant differences were found after a Wilcoxon signed rank test of the AUC for concentration, TAG and FA in CMRF (respectively p=0.82, p=0.54, p=0.38), nor for total cholesterol and phospholipid concentration in the chylomicrons (respectively p=0.89 and p=1.00). The AUC of particle size was significantly higher for the BOV-test drink (p=0.03). The black circles (●) represent the VEG-test drink, the open squares (□) represent the BOV-test drink.

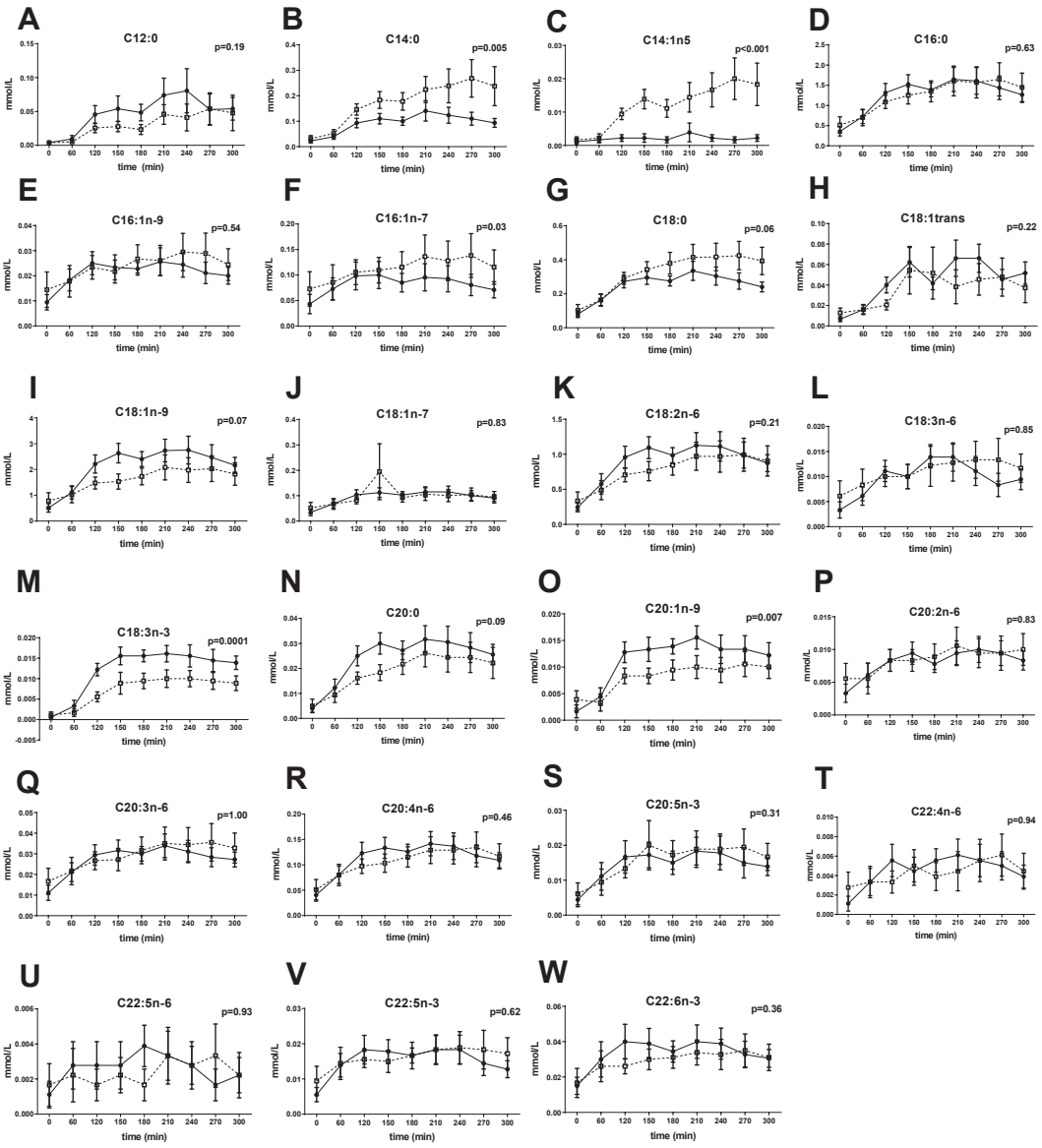

Figure 3: Fatty acid profile in chylomicron-rich fraction (CMRF) of plasma after consumption of a milk drink containing either 100% vegetable fat (VEG-test drink) or a mixture of 67% bovine milk fat with 33% vegetable fat (BOV-test drink) (mean ± SEM, n=18). A) C12:0, B) C14:0, C) C14:1n-5, D) C16:0, E) C16:1n-9, F) C16:1n-7, G) C18:0, H) C18:1trans, I) C18:1n-9, J) C18:1n-7, K) C18:2n-6, L) C18:3n-6, M) C18:3n-3, N) C20:0, O) C20:1n-9, P) C20:2n-6, Q) C20:3n-6, R) C20:4n-6, S) C20:5n-3, T) C22:4n-6), U) C22:5n-6, V) C22:5n-3, W) C22:6n-3. The black circles (●) represent the VEG-test drink, the open squares (□) represent the BOV-test drink. AUC was compared with either a paired t-test or Wilcoxon signed rank test if data was not normally distributed, $p<0.05$ was considered significant.

Fatty acid profile of chylomicrons

Figure 3 displays the FA profile of the CMRF from plasma over time. After consumption of the VEG-test drink the AUC of the postprandial concentrations of C18:3n-3 and C20:1n-9 were significantly higher compared to the BOV-test drink (respectively p=0.0001 and p=0.007). While following consumption of the BOV-test drink significantly higher AUC was observed for C14:0, C14:1, and C16:1n-7 (respectively p=0.005, p<0.001, p=0.03).

The FA profile of the CMRF from plasma at 180 minutes after consumption of the test drinks is shown in **Figure 4**, together with the FA profile of the two test drinks of which normalized percentages are provided.

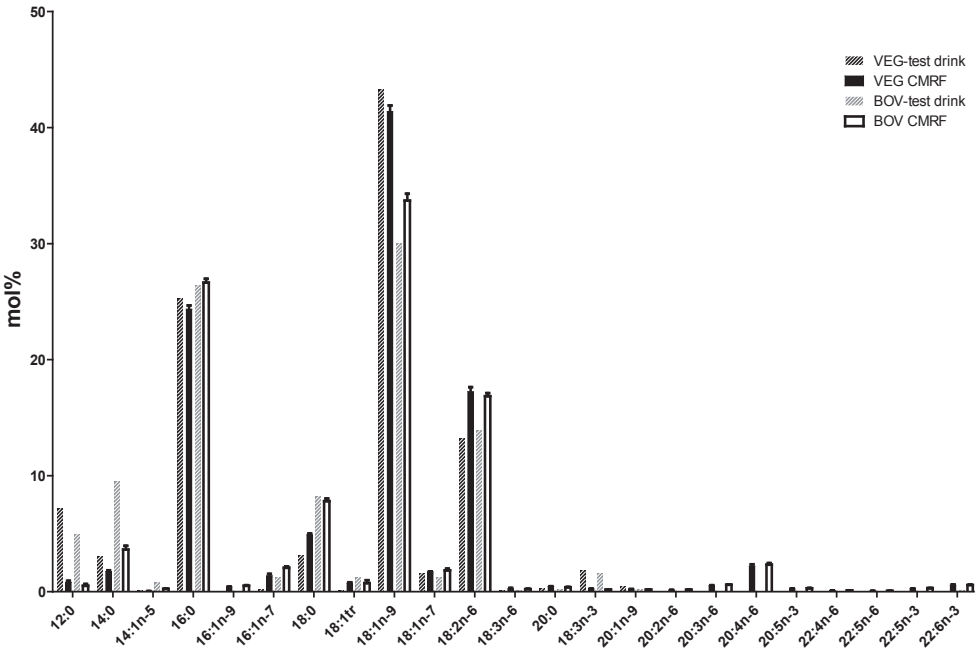

Figure 4: Fatty acid profile in chylomicron-rich fraction (CMRF) of plasma 180 minutes after consumption of a milk drink containing either 100% vegetable fat (VEG-test drink) or a mixture of 67% bovine milk fat with 33% vegetable fat (BOV-test drink) (mean ± SEM, n=18), and the normalized initial fatty acid profile of the VEG- and BOV-test drinks (sum of C12:0-C22:6n-3: 100%). The black bars (▪) represent the percentages in CMRF after consumption of the VEG-test drink, the open bars (□) represent the percentages in CMRF after consumption of BOV-test drink, the striped bars represent the percentages in the test drinks.

Lipoproteins

Figure 5 shows the serum concentrations of the three main classes of lipoproteins, i.e. VLDL, LDL, and HDL. The AUC after consumption of the VEG- and BOV- test drinks were compared, and no significant differences were found (p=0.59, p=0.49, and p=0.08 respectively). The AUC of the particle diameters of VLDL, LDL, and HDL in serum were not significantly different (p=0.65, p=0.05, and p=0.25 respectively), although there was a tendency for an increased AUC of the particle diameter of LDL after consumption of the BOV-test drink.

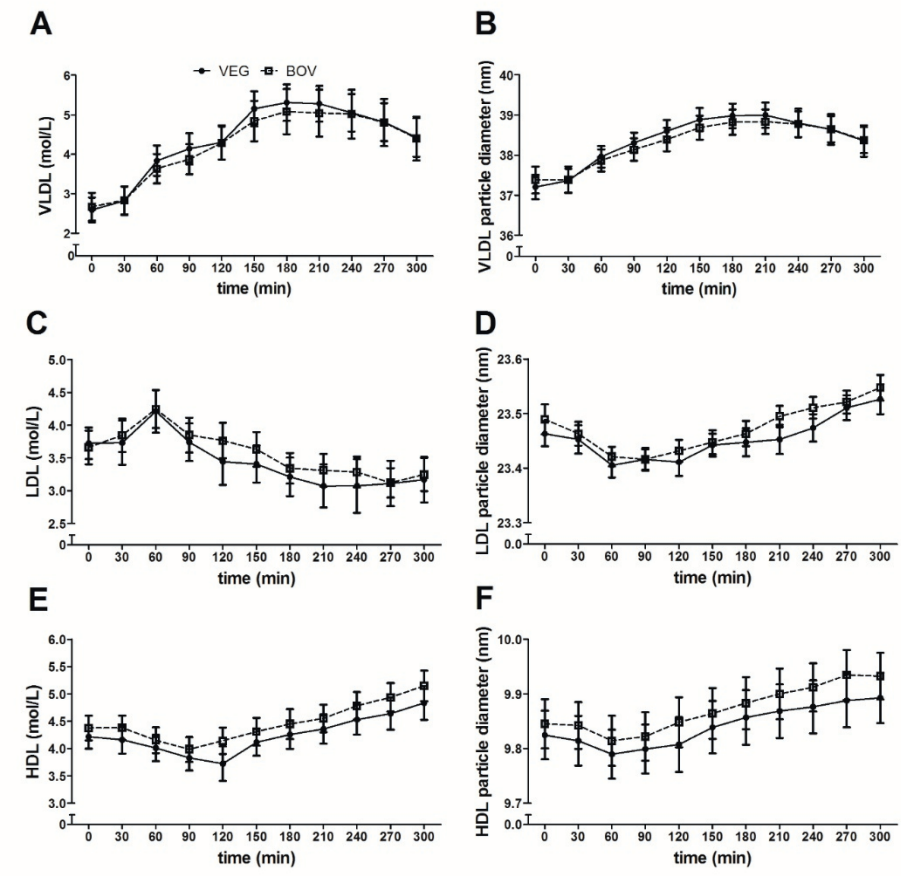

Figure 5: Concentrations and particle diameters of lipoprotein particles. A) Concentration and B) particle diameter of very large density lipoprotein (VLDL) particles, C) concentration, and D) particle diameter of large density lipoprotein (LDL) particles, E) concentration, and F) particle diameter of high density lipoprotein (HDL) particles (mean ± SEM, n=19). The black circles (●) represent the VEG-test drink, the open squares (□) represent the BOV-test drink. AUC was compared with either a paired t-test or Wilcoxon signed rank test if data was not normally distributed. No significant differences were found for AUC of concentration of VLDL (p=0.59), LDL (p=0.49), and HDL particles (p=0.08). The particle diameter of the VLDL (p=0.65), LDL (p=0.05), and HDL (p=0.25) particles were not significant different between the two treatments.

Apolipoproteins

Serum concentrations of apolipoproteins B and A-I were slightly increased 60 minutes after consumption of the test drinks and remained more or less stable afterwards, as is displayed in **Figure 6**. No significant differences were found between the VEG- and BOV-test drinks.

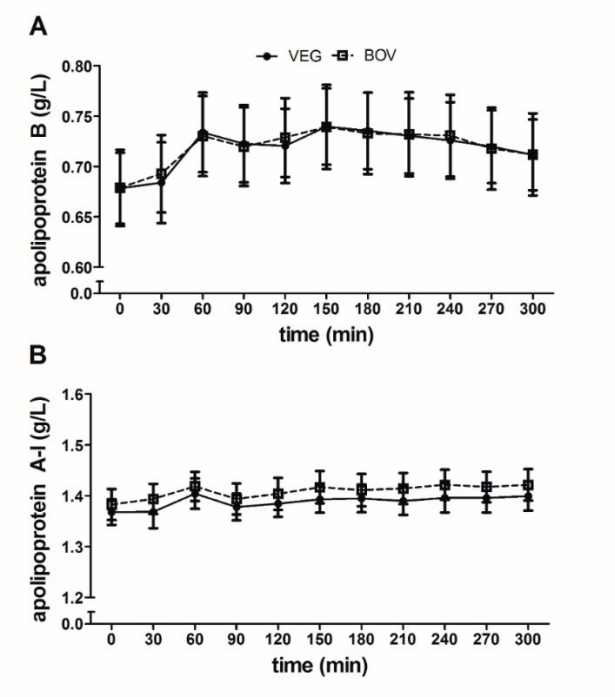

Figure 6: Concentrations of apolipoprotein B (A) and A-I (B) after consumption of two different IFs. The black circles (●) represent the VEG-test drink, the open squares (□) represent the BOV-test drink. A paired t-test did not show significant differences for AUC of concentration of apolipoprotein-B (p=0.98), and apolipoprotein A-I (p=0.17).

Substrate oxidation

Non-protein fat oxidation and glucose oxidation were calculated from VO_2 and VCO_2. No differences in these substrate oxidations were found between the two test drinks (**Figure 7**). During the first 30 minutes an increase in fat oxidation and concomitant decrease in glucose oxidation could be observed. Thereafter, fat oxidation decreased and stabilized from about 150 minutes postprandially onwards. Glucose oxidation increased from 30 minutes to 90 minutes, and then it gradually returned towards baseline.

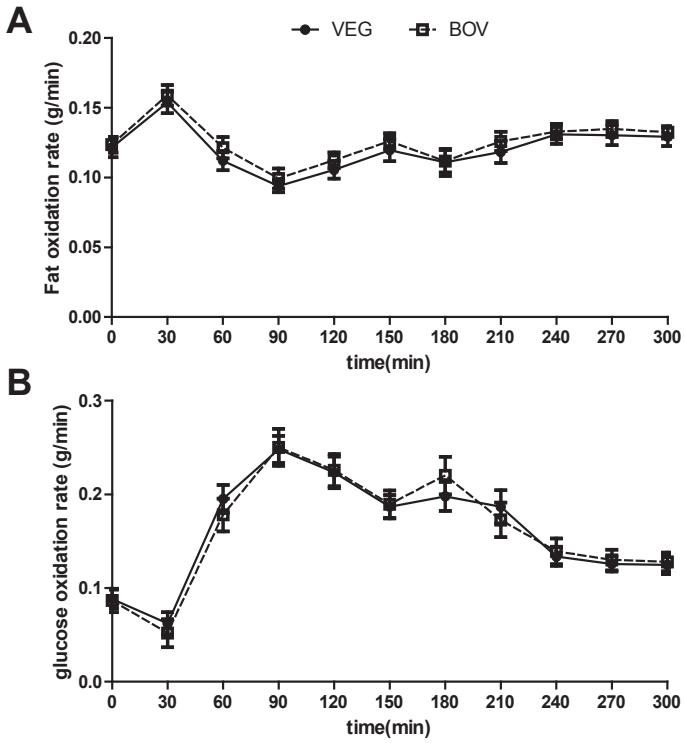

Figure 7: Substrate oxidation after consumption of a milk drink containing either 100% vegetable fat (VEG-test drink) or a mixture of 67% bovine milk fat with 33% vegetable fat (BOV-test drink) (mean ± SEM, n=19). A) Fat oxidation rate, and B) Glucose oxidation rate were not different after a paired t-test of the AUC (respectively p=0.82 and p=0.98). The black circles (●) represent the VEG-test drink, the open squares (□) represent the BOV-test drink.

Ketone bodies

After consumption of the BOV-test drink a transient increase in the serum concentration of acetoacetate and 3-hydroxybutyrate was found, while after consumption of the VEG-test drink the concentrations of the compounds continuously decreased (**Figure 8**). The AUC of acetoacetate and 3-hydroxybutyrate were significantly higher for the BOV-test drink compared to the VEG-test drink (p=0.006 and p=0.01 respectively). The acetone response, measured in breath, did not differ between the BOV- and VEG-test drink.

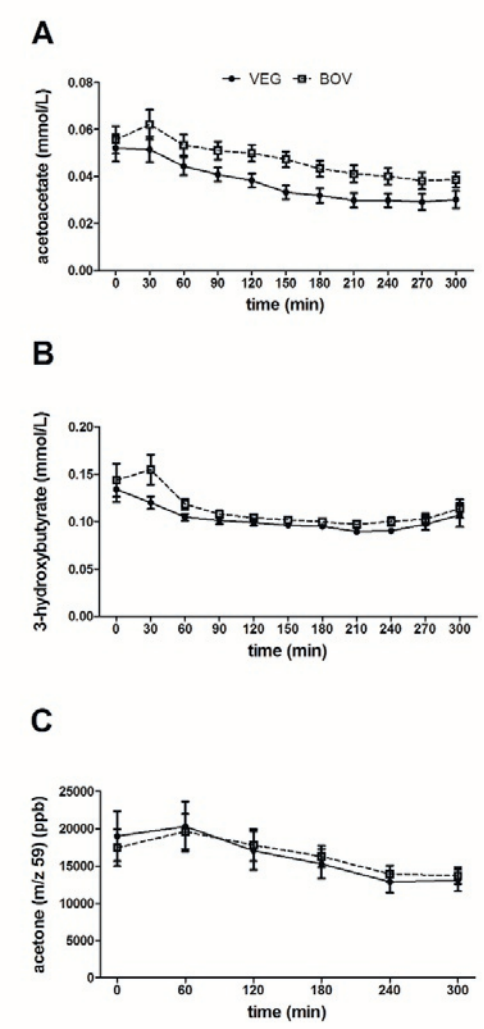

Figure 8: Concentrations of ketone bodies in serum (acetoacetate (A) and 3-hydroxybutyrate (B)) and exhaled air (acetone (C)) after consumption of a milk drink containing either 100% vegetable fat (VEG-test drink) or a mixture of 67% bovine milk fat with 33% vegetable fat (BOV-test drink) (mean ± SEM, n=19). For acetoacetate and 3-hydroxybutyrate the AUC was significantly different (respectively p=0.006 and p=0.01),compared with a paired t-test. The AUC of acetone was not different between test drinks (p=0.88). The black circles (●) represent the VEG-test drink, the open squares (□) represent the BOV-test drink.

Discussion

Using 67% bovine milk fat in a fat blend for IF, with a different TAG structure and FA profile than vegetable fat, did not affect the postprandial lipemic response to the consumption of IF, compared to 100% vegetable fat. Although the size of chylomicrons was slightly increased after consumption of IF containing bovine milk fat (BOV), compared to an IF containing vegetable fats only (VEG), the addition of bovine milk fat did not affect chylomicron concentration nor its total lipid content. The postprandial FA profile in CMRF reflected the difference in FA profile of the respective IFs. The concentration of ketone bodies was increased directly after consumption of the BOV-test drink, which was not found after consumption of the VEG-test drink. No differences between the test drinks were observed for total TAGs, lipoprotein concentrations, or substrate oxidation.

The BOV-test drink contained 51% SFA and the VEG-test drink 40%. The consumption of SFA is thought to result in a higher postprandial lipemic response compared to intake of MUFA.[4] However, despite the higher concentration of SFA in the BOV-drink, no acute effects on postprandial lipemia were observed. This is in line with the findings of Scarillo *et al.*[16], who showed, in young adults, similar postprandial TAG responses to four single high-fat meals differing in SFA and MUFA content. In bovine milk fat, part of the SFA are SCFA and MCFA. Those FAs can be easily absorbed and be transported directly to the liver, without incorporation into chylomicrons.[17] Therefore, not all FAs of the test drink with bovine milk fat would end up in chylomicrons. A study on the effects of chain length of SFA on postprandial lipemia showed that MCFA induced a lower postprandial TAG concentration than LCSFA.[18] Hence, the relatively higher levels of SCFA and MCA may have contributed to the absence of an increase of TAG in CMRF after consumption of the BOV-test drink compared to the VEG-test drink, despite the higher total SFA content.

After consumption of the test drinks an increase in chylomicron particle size was observed. This was similar to the results of Vors *et al.*[19], who used a similar analysis of CMRF after consumption of 40 g anhydrous bovine milk fat. Our results showed a slightly increased particle size after consumption of the BOV-test drink compared to the VEG-test drink. Previous studies showed that the size of chylomicrons mainly depends on the amount of fat ingested, and thus the amount of TAGs available.[20–22] Surprisingly, we did not find a difference in TAG concentrations in the CMRF. Phospholipids are part of the membrane of chylomicrons, and are therefore an important determinant for its size.[23] However, just like FA and cholesterol concentrations, phospholipid concentrations in chylomicrons were similar for the two test drinks. So, this cannot explain the small difference in particle size. FA lengths also influences chylomicron size, longer FAs result in bigger chylomicrons.[24] However, the chylomicrons after the BOV test drink did not contain higher concentrations of longer FAs compared to VEG. Other components of chylomicrons that could determine the size are minor fat-soluble components, such as vitamins, or apolipoproteins. In this study apolipoproteins B and A-I were determined, concentrations were not different after consumption of the two test drinks. Besides apolipoproteins B and A-I,

chylomicrons contain apolipoproteins A-II, A-IV, A-V, C-II, C-III, and E.[20] These were not measured in this study, but may play a role in the particle size difference.

