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Effects of foods containing lipase inhibitory bioactive substances on triglyceride lipolysis and protein digestibility

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Judit Tormási

Budapest

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Doctoral School:

Name: Doctoral School of Food Science

Discipline: Food Science

Head of Doctoral School: Dr. Livia Simon-Sarkadi
Professor, DSc
MATE, Institute of Food Science and Technology
Department of Food Chemistry and Nutrition

Supervisor: Dr. László Abrankó
Professor, PhD
MATE, Institute of Food Science and Technology
Department of Food and Analytical Chemistry

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Approval of the Head of School Approval of the Supervisor

Table of Contents

List of Abbreviations

Abbreviation	Definition
AA	Amino acid
AQC	6-aminoquinoly-N-hydroxysuccinimidyl carbamate
B&D	Bligh and Dyer method
BAA	Bioaccessible amino acid
BB	Baked beef
BC	Baked carp
BTB	Black tea brew
C	Cream
C17:0 FFA	Heptadecanoic acid
C17:0 TAG	Glycerol triheptadecanioc acid
C19:0 ME	Nonadecanoic acid methyl ester
C19:0 TAG	Glycerol trinonadecanioc acid
CB	Coomassie blue dye
$CHCl_3$	Chloroform
CO	Coconut oil
CVD	Cardiovascular disease
DAA	Dispensable amino acid
DAG	Diacylglycerol
DIAAS	Digestible Indispensable Amino acid Score
DP	Dipeptide
EFA	Esterified fatty acid
FA	Fatty acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
GC	Gas chromatography
GC-FID	Gas chromatography with flame ionisation detector
GL	Gastric lipase
GSO	Grape seed oil
GSP	Grape seed powder
HO	Hempseed oil
IAA	Indispensable amino acid
ISTD	Internal standard
IVD	*in vitro* digestion
IVPD%	*in vitro* protein digestibility
LCFA	Long chain fatty acid
LC-MS	Liquid chromatography with mass spectrometry
LO	Linseed oil
MAG	Monoacylglycerol
MCFA	Medium chain fatty acid
MCT	Medium chain triacylglycerol oil
MeOH	Methanol

MFG	Milk fat globule
MFGM	Milk fat globule membrane
NH_2	Amino group
NR	Nile red dye
OO	Olive oil
PDCAAS	Protein Digestibility Corrected Amino Acid Score
PDO	Protected designation of origin
PL	Pancreatic lipase
PP	Polypeptide
PSO	Pumpkinseed oil
RGE	Rabbit gastric extract
RO	Rapeseed oil
RR	Release ratio
SC	Sour cream
SCA	Sour cream analogue
SCFA	Short chain fatty acid
SDS-PAGE	Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SFA	Saturated fatty acid
SFO	Sunflower oil
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SN	Supernatant
SSF	Simulated salivary fluid
TAG	Triacylglycerol
TFA	Total fatty acid
TP	Tripeptide
USFA	Unsaturated fatty acid
WO	Walnut oil

1. Introduction

The main purpose of foods is to provide energy for humans. Energy is produced through several steps of metabolism from nutrients found in the bloodstream, such as amino acids, simple sugars and fatty acids. In order for these molecules to be metabolised by the human body, they must be efficiently released from their food sources and sufficiently absorbed into the bloodstream during human digestion. As a result, digestion has evolved into a multi-step process capable of effective break down of foods, with a goal to release nutrients available for absorption through the intestinal wall, and transferring these molecules into the bloodstream.

Digestion is a chemical, mechanical and enzymatic process where the efficacy of breakdown is dependent on several factors, e.g., activity of digestive enzymes, presence of antinutritive and enzyme inhibitory molecules, just to mention a few. Moreover, digestion and absorption of nutrients are dependent on the composition and structure of the food matrix in which they are found. Interactions within the food matrix both pre and post consumption may alter the composition of the nutrient pool available for absorption at the absorption site of digestion. These changes are not always predictable, at least not from the information on nutrition labels. Therefore, there is a trend in food science and in nutrition to assess nutrient content of the foods or adjust existing and reliable data based on digestibility and absorption properties of nutrients. This trend has already been initiated by the EFSA (European Food Safety Authority), where a Technical report entitled *"Guidance on safety evaluation of sources of nutrients and bioavailability of nutrient from the source*s*"*, published in March 2023, summarises the methods for determining and considering the bioavailability of micronutrients for the development of future guidance.

Although the best and foremost methods to determine bioavailability of food components are still the *in vivo* methods based on human and animal (pig, rat) studies, there is a shift towards more ethically appropriate and methodologically stable means, such as *in vitro* methods. In contrast to *in vivo* methods where bioavailability (nutrients available in the bloodstream to be used by the body) could be determined, the *in vitro* methods are used to assess bioaccessibility (nutrients present in the small intestine that could be absorbed into the bloodstream). Although *in vitro* methods involve some simplification, with the use of well-chosen simulation parameters, they provide relevant and reliable results, which are particularly useful in early-stage research or when determining the availability of substances that are harmful to humans. One of the most commonly used *in vitro* method today is the Infogest method. The protocol is a standardised method, moreover it is the result of an international collaboration, designed to model processes in the first three stages of the gastrointestinal tract: mouth, stomach and small intestine. The results obtained

so far suggest that the method is particularly well-suited for determining the accessibility of both macro-, and micronutrients.

Even though an increasing number of publications use this standard *in vitro* method (Infogest), the published results are not always comparable due to differences in analytical approaches, e.g., the wide range of sample preparation methods and analytes used to determine accessibility of the same nutrients. In addition, the majority of studies so far have focused on qualitative determination, so that the emergence of methods suitable for quantitative determination is still needed. Moreover, until recently most studies only focused on bioaccessibility of individual nutrients ignoring the interaction of nutrients during digestion. Analytical practices providing quantitative data and capable of simultaneous determination of multiple nutrients would not only provide indispensable and useful data on the actual digestibility of foods and certain nutrients, but are also suitable for the specific determination of the effects of influences on digestibility, i.e., food matrix, antinutritive and bioactive substances.

Therefore, my PhD work focused on the development of methods for the fatty acid-specific determination of lipid digestibility and for the amino acid-based determination of protein digestibility and protein quality. Particular emphasis has been placed on the applicability of the proposed methods in determining the effects of bioactive substances and macronutrient interactions. I highlight the versatility of the method through the evaluation of several test foods and dishes.

2. Aims

1) Implementing an integrated platform where the effect of bioactive molecules on bioaccessibility of macronutrients could be simultaneously evaluated. The holistic platform is based on an *in vitro* digestion simulation model matched with specific analytical methods for measurement of each macronutrient bioaccessibility.
2) Creating systematic and easy-to-use routine procedures to serve industrial projects and functional product development by:
    a. Determination of nutritional values and scores (e.g., PDCAAS and DIAAS)
    b. Evaluating effects of known bioactive molecules
    c. Standardization of protocols that are used to describe nutritional value of foods
3) Expanding the knowledge on food science and nutrition, from the point of view of food digestibility.
    a. Lipid and protein digestibility of foods are determined
    b. Contribution of gastric lipase during digestion is evaluated
    c. Effect of certain foods with bioactive compounds on gastric-, and pancreatic lipase activity is tested
    d. Macronutrient interactions are exposed in co-digestion experiments

3. Literature overview

3.1. Overview of gastrointestinal digestion

Foods consumable for humans contain three main nutrient groups, macronutrients: proteins, lipids, and carbohydrates, various micronutrients and minerals, but also antinutrients and toxic compounds could be present. The purpose of the human digestion process is to efficiently break down foods, to extract and absorb required nutrients and to eliminate waste (Hajishafiee et al. 2019). The digestion process includes multiple organs, enzymes and chemical fluids (salt solutions, bile and hormones) each with its distinct function to achieve proper nutrition for humans. In addition, the gastrointestinal tract is the only open-ended vegetative system in the human body thus it is a direct contact between humans and the environment. The organs involved in the system and their functions are shown in the Figure 1.