The FA profile of the test drinks and that of the CMRF, 180 minutes after consumption of the test drinks, were highly similar, which is in line with the general belief that dietary fat is directly used for the production of chylomicrons. Nevertheless, some differences were also evident. The CMRF samples, taken after consumption of the test drinks, seemed to be relatively reduced in C12:0 and C14:0 content compared to the test drinks. FAs <C14 are reported to be more water soluble than larger FA,[25] and are partly taken up directly into the portal vein.[26] This study gives an indication that C14:0, just like C12:0, may partly also be directly absorbed without inclusion in chylomicrons. The relative reduction of C12:0 and C14:0 percentages in CMRF potentially explain the relative enrichment of longer chain FA in CMRF, compared to the FA profile of the test drinks, although it seems that especially C18:1n-9 for the BOV-test drink, and C18:2n-6, C20:3n-6, C20:4n-6, C22:5n-3, and C22:6n-3 for both drinks were particularly enriched in the CMRF. Possibly, this is due to metabolism and desaturation of the FAs in the enterocyte.[27] After a period of fasting the activity of Δ-desaturases is increased in rats.[28] Synthesis of C18:1n-9 from C18:0 by Δ9-desaturase might explain the enrichment of this FA and also the absence of an enrichment of its precursor (C18:0) in the CMRF after consumption of the BOV-test drink. Still it remains unclear why this is only seen for the BOV-test drink. One possibility may be that this occurred in order to maintain MUFA levels, which were present in lower levels in the BOV-test drink compared to the VEG-test drink, as Δ9-desaturase, in adipose tissue, is suggested to be involved in the regulation of MUFA content.[29] Alternatively, a diet-selective desaturase gene activation may occur in the enterocyte. Lipid headgroups have been shown to selectively activate desaturases,[30] and it is tempting to speculate that differential desaturation of free FAs and monoacylglycerols could have occurred. If this would be the case, higher levels of C18:0 at the sn-2 position in the BOV-test drink might have resulted in higher levels of desaturation. Activation of Δ5-desaturase in the enterocytes may clarify the enrichment of C20:3n-6 and C20:4n-6 in CMRF.[31] Furthermore, combined with activation of Δ6-desaturase this could explain the enrichment of C22:5n-3 and C22:6n-3, which might have been synthesized from C18:3n-3, which seems to be abundant in higher percentages in the normalized FA profile of the test drink compared to the CMRF FA profiles. C18:2n-6, linoleic acid, cannot be synthesized from other FAs in humans. It is particularly abundant in the microsomal membranes of enterocytes and may thus have been added to the chylomicrons from the enterocyte.[32]

Another interesting finding of this study, was that directly (i.e., within 30 minutes) after consumption of the two drinks fat oxidation increased with a concomitant slight decrease in glucose oxidation. After 30 minutes postprandially, FA oxidation decreased again and carbohydrates became more predominant. The swift increase in fat oxidation is in line with a dietary intervention performed in rats, which also showed an increase in fat oxidation the first minutes after a high fat meal.[33] This rapid initial increase in fat oxidation might be caused by the oral perception of fat. Sham feeding of fat has been found to increase metabolites, which can be used for oxidation and energy expenditure up to one hour after the sham feeding.[34–36] Another explanation might be the oxidation of FAs by intestinal enterocytes directly after

uptake.[37,38] So, directly after consumption of the test drinks, FAs were mainly used for oxidation and oxidation did not switch to carbohydrates yet. In line with this increase in fat oxidation, an increase in the concentration of the ketone bodies acetoacetate and 3-hydroxybutyrate was observed directly after consumption of the BOV-test drink, but not after the VEG-test drink. This increase was specific for the ketone bodies, since this was not observed for total FAs or total TAG in serum. The exact mechanisms underlying these increased concentrations of ketone bodies remains unclear. It may be related to the higher levels of SCFA and MCFA in the BOV-test drink, as these FAs become immediately available for beta-oxidation due to the fast absorption and mitochondrial uptake independent of the rate limiting carnitine palmitoyltransferase shuttle system.[17] Still, fat oxidation rates were similar after consumption of the two test drinks, contradicting this reasoning. A similar increase in ketone bodies has been found after consumption of medium-chain TAGs (MCT).[39–43] Ketogenesis was especially related to intake of octanoate (caprylic acid),[40] which was present twice as much in the BOV-test drink compared to VEG-test drink. The increase in ketone body concentration might be beneficial for infants. Lucas *et al.* [44] showed that breast-fed infants have higher concentrations of ketone bodies compared to formula-fed infants. Ketone bodies are mostly used as energy source for the heart, brain, and skeletal muscle. [45] In infants, the capacity to oxidize ketone bodies in the brain is higher than in adults.[42] This matches the development of lipogenesis and myelination of the central nervous system.[42] However, whether using bovine milk fat in IF has similar effects on brain development, via formation of ketone bodies, needs further exploration.

In conclusion, in this study we examined the acute effects of two different fat blends in IF on postprandial lipemia in healthy adults. We showed that replacing 67% of vegetable fat with bovine milk fat in IF did not affect the postprandial lipemic response. No differences in absorption, lipoprotein metabolism or substrate oxidation were found. Addition of bovine milk fat to IF affected the FA profile in chylomicrons, and thus the FAs that were presented to peripheral tissues, and also increased serum concentrations of ketone bodies. The latter effect may have been caused by the higher percentages of SCFA and MCFA in bovine milk fat compared to vegetable fat. These differences may have effects on metabolic health, but this needs further investigation. This study provides some insight for effects that can be expected in infants, however, the true acute and longer-term effect in infants cannot be extrapolated from these findings and needs further studies.

Conflict of interest

Jeske Hageman is an employee of FrieslandCampina

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Supplemental information

Table 1. Baseline subject characteristics (mean ± SD, n=19).

	Mean ± SD (n=19)
Age (years)	21.4 ± 2.0
Body weight (kg)	75.9 ± 8.3
BMI (kg/m^2)	22.5 ± 1.6
Fat mass (%)	13.6 ± 2.8

Table 2. Nutrient composition of the two test drinks (per 100 gram).

Per 100 gram	Test drink with 100% vegetable fat	Test drink with 67% bovine milk fat and 33% vegetable fat
Energy (kcal)	527	526
Carbohydrates (g)	56.1	56.1
Fat (g)	29.0	29.0
Of which vegetable fat (g)	29.0	9.5
Of which bovine milk fat (g)	-	19.5
Protein (Nx6.25) (g)	10.5	10.4

Chapter 5

Bovine milk fat in infant formula prolongs satiety: a randomized double-blind cross-over proof-of-principle study in healthy young male adults

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Submitted

Abstract

Background: In infant formula (IF) different fat sources are used; these include several vegetable fats, but currently also bovine milk fat is added. The latter contains short- and medium-chain fatty acids (SCFA and MCFA), which may have an effect on diet-induced thermogenesis (DIT) and satiety.

Objective: To study whether an IF with a fat blend consisting for 67% of bovine milk fat affects DIT and satiety, compared to an IF containing vegetable fats only.

Design: A randomized double-blind crossover proof-of-principle study, using adults, was conducted. Twenty healthy Caucasian males, aged 18-28 years with a BMI between 20-25 kg/m^2 were included. On two separate days, after an overnight fast, basal energy expenditure was determined via indirect calorimetry. After consumption of one of the two test drinks, energy expenditure was analyzed continuously for five hours, and DIT and respiratory exchange ratio (RER) were calculated. Before and every 30 minutes after consumption, venous blood samples were taken, for determination of circulating glucose, secretin, acylated ghrelin, peptide YY (PYY) and cholecystokinin (CCK). Furthermore satiety was scored every 30 minutes.

Results: No differences in DIT or RER were found between the two test drinks. Satiety was prolonged after consumption of the test drink with bovine milk fat. No differences were found in postprandial responses of glucose, PYY and CCK. Postprandial responses of secretin and acylated ghrelin were increased after consumption of the test drink with bovine milk fat, compared to the test drink with vegetable fats only, and correlated with satiety.

Conclusions: An IF containing a fat blend consisting for 67% of bovine milk fat prolongs satiety in healthy male adults. This difference with 100% vegetable fat is not caused by a difference in DIT, but may be related to an increased postprandial secretin response.

Key words: infant formula, bovine milk fat, vegetable fat, diet-induced thermogenesis, satiety, gastrointestinal peptides, proof-of-principle, human, adults

Introduction

Over the last 40 years a rise in childhood obesity has occurred.[1] Early nutrition seems to play an important role in the development of childhood obesity and may even impact on adult obesity.[2,3] Infants have a high energy supply, which is needed for optimal growth and development. On the other hand, rapid weight gain in infancy might result in overweight and obesity later in childhood.[4,5] Breastmilk is the best source of nutrition for infants,[6] and although evidence is inconsistent, breastfeeding might protect against childhood overweight, compared to formula feeding.[7] One of the suggested explanations is a difference in composition between infant formulas (IFs) and breast milk.[7] In this regard, most attention has been paid to protein; IFs are generally higher in protein content than breast milk, and this has been linked to differences in infant growth.[8]

Considerable less attention has been paid to a possible role of fat in IF, probably because fat is the least satiating macronutrient relative to its energy content.[9] However, fat is an important source of energy for infants, about 50% of the energy that an infant consumes comes from fat.[10] In IF different fat sources are used to generate a blend that resembles the fatty acid composition of human milk as close as possible. These fat sources are mostly of vegetable origin, but animal fat sources are also used, including bovine milk fat.[11] Bovine milk fat differs from most vegetable fats because it contains higher levels of saturated fatty acids, including short- and medium-chain fatty acids (SCFA and MCFA).[12] So the addition of bovine milk fat to IF results in a different lipid composition. This may have consequences for its satiating properties, as lipids can differ in their satiating effects. For instance, medium-chain triglycerides (MCT) were shown to be more satiating than long-chain triglycerides (LCT), and ingestion of MCT resulted in a lower subsequent food intake than LCT in healthy adults.[13] Various mechanisms have been suggested to underlie the (differential) effects of dietary lipids on satiety. One is the increase in hepatic fat oxidation and meal induced energy expenditure (diet-induced thermogenesis; DIT).[14] Consumption of MCT has been shown to increase energy expenditure and fat oxidation in adults, when compared to LCT.[15–17] Besides their effect on postprandial energy metabolism, lipids may also induce satiety through their stimulating effects on gastrointestinal peptide secretion. Several gastrointestinal peptides involved in appetite regulation were shown to be affected by intake of fat, including secretin, ghrelin, peptide YY (PYY) and cholecystokinin (CCK).[18–20]

A maximum of 67% bovine milk fat can be used in a fat blend for IF, supplemented with a blend of vegetable fats, to reach preferred linoleic acid levels.[12] In such a blend, the level of SCFA and MCFA would be 7%, compared to only 1% in a commonly used fat blend consisting of only vegetable fats. It is not known whether such a relatively small difference in lipid composition is sufficient to affect energy expenditure and satiety. Therefore, the aim of this study was to determine whether the presence of 67% bovine milk fat in an IF affects DIT and satiety, compared to an IF containing only vegetable fats. Since the intake of formula by infants is heavily influenced by caretakers,[21] and frequent sampling of blood in infants is highly restricted

because of ethical reasons, we chose to study possible effects in a proof-of-principle study in young adults.

Subjects

Twenty healthy Caucasian males, aged 18-28 years with a BMI between 20-25 kg/m^2 were enrolled in this study, from March-June 2018, by the project leader. The main baseline characteristics are displayed in **Table 1**. Males with claustrophobia, (symptoms of) lactose intolerance or cow's milk allergy, known metabolic diseases, autoimmune diseases, gastro-intestinal diseases, cardiovascular diseases were excluded. Smokers or recent smokers, vegetarians, males that consumed more than 21 glasses of alcohol per week, or performed more than 5 hours of strenuous exercise (>6.0 METS) per week, or who donated blood during the two months before the study were excluded. The level of restraint eating was determined with the Dutch eating behavior questionnaire,[22] subjects with a score ≥ 30 were excluded.

Table 1. Baseline subject characteristics (mean ± SD, n=19).

	Mean ± SD (n=19)
Age (years)	21.4 ± 2.0
Body weight (kg)	75.9 ± 8.3
BMI (kg/m^2)	22.5 ± 1.6
Fat mass (%)	13.6 ± 2.8

Methods

Ethics

This study, with acronym MELC, has been conducted at the Human Research Unit of Wageningen University, was approved by the Medical Ethics Committee of Wageningen University (METC-WU), and was conducted according to the declaration of Helsinki. The trial was registered at the Dutch Trial Register (NTR7083). Volunteers received written and oral information. Informed written consent was obtained from all subjects before enrolment.

Study design

Since energy metabolism highly varies between subjects, we chose a double-blind randomized crossover design, involving two days of testing separated by at least one week, for this study. On the day preceding a study day, participants were restricted from exercise, alcohol consumption and use of drugs. A standardized dinner containing 584 kcal, 15.0 g fat, 63.9 g carbohydrates, and 43.3 g protein, was provided to the subjects. After overnight fasting for 12 hours, subjects were transported by car to the research unit. After placement of an indwelling venous catheter in the forearm, participants were asked to lay down on a bed for at least 30 minutes to ensure a resting state. Thereafter, indirect calorimetry was performed using a

ventilated hood system, for determinations of basal energy metabolism. Afterwards, blood samples were taken and a questionnaire with visual analogue scale (VAS) was conducted to determine feelings of hunger, fullness, and prospective food consumption. After these baseline measurements, participants consumed one of the two test drinks. Subsequently, participants were placed under the ventilated hood again and measurements of energy metabolism continued for 5 hours. Every 30 minutes venous blood samples were taken through the catheter and satiety feelings were determined using the VAS questionnaire. At 150 minutes postprandially participants were allowed to drink a glass of water and have a toilet break. During the five hours, participants were allowed to watch television, and were instructed to lie down as still as possible.

Test drinks

IF basepowders, provided by FrieslandCampina, were used to produce the test drinks. The products were isoenergetic and equal in nutrient composition, see **Table 2**. The test drinks only differed in fatty acid profile (**Table 3 and Supplemental Table 1**). This was determined by conversion of fatty acids into methyl esters followed by capillary gas chromatography (NEN-ISO 15884 and 15885). One test drink contained a mixture of vegetable fats only (VEG), the other contained 67% anhydrous bovine milk fat and 33% vegetable fats (BOV). The researchers and participants were blinded as the study products were coded by employees of FrieslandCampina not involved in execution of the study. The powdered product was diluted in lukewarm water, with a ratio of 1:2.1. To correct for the individual difference in energy expenditure of the participants, the total amount of study product to be consumed by participants was based on their estimated total energy expenditure. Basal metabolic rate was calculated using the Harris-Benedict equation, based on age, height, and weight. From this, total energy expenditure was estimated assuming a physical activity level of 1.75. The participants received a test drink of which the energy content represented 30% of their calculated total energy expenditure. Randomization of the treatment was performed with a random sequence generator.

Table 2. Nutrient composition of the two test drinks (per 100 gram).

Per 100 gram	VEG	BOV
Energy (kcal)	527	526
Carbohydrates (g)	56.1	56.1
Fat (g)	29.0	29.0
Of which vegetable fat (g)	29.0	9.5
Of which bovine milk fat (g)	-	19.5
Protein (Nx6.25) (g)	10.5	10.4

Table 3. Fatty acid composition of the two test drinks (in % of total FAMEs).

	VEG	BOV
SCFA	<0.1	2.6
MCFA	1.3	5.2
LCSFA	38.2	43.3
MUFA	44.8	30.1
PUFA	14.8	13.5

FAME: Fatty-acid methyl esters, SCFA: short-chain fatty acids, MCFA: medium-chain fatty acids, LCSFA: long-chain saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids

Energy metabolism

The indirect calorimetry system MAX-II Metabolic System (AEI technologies Inc, USA), with a ventilated hood, was used to determine O_2 consumption and CO_2 production. Every 30 seconds a measurements was performed. Energy expenditure was calculated using the equations of Weir.[23] Measurements were performed at 22.8 ± 0.7 °C. Basal energy expenditure was determined in fasted state, after 30 minutes of rest, for 10 minutes. The average of the last 5 minutes of this measurement was used to calculate basal energy expenditure. By subtracting basal energy expenditure from the energy expenditure that was measured after consumption of the drinks diet-induced thermogenesis (DIT) could be calculated. Averages of DIT were calculated per 30 minutes. Basal energy expenditure and DIT are expressed in kcal/min. To determine the respiratory exchange ratio (RER) volume of O_2 consumption (VO_2) and volume of CO_2 production (VCO_2) were calculated, and divided. Averages of RER were calculated per 30 minutes.

Appetite profile

Subjective assessment of hunger, fullness, and prospective consumption was measured on a 10 cm horizontal visual analogue scale (VAS), anchored with ''not at all'' and ''extremely'', right before consumption of the test drinks and every 30 minutes postprandially until five hours after the drink. The specific questions asked were ''How hungry do you feel?'', ''How full do you feel?'', ''How much could you eat?''. Subjects were instructed to draw a vertical line on the place of the line that best matched how they were feeling.

Blood samples

Blood was collected into sterile evacuated tubes, with either coagulation activators (serum), or EDTA and protease inhibitors (plasma). The blood samples were inverted ten times after which tubes for serum were left at room temperature for 60 minutes to clot before centrifugation (1200g, 10 min, RT) and the other tubes were immediately centrifuged (1200g, 10 min, 4°C). Serum and plasma were obtained and aliquots were stored at -80°C until analysis.

Blood analyses

Concentrations of glucose were determined in serum samples of all time points via NMR analysis (Nightingale Health, Finland). Concentrations of secretin were determined in serum samples from time points 0, 30, 60, 90, 120, and 180 minutes postprandially by ELISA (Cloud-Clone Corp, CEB075Hu). Concentrations of acylated ghrelin were determined in plasma samples from time points 0, 30, 90, and 180 minutes postprandially by ELISA (Merck, EZGRA-88K). Concentrations of PYY and CCK, were analyzed in plasma samples from time points 0, 30, 60, 90, and 180 minutes postprandially by ELISA (Merck - EZHPYYT66K and Sigma - RAB0039-1KT, respectively).

Statistics

This study was calculated to have a statistical power of 90% at p=0.05, to detect a difference in DIT between the two study products of 15 kcal over five hours, with an estimated standard deviation of 20 kcal, when 17 subjects would be included. To negate potential dropouts a total of 20 subjects were included.

Net area under the curve (netAUC) was calculated for DIT and RER, and compared with respectively a Wilcoxon matched-pairs signed rank test and a paired t-test. To be able to compare the two products, the VAS scores were corrected for baseline values, and the scores from 30-300 minutes were analyzed with linear regression. Glucose and gastrointestinal peptides were corrected for baseline values, netAUC was calculated, and the two products were compared by a Wilcoxon matched-pairs signed rank test. Spearman correlation analyses were performed on all data points combined of DIT and VAS scores, and gastrointestinal peptides and VAS scores. GraphPad Prism (version 5.04 for Windows, GraphPad Software, San Diego California USA) was used for all statistical analysis. A p-value below 0.05 was considered to be significantly different. One subject was not included in the data analysis due to non-compliance. VAS scores of one participant were excluded due to missing baseline measurements, therefore the VAS scores could not be corrected for baseline values.

Results

Baseline values of energy expenditure, VAS scores, and concentrations of glucose and gastrointestinal peptides are presented in **Table 4**. At baseline, the energy expenditure, VAS scores and glucose concentrations did not differ between the two treatment days. However, there were differences in gastrointestinal peptide levels at baseline. Secretin and acylated ghrelin concentrations were higher on the day the participants received the VEG-test drink. CCK concentrations were lower on the day the participants received the VEG-test drink. However, these differences were not related to the order of treatment (see **Supplemental Figure 1**).

Table 4: Baseline values (mean ± SEM, n=19 for all except for VAS scores; n=18, compared with a paired t-test)

	VEG	BOV	p-value
Basal energy expenditure (kcal)	2146 ± 60	2169 ± 49	*p=0.54*
Respiratory Exchange Ratio (RER)	0.77 ± 0.01	0.77 ± 0.01	*p=0.73*
VAS score 'hunger' (cm)	6.47 ± 0.5	6.49 ± 0.4	*p=0.97*
VAS score 'fullness' (cm)	2.48 ± 0.3	2.32 ± 0.3	*p=0.59*
VAS score 'prospective consumption' (cm)	6.38 ± 0.4	6.66 ± 0.3	*p=0.35*
Glucose (mmol/L)	3.8 ± 0.1	3.9 ± 0.1	*p=0.14*
Secretin (pg/ml)	483.7 ± 45.7	342.1 ± 38.7	***p<0.001***
Acylated ghrelin (pg/ml)	463.5 ± 61.2	375.2 ± 51.7	***p=0.02***
PYY (pg/ml)	114.4 ± 9.7	115.9 ± 9.8	*p=0.87*
CCK (pg/ml)	112.1 ± 21.1	148.6 ± 25.5	***p=0.046***

Diet-induced thermogenesis and substrate oxidation

After consumption of the drinks energy expenditure increased, as depicted in **Figure 1A.** The netAUC was not different between the two test drinks (VEG vs BOV, respectively 84.7 vs 91.5 kcal, p=0.46). **Figure 1B** shows that 30 minutes after consumption of the test drinks RER started to increase, reaching a maximum after 90 minutes. From then on RER gradually decreased. Both drinks gave a highly similar response in time and the netAUC was not different between the two test drinks (p=0.61).

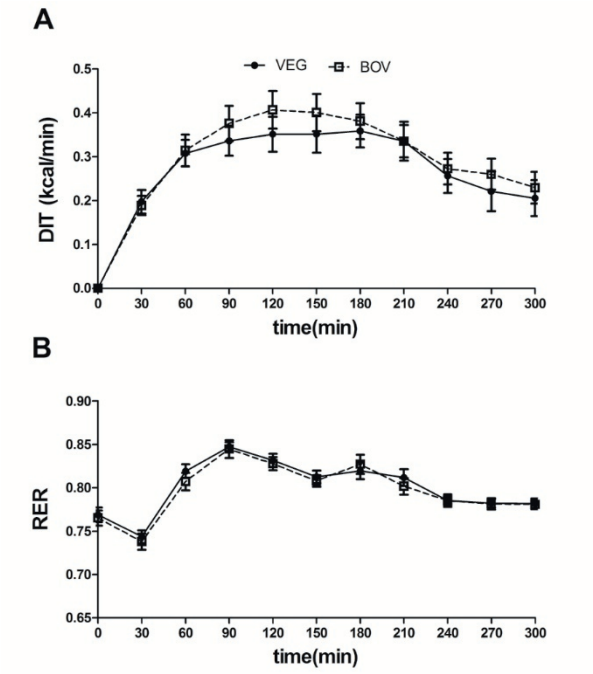

Figure 1: Diet-induced thermogenesis (DIT) (A) and Respiratory Exchange Ratio (RER) (B) after consumption of a milk drink containing either 100% vegetable fat (VEG) or a mixture of 67% bovine milk fat with 33% vegetable fat (BOV) (mean ± SEM, n=19). A Wilcoxon matched-pairs signed rank test of the netAUC of DIT did not result in significant differences (p=0.46), neither did a paired t-test of the netAUC of RER (p=0.61). The black circles (●) represent the VEG-test drink, the open squares (□) represent the BOV-test drink.

Appetite profile

Feelings of appetite were checked at baseline and every 30 minutes after consumption of the two different drinks. After consumption the initial increase in satiety was similar for both test drinks (**Figure 2**). And although the slopes for 30-300 minutes of the three different questions to assess satiety were not significantly different for the feeling of fullness and prospective consumption, the intercept was higher after consumption of BOV-test drink, i.e. it takes a longer time period to return to the baseline score after consumption of the BOV-test drink compared to the VEG-test drink (p=0.02 and p=0.001 respectively). After consumption of the VEG-test drink the feeling of prospective consumption had returned to baseline levels at 258 minutes, while after consumption of the BOV-test drink this could be extrapolated to take 321 minutes. For the feeling of fullness this was extrapolated to 333 minutes for the VEG-test drink and 361 minutes for the BOV-test drink. For the feeling of hunger no differences were observed for the intercept after consumption of the two different drinks (p=0.18).

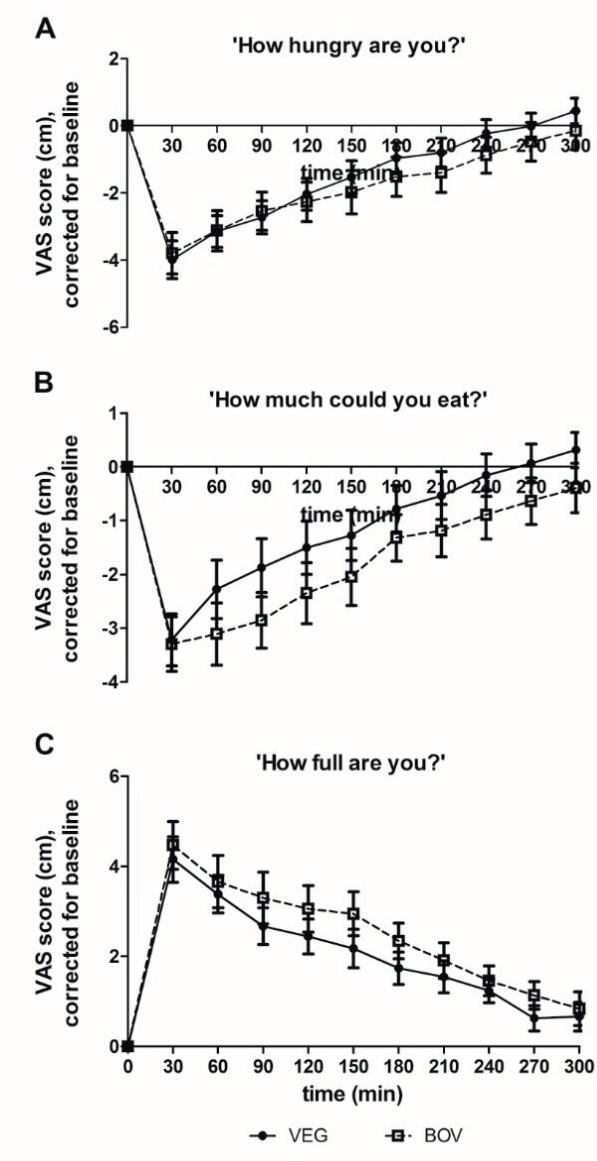

Figure 2: Appetite profile after consumption of two milk drinks containing different fat blends, indicated on a visual analogue scale (VAS) of 10 cm, corrected for baseline values. Feeling of hunger (A), feeling of prospective consumption (B), and feeling of fullness (C) after consumption of a milk drink containing either 100% vegetable fat (VEG-test drink) or a mixture of 67% bovine milk fat with 33% vegetable fat (BOV-test drink) (mean ± SEM, n=18). Linear regression of 30-300 minutes showed no significant differences in slopes for the three questions, however it did show a significant different intercept for prospective consumption (p=0.001) and fullness (p=0.02), not for hunger (p=0.18). The black circles (●) represent the VEG-test drink, the open squares (□) represent BOV-test drink.

Correlation diet-induced thermogenesis and VAS scores

When considering all data points together, the VAS scores indicating satiety were significantly correlated to DIT (**Figure 3**). A negative correlation was observed for DIT and prospective consumption (ρ:-0.15, p=0.003) and for DIT and hunger (ρ:-0.25, p<0.0001). For the feeling of fullness and DIT a positive correlation was observed (ρ:0.25, p<0.0001).

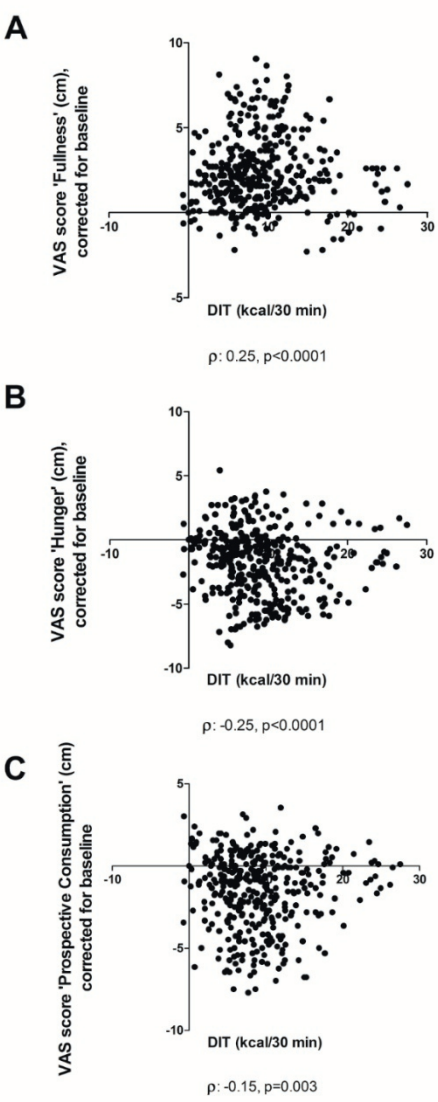

Figure 3: Correlation of diet-induced thermogenesis (DIT) with the visual analogue scale (VAS) scores about feelings of fullness (A), hunger (B), and prospective consumption (C), corrected for baseline. Correlations were tested using Spearman r test.

Gastrointestinal peptides and glucose

Postprandial responses of glucose and gastrointestinal peptides are shown in **Figure 4**. No differences were found in the netAUC of the postprandial responses of glucose, PYY, and CCK after consumption of the two test drinks (respectively p=0.76, p=0.17, p= 0.26, and p=0.39). NetAUC of the postprandial secretin response was elevated after consumption of the BOV-test drink, compared to the VEG-test drink (p<0.01). Acylated ghrelin decreased after consumption of both test drinks, for the BOV-test drink the netAUC of the postprandial response of acylated ghrelin was higher compared to the VEG-test drink (p=0.01).

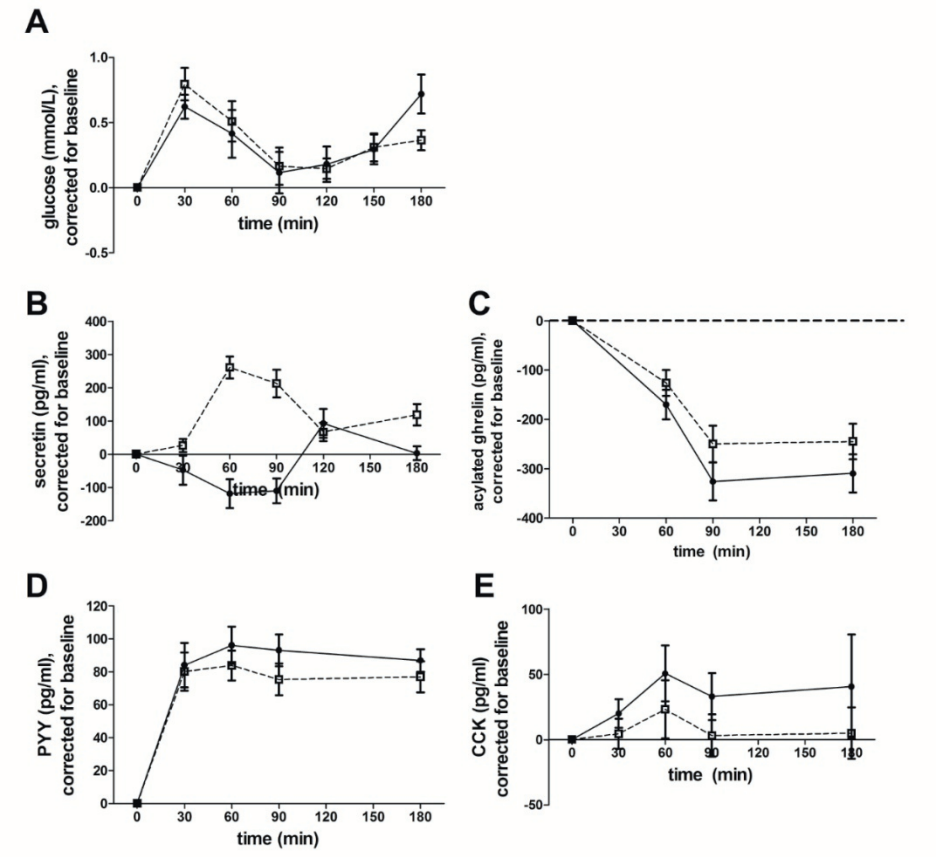

Figure 4: Postprandial responses of glucose and gastrointestinal peptides involved in satiety after consumption of a milk drink containing either 100% vegetable fat (VEG-test drink) or a mixture of 67% bovine milk fat with 33% vegetable fat (BOV-test drink) (mean ± SEM, n=19), corrected for baseline concentrations. Postprandial responses of glucose (A), secretin (B), acylated ghrelin (C), PYY (D), and CCK (E). netAUC was compared using Wilcoxon matched-pairs signed rank test. Significant differences in netAUC were found for postprandial responses of acylated ghrelin (p=0.01) and secretin (p<0.01), and not for postprandial responses of glucose (p=0.76), PYY (p=0.26), and CCK (p=0.39). The black circles (●) represent the VEG-test drink, the open squares (□) represent BOV-test drink.

Correlations postprandial responses of glucose and gastrointestinal peptides and VAS scores

Figure 5 shows the correlations of the postprandial responses of the different gastrointestinal peptides with the postprandial changes in VAS scores on feelings of fullness, hunger, and prospective consumption. Postprandial responses of all five peptides were significantly correlated with the change in VAS scores of feelings of fullness after consumption of the test drinks. A similar result was found for the feeling of hunger, except that CCK did not show a significant correlation. Postprandial responses of secretin, acylated ghrelin, and PYY were significantly correlated with the change in the feeling of prospective consumption.

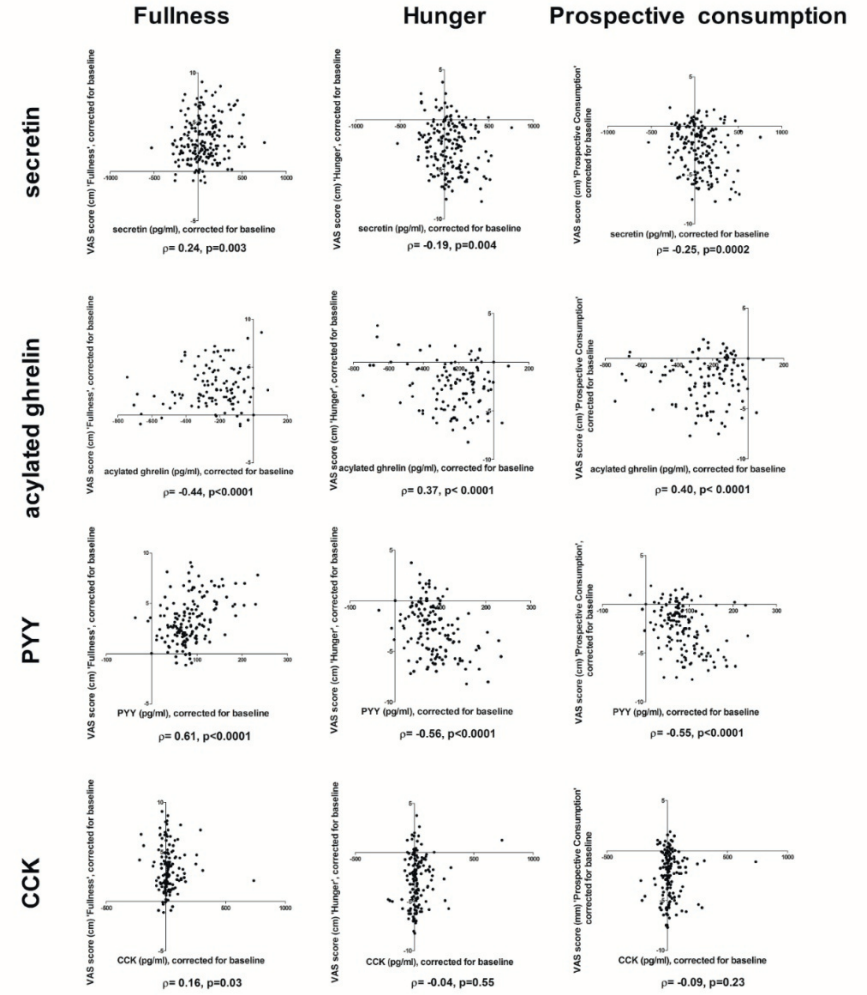

Figure 5: Correlations of gastrointestinal peptides (corrected for baseline) with the visual analogue scale (VAS) scores about feelings of fullness, hunger and prospective consumption, corrected for baseline. Correlations were tested using Spearman r test.

Discussion

This study demonstrates that consumption of an IF containing 67% bovine milk fat and 33% vegetable fat (BOV) prolongs satiety compared to an IF containing 100% vegetable fat only (VEG). No significant differences in DIT and RER were found between the two IF products. Postprandial secretin and acylated ghrelin responses were affected by the difference in fat blend and were correlated to postprandial changes in VAS scores.

The prolongation of satiety upon consumption of the BOV-test drink was reflected by the longer period of time that was needed for the VAS scores, representing fullness and prospective consumption, to return to baseline values than after consumption of VEG-test drink. This study examined several potential mechanisms underlying this satiating effect, including DIT, which is generally considered to be an important satiety mechanism.[24] Our results support the involvement of DIT in appetite regulation, as postprandial changes in appetite profiles correlated with DIT. However, DIT and fat oxidation are unlikely to explain the prolongation of satiety after consumption of the BOV-test drink. Even though DIT was slightly higher over most of the time course after consumption of the BOV-test drink, total DIT over the 5 hour period was not significantly different from the VEG-test drink. Furthermore, fat oxidation did not differ between treatments, as the RER showed a similar profile in time. Animal studies showed that hepatic oxidation of dietary fat is satiating, while storage of fat is not.[25] Previous studies that showed an increased DIT and reduced RER after consumption of MCT vs LCT examined considerably higher levels of MCFA (at least 12%).[15,16] The similar DIT and RER of the two test drinks, despite a higher SCFA and MCFA content for the BOV-test drink, may be explained by the relatively small difference in SCFA and MCFA between the two test drinks, which was just 6%, which is the maximum difference that can be reached by a realistic addition of bovine milk fat to IF.

Several gastrointestinal peptides have been suggested to be involved in satiety, which is supported by our results, since postprandial responses of secretin, acylated ghrelin, and PYY were correlated with the postprandial changes in appetite profiles, when all data points were combined. The postprandial response of CCK however, was only correlated to the change in feeling of fullness. So in this study postprandial response of CCK is a less good indicator of satiety than the responses of the other circulating peptides that were determined. Unexpectedly, the basal concentrations of secretin, CCK, and acylated ghrelin were different for the two treatments. However, these differences are not unambiguously pointing to a different physiological state on the different study days. The higher basal CCK and reduced acylated ghrelin concentrations before BOV-test drink may suggest that our subjects were not in an absolute post-absorptive state, while the lower secretin levels indicates the opposite. Furthermore, no differences in basal VAS scores, energy expenditure and RER were observed, indicating a similar prandial state. The reason for the difference in basal concentrations remains unknown, as differences in basal concentrations were not due to treatment order.

As expected, ingestion of the test drinks increased circulating concentrations of CCK and PYY. However, the difference in fatty acid profile of our test drinks, including different levels of SCFA

and MCFA, did not result in significant differences in postprandial responses of CCK and PYY. A recent review indicated that PYY and CCK are especially stimulated by LCFA.[18] Whether these PYY and CCK responses are different for specific LCFA is not completely clear; still, it is suggested that mono-unsaturated fatty acids (MUFA) induce a lower response of PYY than saturated fatty acids (SFA).[18] Our results, showing similar postprandial PYY responses despite different MUFA levels are not in line with these findings. The difference in LCFA profile between BOV and VEG may have been too small to result in differential stimulation of PYY or CCK. This also implies that the prolongation of satiety that we observed after consumption of the BOV-test drink is therefore not likely to be caused by differences in postprandial CCK and PYY responses. Our result agrees with another study showing that consumption of MCT reduced food intake, which was not related to changes in PYY.[26]

Of the gastrointestinal peptides that were examined, ghrelin is the only one that stimulates food intake.[27] The main circulating active form of ghrelin in humans is octanoyl-ghrelin,[28] and consumption of MCFA leads to a faster acylation of ghrelin than LCFA, possibly through direct supply of acyl units.[29,30] Accordingly, several studies show that concentrations of acylated ghrelin were less reduced less after consumption of MCT compared to LCT.[26,30–32] This may provide a possible explanation for the different postprandial responses of acylated ghrelin to our two test drinks: after consumption of the BOV-test drink, that contained higher levels of MCFA, circulating levels of acylated ghrelin were less decreased than after the VEG-test drink. Another explanation might be the higher concentrations of the MUFA C18:1 and the lower concentrations of SFA in the VEG-test drink, since it has been shown that MUFA suppress ghrelin secretion more than SFA.[18]

Since we found that the BOV-test drink prolonged satiety, while at the same time induced a lower decrease in circulating acylated ghrelin, the difference in the postprandial response of acylated ghrelin is unlikely to be the cause for the difference in satiety of the two test drinks. It is worth mentioning that the relation between postprandial ghrelin and satiety is not clear yet. While pre-prandial administration of ghrelin was shown to stimulate food intake,[33] postprandial suppression of ghrelin was not related to intermeal intervals.[34]

The other gastrointestinal peptide that differed in postprandial response to the two test drinks was secretin. Secretin is mainly known for its function to stimulate pancreatic secretion to neutralize the pH of the gastric content that reaches the duodenum,[35] so a difference in secretin might point at a difference in gastric emptying. Although we did not determine gastric emptying directly, the concentrations of glucose in serum provide an indication.[36] Since there is no difference in glucose concentrations after consumption of the drinks, we assume that there also was no difference in gastric emptying.

Probably, other mechanisms are involved in the different secretin responses to the different lipid composition of the two test drinks. Secretin secretion can be induced by fatty acids via intracellular calcium signaling.[37] CD36 was identified as the relevant fatty acid sensing receptor.[38] However, CD36 is mainly involved in the uptake of triglycerides and LCFA, and as the BOV-test drink contained lower levels of LCFA, this is an unlikely explanation for the

increased secretin response. The SCFA receptor FFAR3 co-localizes with enteroendocrine cells producing secretin,[39] and, therefore, could possibly be involved in the higher response of secretin to the test drink containing bovine milk fat. However, as the role of FFAR3 in secretin secretion is still obscure, future studies are needed to confirm this.

Recently, it was found that secretin also plays another role: regulating appetite and food intake.[40] The increased secretin responses to the BOV-test drink may therefore provide an explanation for the prolonged feelings of satiety. Secretin was found to induce thermogenesis in brown adipose tissue (BAT) by which satiation is initiated.[40] Indeed, when taking all data points together, we showed that the postprandial secretin response correlated to the change in satiety. However, as we did not observe differences in DIT and RER between the two test drinks, a possible difference in the induction of BAT thermogenesis by the different secretin responses was either too little to detect or did not occur in our study. A potential explanation for this is that the BOV-test drink contained higher concentrations of SCFA and MCFA, which are less capable of inducing uncoupling protein 1 (UCP1).[41] The fatty acid composition of BOV may thus counteract the effects of higher secretin levels on UCP1 activation. These results are in agreement with an animal study that showed no relation between secretin and whole body energy metabolism.[42] Still, our results suggest that secretin also acts on satiety by alternative mechanisms.

Such an alternative mechanism may be a direct activation of vagal afferent pathways. Vagal afferent fibers are present in the GI tract, close to the epithelium.[43] Secretin has shown to induce brain activation[44] and to inhibit gastric motility[45] through stimulation of these vagal pathways. As these neural pathways are also involved in the satiating properties of CCK,[46] and hepatic fat oxidation,[47] it may be hypothesized that they also mediate the satiating effects of secretin. It should be noted that vagal afferent pathways can also be activated directly by specific fatty acids, in particular butyrate.[46,48] This mechanism may underlie the satiating capacity of orally administrated butyrate,[49] and could therefore contribute to the prolonged satiety after the BOV-test drink, that contained higher levels of this specific SCFA. Moreover, acetate, a shorter SCFA, might be able to cross the blood-brain barrier, and reduce appetite through a direct effect on hypothalamic satiety signaling pathways.[50] So, besides the effects of the fatty acids profile on DIT or gastrointestinal peptide secretion, it may also more directly influence appetite through direct effects on vagal afferent or even hypothalamic pathways. Of course, to what extent whether these mechanisms, particularly affected by SCFA, contributed to the prolonged satiety after the BOV test drink remains unclear.

Conclusion

This study showed that addition of the representative maximum percentage of bovine milk fat to IF prolonged satiety and increased postprandial responses of secretin and acylated ghrelin, compared to an IF containing vegetable fats only. The prolongation of satiety may be explained by the increased secretin response, possibly through stimulation of vagal afferent pathways, or perhaps through direct effects of SCFA present in bovine milk fat. Whether these results can be translated to infants, and whether this prolongation of satiety influences food intake needs further research.