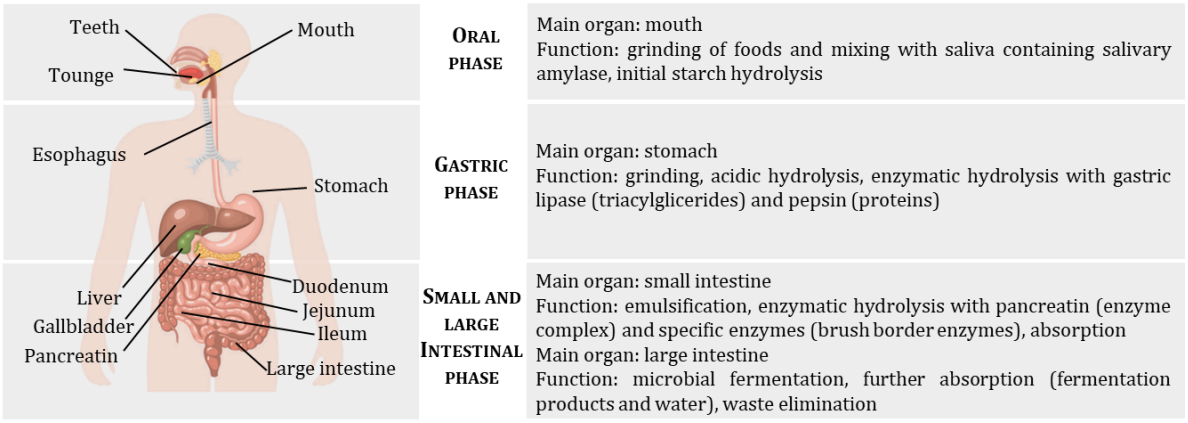

Figure 1. Organs of digestion, main phases of digestion, their main organs, and their functions *(Original image).*

Digestion starts in the mouth. With the help of the teeth and tongue, the bite taken from a food is broken down to get smaller morsels and larger surface area. The chewing is supported by the saliva produced by salivary glands in the mouth which moistens the particles. Saliva also contains the first digestive enzyme, salivary α-amylase, which starts to break down starch molecules by cleaving the α(1,4) interchain bonds creating smaller saccharides (maltose) and dextrines (with α(1,6) bonded side chains). The bolus than is swallowed and through the oesophagus it is transferred into the stomach.

The fasted pH of the stomach is around 1-2. After the bolus arrives in the stomach, the inherently low pH of the organ increases and the stomach wall starts to secrete hydrochloric acid to lower the

pH and to find homeostasis again. In the stomach all processes are dependent on the pH since the enzymes acting here all have different optimal pH. Salivary α-amylase enters the stomach with the swallowed food and at the higher pH it is still active. However starch digestion gradually stops as the pH lowers (ideal pH of salivary α-amylase is 6-7), therefore it is still active at pH 3.8 (Freitas et al. 2018; Mackie et al. 2020). As the pH lowers, protein and lipid digestion become the main processes at play. In parallel to hydrochloric acid, gastric wall also produces the digestive enzymes of the gastric phase, the pepsinogen (pre-enzyme) and gastric lipase. Gastric lipase has an activity range between pH 2-7 (optimum 5-5.4; (Sams et al. 2016), thus initial lipid digestion starts in the stomach. However, the main process in the stomach is the protein digestion by pepsin. Pepsin is activated at pH 2 from pepsinogen excreted by the stomach wall. Activated pepsin then breaks down proteins into peptides. When the swallowed bolus is degraded enough that the particle size of the chyme in the stomach reaches under 3 mm, it could exit through the pylorus into the small intestine.

The small intestine is divided into three main parts with different functions. The first part is the duodenum, this is where the chyme enters from the stomach. Here the acidic gastric secretion is mixed with the alkaline media of the intestine. The pH elevates with the production of hydrogen carbonates until pH 6-6.5 is reached. In the duodenum, pepsin is inactivated. However, gastric lipase might be reactivated after optimal pH is reached. In addition, pancreas secretes a mixture of digestive enzymes into the duodenum, containing trypsin, chymotrypsin, pancreatic amylase, pancreatic lipase, and co-lipase (Mackie et al. 2020). Trypsin and chymotrypsin are responsible for the hydrolysis of the peptides into smaller units, such as oligo-, tri-, dipeptides and amino acids. Pancreatic amylase continues the starch hydrolysis started in the mouth and as a result di- and monosaccharides are formed from digestible starches. Pancreatic lipase hydrolyses lipid (triacylglycerols) molecules with the aid of co-lipase and bile acids (secreted by the gallbladder). Lipid digestion produces free fatty acids, mono-, and diacylglycerides. In the second part of the small intestine, the jejunum, the breakdown of nutrients is continued. In addition to main enzymes, the enterocytes of the jejunum also produce brush border enzymes. These are more specific enzymes only able to cleave specific bonds in specific molecules e.g., maltase, lactase, sucrase. In parallel to the further hydrolytic reactions, absorption of accessible molecules starts as well. The molecules accessible to be absorbed, go through the intestinal wall (by active or passive transport) into the blood circulation. In the last part of the small intestine (ileum), absorption is the main mechanism to happen. The unabsorbed material reaches the large intestine, where water and the further utilizable molecules – that are created by microbial fermentation – are absorbed, and unnecessary materials are eliminated.

3.2. Bioaccessibility, bioavailability, and bioactivity

In order to understand how the foods that we eat affect metabolomic processes of human beings, besides the mechanisms of digestive processes i.e., breakdown of foods into molecules ready to be absorbed, it is necessary to know the steps of nutrition as well.

Depending on the level of nutrition three main definition could be introduced: bioaccessibility, bioavailability and bioactivity (Figure 2). Bioaccessibility gives the amount of molecules in the small intestine that are in a suitable form to potentially pass through the intestinal wall, i.e. to be subjected to passive and active transport processes. The part of the bioaccessible molecules that passed the small intestinal wall represent the absorbed or bioavailable fraction. The concentration of a given compound or its metabolite at the target organ describes bioavailability. Bioactivity is the actual action of molecules that trigger biological functions. In addition, bioefficacy describes the effectiveness of a bioactive compound to impact certain process, i.e., the rate or how fast the effect could be achieved, or the extent of the impact provided by a bioactive. Hence bioaccessibility and bioavailability are prerequisites of the biological effect and thus critical for bioefficacy (Holst and Williamson 2008).

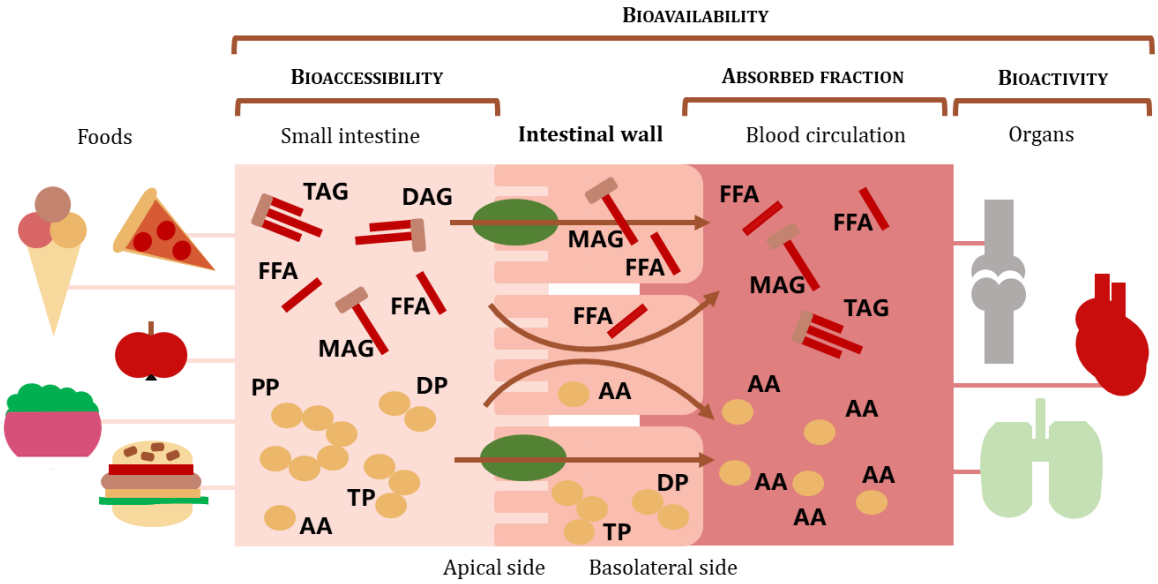

Figure 2. Levels of nutrition: Bioaccessibility, bioactivity, and bioavailability. TAG: triacylglycerols, DAG: diacylglycerols, MAG: monoacylglycerols, FFA: free fatty acids, PP: polypeptides, TP: tripeptides, DP: dipeptides, AA: amino acids *(Original image).*

The evaluation of these attributes is based on different methods. The bioefficacy, bioactivity and bioavailability – since these are defined after uptake – are usually determined with more extensive

in vivo procedures based on human and animal models or various cell and *ex-vivo* cultures (Rein et al. 2013). With all advantages of the *in vivo* methods i.e., processes are observed in physiological conditions, interpersonal differences could be noticed, these are costly, and labour intensive and ethically restricted methods needed to be performed during a long period of time to get consistent data.

More recently, there is shift to *in vitro* methods by assessing bioavailability through bioaccessibility. Since there is a good correlation between the amounts of bioaccessible molecules (present at the site of absorption) and the amounts of bioavailable molecules (passed through the intestinal wall), macronutrient bioaccessibility from *in vitro* digestion simulations is used to determine real nutritional value of foods (Fernández-García et al. 2009). There are several factors influencing real nutritional value of a food, such as composition, structure, pre-treatment (e.g., raw material handling, processing steps, storage, kitchen techniques used), amount consumed, interactions between different food components (e.g., antinutrients in plant materials) or different foods (e.g., eaten as a meal) (Dima et al. 2020).

In my work, the main focus is on the assessment of the bioaccessible fraction of nutrients, most particularly on the bioaccessibility of lipids and proteins. In the next chapters, lipid and protein digestion is explained in detail, methods for modelling digestion simulation to obtain a physiologically relevant bioaccessible fraction are introduced, and analytical tools to determine amount of lipids and proteins are described. Carbohydrate digestion is outside of the scope of my work so hereafter the focus is on lipids and proteins only.

3.3. Digestion of lipids and protein

It is evident that nutrient composition of consumed foods are not necessarily equal of the nutrient composition of the bioaccessible fraction obtained after digestion. In order to more deeply understand the mechanisms guiding lipid and protein digestion, the main focus of this section will be on enzymatic reaction pathways.

3.3.1. Lipids

Generally, every molecule is a lipid if soluble in non-polar solvents but not in water. Lipids could be classified by four factors i.e., physico-chemical properties shown at ambient temperature, polarity, structure and necessity. Foods consumable for humans mainly contain acylglycerides, which are derived lipids, made from fatty acids and glycerol. There are a few different lipids that are a part of human diet, such as phospholipids and sterols, and some of them are necessary for

proper nutrition e.g., cholesterol, phosphatidylcholine and fat-soluble vitamins (Akoh and Min 2002).

Edible lipids can derive from animal or vegetable origin as well. These lipids are primarily consist of triacylglycerols (TAG; >95%, Field and Robinson, 2019). TAGs contain three fatty acids (FA) attached to a glycerol backbone with three ester bonds (Figure 3).

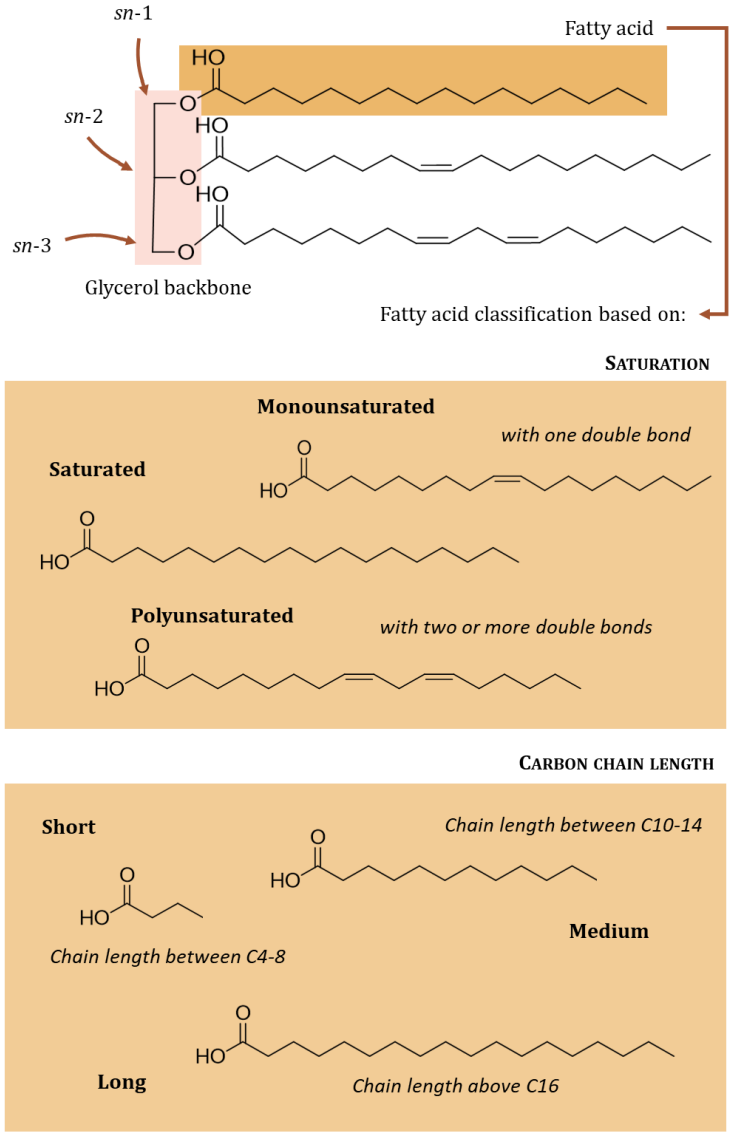

Figure 3. Structure of triacylglycerols (TAGs), nomenclature of fatty acids on triacylglycerols, and classification of fatty acids based on saturation ad carbon chain length *(Original image).*

The physico-chemical and digestive properties of lipids are dependent on fatty acid composition. Fatty acids are classified by i) chain length: short (C4-8), medium (C10-14) and long (C16-)

chained FAs (Zaravela et al. 2021), ii) saturation: saturated (no double bonds in the chain) or unsaturated (monosaturated: one double bond; polyunsaturated: two or more double bonds). Unsaturated FAs could have two conformations, *cis* (side chains are close) and *trans* (side chains are far), which influences the structural and nutritive properties of lipids. Generally, saturated and *trans* unsaturated fatty acids are considered to have negative health effects as they are linked to the development of cardio vascular diseases (Eshak et al. 2018; Longhi 2019).