Acknowledgments

Conflict of interest: Jeske Hageman is an employee of FrieslandCampina. Other authors report no conflict of interest. FrieslandCampina financially contributed to this study.

Authors' contributions: JHJH, JK and AGN designed the research, interpreted the data and edited the manuscript, JHJH, RF and JAAH conducted the research, JHJH analyzed the data and wrote the manuscript, AGN has primary responsibility for final content. All authors read and approved the final manuscript.

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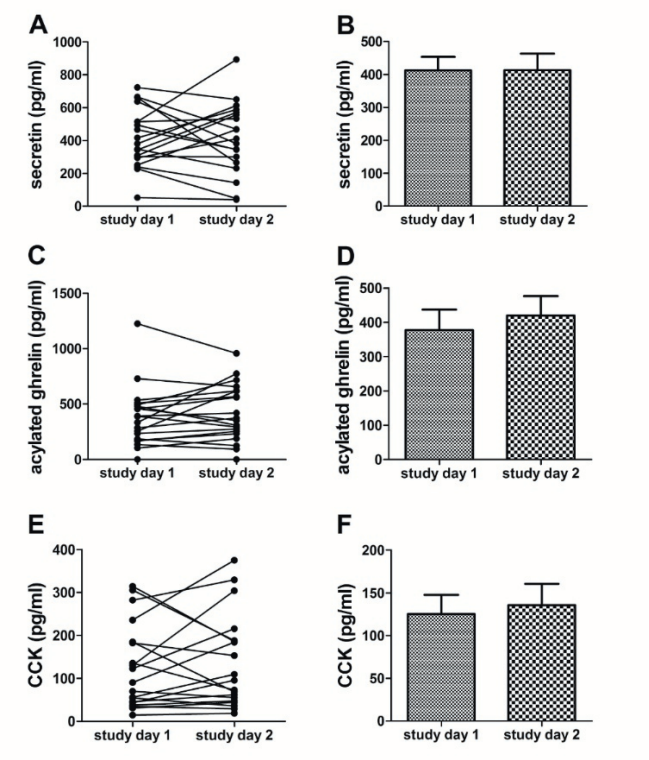
Supplemental information

Supplemental Table 1. Fatty acid composition of the two test drinks (in % of total FAMEs).

	VEG- test drink	BOV-test drink
C4:0	<0.1	2.6
C6:0	<0.1	1.6
C8:0	0.6	1.2
C10:0	0.5	2.1
C11:0	<0.1	0.3
C12:0	7.0	4.3
C14:0	3.0	8.2
C14:1n-5	<0.1	0.7
C15:0	<0.1	0.7
C16:0	24.7	22.8
C16:1n-7	0.2	1.1
C18:0	3.1	7.1
C18:1 trans	<0.1	1.1
C18:1 n-9	42.3	25.9
C18:1n-7	1.6	1.1
C18:2 n-6	12.9	12.0
C18:3 n-3	1.8	1.4
C18:3n-6	0.1	<0.1
C20:0	0.3	0.2
C20:1n-9	0.5	0.2



Supplemental Figure 1: Mean concentrations of secretin (p=0.99) (A-B), acylated ghrelin (p=0.41) (C-D), and CCK (p=0.58) (E-F) on both study days.

Chapter 6

Application of volatile organic compound (VOC) analysis in a nutritional intervention study: differential responses during five hours following consumption of a high- and a low-fat dairy drink

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Abstract

Scope: Exhaled volatile organic compounds (VOCs) are a possible relevant target for non-invasive assessment of metabolic responses. Using a breathomics approach, we aimed to explore whether lipid intake influences VOC profiles in exhaled air, and to obtain insight in intra- and inter-individual variations.

Methods and results: Three human interventions were performed. In the first, 12 males consumed a high-fat drink on three study days. In the second, 12 males received a high- and a low-fat drink on six days. In the third, three volunteers consumed the high-fat drink again for tentative compound identification. Participants were asked to exhale, for five hours postprandial, with 15-20 minute intervals, into a proton-transfer-reaction MS, and VOCs in exhaled air were measured. Consumption of a drink altered the VOC profile, with considerable inter-individual variation and quantitative intra-individual differences between days. Consumption of two different drinks resulted in a distinct VOC profile, caused by several specific m/z values. Most of these compounds were identified as being related to ketone body formation and lipid oxidation, showing an increase in the high- versus the low-fat drink.

Conclusion: Exhaled VOCs have the potential to assess differences in metabolic responses induced by nutrition, especially when day-to-day variation can be minimized.

Keywords: breath analysis; breathomics; inter- and intra-individual variation; lipids; volatile organic compounds (VOCs)

Introduction

Currently, effects of nutrition or specific food components on absorption or metabolism are mostly studied via biomarkers measured in sampled blood. However, in specific target groups like infants, children or elderly, this invasive method is not always desirable. To reduce the burden on participants of nutritional intervention studies, there is a need for non-invasive assessment methods. Analysis of exhaled compounds, breathomics, may be such an interesting non-invasive method. With each exhaled breath, a mixture of molecules is released into the air.[1] Some of these molecules are volatile organic compounds (VOCs), which are partly derived from the body's internal metabolism.[2] Approximately 200 VOCs are present in a breath sample analysed with GC-MS, which differ per individual.[3] VOCs in exhaled air have been related to volatile components present in blood, since several studies showed similar VOCs being present in blood and in exhaled air, in similar concentrations.[4,5] Analysis of the composition of the VOCs present in exhaled air can therefore give an indication about the physiological processes that occur in the body.[1,2,6] Diseases and health status have been shown to influence the profile of VOCs in exhaled air.[6–8] Specific examples suggest that VOC patterns in exhaled air indeed may reveal metabolic pathways impacted by nutrition. For instance, during fasting acetone is measured in exhaled air,[9] which is likely to reflect increased ketogenesis. Furthermore, a recent study shows that consumption of two different infant formulas results in a different postprandial VOC pattern.[10]

The use of VOCs analysis to assess direct effects of nutrition on metabolism comes with some challenges. One of those is the qualitative and quantitative inter-individual and intra-individual variation in the VOCs in exhaled air.[11] Concentrations of VOCs may vary considerable between individuals,[3] for example due to smoking, gender, age and BMI.[11,12] The intra-individual variation has received little attention so far. Changes over time within subjects over several exhalations,[13] several hours,[14] or several days[15] have been reported , but, to our knowledge, day-to-day variation in response to a meal has not been investigated.

The suitability of VOCs analysis to study metabolic responses in a nutritional intervention study is depending on the magnitude of effects of a nutritional challenge on VOCs, relative to the inter-individual, and, more importantly, the intra-individual variation in VOCs in exhaled air. One objective of this study was therefore to study intra-subject variation in VOCs in exhaled air after a nutritional intervention. Since it should also be possible to discriminate between nutritional interventions, another objective was to establish whether the consumption of a high-fat versus low-fat dairy drink resulted in a different VOCs profile in exhaled air. Furthermore, we aimed to tentatively identify affected VOCs for insight in potentially affected metabolic pathways.

Experimental section

Subjects

Both for intervention 1 and 2, 12 healthy male volunteers (intervention 1: 22.8 ± 3.0 years of age, intervention 2: 22.5 ± 2.7 years of age) from Wageningen and surroundings were enrolled. To avoid confounding factors such as differences in gender, age and BMI [12], only healthy males aged 18-35 years, with a BMI between 22-25 kg/m2 and with a relative body fat mass between 8 and 15% were included. Smokers, or subjects who smoked in the past, were excluded. The main subject characteristics are presented in **Table 1**. Informed consent was obtained from the volunteers before their involvement in the intervention. The Wageningen University ethics committee (METC-WU) (NL56722.081.16) approved this study protocol. The study (Breath Taking) was registered at the NTR (NTR5974). The study was conducted in accordance with the principles of the Declaration of Helsinki (Fortaleza, Brazil 2013).

Table 1: Subject characteristics (n=12) (mean ± SD)

	Intervention 1	Intervention 2
Age (years)	22.8 ± 3.0	22.5 ± 2.7
Body weight (kg)	79.6 ± 6.7	74.8 ± 6.4
BMI (kg/m^2)	23.4 ± 1.3	22.4 ± 1.0
Fat mass (%)	13.0 ± 2.2	12.3 ± 2.2

Methods

Intervention 1: Subjects were restricted from high-intensity exercise, alcohol consumption and usage of recreational drugs and medication the day before a study day. The evening preceding a study day, the subjects consumed a standardized dinner (see **Table 2**) followed by a 12 hours overnight fast. The next morning subjects consumed 500 ml of a high-fat dairy drink (see **Table 3**) within a period of five minutes. Exhaled air was measured before consumption (baseline) and every 15 minutes after consumption until five hours postprandial. The subjects were asked to breath into a mouthpiece connected to a breath sampling device (Buffered End-Tidal breath sampling inlet, Ionicon Analytik G.m.b.H., Innsbruck, Austria),[16] which was connected to a proton transfer reaction-mass spectrometer (PTR-MS) (Ionicon Analytik G.m.b.H., Innsbruck, Austria) for immediate and online analysis of breath samples. Mass-to-charge ratios (m/z) of m/z 21 to 160 were determined, with a dwell time of 0.1 s mass^{-1}, at a drift pressure of 2.20 mbar and chamber and inlet temperature of 60 °C. Per time point three replicate analyses of 10 cycles were performed. In between measurements, the volunteers were asked to keep in a seated, rested position. Ambient air was analysed in between all measurements to determine whether any fluctuations over time occurred. This procedure was repeated three times, on different days with at least one day of wash-out in between.

Table 2: Composition of the standardized dinner

Per portion	Standardized dinner
Kcal	876
Protein (g)	33.3
Fat (g)	37.0
Carbohydrates (g)	94.9

Table 3: Composition of the dairy drinks. Protein, carbohydrate and fat sources were identical in both products, different amount of fats were added to the drinks resulting in a high-fat and low-fat dairy drink with different energy contents.

Per 100 ml	High fat dairy drink	Low fat dairy drink
Kcal	124.8	36.5
Protein (g)	3.7	3.7
Fat (g)	9.9	0.1
Carbohydrates (g)	5.2	5.2

Intervention 2: In the second intervention, the same procedure as described above for intervention 1 was followed, except that two different nutritional interventions were applied: one consisting of a high-fat dairy drink and the other of a low-fat dairy drink (**Table 3**). In a cross-over design, participants received the low-fat and high-fat dairy drink three times each, all in randomized order, on six different study days, which were separated by at least one day of wash-out.

Intervention 3: To be able to tentatively identify specific VOCs related to the intake of the dairy drinks, three participants of the first two interventions were invited for another study day. The same procedure as that of the previous interventions with consumption of the same high fat dairy drink was used, except that breath measurements were performed every 20 minutes postprandial, and the breath sampling device was coupled to a PTR-Quadrupole interface Time of Flight-MS (PTR-QiToF-MS) (Ionicon Analytik G.m.b.H., Innsbruck, Austria), which provides an more accurate mass to enable tentative compound identification. Ionization of H_3O^+ was performed under drift tube voltage of 900 V, and an inlet temperature of 60 °C. Mass-to-charge ratios (m/z) of m/z 17.002 to m/z 445.123 were determined in all exhalations, with a dwell time of 0.4 s $mass^{-1}$.

VOCs in dairy drink: To tentatively identify the VOCs present in the dairy drinks, the drinks were transferred to a glass bottle with a mechanical stirrer and the headspaces of the low-fat (n=2) as well as the high-fat dairy drink (n=4) were measured using PTR-MS (Ionicon Analytik G.m.b.H., Innsbruck, Austria). Mass-to-charge ratios (m/z) of mass range m/z 21 to 160 were determined in 5 cycles, with a dwell time of 0.1 s $mass^{-1}$, at a drift pressure of 2.20 mbar, inlet flow of 54 ml/min, and chamber and inlet temperature of 60 °C.

Data pre-processing

Prior to statistical analysis of the data of intervention 1 and 2, several steps of pre-processing were performed. For these studies, the raw counts were converted into ppbv (parts per billion by volume). m/z 32 (O2+) and m/z 37 (water cluster ion) associated with the PTR-MS ion source were removed from the dataset. The correlation of the different replicates per time point was checked and uncorrelated cycles were removed. The analytical level of detection (LOD) of the PTR-MS was determined as three times the signal to noise ratio of blank m/z values in 4350 ambient air samples, which was 0.187 ppbv. Denoising was performed by replacing all concentrations below 0.2 ppbv (i.e. slightly above the analytical LOD) with 0.2 ppbv and the m/z values with 80% or more of the measurements being 0.2 ppbv were removed from the data set. Prior to Principal Component Analysis (PCA), m/z values were logtransformed and scaled. To remove variation caused by subjects the m/z values were corrected for baseline values and meancentered per subject. To obtain a global understanding of the variation in the m/z values of intervention 2 related to the time points and the two treatments, baseline corrected m/z values were averaged across the different participants for the two treatments separately. PTRwid was used to execute mass scale calibration and peak extraction of the data of intervention 3, gathered via PTR-QiToF-MS.[17]

Statistics

Intervention 1

A PCA of the data of intervention 1 was performed and a PCA score plot was created in Rstudio (version 1.1.456 for Windows, RStudio Inc.) using R (version 3.5.1 for Windows) and package FactoMineR, to investigate the differences between participants (uncorrected for baseline and without meancentering per subject), the effect of the meal and the effect of study days. For each m/z value, net area under the curve (AUC) was calculated using GraphPad Prism (version 5.04 for Windows, GraphPad Software, San Diego California USA), and the different study days were compared using repeated measures ANOVA. A p-value <0.05 was considered to be significantly different.

Intervention 2

For each m/z value, net area under the curve (AUC) was calculated using GraphPad Prism (version 5.04 for Windows, GraphPad Software, San Diego California USA), and the two dairy drinks were compared using paired t-test. A p-value <0.05 was considered to be significant different. PCA was used to obtain a low dimensional summary of all m/z values for the two treatments and time points. The change over time of each m/z value was modelled using a segmented linear regression for each participant and measurement day separately, using the R-package segmented.[18] Segmented regression allowed for modelling each m/z curve by two individual linear regression lines connected by a flexible breakpoint. This allowed to mimic a steadily change in intensity followed by a period of constant intensity. This way, each m/z curve was summarized by two intercepts, two slopes and a breakpoint. For each m/z value and drink, the change in intensity in time was plotted using the participant and measurement day

averaged data. Super positioned in this overview are the averaged segmented regression models. The effect of the different drinks was tested on the 2 different intercepts and 2 different slopes with the following mixed model:

response ~ TypeDrink + (1| MeasurementDay / Participant)

Where *TypeDrink* was the fixed treatment effect and *(1| MeasurementDay / Participant)* was a random term taking into account that participants were nested within measurement days. A p-value <0.05 was considered to be significantly different.

Intervention 3

The data of this intervention was used to provide responses with a more accurate m/z values to enable tentative compound identification. A profile comparison was performed of those more accurate m/z values and the m/z values that showed a different response over time between the two drinks in intervention 2. Thereafter, ChemCalc was used to obtain corresponding molecular formulas,[19] and the Human Metabolome Database (HMDB) and literature was used to derive possible compounds from these formulas.[20]

VOCs in dairy drink

The averages of five cycles were calculated and thereafter averages and standard deviations of the two different dairy drinks were calculated. M/z-values with a ppbv above 0.2 were selected and compared. When concentrations of a certain mass differed more than the standard deviation multiplied by two between the two dairy drinks, the concentrations were considered to be different.

Results

Participants

Intervention 1 was performed to gain insight in intra- and inter-individual differences causing variation in VOC profiles. PCA analysis of the VOC concentrations in exhaled air in fasted and postprandial state showed that, despite some overlap, the 12 subjects formed separate clusters based on their VOC profile (**Figure 1A)**. In fasted state the different m/z values, e.g. m/z 59, differed between subjects (**Figure 1B**). Although the concentrations after correction for baseline still showed quantitative differences, the trends over time were similar for the participants **(Figure 1C**).

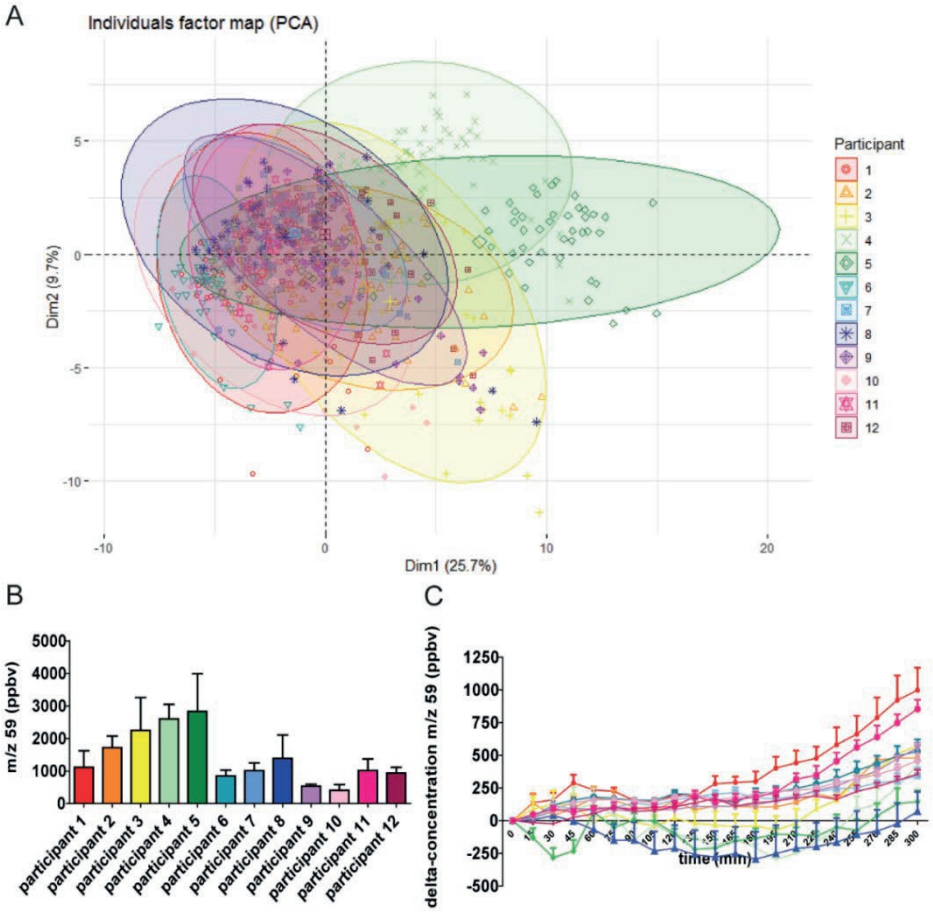

Figure 1. The VOC profiles in exhaled air of different subjects. Subjects were followed over time, VOCs were measured online with PTR-MS in fasted and postprandial states. **(A)** PCA score plot of 756 breath-o-grams performed on 71 VOCs excreted in exhaled air from 12 different subjects. Each colour represents a different participant. The subjects form separate clusters. (logtransformed, scaled data from intervention 1) **(B)** Absolute concentrations (ppbv) of m/z 59 in fasted state, average of three days (mean±SD). **(C)** Concentrations of m/z 59, corrected for baseline concentrations, for the 12 different subjects, average of three days (mean±SD).

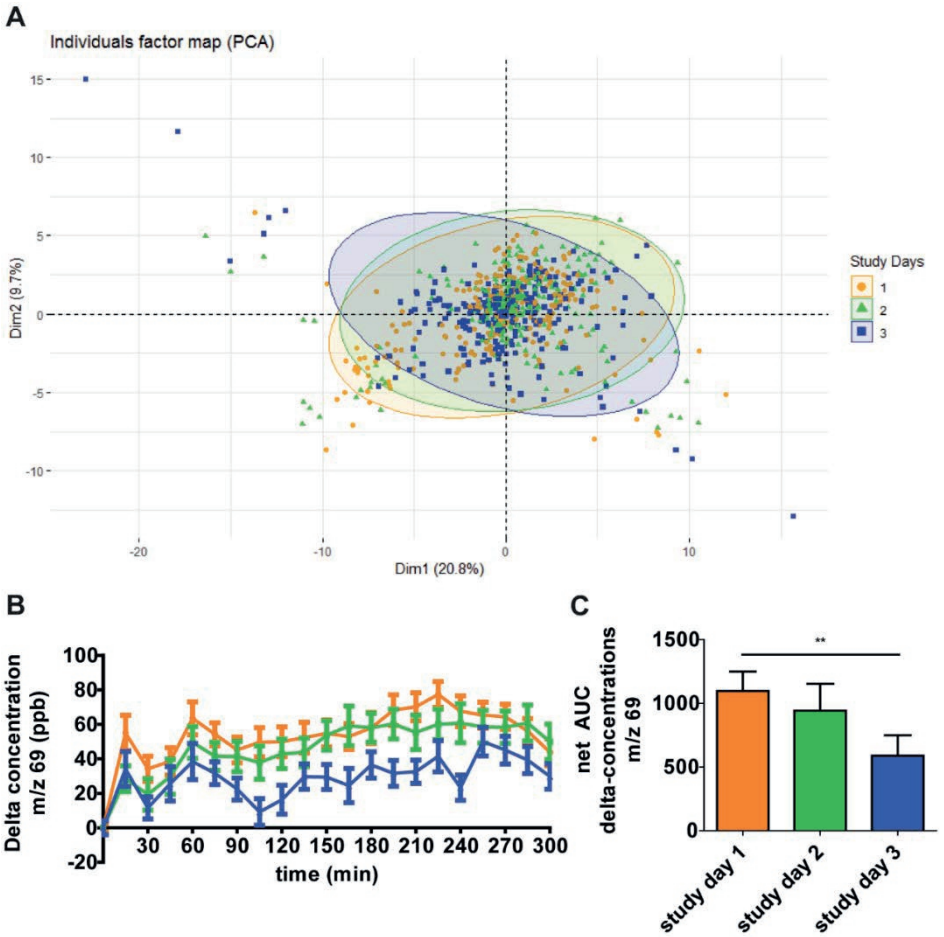

Figure 2. Variation in VOC profile in exhaled air on different study days. **(A)** PCA score plot of 756 breath-o-grams on 71 VOCs excreted in breath from 12 different subjects. The different study days are represented, no separate clusters were observed. (data of intervention 1: cleaned data of all VOCs, corrected for baseline and meancentered per participant) **(B)** Concentration of m/z 69 in exhaled air, corrected for baseline, on the three study days (mean±SEM). Mean basal concentration (±SEM) was 128.7 ± 8.4. **(C)** net Area under the Curves (AUC) of the concentrations of m/z 69 corrected for baseline on the three study days. Statistical analysis was performed using repeated measures ANOVA (p=0.0074), ** p<0.01. The orange symbols, line and bar represent study day 1, the green symbols represent study day 2, and the blue symbols represent study day 3.