The goal of lipid digestion (lipolysis) is to release TAGs from food matrix and to hydrolyse TAGs into free fatty acids (FFA) and monoacylglycerol (MAG). Although there is controversy about the effectiveness or even on the existence of lingual lipase, it is generally accepted that it is not a major contributor of preduodenal lipase activity. However some research implies that lingual lipase could be more important in infant digestion before pancreatic lipase activity reaches its optimum. (N'Goma et al. 2012; Brodkorb et al. 2019). Therefore, relevant part of lipolysis starts at the gastric compartment effectuated by gastric lipase (GL), which is responsible for the 5-40% of overall lipid digestion in humans (Lichtenstein and Jones 2012). As mentioned before, GL is secreted by the wall of the stomach and has the optimum activity between pH 2-7, making it an acidic lipase. In order to properly function, GL does not require bile acids or co-lipase. Gastric lipase is a stereoselective enzyme cleaving *sn*-3 fatty acids (FA nomenclature is shown on Figure 3) of the TAG. Depending on the environment, it might show reverse stereoselectivity meaning that in the presence of *sn*-1,2-diacylglycerides (DAG) *sn*-1 FAs, and in presence of *sn*-2-monoacylglicerides (MAG) *sn*-2 FAs could be cleaved from glycerol backbone (Carrière et al. 1997). It is important to mention that gastric emptying time is dependent on fat content (also on protein content) of a meal (Goetze et al. 2007). Foods with higher lipid (and protein) content need more time to pre-digest which slows down gastric motility. Initial breakdown of lipid (and protein) molecules is a main factor in further transition time as well.

After gastric hydrolysis, the pool of partially hydrolysed lipid molecules enters the duodenum and secondary lipolysis starts. Once the pH is settled, pancreatic enzymes (pancreatic lipase and co-lipase) and bile acids (from gallbladder) are activated. Pancreatic lipase (PL) is a stereo-, and regioselective enzyme. Stereoselectivity in this case means *sn*-1 and *sn*-3 preference with an affinity for symmetrical TAGs (regioselectivity) (Benito-Gallo et al. 2015). Pancreatic lipase has further characteristic limitations. Ester bonds only could be cleaved if bile acids and co-lipase are present to aid emulsification and to disrupt the aqueous surface, respectively. All these criteria should be met for efficient pancreatic lipolysis. Free fatty acids (FFAs) and monoacylglycerides could be subjected to transport through the intestinal wall. Short and medium-chain fatty acids, MAGs with short and medium-chain fatty acids and glycerol molecules could be absorbed into the

blood circulation via capillary actions. Longer-chain fatty acids and MAGs with long-chain fatty acids are transported into the small intestinal cells (enterocytes) where triacylglycerol are formed and transported in chylomicrons into the lymph vessels (Pan and Hussain 2012). Lipids provide a rapid energy source and if needed storage, substrates for hormone synthesis and regulate several metabolic pathways (National Research Council (US) Committee on Diet and Health. 1989).

In most foods, lipid digestion is generally high (around 90%) at the end of small intestine (Lichtenstein and Jones 2012), thus studies of lipid digestion mainly focus on i) kinetics, or ii) final extent of hydrolysis at specific end-points of various scenarios, e.g., how the lipid source effects lipolysis, and how to affect lipid hydrolysis with the modification of the lipid source (Duijsens et al. 2022). In my work, I provide examples to prove there is the need for evaluating the extent of lipid digestion on the fatty acid level as well.

3.3.2. Proteins

Proteins are the main dietary source of nitrogen and essential amino acids , which are required by the body for protein synthesis to enable e.g., tissue growth and maintenance (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) 2012). These are complex macromolecules built from amino acids (AA) attached through peptide bonds. All amino acids have the same base structure and they are all zwitterionic i.e., in solution – depending on the pH – amino group can be positively and carboxyl group can be negatively charged. There are several ways to classify amino acids based on i) charge properties, ii) metabolic properties, iii) nutritional necessity, iv) chemical properties. Categorization based on chemical properties – as well as three letter abbreviations – and necessity of amino acids (essential and non-essential) relevant to human nutrition showed on Figure 4.

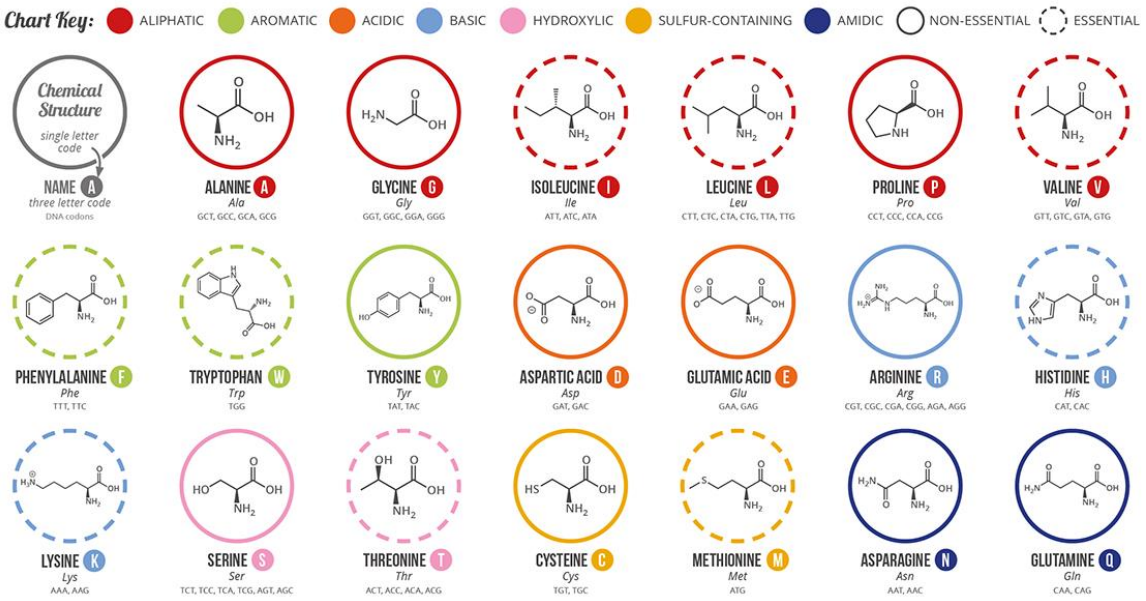

Figure 4. Chemical structure of amino acids, three letter abbreviations and categorization based on chemical properties, and necessity of amino acids (essential and non-essential) relevant to human nutrition (*Adapted from: Compound Interest*).

Amino acids (primary structure) bonded with peptide bonds to form peptide chains arranged into α-helix and β-sheets (secondary structure). The chains linked through unarranged structural parts called random coil (tertiary structure). Tertiary structure is stabilized with secondary interactions (hydrogen bonds, disulphate links, and hydrophobic interactions). Final structure is achieved with the arrangement of multiple tertiary structures (quaternary structure). Proteins – as described at lipids –could have different amino acid composition and structure based on their origin (animal or plant) (Alberts et al. 2002).

During protein digestion (proteolysis) the goal is to disassemble higher structures and release amino acids. Protein digestion also starts in the stomach. The first enzyme involved is pepsin. Pepsin is created from a pre-enzyme (pepsinogen) secreted by the gastric wall by the activation of acidic environment. Optimal pH of pepsin is 1.5-2 (Heda et al. 2022). There is no specific cleavage site known for pepsin however it favours bonds between hydrophobic AAs (Phe, Trp, Tyr, Leu, Met) and disfavours bonds near positively charged AAs (His, Lys, Arg) (Hamuro et al. 2008). Peptides and amino acids formed inside the stomach then enter the small intestine where trypsin and chymotrypsin (from pancreatic juice) continue the protein hydrolysis. Trypsin is also activated from its pre-enzyme called trypsinogen and breaks down proteins into smaller peptides at a specific cleavage site, i.e., between lysine and arginine. Chymotrypsin – activated from

chymotrypsinogen - only acts in the presence of trypsin. It could breakdown both proteins and peptides via preferred pathways involving bonds between hydrophobic AAs (Tyr, Trp, Phe). Specific bush border enzymes (peptidases) are also involved in the proteolysis.