Variation in study days

Figure 2A shows a PCA plot of the three different study days for each participant. After pre-processing to remove the inter-individual variation, the intra-subject variation was examined by comparing differences between study days. The total VOC profiles on the three study days overlaid each other; no separate clusters were formed. For the m/z values that changed with time, for example m/z 69, a similar qualitative profile after consumption of the high-fat dairy drink was observed on the different study days (**Figure 2B**). Quantitatively, the three study days resulted in somewhat different net AUCs for some of the components, as is shown for m/z 69 ($p=0.0074$) **(Figure 2C**).

Effect of nutrition

Besides insight in inter- and intra-subject variation, intervention 1 also showed a time response for some specific m/z values. **Figures 3A-F** display specific m/z values with a different postprandial response. M/z 33 was found to decrease after consumption of the high-fat drink. Some VOCs showed a gradual increase over time, such as m/z 59 or m/z 77, while others directly increased after consumption, such as m/z 55 or m/z 69. Some VOCs, such as m/z 45, showed a peak in concentration right after consumption of the milk.

Differences upon the intake of different drinks

In intervention 2, two different dairy drinks were provided to study the effect of a difference in lipid intake. **Figure 4A** shows a PCA score plot in which the two drinks formed separate clusters, indicating a difference in VOC profile between the two milk drinks. The two drinks were separated essentially along PC2, which explained almost 23% of the variation in the data. A similar trend in time was visible along the PC1 axis from 15 to 300 minutes after consumption of the drinks. The corresponding biplot of all m/z values (**Figure 4B)** shows which m/z values were mostly influenced by PC1 (horizontal change) or were mostly affected along PC2 (vertical change).

To further investigate whether specific m/z values showed different time responses between the two dairy drinks, segmented regression was used. The segmented regression model summarizes each curve in two intercepts and two slopes. These parameters were used to compare the two dairy drinks using mixed model analysis. **Table 4** shows the results of this analysis and **Figure 5** displays the time profiles of selected m/z values. An overview of all m/z values is present in the **Supporting Information Figure 1**.

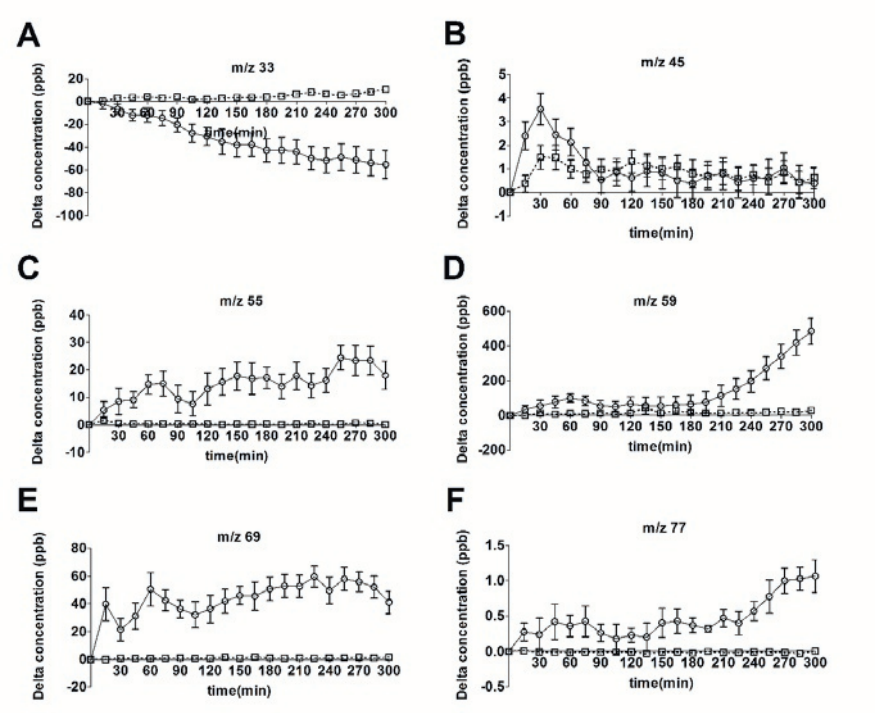

Figure 3. Effect of a high-fat drink on the VOC profile in exhaled air (A-F) Concentrations of m/z 33, m/z 45, m/z 55, m/z 59, m/z 69, and m/z 77 in exhaled air, corrected for baseline (full line, ○), and ambient air (dotted line, □) (mean±SEM) (data from intervention 1: mean of 12 participants). Mean basal concentrations (±SEM) were: A) 223.0 ± 28.8, B) 31.3. ± 0.5, C) 107.7 ± 6.4, D) 1392 ± 232.5, E) 128.7 ± 8.4, F) 2.4 ± 0.4.

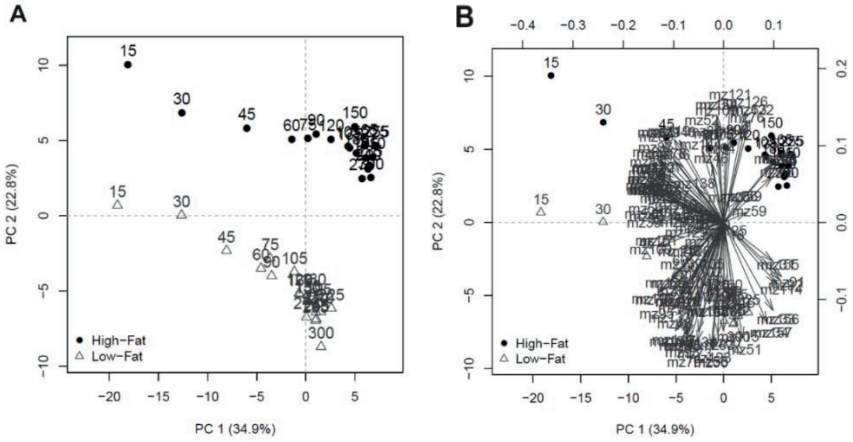

Figure 4. Different effects of high-fat vs low-fat dairy drinks on VOC profile in exhaled air. PCA plot of 1486 breath-o-grams on 100 VOCs excreted in breath from 12 different subjects (A) score plot, (B) including loading plot. The black dots (●) represents high-fat milk, and the grey triangles (Δ) represent low-fat milk. (data of intervention 2: corrected for baseline and meancentered per participant).

Table 4: P-values of VOC m/z values where a significant difference in response to the high-fat vs low-fat dairy drink was observed for one of the parameters derived from segmented regression analysis, analysed using a mixed model analysis (p-values below 0.05 were considered to be significant, non-significant results are not shown).

VOC	Intercept 1	Slope 1	Intercept 2	Slope 2
m/z 56		0.013		
m/z 59		0.009		
m/z 60		0.005		
m/z 79			0.046	
m/z 87		0.009		
m/z 92			0.009	0.018
m/z 100			0.007	0.028
m/z 122		0.047		

Time responses of 11 m/z values were different after the consumption of the high-fat and the low-fat dairy drink. M/z 59, m/z 60, m/z 77 and m/z 87 showed a similar time profile. M/z 59, m/z 60 and m/z 87 displayed a first slope of the model that significantly differed between the high-fat and low-fat dairy drink, with an positive slope for high-fat milk and a negative slope for low-fat milk. Although for m/z 77 no significant difference between the two drinks was found for the first slope the two drinks gave a significant different net AUC for this m/z, similar to m/z 59, m/z 60 and m/z 87 (**Figure 6**). For m/z 56 and m/z 92 a slight decrease in time was found. After the consumption of the high-fat dairy drink, compared to the low-fat dairy drink, the concentrations of those two compounds were slightly reduced. The first slope of m/z 56 was significantly different. For m/z 92, the second intercept and second slope were significantly different between the drinks. Another compound that showed the same differences as m/z 92, was m/z 100. However, this compound displayed a different profile in time; the concentrations were less reduced after consumption of the high-fat drink compared to the low-fat drink. M/z 79 and m/z 117 both showed a peak in concentration at 15 minutes postprandially. After consumption of the two drinks the second intercept of m/z 79 was significantly different. For m/z 117 the net AUC was significantly higher after consumption of the high-fat drink compared to the low-fat drink. A concentration peak was also found for m/z 107. However, for this compound a different time response was observed, as the concentration only increased after consumption of the high-fat drink and after consumption of the low-fat drink a decrease in concentration was observed. The compound with m/z 122 displayed a significant different first slope between the time profiles of the two drinks; directly after consumption, the time profiles were inverse for the two dairy drinks.

Figure 6 shows the compounds with an AUC that is significantly different between the low-fat and the high-fat drink (m/z 59, m/z 60, m/z 77, m/z 87, m/z 107 and m/z 117). For all six components a higher net AUC was found after consumption of the high-fat drink. This pattern was observed for nearly all participants.

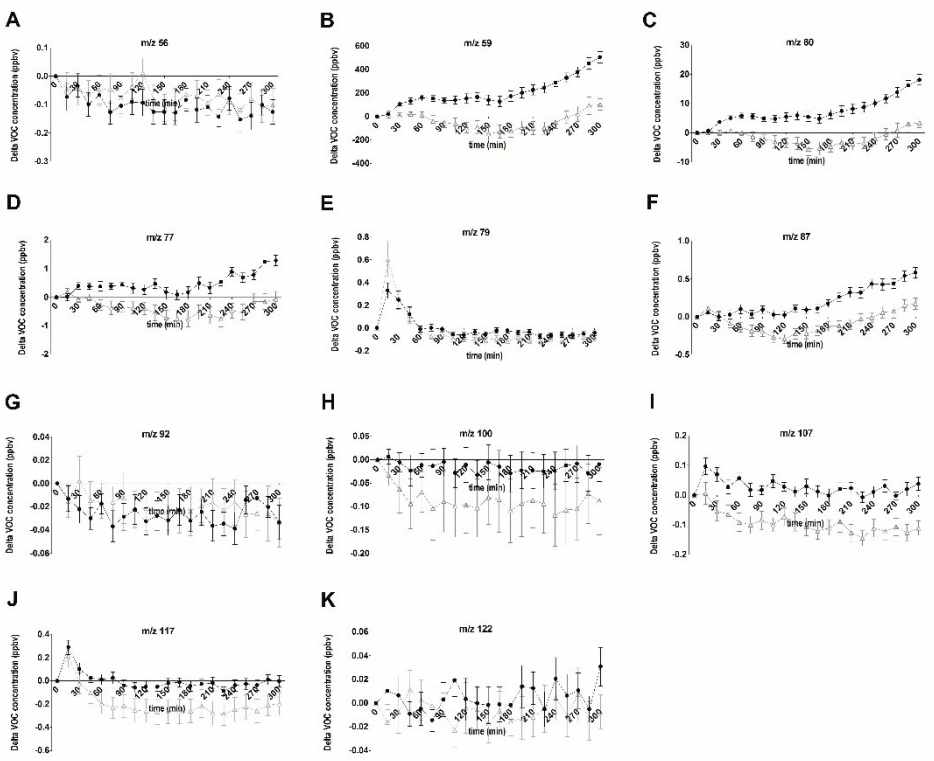

Figure 5. Postprandial VOC m/z value concentrations, corrected for baseline, in response to high-fat and low-fat dairy drink. Concentrations of those m/z response curves of exhaled VOCs that revealed a difference between the two dairy drinks are shown (mean±SEM). A) m/z 56, B) m/z 59, C) m/z 60, D) m/z 77, E) m/z 79, F) m/z 87, G) m/z 92, H) m/z 100, I) m/z 107, J) m/z 117, and K) m/z 122. The black line (●) represents the high-fat dairy drink and the grey line (Δ) represents the low-fat dairy drink. (data from intervention 2: mean of 12 participants). Mean basal concentrations (±SEM) for the low-fat and high-fat dairy drink were respectively: A) 0.92±0.06, 0.91±0.05, B) 1141±177.10, 1046±126.6, C) 40.35±6.42, 36.51±4.61, D) 3.35±0.58, 3.00±0.42, E) 0.34±0.03, 0.31±0.02, F) 1.23±0.10, 1.27±0.06, G) 0.25±0.02, 0.25±0.01, H) 0.34±0.07, 0.25±0.04, I) 0.40±0.02, 0.27±0.01, J) 0.60±0.12, 0.41±0.04, K) 0.25±0.01, 0.24±0.01.

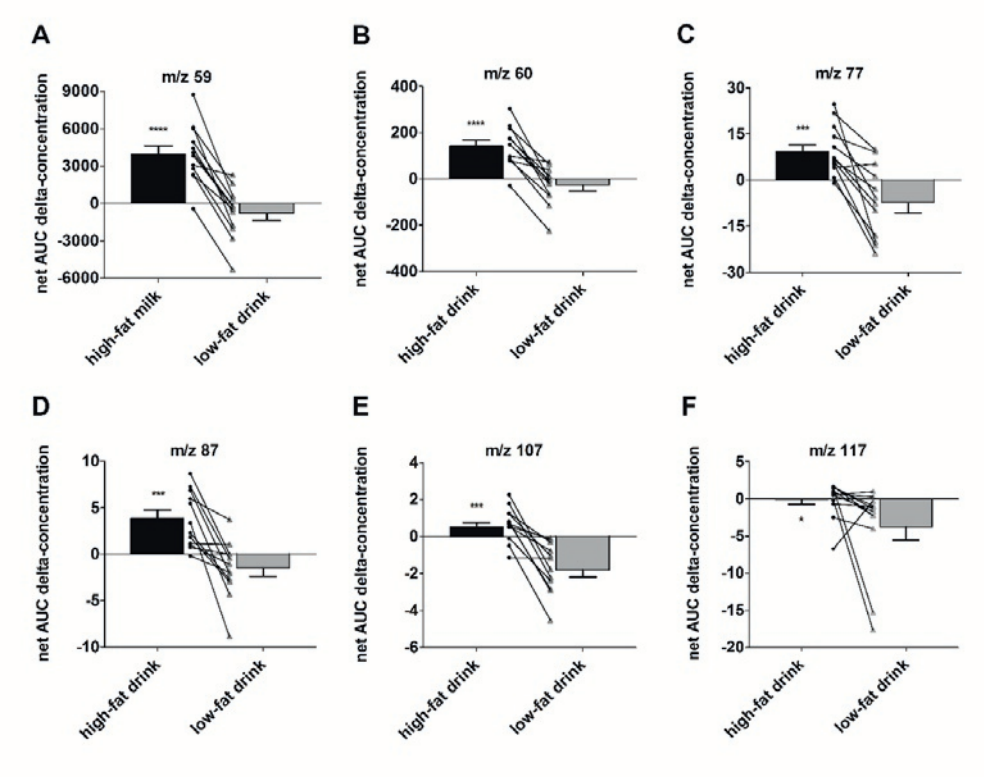

Figure 6. Difference in net AUC of concentrations, corrected for baseline, in exhaled air after two different drinks. Net area under the curve (AUC) (mean ± SEM) and difference in net AUC in response to the high-fat and low-fat dairy drink of concentrations, corrected for baseline, of A) m/z 59, B) m/z 60, C) m/z 77, D) m/z 87, E) m/z 107, and F) m/z 117 in exhaled air. The net AUC were compared using a paired t-test or, when the data were not normally distributed, Wilcoxon matched-pairs signed rank test, $p<0.05$ was considered to be statistically significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. (data from intervention 2: mean of 12 participants).

Tentative identification of VOCs

By alignment of the time responses of the m/z values of intervention 3 to the data of intervention 2, more accurate m/z values were obtained for those m/z values that were significant different after consumption of the high-fat and the low-fat dairy drink. This was used to determine probable molecular formulas and possible metabolites for these m/z values (**Table 5**).

Table 5: Possible metabolites related to m/z values differently influenced after consumption of a high-fat versus a low-fat dairy drink. Results of the PTR-MS intervention 2) and PTR-QiToF-MS (intervention 3) were compared, m/z values were converted to molecular formulas using Chemcalc.[19] Potential compounds were tentatively identified using the Human Metabolome Database (HMDB)[20] and literature (references provided).

m/z	m/z (PTR-QiToF-MS)	Molecular formula	Potential compounds	Ref
56	56.044	$C_3H_5N \circ H+$	fragment of heptanal or hexanal	21
59	59.049	$C_3H_6O \circ H+$	acetone	HMDB
60	60.052	$^{13}C_3H_6O \circ H+$	isotope of acetone	21
77	77.059	$C_3H_6O \circ H+ + H_2O$	H_2O cluster of acetone	22
79	79.039	$C_6H_6 \circ H+$	benzene	23
87	87.081	$C_5H_{10}O \circ H+$	2-pentanone 2-methylbutanal pentanal 3-methylbutanal (isovaleraldehyde)	24 25 26 27
92	92.049 / 92.059	$C_6H_5N \circ H+$	unknown	
100	100.04	$C_4H_5NO_2 \circ H+$	(R)-dihydromaleimide, NCCOOC2H5 (ethyl cyanoformate) methyl cyanoacetate 3-cyanopropanoic acid	HMDB
107	107.038	$C_3H_6O_4 \circ H+$	glyceric acid	HMDB
117	117.054	$C_5H_8O_3 \circ H+$	5-oxopentanoic acid alpha-ketoisovaleric acid acetoxyacetone,	HMDB
	117.091	$C_6H_{12}O_2 \circ H+$	caproic acid	HMDB
			ethyl 2-methylpropanoate 2-methylpropyl acetate isopropyl propionate methyl (S)-2-methylbutyrate	HMDB
122	122.061	$C_7H_7NO \circ H+$	3-acetylpyridine	HMDB
	122.104	$C_8H_{11}N \circ H+$	(1/2)-phenethylamine 5-ethyl-2-methylpyridine 2-propylpyridine 2-phenylethanaminium	HMDB

VOCs in milk

VOCs in the headspace of the high-fat and low-fat dairy drinks were analysed (**Figure 7**). A concentration above 0.2 ppbv was found for 28 m/z values in both drinks. Almost all of these m/z values were present in higher concentrations in the headspace of the high-fat dairy drink compared to the low-fat drink.

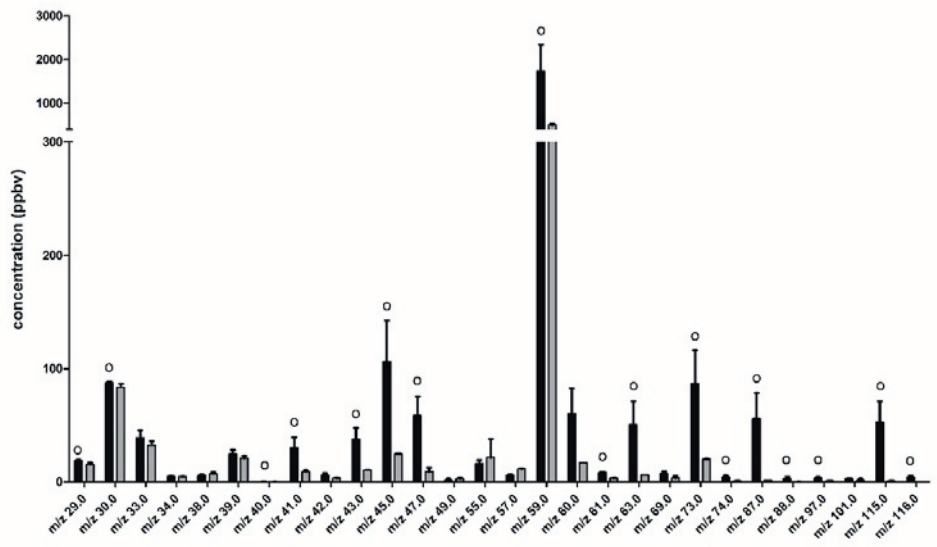

Figure 7. VOCs from the headspace of the high-fat and low-fat dairy drinks (mean ± SD) (black bars represent the high-fat dairy drink and the grey bars represent the low-fat dairy drink, ° difference between the two dairy drinks was larger than 2*SD).

Discussion

This study showed that nutrition altered the exhaled VOC profile and that the consumption of two dairy drinks with a different fat percentage resulted in a different postprandial VOC profile. This implies that analysis of VOCs in exhaled air can be used to non-invasively assess responses to nutritional interventions. However, there was substantial variation between and within subjects, which needs to be taken into account for study design and data analysis.

Inter-individual variation

In this study we observed high inter-individual variation for VOC profiles, which is in line with previous findings.[3] We attempted to minimize inter-individual variation by including only males within a specific age and BMI range, since it was recently shown that BMI, gender and age influence the VOC profile.[12] However, we still found substantial differences in the VOC profiles of the different participants of intervention 1. Using pre-processing, we were able to remove a substantial portion of this variation.

Intra-individual variation

Although the total postprandial VOC profile was similar on three different study days, we observed intra-individual variation as for some m/z values the three study days differed quantitatively. M/z 69 is an example, which showed significant differences between study days, independent of the intervention. In accordance with previous literature,[28,29] this indicates that there is day-to-day variation in VOC concentrations. So, despite exclusion of alcohol consumption and use of drugs and standardization of exercise and dinner the day preceding a study day as well as standardization of the execution of the measurements, we still found intra-subject variation. The source of this variation is still unknown. For our second intervention multiple repetitions of each treatment were included as a solution. This, however, is not optimal, since the method was intended to reduce the burden for the participants.

Effect of nutrition

This study showed a clear effect of nutrition on the VOC profile. From the second intervention, it can be concluded that consumption of two drinks differing only in the amount of fat resulted in a different VOC profile; about 23% of the total variation in the data set can be explained by the difference in lipid content. Some individual VOCs were found to contribute to these different profiles, as different time responses after consumption of the two different drinks were found. Intervention 1 also clearly showed an effect of nutrition on some specific m/z values. For these m/z values, different profiles with time could be identified. These may be derived from different metabolic pathways. Some VOCs, such as m/z 45, showed an increase directly after consumption of the milk. These likely are compounds related to aroma/flavour in the milk, which are consumed and directly exhaled. Indeed, m/z 45 is also found in the headspace of the high-fat milk.

Effect of lipid ingestion

The amount of ingested fat clearly affected the profile of VOCs. This could reflect effects on substrate metabolism, as a fat load was shown to profoundly influence substrate utilization.[30] This was further supported by the tentative identification of the affected VOCs. M/z 59 was identified as acetone, and since m/z 60 and m/z 77 showed a very similar pattern with time, these most likely were an isotope and a water cluster of acetone, respectively, as was also suggested by Herbig *et al*. (2009).[31] The strong increase three hours after the consumption of the dairy drinks in acetone and related compounds suggested an increase in ketogenesis, which is in agreement with previous research.[32] An increase in breath acetone has been related to an increase in fat oxidation.[33] A previous study showed that fat oxidation increased above fasting levels 150 minutes after consumption of a high-fat meal.[34] So, it is very likely that in the present study fat oxidation started to increase 180 minutes after the consumption of the dairy drinks. Since m/z 87 displayed a similar response curve as m/z 59, it is likely also a member of the ketone family, such as 2-pentanone (**Table 5**). This ketone was, just like acetone, present in higher concentrations in exhaled air compared to ambient air,[24] and was also found to be increased in fasting subjects.[35] It is hypothesized to be formed during beta-oxidation of caproic

acid, at least in *Penicillium roqueforti*.[35] Whether this process also occurs in humans is not known yet. The early increase in ketones after the consumption of the high-fat drink might come from VOCs present in the milk, being immediately exhaled upon the diary drink intake. Indeed, m/z 59, m/z 60, and m/z 87 were present in higher concentrations in full-fat milk compared to skimmed milk, as were some key butter aroma compounds.[36]

After consumption of the high-fat drink an increase in m/z 107 was found, while this m/z value decreased after consumption of the low-fat drink. This compound might have been glyceric acid; an oxidation product of glycerol. It was previously shown that consumption of dietary fat increases fat oxidation directly,[34] the increase of glyceric acid might be a reflection of that. Another VOC which might be related to fat oxidation is m/z 56, which is possibly a fragment of hexanal. Hexanal is a breakdown product of linoleic acid oxidation. Surprisingly, m/z 56 decreased after consumption of the drinks, while it was expected that fat oxidation would increase after this high fat intake. The slight decrease may be explained by low levels of linoleic acid in bovine milk fat,[37] providing a relatively lower amount of substrate that is oxidized into hexanal. m/z 117 might have been caproic acid (C6:0), a (combination of) short fatty acid ethyl ester(s), or metabolites related to amino acid degradation. Since participants were not allowed to consume alcohol on the day before the intervention, it is more likely that m/z 177 corresponds to caproic acid or metabolites related to amino acid degradation were exhaled, rather than to ethyl esters. Caproic acid is one of the medium-chain fatty acids present in milk fat.[37] This fatty acid is released during digestion in the stomach, and is very volatile. Potentially part of this fatty acid might have been exhaled before it could be absorbed. However, the increase in m/z 117 after the high-fat and the low-fat milk was similar. Since the low-fat milk contained much lower concentrations of caproic acid, this is therefore a less likely explanation. Since m/z 117 showed a direct peak after consumption of the milk it might also have been an aroma/flavour compound present in milk fat. However, as m/z 117 was not detected in the headspace of the milk products, it should then be released after digestion. The other m/z values were not identified or were difficult to link to metabolism. Altogether, this study showed that when nutrition influences metabolism, this might also be represented as VOCs in exhaled air.

VOCs in nutrition studies

Due to the great potential in clinical diagnostics and exposure assessment, analysis of VOCs in exhaled air has attracted a great deal of attention in the latest years.[1] Especially for vulnerable target groups this seems a promising method. In a recent study, GC-MS was used to analyse VOCs patterns in exhaled air after consumption of two different infant formula products.[10] Eight VOCs were significant different, only at the final time point, i.e., 240 minutes after consumption of the products.[10] In that study, participants received both treatments only once, so, based on our study, a considerable intra-subject variation may have prevented identification of additional differentially affected VOCs. Although exhaled air is less complex than urine or blood samples, standardization and reproducibility have proven to be more difficult. [11] This present study gave some additional insights in the inter- and intra-subject variation. For now some of this variation can be corrected for by choosing proper study designs, such as a cross-over

design and multiple repetitions, and pre-processing of the data. Further research could focus on how to minimize intra- and inter-individual variation, for example by standardizing exercise on a study day, by similar transportation to the study site, and by controlling exercise on multiple days before a study day. Since microbiota also produce VOCs,[14] perhaps the diet, and therefore substrate for microbiota, on the whole day or several days before a study day could be controlled for. This present study showed that although these types of variation are present, the effect of nutrition on the VOC profile could clearly be assessed. A next step would be to relate VOCs in exhaled air with metabolites in blood samples, to verify whether these compounds are endogenous and to examine the link with metabolism in order to study whether VOCs can be used as biomarkers to study metabolic outcomes.

Conclusions

This study showed that nutrition, despite a considerable inter-and intra-individual variability, had a strong influence on the post-prandial exhaled VOC profile. Increasing the fat load affected specific VOCs that, based on their m/z values, are likely to be produced by metabolic pathways related to fat oxidation, such as ketogenesis and glycerol oxidation. Therefore, the analysis of VOCs can be considered a promising non-invasive method to study energy metabolism, which should be further explored for use in nutritional intervention studies. High inter-individual variation still complicated VOCs analysis and may be controlled for by choosing a right study design and data processing. Important next steps to move this promising new technology towards practical application, are studies on how to reduce intra-individual variability, to link exhaled VOCs with biomarkers in blood, and to connect VOC responses to metabolic outcomes. Nevertheless, the information provided can to help to understand sources of variation in VOC analyses aiming at other goals, such a diagnosis of disease. By showing that VOCs in exhaled air can be used to discriminate between intake of drinks differing only in the amount of fat, we exemplify its potential use as a non-invasive tool in nutritional assessment.

Author contributions

The author's responsibilities were as follows: AGN, JHJH, JK, and SMvR designed the research; JHJH conducted research; JHJH and JAH analysed data; JHJH wrote the paper, JK had primary responsibility for final content, all authors: critically revised the paper and read and approved the final manuscript.

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Conflict of interest: Jeske Hageman is an employee of FrieslandCampina**.** Other authors report no conflict of interest. FrieslandCampina financially contributed to these studies.

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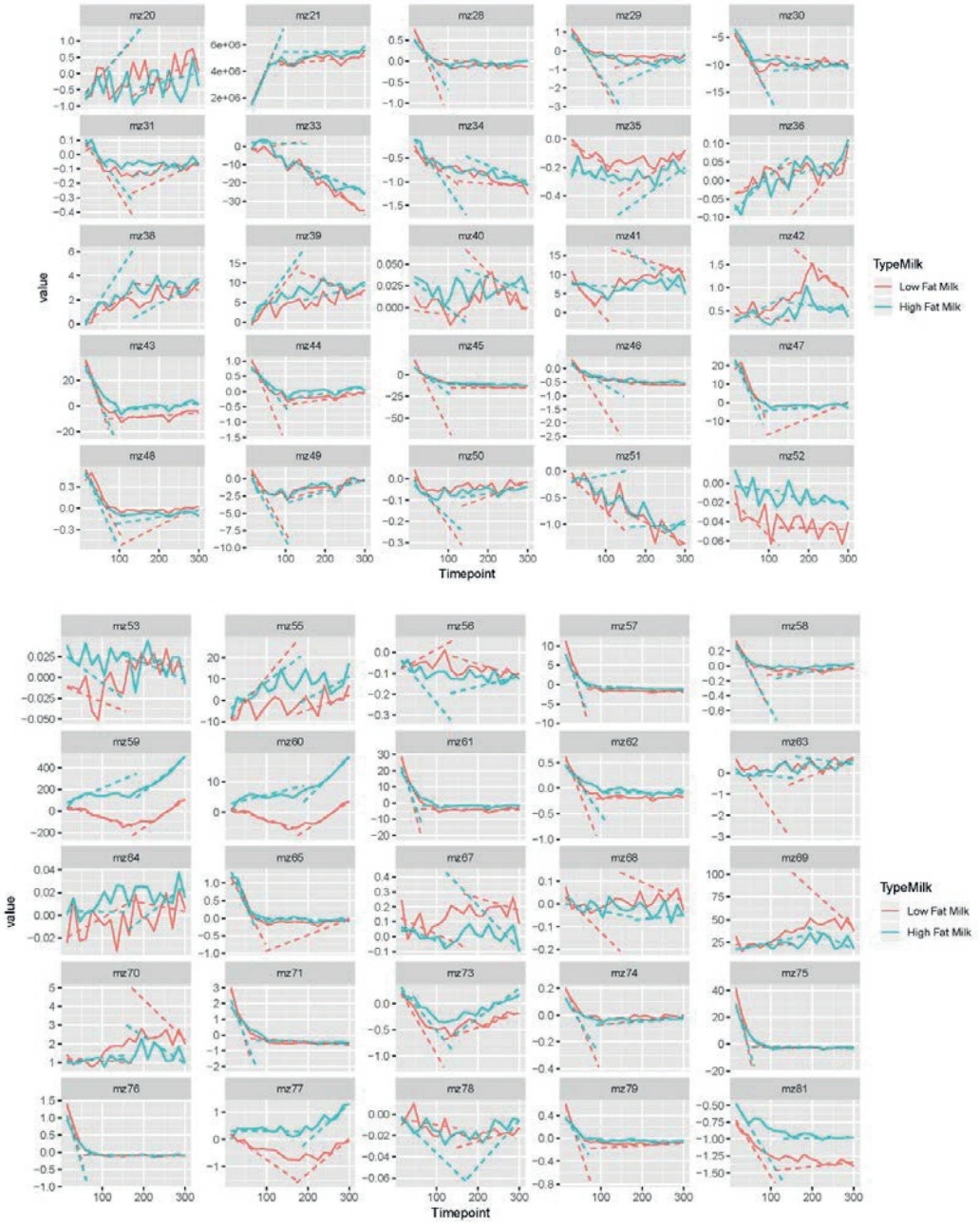
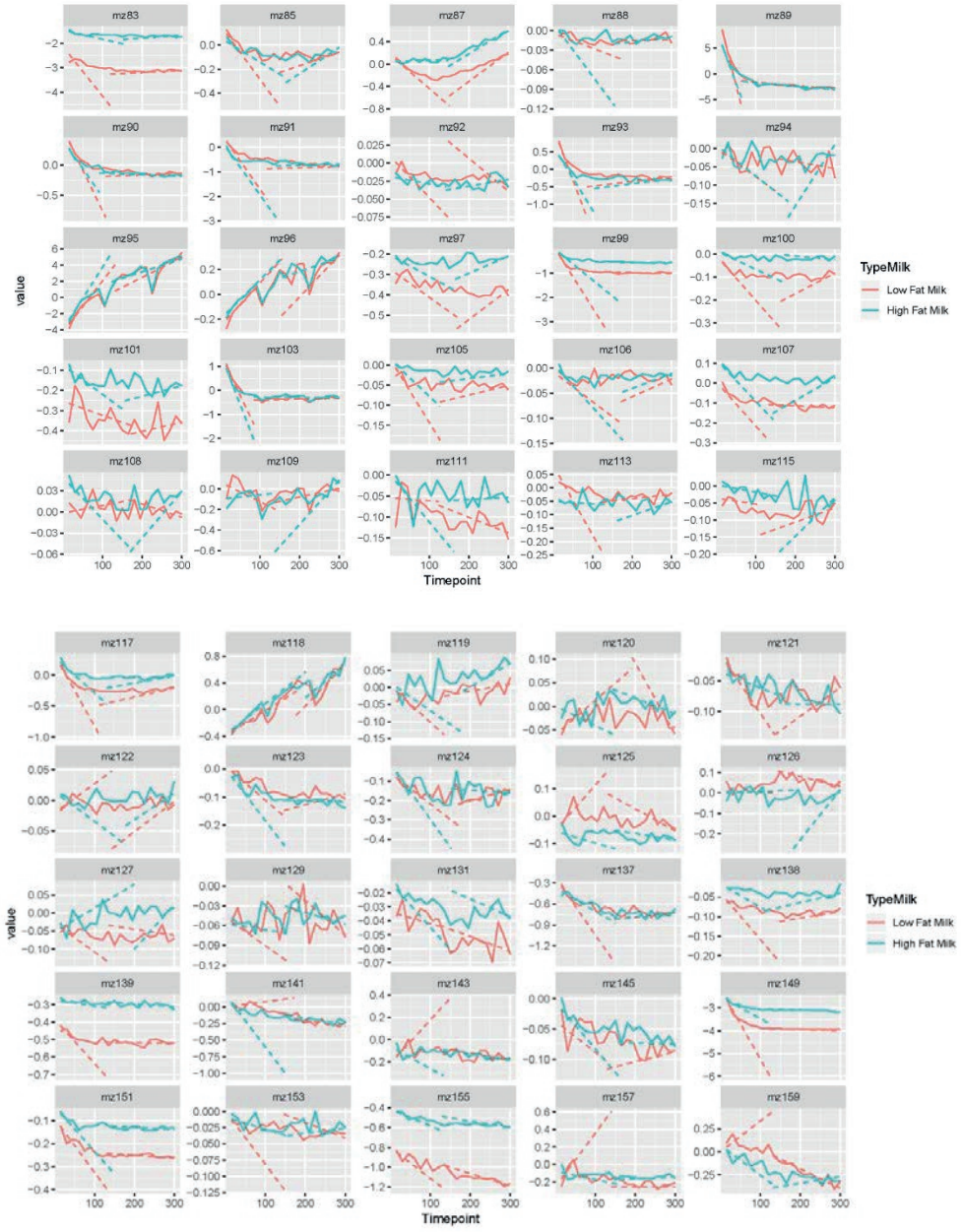
Supporting information





Supplemental Figure 1. Overview of segmented regression lines of VOCs measured in exhaled air after consumption of high-fat and low-fat dairy drinks, from 15 – 300 minutes postprandial. The green lines represent the high-fat dairy drink and the red lines represent the low-fat dairy drink, averages of 12 subjects are shown.

Chapter 7

General Discussion

The aim of this thesis was to get some insight in whether an infant formula (IF) with a fat blend containing anhydrous bovine milk fat affects digestion, absorption, and metabolism, compared to an IF containing a fat blend consisting of vegetable fats only. The second aim was to study whether the analysis of exhaled air can be used to assess metabolism of fat. Bovine milk fat is already used in the fat blend of some IFs, in different concentrations.[1] So far, no studies have been reported to study the effect of an IF with anhydrous bovine milk fat on infant's health compared to a similar IF with only vegetable fats. In our studies we used the maximum realistic percentage of bovine milk fat that can be added to IF; i.e. 67%. Our hypothesis was that differences in the fatty acid (FA) composition and triacylglycerol (TAG) structure between the fat sources would result in a difference in digestion, absorption, and postprandial metabolism. The main findings of this thesis are represented in **Figure 1**.

Results of this thesis

This thesis showed that the differences in FA profile and TAG structure between an IF with 67% bovine milk fat and an IF with 100% vegetable fats (described in **chapter 2**) did not influence total digestion, *in vitro*. The total amount of FA released was similar for both IFs. However, as shown in **chapter 3**, the difference in FA profile and positioning of fatty acids (FAs) at the glycerol backbone did influence the release of type of FAs during digestion. When such IF products were administered to healthy adults no differences were found in chylomicron concentrations or content (**chapter 4**). This indicates that the differences in free fatty acid (FFA) profile did not influence total absorption, in healthy adults. The particle size of chylomicrons was slightly increased after consumption of bovine milk fat. However, this was not related to lipid content, so this is probably caused by other factors such as apolipoproteins or minor components in chylomicrons, like fat-soluble vitamins. The lipoprotein metabolism was not affected by the difference in fat source, indicated by similar concentrations of VLDL, LDL, and HDL. However, after consumption of the IF containing 67% bovine milk fat the concentration of ketone bodies in the serum was immediately increased. This might indicate that short-chain fatty acids (SCFA) and medium-chain fatty acids (MCFA), present in bovine milk fat, are indeed released from the TAGs very fast, followed by a rapid absorption and transportation to the liver for oxidation.

Furthermore, the difference in FFA did affect satiety, as described in **chapter 5**. After consumption of the IF with 67% bovine milk fat, the feeling of fullness and prospective consumption were prolonged compared to the IF with 100% vegetable fats. The different fat sources did not result in a different postprandial energy expenditure; the diet-induced thermogenesis (DIT) was similar after consumption of the two different products. So this did not explain the difference in satiety. The postprandial responses of the gastrointestinal peptides secretin and acylated ghrelin were influenced by the different fat sources. Concentrations of acylated ghrelin were less suppressed after consumption of bovine milk fat. Since, ghrelin is mainly acylated by MCFA, the higher concentrations of MCFA in the IF with bovine milk fat, compared to the IF with the vegetable fat blend, may explain the difference in acylated ghrelin concentrations after consumption of the two different fat sources. As acylated

ghrelin is thought to stimulate food intake, this difference cannot explain the prolonged satiety that was observed. Secretin was increased after consumption of bovine milk fat, and might have played a role in the prolonged satiety via activation of the nervus vagus.

The second aim of this study was to assess the suitability of the method of breath analysis for nutritional intervention studies, to get more insight in the metabolism of fat. **Chapter 6** described the human trials that were performed. With a first study we showed that consumption of a meal resulted in a change in volatile organic compound (VOC) profile in exhaled air. It also provided insights in the inter- and intra-subject variation. Despite a specific target population we found substantial differences in VOC profiles of different participants. Also, although the total postprandial VOC profile was similar on different study days, for some m/z values we observed quantitative differences. In a second study we indicated that two different meals, low-fat and high-fat, had a different effect on the VOC profile, indicating that the method is suitable to detect differences in dietary exposure. VOCs that were affected could be linked to lipid metabolism.

This thesis showed some differences in postprandial metabolism after consumption of different infant formulas containing different fat sources. It seems that these differences were mainly due to the difference in FA profile and not due to differences in TAG structure between these fat sources. SCFA and MCFA may play a major role in the differences that were observed.

Potential role of SCFA and MCFA

Effects of butyrate

The main SCFA present in bovine milk fat is butyrate (C4:0), which is also present in human milk.[2,3] SCFA are known to be easily absorbed and transported to the liver for fast oxidation. Most knowledge about SCFA is derived from research on SCFA produced by microbiota. Less is known about the effects of dietary SCFA on health. A limited number of studies examined the effect of oral butyrate on metabolic disorders.[4,5] Oral supplementation, but not intravenous administration, of butyrate decreased food intake.[5] Long-term supplementation of butyrate prevented development of obesity and increased insulin sensitivity.[4,5] This was probably related to an increased fat oxidation and activation of brown adipose tissue (BAT).[4,5] Furthermore, butyrate concentrations similar to those found in breast milk reduced inflammatory and allergic responses.[6] An *in vitro* study showed that butyrate had inhibitory effects on inflammation and lipolysis in the interaction between macrophages and adipocytes.[7] The presence of butyrate in human milk was negatively associated with infant weight and change in BMI between 3 and 12 months, and BMI at 12 months of age.[8] Prentice *et al.*[8] hypothesized that this might be due to increased thermogenesis by activation of BAT. Butyrate might also have direct effects on vagal afferents, and may induce satiety via this pathway.[9,10]

However, it is not known whether butyrate present in bovine milk fat can exert these effects in humans. Gastric lipase is known to have a preference for shorter-chain FAs,[11,12] and SCFA can be absorbed by gastric cells.[13] The results of the *in vitro* digestion study (**chapter 3**) showed

that not all butyrate of bovine milk fat and human milk fat was released in the gastric phase, but that is was partly released during the duodenal phase as well. This might indicate that *in vivo* butyrate is also only partly released in the stomach, and part might reach the small intestine. In the small intestine, butyrate is quickly absorbed from the lumen,[14] since it does not need to be incorporated into micelles. Perhaps its presence can act upon vagal afferent fibres and enteroendocrine cells. After absorption from the lumen SCFA, such as butyrate, can be used for oxidation in the enterocytes or can be transported to the liver for oxidation.[15] In the MELC study (**chapter 4**) we showed that directly after consumption of IF with bovine milk fat concentrations of ketone bodies increased. This might have been caused by beta-oxidation of SCFA and MCFA in the liver. However, whether butyrate from bovine milk fat reaches other peripheral tissues and can act upon them is not known. The role of butyrate in IF on infant's health should be further investigated.

Effects of MCFA

MCFA are not only present in bovine milk fat, vegetable fat also contain MCFA. However, the concentrations in bovine milk fat are much higher. Therefore, more MCFA are released from bovine milk fat compared to vegetable fat. This might play role in the differences we observed between the two IFs. For example in the difference in acylated ghrelin concentrations we found after consumption of the IFs. The higher concentrations of MCFA, especially caprylic acid (C8:0), might be the reason for this difference. Just like SCFA, MCFA can be absorbed directly and are transported to the liver via the portal vein for oxidation.[15] Consumption of medium-chain TAGs (MCT) is known to stimulate DIT, compared to consumption of long-chain TAGs (LCT).[16–19] Longer-term studies also show a reduced body weight after consumption of MCT vs LCT.[20–23] Van de Heijning *et al.*[23] studied the effect of high levels of MCFA, especially C8:0 and C10:0, in an early diet in two animal studies. After the early diet, mice and rats were fed a western-style diet. High levels of MCFA reduced the fat mass in adult mice with almost 30%. No differences were found in adipocyte number, but size was decreased.[23] This is in line with findings from Baba *et al.*[24] It seems that MCFA stimulate lipolysis, either via increasing the level of lipoprotein lipase,[22,25] and/or via activation of cAMP-dependent protein kinase in white adipose tissue.[22]

The presence of MCFA in the diet might also have an effect on microbiota and pathogens. MCFA were found to stimulate the growth of Bifidobacteria and Lactobacilli *in vitro*.[26] Especially C10:0 showed to have an antibacterial function against specific bacteria *in vitro*.[27,28] Whether addition of MCFA to IF, via bovine milk fat, also exerts these effects in infants should be studied.