At the end of protein digestion various length of peptide chains could be accessible to intestinal absorption. Presently there is no consensus on the length of peptides able to go through the intestinal wall. It is recognised that small molecules, such as tri-, dipeptides and amino acids are definitely bioaccessible and there are pathways to transport these into small intestinal cells (enterocytes) (Rieder et al. 2021). In the enterocytes, tri- and dipeptides are disassembled and go through with the blood stream as amino acids. Evidently longer peptide chains (5-7 AAs) could be found in the blood stream (Carl et al. 2014) however transport pathways are not identified yet. One way could be through more opened or damaged tight junctions. Tight junctions are openings between intestinal cells which could be loosened by various inflammatory factors. The advantage of this pathway is that bioactive peptides (10-22 AAs) that have ameliorating effects could enter the blood circulation however on the downside longer peptides are also known as allergenic agents. Absorbed amino acids could act in various ways in the human body based on quantity, quality, and needs of it, such as become energy source, or other nitrogen containing compounds.

Nutritional value of protein sources is dependent on their amino acid composition. Amino acids could be categorized based on nutritional necessity, i.e., whether the body could synthetize them *de novo* or not. These categories are: i) essential or indispensable (IAAs) and ii) non-essential or dispensable (DAAs). Also, there are some AAs that can be conditionally indispensable, such as arginine, cysteine, glutamine, glycine, proline, and tyrosine. Indispensable and conditionally indispensable AAs cannot be synthetized (or not at a sufficient level) in the body, therefore these AAs should be supplemented from the diet (Wu, 2009). Recommended dietary intake for proteins is given as 0.83 g protein per kg body weight per day (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) 2012). Generally, foods with high protein content are noted as “good protein sources”. However, this assessment is based on only amount of protein not on composition.

In recent years, there has been a shift towards amino acid-based evaluation of protein quality. Amino acid-based needs have been recognized and an ideal protein content were set to meet AA requirements of the human body. The requirements are based on 100% digestibility, and set for all age groups. Digestibility of AAs is defined as the proportion of ingested proteins that is hydrolysed into amino acids, di- and tripeptides (DP and TP), which are available for absorption. In 2007, PDCAAS (Protein Digestibility Corrected Amino Acid Score) was introduced to assess digestibility of food proteins (FAO/WHO/UNU, 2007) . PDCAAS considers the limiting AA of the food (lowest ratio compared to ideal protein composition) and the faecal digestibility of total

protein content. Since 2013 however, the DIAAS (Digestible Indispensable Amino Acid Score) is recommended for evaluation of food protein quality (FAO 2013). DIAAS differs from PDCAAS in three key points: i) digestibility is calculated from ileal samples not at the faecal level, ii) instead of nitrogen-based protein content determination, accessibility of each individual amino acids is determined, iii) DIAAS value can be over 100 however PDCAAS have to be truncated. DIAAS is calculated as minimum of the ratio of digestibility adjusted content of each AAs compared to the ideal protein composition. Thus, DIAAS gives a more detailed description on protein digestibility than PDCAAS.

3.4. Digestion simulation

As seen above digestion is a complex process. Researchers have been trying to learn and understand the underlying mechanisms for more than 100 years. Rapid advancement in this field have been seen in the last two decades which could be stemmed back to the development and widespread of the *in vitro* digestion (IVD) simulation models. Although *in vitro* methods still have physiological limitations, they are usually cheaper to perform and considered an ideal tool for applications like, early-stage nutritional studies, mechanistic investigations, or assessment of toxicity. These models vary from the basic – single enzyme hydrolysis in controlled environment– to the much more advanced – automatically regulated multi-enzyme methods with absorption and microbial fermentation (Molly et al. 1993; Wickham et al. 2009; Kong and Singh 2010; Dupont 2016). Notwithstanding the recent shift towards more complex IVD methods, the possibilities and advantages of more simple digestion methods should not be overlooked for mechanistic understanding or for sample screening purposes. Since the information retrieved from a simulation experiment depends on the applied conditions, the appropriate *in vitro* protocol should be chosen depending on the research question (Duijsens et al. 2022).

In vitro methods can be classified into dynamic and static models. Dynamic models include ways to simulate the *in vivo* circumstances better, in one or more parts of the gastrointestinal tract. Simulation usually includes more realistic mechanical agitation (TIM-1 or IViDiS) or dynamic transitions e.g., gastric emptying is added (DGM or HGS) or absorption of bioaccessible molecules (TIM-1). There are also methods that include the large intestine as well (DIDGI, SHIME). Advantages of dynamic models i.e., *in vivo* like and more reliable results, are usually overwritten by the fact that they are expensive, time-consuming, unique equipment and highly qualified personnel is needed for running these simulations. Static models are simpler thus more easily applicable for a large number of laboratories. These methods also could include one or more phases

of digestion and for all phases exact parameters are given which are needed to be monitored (Fernández-García et al. 2009; Wickham et al. 2009; Ulleberg et al. 2011).

One of the most popular standardized digestion simulation methods recently is the static *in vitro* Infogest protocol. The Infogest method published in 2014 after 3 years of systematic development from the Infogest international network. The group was the part of the COST Action project between 2011 and 2015 and since then works as an open network for those whose research objective is to improve health properties of foods by sharing knowledge on digestion process. The Infogest gathers more than 450 scientists from 150 institutes and 45 countries worldwide. The first version of method was published in 2014 (Minekus et al. 2014). Improvements were made until 2019 and discussed in a Nature Protocols article (Brodkorb et al. 2019). The goal of the authors was to establish a general and controllable *in vitro* digestion simulation model that could be used anywhere in the same way thus the published results will be comparable to each other enlarging the data on food digestibility.

The protocol models all three phases of the upper gastrointestinal tract, the mouth, the stomach, and the small intestine (Figure 5).

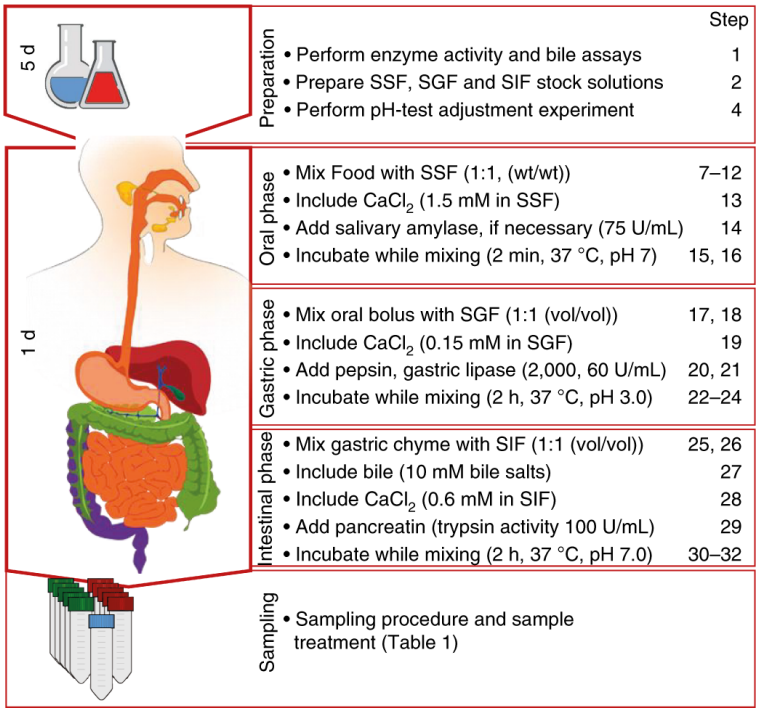

Figure 5. Timing and flow diagram of the Infogest *in vitro* digestion simulation method used for digestibility studies of foods. Expected time frame (left) and stages and corresponding step numbers in the procedure (right) are given. SSF, simulated salivary fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid *(Adapted from Brodkorb et al., 2019)*.