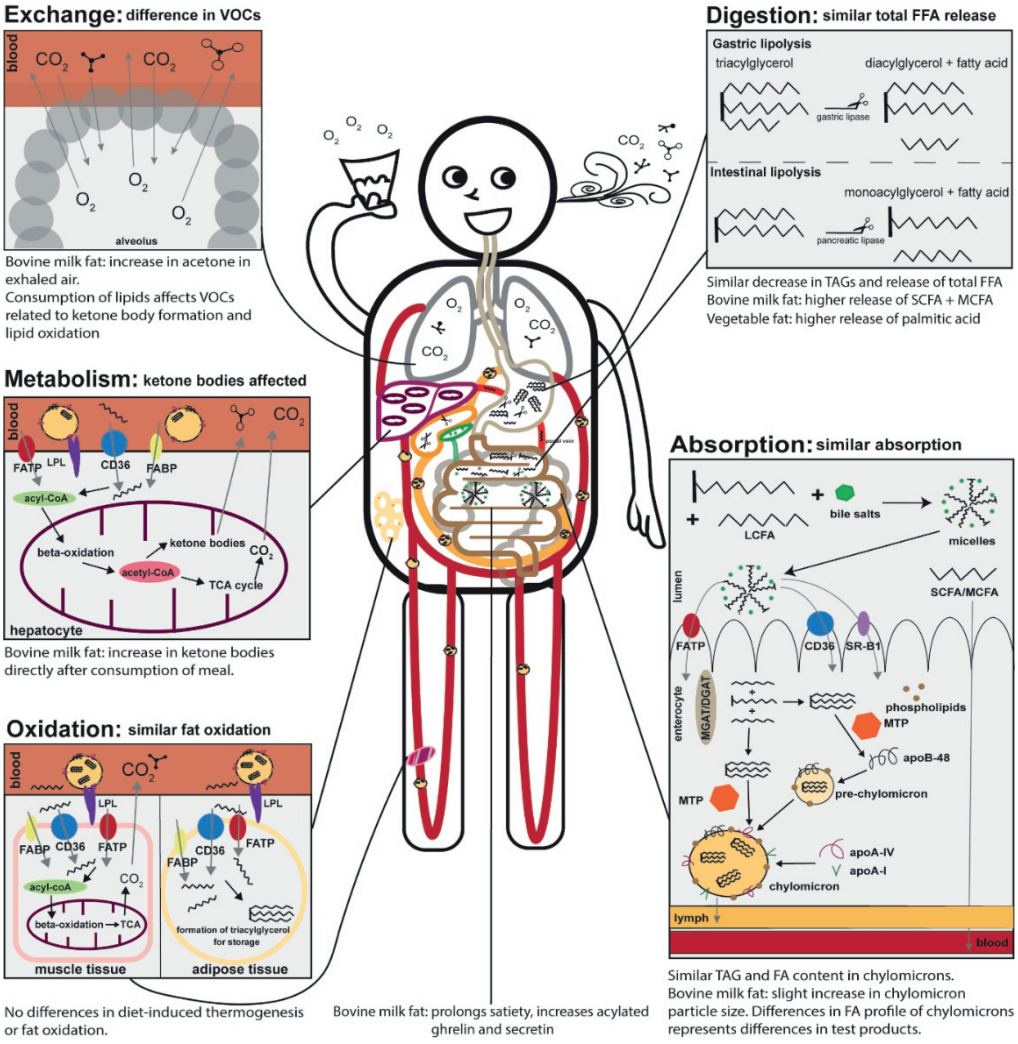


Figure 1: Main findings of this thesis

Translation of findings from adults to infants

To study the effects of nutrition on metabolism in healthy infants is challenging, since the use of invasive methods is restricted due to regulations[29,30] and ethical restrictions. Therefore, in this research the effects of the addition of bovine milk fat to IF on lipid and energy metabolism were studied in young male adults. However, these results may not be directly translatable to infants, since there are some differences between adult and infant physiology.

Digestion of fat

The lipases that are present in infants are similar to those present in adults. In adults, the main enzyme involved in lipolysis is pancreatic lipase.[31] However, the concentrations of pancreatic lipase, and bile salts, are much lower in new-borns, until the first year of life.[31,32] Therefore, in infants gastric lipase plays a bigger role in digestion than it does in adults.[31] Gastric lipase is active in a broad pH range, but its optimum is at a pH between 5.4 and 5.8.[33] In infants the pH in the stomach and small intestine is somewhat higher compared to adults.[34] In the stomach this is around 5.5,[35] so gastric lipase can act at full capacity. About 10% of the total lipolysis occurs in the stomach.[31] This enables rapid hydrolysis of the TAGs in the intestine,[36] since the release of FAs in the stomach increases the binding of lipase to the fat globule.[36] Another difference between infants and adults is the gastric emptying time. In adults, after consumption of a liquid meal, the gastric emptying half-time is 10-60 minutes, while in infants this is longer with 76-87 minutes for IF and 48-61 minutes for human milk.[37] The gastric emptying rate to the duodenum is therefore higher in adults compared to infants, respectively 2 kcal/min versus 0.63 kcal/min.[37] In the small intestine of infants the pH is around 6.2, while in adults this is around 5-5.5.[37] Since the pH in the small intestine of infants is a bit higher, gastric lipase preserves part of its activity there.[33] In this way the lower concentrations of pancreatic lipase are partly compensated. Although in infants the intestinal motility is not mature yet, the transit time is close to that of adults.[37]

Although there are differences in fat digestion between infants and adults, it is a very efficient process in both age groups. The results of our *in vitro* study (**chapter 3**) indicate this efficiency as well. Therefore, it is unlikely that the differences in the digestion process between adults and infants will influence the release of FAs. Our *in vitro* results also indicate that the difference in fatty acid composition and TAG structure do not affect total lipolysis.[38,39] Other studies showed no differences in gastric emptying in infants after consumption of MCT and LCT containing products,[40,41] therefore it is not expected that the difference in fatty acid composition and TAG structure affect gastric emptying. The size of the milk fat globule probably has a much larger impact on gastric emptying.[42,43]

Absorption of fat

The fat absorption in infants is a bit lower compared to adults, however already very efficient.[44] In healthy adults less than 5% of the dietary fat is excreted in feces.[31] In term infants fed standard IF, the fat absorption is around 90%.[45–48] The intake of fat per kilogram bodyweight is three to five times higher in infants compared to adults.[31] So the enterocytes of infants seems to be very capable of handling the absorption of the large lipid load. The consumption of fat by infants increases the expression of apoliprotein-B48 and microsomal triglyceride transfer protein (MTP) in both the ileum and jejunum.[31] So just like adults, infants produce chylomicrons to deliver lipids to peripheral tissues.[31] Since the fat absorption in infants is slightly lower compared to adults, probably in infants somewhat less FAs will be absorbed from IF compared to adults, but likely the difference will be very small. The process of absorption and transportation seems to be similar for infants and adults.

Energy metabolism

Since infants need a lot of energy for growth and development, there is a well-known difference in energy requirement per kilogram bodyweight between infants and adults.[49] In the first 6 months of life, the growth of an infant is most intense, and the energy requirement is around 460 kJ/kg bw/day, while at 1 year of age this is reduced to about 335 kJ/kg bw/day.[50] In comparison, the basal energy requirement for an adult is 105 kJ/kg bw/day.[51,52] Not only the total energy requirement differs between adults and infants, there are also differences in the tissues that require that energy. In infants, most of the energy is needed for the brain, 53% of basal metabolism, while in adults less than half of that is required, 24% of basal metabolism.[53] The tissue with most energy requirement in adults is skeletal muscle tissue, which take up only 8% of basal metabolism of infants, while in adults this is 28%.[52,53]

The thermic effect of food, DIT, is the increase in energy expenditure after consumption of a meal that is needed for processing, storing, and the metabolic effects of the nutrients.[54,55] Just like basal energy metabolism, DIT also seems to decrease with age.[54,56] With increasing age of school children the thermic effect to a similar bolus of glucose already decreased.[53] BAT partly contributes to DIT.[55] BAT is filled with mitochondria which harbour uncoupling protein 1 (UCP1) in their inner membrane, which facilitates thermogenesis.[57] The presence of fatty acids released from lipoproteins trigger thermogenesis in brown adipocytes.[58,59] BAT is present in all infants, and is known to decrease with age.[53] So, probably in infants the effects of fat on DIT is larger compared to young male adults. Thus perhaps in infants a difference in DIT after consumption of the two different IFs tested can be observed.

Satiety

In a proof-of-concept study, described in **chapter 5**, we studied the effects two IF products with different fat blends on satiety. DIT was found to play a role in this process. Especially hepatic fat oxidation and thermogenesis by BAT are known to play a role in satiety.[21,60] As infants can switch faster from carbohydrate to fat oxidation,[61] and as BAT plays a bigger role in infants, the effects of FAs on satiety might be larger in infants compared to adults.

Since there are physiological differences between adults and infants, the findings of the proof-of-concept study performed in this thesis cannot be translated directly to infants. However, as the processes involved in fat digestion, absorption and metabolism are very similar in infants and adults this study provides some insight in the effects that might occur in infants. A clinical trial with infants should be executed to confirm whether the IFs tested indeed show similar results in infants. With the findings of the proof-of-concept study these trials can be designed in a more targeted manner and provides arguments to make a well-balanced ethical decision.

Non-invasive methods

Intervention studies with healthy children can only be performed if risks and burden are minimal.[29,30] Therefore, invasive methods, like blood sampling, should not be used in healthy infants. To be able to study effects of nutrition in minors non-invasive methods should be established. In the last two decades several wearable sensory systems and intelligent textiles have been developed for infants.[62] Examples are wearable sensor systems that are able to measure electrocardiography,[63] heart rate, respiration, and temperature.[64] Furthermore, sensors for detection of movements and prone position have been tested.[65–68] Furthermore, a device with pressure sensors has been developed to determine infant's grip strength, which is in correspondence with physical development.[69] Trials have been performed in which carbon dioxide (CO_2) sensors were placed in a crib, to monitor respiration.[70] Even intelligent diapers are available, which indicate when a change to a fresh diaper is required.[71] Most of these examples provide information about clinical outcome parameters, but not about metabolic changes.

Breath analysis

The method of breath analysis is used in clinical research settings to examine biomarkers that could predict diseases like asthma, lung cancer, Crohn's Disease, and inflammatory bowel disease.[72–76] Breath analysis is an interesting non-invasive method to study metabolites produced endogenously. Nutrition is thought to result in more subtle changes in exhaled air compared to a different stable physiological state. In the Breath Taking studies, described in **chapter 6**, we examined whether metabolic differences caused by a nutritional intervention are reflected in exhaled air. As discussed in **chapter 6** two drinks differing in fat percentage resulted in differences in the exhaled VOC profile, and these could also be linked to lipid metabolism. A recent study compared the total VOC profile in exhaled air of adults after consumption of two different IFs, which only differed in lipid droplet size and coating.[77] A couple of VOCs were different after consumption of the two products, and are thought to give insight in metabolic effects.[77] Although the protocols should be optimized, breath analysis can be used to get insight in metabolic changes after a nutritional intervention. This non-invasive method has already been applied successively to pre-school aged children,[72,78,79] and even in infants.[80–82] A face mask connected to ventilation non-rebreathing valve system connected to a sample bag was used to sample exhaled air of infants.[80,81]

The question is whether the VOCs in exhaled air also reflect the serum concentration of metabolites. Several studies have been performed in which VOCs were measured both in breath and blood samples.[83–86] Breath samples were shown to reflect the serum concentrations of metabolites. In the MELC study, **chapter 4,** we determined ketone bodies in serum as well as acetone in exhaled air. We found that acetone in exhaled air and its precursor acetoacetate in serum are highly correlated, as shown in **Figure 2**. So our findings also indicated that VOCs are a good representation of endogenous metabolites.

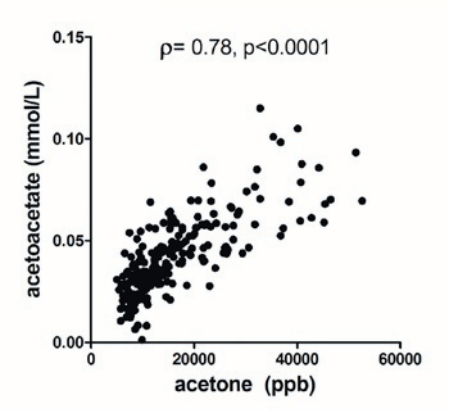


Figure 2: Correlation between acetone in exhaled air and acetoacetate in serum (n=19). A Spearman correlation analysis was performed, and acetoacetate in serum was significantly correlated to acetone concentrations in exhaled air ($p<0.0001$).

Since there is no standardization of procedures and analytical methods, there is a wide variation in results of different studies, which complicates comparison.[87] Several methodologies regarding analysis and data processing are available and used by different research groups.[88] Besides that, also the type of air sample that is collected or analysed can differ. Three different types of exhaled breath can be sampled: late expiratory, end-tidal, and mixed expiratory, as presented in **Figure 3**.[89] A mixed expiratory breath sample represents total exhaled breath including 'dead space air'. To increase the concentration of endogenous VOCs and to reduce that of exogenous VOCs, in late expiratory breath samples the initial portion of exhaled air is discarded so that the dead air is not included. Sometimes this is time-controlled, however, an optimal exclusion time is not known.[89] End-tidal breath samples, also called alveolar breath, contains air from deeper in the lungs, which contains the highest concentration of CO_2 and endogenous VOCs and the lowest concentrations of exogenous VOCs.[90] Most studies sample either late or mixed expiratory breath.[89]

In our studies we used proton-transfer-reaction mass spectrometry (PTR-MS) to analyse samples directly (online) or air sampled in bags (offline). Currently, the most commonly used technique for breath analysis is GC-MS, because of its reliability in identification of compounds.[91,92] Other frequently used methods are sorbent-containing thermal desorption (TD) tubes, solid-phase micro-extraction (SPME), and needle trap devices (NTDs).[89] Disadvantages of PTR-MS are that identification of compounds is more difficult compared to GC-MS. However, with addition of time-of-flight (TOF) this is greatly improved.[93] An advantage of the PTR-MS is that measurements can be performed online, since no pre-concentration step is required, as is the case for the other analysis methods.[91,94] However, the PTR-MS is difficult to move. Offline sampling of exhaled air is therefore more flexible to use in a research setting.

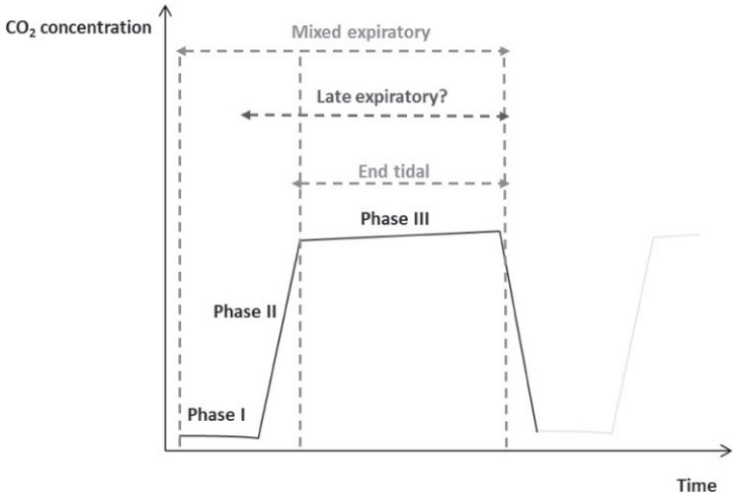


Figure 3: Different phases, with different concentrations of CO_2, in exhaled air, from Lawal *et al.*[89]

To sample air samples for offline measurements, different storage containers are used; polymer bags, aluminium bags, glass vials, and several breath samplers.[89] Especially Tedlar bags are used for sampling, since those are most stable during storage, have the lowest background emission, and can be reused.[95] To what extent offline sampled exhaled air, in Tedlar bags, reflect online samples is not known. We compared offline sampling in Tedlar sample bags with direct online analysis using a PTR-MS. This was part of study 1 which is described in **Chapter 6**. Twelve healthy young males were asked to exhale in a buffered end-tidal online sampling system[96] coupled to a PTR-MS for an online measurement. Directly afterwards, subjects were asked to also exhale into a sampling system which was developed to fill a sample bag. When participants exhaled into this sampling system, a pump was activated which released the first exhaled air in the ambient air, after two seconds the exhaled air was sampled in a Tedlar sample bag. To prevent condensation the sample bags were stored at 37°C. After completion of the study day the VOCs present in the sample bags were determined using the PTR-MS. We observed that most VOCs that were found in the online measurements of exhaled air were also traceable in the sample bags. However, the concentrations in the online measurements were much higher than those in the sample bags, as shown in **Figure 4.** The difference between online and offline measurements could be explained by the loss of VOCs during sampling and storage. Beauchamp *et al.*[97] found that components can be absorbed by or diffuse through the walls on the bag. Another explanation could be the difference in the type of sample. For the online measurement we made use of the buffered end-tidal (BET) sampling system. In this sampler the initial breath, the dead-space air, is quickly removed from the tube. The last 40 ml of the exhaled breath remains buffered in the sampling tube, which enables the measurements of the end-tidal fraction.[96] For the offline sampling, we discarded the first two seconds of exhaled breath, so here we sampled late expiratory. End-tidal samples are known to have higher concentrations of endogenous VOCs compared to late expiratory samples.[90] Furthermore, our off-line sampling

system was not heated, which caused some condensation. Sample humidity is a cause for decline in VOCs concentrations.[95,97]

Figure 5 shows the concentrations of the main VOCs in exhaled air determined online or sampled offline, corrected for baseline concentrations. Although these six VOCs can all be determined in the sample bags, for four components the response of the meal is not detectable. The response of m/z 33 and m/z 59 is very similar for the online and offline measurements. So metabolic changes after a nutritional intervention can also be found with offline sampling. However, the method should be optimized. One way to do this is by using black-layered Tedlar bags.[98] To make sure the sampling system is heated, so no condensation occurs, and to analyse the bags as quickly as possible, at least within 6-10 hours.[95,97]

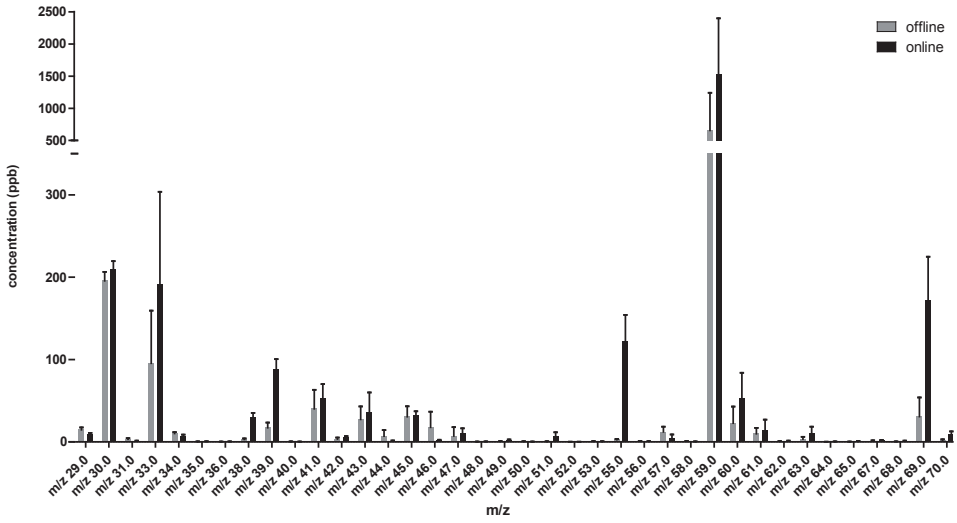


Figure 4: Average concentrations of volatile organic compounds (VOCs) in exhaled air determined directly online (black bars) or sampled offline in samples bags (grey bars) (n=12).

All in all, breath analysis seems a very promising method to get some more insight in metabolic changes after short-term intake of nutrition. This method would gain even more potential if identification of compounds associated with specific m/z values advances. To improve the comparison of different studies, consensus procedures would be very beneficial. The International Breath Research community is working on a standardized way of reporting breath sampling methods for improved comparability of results.[99,100] In infant trials, a PTR-MS could be coupled to an incubator or face mask for online measurements, or offline samples could be taken. When specific metabolites, such as acetone, are of interest, specific sensors for these components could be developed, and those could be placed in a crib or be used as a wearable sensor. Wearable VOCs sensors have already been developed.[101,102]

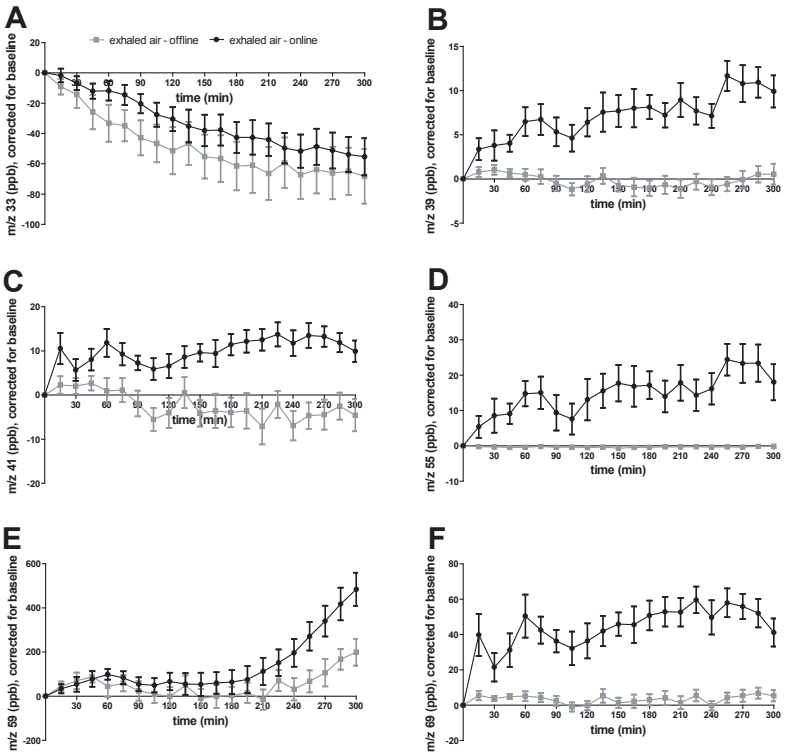


Figure 5: Concentrations of the main VOCs in exhaled air, m/z 33 (A), m/z 39 (B), m/z 41 (C), m/z 55(D), m/z 59 (E), m/z 69 (F), corrected for baseline concentrations, in exhaled air measured directly online (black circles) or measured after offline sampling in sample bags (grey squares) (n=12).

Human study design

In the intervention studies performed for this thesis a cross-over design was used. To study the short-term effects of nutrition this is the preferred design.[103] Since there is high variation in metabolic states between subjects,[104] this can be best accommodated by a cross-over design. However, the within-subjects variations of metabolites can also be substantial.[105] Since nutrition usually induces subtle differences in metabolism it is important to minimize within-subject variation.[106] As exercise influences the metabolic rate, we tried to minimize variation caused by differences in exercise by asking subjects to restrain from exercise the day before a study day. Furthermore, recreational drugs, such as alcohol and cannabis, affect metabolism,[107–109] therefore subjects were also asked to restrain from alcohol and drugs. To reduce effects of dinner preceding a study day, we supplied a standardized dinner. After consumption of the dinner participants were asked not to eat and drink anything, except for water until 10 pm. In this way we tried to get subjects in a similar metabolic condition, fasted. In order to avoid diurnal variations in metabolites, study days were started at the same time of the day.[110,111] Even though these restrictions were applied, the baseline concentrations of the different study days

of the MELC study were still different for some outcomes (**chapter 5**). Furthermore, the Breath Taking study showed that there were differences in VOC response to the same meal on different days (**chapter 6**).