Standard digestion time, temperature, pH, ion-, and enzyme concentrations are given to each phase, and mechanical agitation is modelled by an overhead shaker. For easier implementation, step by step instruction are given in the papers along with the appropriate methods chosen for activity measurements for the used enzymes; salivary α-amylase (E.C.3.2.1.1), porcine pepsin (E.C. 3.4.23.1), rabbit gastric lipase and porcine pancreatin (E.C. 3.1.1.3). Bioaccessible molecules can be identified and quantitated from the small intestinal digesta at the end of the simulation by various analytical methods. The method has two main limitations. The first is that the absorption is not modelled, thus only bioaccessibility could be determined, and however data obtained after Infogest digestion simulation correlates with the *in vivo* results (Egger et al. 2017). The second is that it is not recommended to use for the evaluation of digestion kinetics although several authors have published data in this area based on the Infogest method (Mat et al. 2016; Infantes-Garcia et al. 2021; Guevara-Zambrano et al. 2022). In addition, a semi-dynamic version of Infogest – by adding gastric emptying to the model – was published as well (Mulet-Cabero et al. 2019).

Digestion simulation is a relatively new area in food science. Lot of simulation methods were created in the last two decades all having their advantages and disadvantages. However, standardization of this area is a necessary step to grow the knowledge on food digestibility and its mechanisms. The harmonization of digestion methods such as the standardized Infogest protocols can play a notable role in food digestion research and the development of tailored foods for all different strata of the population (Duijsens et al. 2022). It is relatively easy and cost-effective to implement and use, digestibility of several components of foods can be determined, and even bioaccessibility of toxic compounds could be assessed. These reasons led me to choose this method for my work.

3.5. Factors affecting food digestion and bioaccessibility

Food is both physically and nutritionally complex, which will affect digestion and absorption and may lead to interactions within the food matrix both pre and post consumption that alter its bioactive properties. Such interactions can alter the extent and kinetics of nutrient absorption in ways that are not always predictable, at least not from the information on nutrition labels.

3.5.1. Food matrix

Evidence from recent studies has shown that the food matrix can modify the nutritional properties of a food (Mackie, 2017). Several studies have recently investigated the impact of the food matrix on the digestibility of lipids using either *in vitro* or *in vivo* digestion models. The availability of the lipid substrate for lipases is determined not only by lipid droplet size (Michalski et al. 2013),

the oil-water interfacial area, but also by the interfacial structure (Corstens et al. 2017) and the food form (Dias et al. 2019). Moreover, when oil droplets are dispersed in a solid-like food matrix (e.g., cheese or strained-type yogurt), the structure of the surrounding food matrix becomes the dominant factor controlling digestion (Mengucci et al. 2022). These properties not only affect the rate and extent of lipid digestion but also these emulsions could serve as a carrier for other molecules, like lipid-soluble vitamins. A report from Tan *et al.*, underlines that size of the droplets in oil-in-water emulsions can significantly impact the bioaccessibility in β-carotene-loaded oil-in-water emulsions (Tan et al. 2020).

Similarly, if proteins are present in a structured matrix or a clot-like structure is formed in the gastric environment, gastric juice needs to penetrate this structured matrix to digest the protein. A 2–10 reduction factor for the diffusion coefficient of pepsin has been measured in a structured matrix as compared to water. The diffusion of pepsin is one of the limiting factors in the digestion rate of a structured food matrix (Capuano and Janssen, 2021). Moreover, proteins can form supramolecular assemblies also because of thermal treatment. The formation of aggregates may hide peptide bonds from proteases compared to denatured but isolated molecules (Mengucci et al. 2022).

3.5.2. Interplay of macronutrient digestion

There are substantial amount of work on digestibility and accessibility of individual macronutrients. However, foods are typically not consumed individually, thus the simultaneous release of multiple macronutrients could affect one another. Nevertheless, this gap has already started to get filled. In a study by Vanhatalo *et al*. digestibility of starch and glycaemic response was compared for two pasta products, couscous, and bread. It was showed that preservation of the pasta structure during mastication and gastric digestion explains slower starch hydrolysis and, consequently, lower postprandial glycemia compared with bread or couscous prepared from the same durum wheat semolina flour in healthy adults. It was also shown that the strong protein network of pasta is slowly digested and this contributes to the protection of granules from enzymatic hydrolysis. Additionally, it was shown that although couscous had a strong protein network, due to its small granule size, it was more prone to enzymatic action, thus resulting in smaller particles compared with the pasta boluses after *in vitro* digestion (Vanhatalo et al. 2022). In a recent work made by Guevara-Zambrano *et al*., plant-based shakes were prepared by various processing method resulting in different droplet size. Besides impact of microstructure on digestion kinetics, lipid and protein digestion was studied. Based on small intestinal digestion kinetics they showed a significant impact of protein aggregates on lipid digestion kinetics but no significant effect of lipids on protein digestion kinetics (Guevara-Zambrano et al. 2022).

3.5.3. Bioactive food components

Over the past decade, there has been a dramatic increase in the number of studies focused on investigating the bioactivity and potential health benefits of a range of non-nutritive bioactive food components. Emerging evidence shows that, although bioactive food components are not essential to life, they may confer a range of effects that support and improve health (Ellwood et al. 2014).

In addition to the much-studied primary effects of bioactive active ingredients, the secondary biological effect appearing through the effect on digestive enzymes can also be of great importance. For instance, lipase inhibitors have become an important means of clinical obesity treatment (Liu et al. 2020).

Obesity and associated diseases such as metabolic syndrome, cardiovascular disease, type 2 diabetes, are unarguably among the most important health concerns (Ng et al. 2014; Tune et al. 2017; Koliaki et al. 2019; World Health Organization. Regional Office for Europe 2022). The imbalance between the calories consumed with food and the energy expenditure of the body plays an important role in the development of obesity (Cercato and Fonseca 2019). The contributions of both total dietary fat intake and percentage of energy intake from fat to obesity have been shown by many studies (Wang et al. 2020; Wu et al. 2022). However, others have also indicated that this observation cannot always be confirmed at the population level (Forouhi et al. 2009) or divergencies were found for some sociodemographic groups (Raatz et al. 2017). These findings may support the rather fatty acid-dependent than generic associations between dietary fat intake and body weight, which is also shown in a number of studies (Beulen et al. 2018; Suara et al. 2020).

Among the many approaches applied in clinical obesity therapy, drug treatments using lipase inhibitors have become an important means of treatment (Liu et al. 2020). By inhibiting the liberation of fatty acids, the bioaccessibility of dietary lipids can be modulated. (de la Garza et al. 2011). In Europe and in the US currently the only authorized medicine for lipase inhibition used in clinical treatment of obesity is Orlistat (lipstatin, Xenical®). This works as a reversible inhibitor for both gastric and pancreatic lipase inhibiting the triacylglycerol hydrolysis but presents some undesirable effects (Spínola et al. 2020). Therefore, there is an interest in finding alternative, natural, and milder solutions for reducing lipid digestibility of high fat foods.

Alternatively, natural lipase inhibitors present in our food commodities and are consumed over longer periods of time as a part of our everyday diet, can also play an important role in reduction of dietary lipid bioaccessibility. Consequently, by inhibiting the absorption of fatty acids and thereby reducing the accumulation of fatty acids in the body, they can be considered as a safe alternative to control lipid bioavailability (de la Garza et al. 2011; Rajan et al. 2020; Kumar and

Chauhan 2021). Therefore, from a dietary point of view, a viable option could be food pairing of high fat foods with foods containing natural lipase inhibitors.

There are numerous plants that contain natural compounds including β-lactones and some botanical foodstuffs – plant extracts and plant metabolites, mainly polyphenols and saponins as well as peptides and some dietary fibres are also associated with lipase inhibitory effects (Bialecka-Florjanczyk et al. 2018). Promising examples are foods containing high concentration of proven bioactive components e.g., i) polyphenols, such as rosemary (Bustanji et al. 2010), several types of tea cultivars (Nakai et al. 2005; Chen et al. 2018), pomegranate leaf (Yu et al. 2017) or grape seed (Moreno et al. 2003), ii) terpenes, such as sage (Ninomiya et al. 2004) or gingko biloba (Bustanji et al. 2011), or iii) polysaccharides, such as apple or citrus fruits (Aguilera-Angel et al. 2018). It should be noted that the mechanism of action of different agents resulting reduction of lipid bioaccessibility via lipase inhibition can be highly different. Some of them – such as Orlistat – can bind directly to the active site of PL, whereas natural compounds are more likely to act indirectly, e.g., by interacting the oil-water interface (Zhang et al. 2021).

The mechanisms of food disintegration in the gastrointestinal tract in relation to food structure remain unclear. Increasing knowledge of the relationship between food structure/matrix design and the rates of nutrient digestion will be vital for meeting the challenge of producing foods explicitly for a new generation of health attributes (Singh et al. 2015).

3.6. Analytical methodology for the assessment of lipid and protein digestibility

Human digestion provides nutrients and energy to the body through efficient disintegration of macromolecules entrapped by the food matrix into molecules available for absorption. Availability of nutrients is dependent on the accessibility of absorbable molecules in the digestive system. Lipids and proteins are two of the three main sources of energy and nutrients to humans. The digestion of these macronutrients is both complex enzymatic hydrolysis processes placed in the same compartments of the gastrointestinal tract. Although structure of foods is the primary factor that guides gastric and subsequently intestinal digestion, difference in microstructure is affected by the origin of macronutrients, i.e., protein/fat source and composition can have a huge impact on overall digestibility of lipids and proteins.

Until recently, lipolysis studies mainly focused on intestinal digestion behaviour and lipid digestibility of oils (Ji et al. 2019; Ye et al. 2019), emulsions (Ye et al. 2010; van Aken et al. 2011; Giang et al. 2015; McClements 2015) or high fat foods (Calvo-Lerma et al. 2018; Hiolle et al. 2020). These studies use several methods for evaluation of digestible lipid content. Most authors

use pH stat method for quantification of free fatty acid content during small intestinal digestion. The small intestinal digesta is titrated with NaOH solution which indicates the amount of FFAs generated. Besides that, spectrophotometric kits are available for measuring FFA content of the digesta. Fatty acid-specific analysis could be made from digesta after extraction of lipid compounds and derivatization. Direct derivatization with highly toxic boron trifluoride or non-standard internal methods are usually used however these methods are not recommended or not generally used. Lately more complex, kinetics-based approach have been made to explore gastric and intestinal lipid digestion using model emulsions to understand reaction pathways during digestion of lipids (Infantes-Garcia et al. 2020, 2021). However, effect of lipolysis taking place in real foods or meals on protein digestibility of the co-consumed foods (and vice-versa) has not yet been described.

In parallel, protein digestibility is usually evaluated in a simplified *in vitro* digestion environment i.e., usually only proteases are added, no gastric lipase is added to the simulation (Sousa et al. 2020; Qazi et al. 2021). However, during gastric digestion, gastric conditions not only influence the protein degradation but the initial lipolysis of complex food matrices or meals (Kenmogne Domguia, 2012). The protein-lipid interaction in the stomach is an important factor to consider during gastric digestion which could influence both lipid and protein digestion. Protein digestibility evaluation using the Infogest method mainly focused on qualitative analyses in the past. Identification of hydrolysed peptides have been done by SDS-PAGE or size exclusion chromatography (SEC) or liquid chromatography coupled with mass spectrometry (LC-MS). For quantification of *in vitro* protein digestibility, the OPA (ortho-phthalaldehyde) method is used (Kopf-Bolanz et al. 2012) however direct analysis from small intestinal digesta will not give valid information on extent of digestion since it contains various hydrolysis products (such as di- or tripeptides besides amino acids). Efforts to study protein-lipid digestibility in a more physiologically relevant environment have been made by Guevara-Zabrano and colleagues with the inclusion of rabbit gastric lipase in the *in vitro* digestion simulation on fat and protein digestibility evaluation of plant protein shakes (Guevara-Zambrano et al. 2022).

Another aspect of digestibility studies is structural analysis. Several microscopic methods are used for visualisation of macronutrient (lipids and proteins) behaviour during digestion. Confocal microscopy and scanning electron microscopy are two of the most used techniques (Qazi et al. 2021; Ye 2021).

4. Materials and methods

4.1. Materials

For digestion protocol: porcine α-amylase (EC 3.2.1.1), porcine pepsin (E.C. 3.4.23.1; ≥2,500 units/mg protein (E1%/280), porcine pancreatin (E.C. 232.468.9; 8×USP), and porcine bile extract (E.C. 3.1.1.3) was purchased from Merck/Sigma-Aldrich. Rabbit gastric lipase (RGE15) was purchased from Lipolytech Ltd. FAME mixture (CRM47885) and internals standards; glyceryl trinonadecanoate (C19:0 TAG; >99%), methyl nonadecanoate (C19:0 ME, analytical standard), glyceryl triheptadecanoate (C17:0 TAG) and heptadecanoic acid (C17:0 FFA) were also from Merck/Sigma-Aldrich. L-serine standard was purchased from Reanal Ltd. Reagents and solvents were of analytical purity. Chloroform was purchased from Carlo Erba Reagents (for analysis, stabilized with ethanol), methanol (for HPLC, LC-MS grade) and hydrochloric acid (37%) from VWR International, isooctane from Fisher Scientific (>95%). High purity water (>18 $M\Omega cm^{-1}$) was prepared by a Millipore Elix Essential 3 UV Water Purification System (Merck-Millipore). Orlistat (>98% solid) were purchased from Sigma-Aldrich. Certified Reference Material CRM3252 (protein drink mix) from NIST was used.

4.2. Samples and sample preparation

4.2.1. Food samples

Samples were specifically chosen for i) testing the analytical methods designed for evaluating lipid and protein digestibility, ii) evaluating macronutrient interactions during digestion of high fat and protein foods, iii) examine inhibition efficacy of bioactive substances on lipid digestibility of the tested foods. Carp from Akasztó (a PDO food), ground beef (20% fat content; *Húsfarm fresh ground beef*), cream (30% fat content; *TOLLE UHT cream*), sour cream (20% fat content; *Milfina sour cream*; ingredients: cream, bacterial culture), sour cream analogue (20% fat content; *Hazai és Finom "Finomföl"*; ingredients: skim milk, milk protein concentrate, palm oil, bacterial culture), and durum wheat pasta (*Gyermelyi Vita Pasta*) was chosen as test matrices. Carp fillets from Akasztó were provided by Fishmarket Ltd. (Budapest, Hungary). Other food products were purchased commercially in local shops. Carp and ground beef were baked before digestion experiments for 20 mins in a 200 °C oven , after cooling baked carp (BC) and baked beef (BB) were homogenized in a meat grinder (Moulinex HV4), three times. Durum wheat pasta was cooked according to the packaging instructions. The 500 g dried pasta was placed in 5 L water boiling water with 1 g/L salt for 8 mins, after cooling cooked pasta (CP) was homogenized in a meat grinder (Moulinex HV4) three times. Baked and cooked samples were stored at -80 °C and thawed

before experiments. Cream (C), sour cream (SC), and sour cream analogue (SCA) were used after thorough mixing. Dairy products were always purchased fresh and used right after opening. One of the co-digestion studies on mapping the interplay between lipid and protein digestibility were done with eleven types of edible oils. These oils: sunflower oil (SFO), MCT oil (MCT), pumpkinseed oil (PSO), walnut oil (WO), hemp oil (HO), olive oil (OO), linseed oil (LO), coconut oil (CO), sesame oil (SO), grape seed oil (GSO), rapeseed oil (RO) were bought in local supermarkets.