Winnike *et al.*[106] studied the effect of dietary standardization on variation in serum metabolome for 14 days. A single day of dietary standardization appeared to be sufficient to reach some normalization.[106] Fasting glucose and lipid concentrations were more normalized after 24 hours on a standardized diet.[106] In the MELC study, we only standardized the dinner on the evening before a study day. This resulted in similar fasting glucose and lipid concentrations on both study days. However, basal concentrations of gastrointestinal peptides were different on the two different study days. In order to further minimize within-subject variation perhaps not only the dinner should be standardized on the day before a study day, but the whole diet on the preceding day should be standardized. This could be done by handing out breakfast, lunch, dinner, drinks, and snacks to participants, or the subjects can be asked to come to the research facility to consume all the meals there. Perhaps having the different study days on the same weekdays might also reduce variation, since the activities on the day before are more likely to be similar. However, this can increase the difficulty of planning an intervention study. The ideal situation would be to have subjects present at the research facility the entire day prior to a study day, so that diet, activities, and duration of rest can be controlled. Another option is to use more methods to check compliance of the subjects to the provided guidelines, such as an ethanol measurement before a study day or providing accelerometers. However, these options increase the burden for participants, and are costly, and therefore not often used. To sum up, it is difficult to find a balance between the perfect study design, the burden for participants, and the costs.

Data analysis

When more than two serial measurements are taken in a study, a cross-over design brings more complicated analysis.[112] There are numerous options of statistical methods that could be used. Since the serial time points are not independent it is inappropriate to test all time points separately.[113] A commonly used and accepted method is to derive a summary measure from the time series, such as a slope, area under the curve (AUC), or maximum peak.[103,113] In this way a more straightforward statistical method, such as a paired t-test, can be applied. For pharmacological studies and for example oral glucose tolerance tests this works very well, as one peak is seen in time, and thereafter concentrations return to baseline. However, effects of a meal mostly show different sorts of responses with time. When a metabolite shows a similar profile in time after two different nutrition intervention then using a summarizing measure, like AUC, is reasonable. However, when a delayed or different response over time is found, the use of one summarizing measure does not give a representative picture of the data. In such cases the AUC could be similar, but the response in time very different. To be able to get a better representation of the complete data, multiple summarizing measures could be used, such as a combination of AUC, maximum peak, and time to peak. Since each participant has a typical curve which can be different from the mean, the summarizing measures should be calculated

per participant, and then all participants together can be tested with a paired t-test or Wilcoxon signed rank test.[113]

More sophisticated methods that can be used for analysis of cross-over design studies with repeated measures are repeated measures analysis of variance (ANOVA) or mixed models. These methods provide results that are more difficult to understand compared to summary measures, and therefore more difficult to use and to interpret the data.[113] Repeated measures ANOVA can be used to compare group means of a dependent variable across time points.[114] Time can be used as the within-subject factor and a participant specific variable as between-subject factor. Time-based data, however, do not always confirm to the assumption of sphericity: the assumption that the variances of the differences between all combinations of related groups are equal.[115] When this assumption is not met this will increase the Type I error, which results in false positives.[115] When using a cross-over design the number of subjects and time points should also be taken into account when choosing for an repeated measures ANOVA. A high number of time points and a relatively low number of subjects might result in a higher within-subject than between-subject variation. Another disadvantage of the repeated measures ANOVA is that it cannot handle missing data.[114,115] In case of missing data, a subject will be removed from the data set entirely. Another method, that is able to deal with missing data, is the mixed model. General linear mixed models use group means as fixed effects and individual subject variables as random effects.[114] The main differences between these two methods is that repeated measures ANOVA puts weight on quantitative differences between time points, and on between-subject differences across time, while mixed model puts emphasis on patterns of change and individual differences. A combination of summarizing measures and mixed model can also be used. For the Breath Taking study we used segmented regression to model each component into two separate linear regression lines with a flexible breakpoint. In this way an intercept and slope for both lines were calculated. These were then compared between the two treatments with a mixed model.

Despite all concerns mentioned above, the most important thing for the design of a human study is of course the research question. By clearly formulating this, the most suitable outcome measure and statistical method can be chosen. It is advisable to include a statistician early in the design of a human study, so that the optimal amount of measurements and subjects can be included.

Further research

Options for infant trials - To study the effect of addition of anhydrous bovine milk fat to IF on infant health, ultimately trials with infants should be performed. A cross-over design in which infants consume both an IF containing bovine milk fat and an IF without bovine milk fat could be conducted. As indicated above, there are severe limitations regarding invasive methods. Therefore, it is difficult to determine effects on metabolism in infants. As we have indicated, breath analysis might be an interesting method to study effects of nutrition on metabolism in infants. This could be combined with a more classical clinical trial in which faeces is sampled. The fatty acid levels in faeces can be measured, and can give an indication of the FAs excreted,

and thus indirect of the FAs absorbed. Furthermore, the calcium soaps should be determined to check whether the FAs are excreted as insoluble soaps. Both MCFA and the TAG structure have been shown to stimulate the growth of Bifidobacteria and Lactobacilli.[26,116] Therefore, faecal samples could also be used to study the effects of bovine milk fat on microbiota. Besides these analyses in faeces, it would be interesting to study the short- and long-term effect of consumption of IF with bovine milk fat on VOC profile in exhaled air, to get some insights in metabolic effects. For example, acetone concentrations could be studied. Furthermore, it would be interesting to use other non-invasive methods to get some insight into the comfort of infants, such as sensor to assess bowel movements, cramping, and crying time.

Non-invasive measurements – In this thesis we established that breath analysis can be used to study effects of nutrition on metabolism. There are also other biofluids which are a good source of biomarkers.[117] Urine is regularly used in nutritional studies to study metabolites, however, in infants is it more complicated to sample this compared to adults. Other biofluids might be of more interest for clinical trials with infants. For example, tear fluid can be used to perform proteomic and lipidomic analysis.[118] Saliva is not only a good source of DNA, but can also be used to determine oxidative stress levels[119] and the concentration of the stress hormone cortisol.[120] The latter one can also be determined in sweat.[121] Wearable patches have been developed to measure several metabolites in sweat.[122,123] Mineral status can be studied via nails or hair samples.[117] Dried blood spot sampling, which is less invasive compared to venous blood sampling, can be used for several measurements of e.g. fatty acids, amino acids, and immunoglobulins.[124] It is of interest to study biofluids that can be sampled non-invasively even further for their use in nutritional intervention studies.

Ketones - The effect of ketone bodies on growth and development are not well known. Increased concentrations of ketone bodies might be beneficial, since they can act as energy source for the brain. For patients with epilepsy or Alzheimer's Disease favourable effects on cognition have been found after consumption of a ketogenic diet.[125–127] So perhaps increased concentrations of ketone bodies can also be beneficial for cognitive development. It is of course very difficult to study this in infants. Therefore, an animal study could be executed to study the effect of a bovine milk fat-rich diet compared to a diet based on vegetable fats without SCFA and MCFA in early life on concentrations of ketone bodies, which can be determined non-invasively in exhaled air, and the relation with cognitive function, which could be studied via e.g. the intellicage system, object location memory task, and radial-arm maze procedure. In a observational human study in which cognitive development is studied, ketone bodies could be determined, in serum or exhaled air, to examine whether a correlation is present.

LCSFA positioning - In the MELC study, described in **chapter 4,** we found that the FA profile of the chylomicron-rich fraction of plasma reflected the FA profile of the test drink. We did not test the positioning of the FAs at the glycerol backbone in these samples. However, the positioning of LCSFA on the glycerol backbone seems to be contained during absorption.[128] Since in human milk almost all LCSFA are positioned at the sn-2 position, there might be a reason behind this. Unesterified LCSFA have been found to have pro-inflammatory effects on peripheral tissue cells,

while this was not found for oleic acid.[129–134] Therefore, the positioning of LCSFA on the sn-2 position and oleic acid at the outer positions might prevent inflammation in peripheral tissues.[135] It would be interesting to test this using specific fatty acids or specifically designed TAGs *in vitro* or in an animal intervention. Another option is to conduct another proof-of-concept study with adults, in which TAG structures as well as inflammatory markers could be determined in serum.

Satiety - As discussed above we observed that satiety is prolonged after consumption of an IF containing 67% bovine milk fat. In our study we determined satiety with the use of visual analogue scales (VAS). Our study set-up was not suitable to study the effects of this prolonged satiety on food intake. A follow-up study with adults could be performed to study the food intake e.g. 120 minutes after consumption of the two different IFs. Whether the use of bovine milk fat in IF also affects eating behaviour and weight development in infants could be studied in a longer term clinical trial in which energy intake, growth, and weight can be followed. To study eating behaviour of infants a questionnaire has been developed which can be filled in by caretakers.[136] However, the intake of IF is heavily influenced by caretakers.[137] Therefore, caretakers should be instructed to pay attention to satiation cues to let the infant control the intake.

Conclusions

Overall, this thesis showed that the TAG structure and minor component composition of bovine milk fat is more similar to human milk fat than vegetable fat. The difference in TAG structure influenced the type, but not the total amount of FA released during *in vitro* digestion. Furthermore, the total absorption of the FAs and energy expenditure were not affected, in healthy adults. The differences in the fat blends with either 67% bovine milk fat and 33% vegetable fat or 100% vegetable fat resulted in some differences in postprandial metabolism. Chylomicron size was slightly higher after consumption of bovine milk fat. However, this was not related to an increased lipid content. The FA profile of the chylomicrons reflected the FA profile in the IFs. Concentrations of ketone bodies were increased immediately after consumption of the IF with bovine milk fat. These differences in the FA profile in the chylomicrons and concentrations in ketone bodies might have different effects on peripheral tissues. It needs to be determined whether this is the case and whether this has any health consequences. Furthermore, the addition of bovine milk fat to IF prolonged satiety, and increased concentrations of secretin and acylated ghrelin. So the use of bovine milk fat in IF might affect the eating behaviour of infants, and perhaps may reduce excess energy intake. It is tempting to speculate that this may contribute to the prevention of overweight and obesity later in life.

In addition, this thesis showed that the non-invasive method of breath analysis can be used in nutritional studies to provide insight in metabolism, although reduction of day-to-day variation is necessary, and more studies should be performed to get additional insight in this.

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Summary

Human milk is the best nutrition available for infants. However, in some situations breastfeeding is not an option. In that case, infant formula (IF) is the best alternative. An important component of IF is fat, which delivers about 50% of the energy for an infant. With the digestion of fat, free fatty acids are released. Those fatty acids are absorbed into the epithelial cells and are either oxidized, transported to the liver via the portal vein or packed into chylomicrons to be transported to peripheral tissues and the liver. In the peripheral tissues, the fatty acids can be used for beta-oxidation or storage.

Different fat sources are used in IF; mostly a combination of vegetable fats is used. Nowadays bovine milk fat is used more often in IF. Because of the preferred level of linoleic acid, maximal 67% bovine milk fat can be used in a fat blend for IF. When using bovine milk fat a wider variety of fatty acids is introduced, including short- and medium-chain fatty acids (SCFA and MCFA). Furthermore, the triacylglycerol (TAG) structure of bovine milk fat differs from that of the in IF common used vegetable fats. Research focused on specific lipids has shown that a difference in fatty acid length may impact the activity of gastrointestinal lipases and therefore lipolysis. Furthermore, medium-chain TAGs have been reported to increase energy metabolism and satiety. The TAG structure has been reported to be important for absorption of fat and may also affect postprandial metabolism. Whether bovine milk fat in IF exerts different effects on digestion, absorption, and metabolism, compared to vegetable fats, is not known yet. Therefore, the **first aim** of the thesis was to study the digestion, absorption, and metabolism of different fat sources used in infant formula; anhydrous bovine milk fat and vegetable fats.

Volatile organic compounds (VOCs) in exhaled air can reflect metabolic aspects of physiology, such as specific diseases. Some indications exist that VOCs may also reflect metabolic effects of nutrition. Since it is difficult to perform invasive studies in infants, analysis of VOCs in exhaled air could provide a non-invasive approach to study effects on metabolism of the addition of bovine milk fat to IF. This was explored in the **second aim,** which was to study whether the analysis of exhaled air can be used to assess metabolism of fat.

A clear overview of differences and similarities between human milk fat and fat sources used for IF is lacking. Therefore, in **chapter 2**, we reviewed the fatty acid composition and triglyceride structure of human milk fat, vegetable fats, and bovine milk fat. Furthermore, an overview is provided of the known and potential health effects of bovine milk fat components that have been described in literature.

Whether using bovine milk fat in a fat blend for IF, and therefore introducing more SCFA and MCFA, affect lipolysis in a different manner than a fat blend containing solely vegetable fat affects lipolysis is unknown. Therefore, we studied the release of free fatty acids of an IF containing 100% vegetable fat with an IF containing 67% bovine milk fat and 33% vegetable fat, and human milk as described in **chapter 3**. A static two-phase *in vitro* digestion model with infant conditions was used. We did not find any differences in the total level of free fatty acids released from the three different products. During lipolysis more SCFA and MCFA were released from the IF with bovine milk fat, compared to the IF with only vegetable fats, which is in line with the fatty acid profile of the two fat blends. In the IF with bovine milk fat less palmitic acid

was released compared to from the one containing vegetable fat only. From human milk even less long-chain saturated fatty acids (LCSFA) were released. So the difference in fat source in IF did not change the total amount of lipolysis, but it did change the profile of free fatty acids released. The type of fatty acids released is important for their absorption, as LCSFA have the ability to form insoluble complexes with calcium in the lumen, which will be excreted.

SCFA and MCFA have been reported to increase energy expenditure, since they can directly be absorbed from the lumen, independent of transportations via chylomicrons, and transported to the liver via the portal vein, where they can be used for oxidation. Whether the usage of bovine milk fat, and thus the inclusion of more SCFA and MCFA, in IF affects lipid and energy metabolism is not known yet. We studied the effects of an IF containing a fat blend with 67% of bovine milk fat and an IF with a fat blend containing 100% vegetable fat on lipid and energy metabolism. Since invasive methods are needed to get insight in metabolic alterations we performed a proof-of-concept study in healthy young adult males.

In **chapter 4** the results of our study on the effects of the different fat sources on lipid metabolism are described. No differences in absorption, lipoprotein metabolism or substrate utilisation were found. Chylomicron size was found to be slightly increased after consumption of IF with bovine milk fat. However, this was not caused by an increased lipid content. The fatty acid profile of chylomicrons formed postprandial reflected that of the different fat sources consumed. Directly after consumption of the IF with bovine milk fat an increase in circulating ketone bodies was observed. **Chapter 5** reports our finding that using bovine milk fat in the fat blend of an IF prolongs satiety. This was not caused by differences in diet-induced thermogenesis. However, this might be caused by an increased postprandial secretin response, possibly via stimulation of vagal afferent pathways.

With the absorption and oxidation of fatty acids several metabolites are formed. Some of these metabolites are excreted via exhaled air; VOCs. Analysis of these VOCs is being used in clinical settings to determine biomarkers for several diseases, like lung cancer and asthma. This method has also made its way to nutrition studies. The effect of several long- and short-term nutrition intervention studies on VOCs has been explored. One of the challenges for using VOCs analysis in these type of studies is the inter- and intra-subject variability. The day-to-day variation in response to a meal was not studied before. In this thesis we explored the intra-individual variation of VOCs in exhaled air after a meal, to determine whether the measurement of VOCs in exhaled air with the online method PTR-MS could be a suitable method for nutritional intervention studies. **Chapter 6** describes several interconnected human trials that were performed. The first study showed that consumption of a meal leads to a different VOC profile in the five postprandial hours examined. However, it also showed quantitative differences between study days, although trends over time were similar. Therefore, in a second study the effects of two different meals, a low-fat and a high-fat dairy drink, were determined on several days. This study indicated that consumption of different meals resulted in a different VOC profile in exhaled air. VOCs that were affected could be linked to lipid metabolism.

In **chapter 7** the results of this thesis are discussed and put in perspective. The translation of the results obtained in adults to infants is discussed. Furthermore, the complexities of the method of breath analysis are discussed. Recommendations for future research are provided.

The **first aim** of the thesis was to study the digestion, absorption, and metabolism of different fat sources used in infant formula; anhydrous bovine milk fat and vegetable fats. We answered the research questions by conducting *in vitro* lipolysis experiments and *in vivo* proof-of-concept trials in adults. We observed that there is no difference in total lipolysis of IF containing a mixture of bovine milk fat and vegetable fat and IF with vegetable fats. However, the type of fatty acids released were different, which was in line with the differences in fatty acid composition of the IFs. These differences in fatty acid release did not result in a difference in fat absorption rate or energy expenditure. The difference in fatty acid profile was reflected in the fatty acid profile of chylomicrons. Using 67% anhydrous bovine milk fat in IF did prolong satiety compared to an IF with a fat blend consisting for 100% of vegetable fats. Prolongation of satiety might reduce energy intake and eating behaviour in infants. The difference in fatty acid profile in chylomicrons might have different effects on peripheral tissues to which they are presented. More studies are needed to examine those effects.

The **second aim** was to study whether the analysis of exhaled air can be used to assess metabolism of fat. With our studies we provide some insight in the inter-and intra-subject variation. Despite these variations, a different VOC profile could be observed between two different meals differing in fat content. Although more research is needed to reduce the intra-subject variability, the method of breath analysis seems to be a promising method to use in nutritional intervention studies in order to get more insight in metabolism.

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About the author

Curriculum Vitae

Jeske Helena Johanna Hageman was born on June 18th 1989 in Oosterhout, the Netherlands. After graduating from the secondary school (gymnasium) Cambreurcollege in Dongen in 2007, she started the BSc program 'Nutrition and Health' at Wageningen University. She included the minor in 'Nutritional aspects of Immunology' in her studies. In 2010 she received her bachelor degree. She continued with the MSc program 'Nutrition and Health', specialisation 'Nutritional Physiology and Health Status'. She followed courses at the University of Wisconsin in Madison (US). She conducted a MSc thesis in the Nutrigenomics group at Wageningen University, during which she studied the effects of flavanol-rich chocolate on the vascular function, as part of a clinical study. Furthermore, she set up a sensory test to investigate the acceptance of the taste of the flavanol-rich chocolate. She concluded her studies with an internship at the Regulatory and Nutritional Science team of Mead Johnson Nutrition in Nijmegen, at which she performed a literature research on the effects of dietary long-chain polyunsaturated fatty acids on respiratory illness in infants and children, which resulted in a scientific review. In 2012 she received her master degree.

After graduation Jeske started as junior researcher Nutrition and Health at FrieslandCampina Domo in Wageningen. In 2014 she started her PhD project in the Human and Animal Physiology lab at Wageningen University. The PhD project focused on the digestion, absorption and metabolism of different fat sources used in infant formula, and non-invasive methods to study this, under the supervision of prof. Jaap Keijer and dr. Arie Nieuwenhuizen. During her PhD project, she joined the educational programme of the Graduate School VLAG. She attended several (inter)national courses and conferences, and supervised several students during their MSc thesis.

Currently, she is working as Development Specialist at FrieslandCampina, as a team member of the Global Nutrition Development team, which works on the substantiation of health benefits of FrieslandCampina products or ingredients and internal and external communication about those health benefits.

List of publications

Hageman J.H.J., Danielsen M., Nieuwenhuizen A.G., Feitsma A.L., Dalsgaard T.K., Comparison of bovine milk fat and vegetable fat for infant formula: implications for infant health. International Dairy Journal, 2019, 92, 37-49

Hageman J.H.J., Keijer J., Dalsgaard T.K., Zeper L.W., Carrière F., Feitsma A.L., Nieuwenhuizen A.G., Free fatty acid release from vegetable and bovine milk fat-based infant formulas and human milk during two-phase *in vitro* digestion. Food&Function, 2019, 10, 2102-2113

Hageman J.H.J., Keijer J., de Wit B., Tvrzicka E., Nieuwenhuizen A.G. The effect of partly replacing vegetable fat with bovine milk fat in infant formula on postprandial lipid metabolism: a proof-of-principle study in healthy young male adults. *Advanced draft*

Hageman J.H.J., Keijer J., Frencken R.G.G., Homan J.A.A., Nieuwenhuizen A.G. Bovine milk fat in infant formula prolongs satiety: a proof-of-principle study in healthy young male adults. *Submitted*

Hageman J.H.J., Nieuwenhuizen A.G., van Ruth S.M., Hageman J.A., Keijer J., Application of volatile organic compound (VOC) analysis in a nutritional intervention study: differential responses during five hours following consumption of a high- and a low-fat dairy drink. Molecular Nutrition and Food Research, 2019, 63, 1900189

Overview of completed training activities

Discipline specific activities

ISSFAL conference, Stockholm, ISSFAL, 2014

Dairy Technology training, FrieslandCampina, 2014

Dutch Nutritional Science Days, NWO, 2014&2015&2018 *including oral presentation*

R&D Basics/Playfield of R&D, FrieslandCampina, 2014

Training in digestion models, CNRS Marseille, 2014

4th International Conference on Food Digestion, Naples, Infogest 2015

Symposium Immunity in early life, FrieslandCampina, 2015

International Association of Breath Research Summit, Vienna, IABR, 2015

Energy metabolism and body composition course, VLAG, 2016

Workshop ICFD, Wageningen, Infogest, 2016

ICH GCP WMO course, Profess, 2016

ISSFAL conference, Stellenbosch, ISSFAL, 2016 *including posterprentation*

Minisymposium ZOOG, FrieslandCampina, 2016

STW project symposium, FrieslandCampina, 2016

5th International Conference on Food Digestion, Rennes, Infogest, 2017 *including posterpresentation*

WMO/GCP course, GCP central, 2018

5th International Conference on Nutrition&Growth, Paris, N&G, 2018

General courses

PhD week, VLAG, 2014

Reviewing a scientific paper, Wageningen Graduate Schools, 2015

Effective behaviour in your professional surroundings, Wageningen Graduate Schools, 2015

PhD workshop carousel, Wageningen Graduate Schools, 2015&2017

Introduction to R for Statistical Analysis, PE&RC, 2016

Data Management, Wageningen Graduate Schools, 2017

Applied Statistics, VLAG, 2017

Big data in Life Sciences, VLAG, 2017

Scientific Publishing, Wageningen Graduate Schools, 2017

Career assessment, Wageningen Graduate Schools, 2018

Optional activities

Preparation research proposal, 2014

Attending scientific and PhD meeting at Human and Animal Physiology Department, 2014-2019

Colophon

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