4.2.2. Preparation of bioactive rich foods

Bioactive rich foods with *in vitro* assay-proven inhibitory effect on pancreatic lipase were chosen to test their ability in a more complex simulation of digestion i.e., Infogest digestion simulation. Experiments were carried out with either direct addition of bioactive containing food (rosemary, grape seed powder) or after extraction of bioactive compounds with food safe methods (tea, brewed).

Effect of rosemary was tested on baked carp lipids. Whole rosemary spice was bought in a local shop and was added as a whole spice. Carp fillets were covered with rosemary (5 w/w%) before baking (200 °C for 20 mins). Effect of grape seed powder (GSP) was tested on cream and baked beef. Grape seed powder was provided by Bock Vineyard Ltd. (Villány, Hungary). Grape seed powder was added to test matrices before digestion experiments, separately. Black tea (Himalayan Spring FF 2022 No.601) with high tannin content were bought commercially. Effect of tea was evaluated as brewed tea (aqueous extract). For the extraction of bioactive compounds from tea 0.2 g of tea leaves were measured into 50-mL round bottom flasks and 50 mL distilled water was added (4 mg/mL). Sample was heated on sand for 1 hour (with water cooler system attached to prevent evaporation). After cooling extracts were sieved on paper sieve and collected filtrate was completed to 50 mL. Black tea brew (BTB) was stored in -80°C until use. Thawed BTB was added separately to fat sources before digestion experiments.

Tea species with high tannin content was chosen from several products after determination of tannin content with MSZ 20685:1980 (Magyar Szabványügyi Hivatal, 1980). Determination of tannin content is not part of thesis, results are not detailed. Determination of tannin content was carried out by Eszter Benes which is kindly acknowledged.

4.2.3. Preparation of the protein free biscuit

In protein digestibility experiments a protein free biscuit was used as blank. Recipe of this cookie is detailed in the work of Moughan et al. (2005). Ingredients, such as purified corn starch (40.8 g), sucrose (15.7 g), cellulose (4.9 g), baking powder (0.7 g), ground ginger (0.5 g), and protein free

margarine (36.9 g) were mixed together to form a uniform dough. The dough was divided into three portions (35 g) and was baked for 30 min at 175 °C. The baked biscuits were ground with mortar and pestle and were stored on -80 °C until use.

4.3. Methods

4.3.1. Moisture content

Moisture content of the meat products was determined according to the ISO 1442:2000 method (ISO 1442:2000; Meat and meat products. Determination of moisture content. (Reference method), 2000). Other samples were measured by oven-drying at 103±1 °C using a Memmert UNE300 drying cabinet. Determination of moisture content was carried out in triplicates.

4.3.2. Determination of fat content and fatty acid composition

4.3.2.1. Meat products: baked carp and baked beef

For evaluating total fat content of baked carp (BC) and baked beef (BB), the standard method available for meat and meat products were used (ISO 1444:2000; Meat and meat products. Determination of free fat content., 2000). Briefly, 5 g (±0.001 g) sample portions were measured into ceramic bowl and mixed with 15 g of acid-washed quartz sand. Mixture was dried until weight equilibrium to remove excess moisture before fat extraction. Dried sample was transferred into a paper sleeve and placed into a 50-mL Soxhlet devise. Extraction was carried out with 100 mL of petroleum ether into a previously measured round-bottom flask for 6 hours. After cooling, solvent was evaporated and weight of dried (at 103±1 °C) fat content was measured. Fat content of sample was given in g fat/100 g food product unit. Determination of fat content of meat products was carried out in triplicates.

4.3.2.2. Dairy products: cream, sour cream, and sour cream analogue

Total fat content of dairy products (cream (C), sour cream (SC), and sour cream analogue (SCA)) was determined according to ISO 2450:2008 (ISO 2450:2008; Cream - Determination of fat content - Gravimetric method (Reference method), 2009). In brief, 10 g (±0.001 g) of cream, sour cream or sour cream analogue was measured into a Mojonnier-dish, 2 mL ammonium solution (25%) and 10 mL ethanol (95%) were added and shaken gently. Extraction was carried out in three rounds with diethyl ether and n-pentane, first with 25-25 mL, then with 15-15 mL for the second and third round. Extraction time was 1 minute and 30 seconds for all three rounds. Organic phases were collected in a pre-weighed flask and evaporated. Extracted fat was dried at 103 °C ±1 °C using a Memmert UNE300 drying cabinet, and weight of dried (at 103±1 °C) fat content was

measured. Fat content of sample was given in g fat/100 g food product unit. Fat content determination of dairy products was carried out in triplicates.

4.3.2.3. Extraction of total fat content with Bligh & Dyer method (Bligh and Dyer, 1959)

Five grams of accurately weighed (±0.001 g) sample portions (in the form to be subjected for digestion) were measured into 50-mL centrifuge tubes with screw tops. Required volumes of extractants are dependent on the moisture content, thus moisture content of the sample were previously measured. The chloroform/methanol/water ratio in the first step should be 1:2:0.8, where the moisture content of the sample gives the water ratio. Thus, in the first step, if the moisture content of the test sample is for instance 60% 3.75 mL chloroform ($CHCl_3$) and 7.5 mL methanol (MeOH) should be added and suspension should be vortexed for 2 minutes. Next, the sample was amended with the same volume of chloroform as in the first step and vortexed for 30 seconds. Then, then same volume of distilled water was added and vortexed again for 30 seconds. Two phase solution was separated via centrifugation at 3700 g for 20 minutes. Lower chloroform phase was pipetted into round-bottom flask with known tara weight obtained after drying until constant mass at 103±1 °C. Solvent was then evaporated with a rotary evaporator, then flask was oven dried at 103±1 °C until constant mass. Fat content was calculated from net dry mass of extract divided by the weight of the test portion. Extraction of the fat content of the foods with the Bligh & Dyer method (B&D) method was carried out in triplicates.

4.3.2.4. Fatty acid composition of extracted fats

To determine fatty acid composition "Rapid method" from ISO 12966-2:2017 standard was used (ISO 12966-2:2017, Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters — Part 2: Preparation of methyl esters of fatty acids, 2017). From the dried fats (both ISO and B&D extracted) 10-15 mg (±0.1 mg) was measured into a 15-mL screw-cap centrifuge tube and was dissolved in the mixture of in 900 µL isooctane and 100 µL C19:0 TAG (glycerol trinonadecanoic acid) internal standard (ISTD) solution (1 mg/mL in $CHCl_3$). To methylate esterified components, 100 µL 2 M potassium hydroxide (dissolved in methanol) is added and vigorously vortexed for 1 minute. After the reaction, the sample becomes opaque, which is cleared after 2 minutes of resting. Then, 4 mL of saturated (40 g/100 mL) sodium chloride solution is added and vortexed. Two phase solution is centrifuged at 3700 g for 10 min before transferring the upper phase to a micro centrifuge tube already containing 0.5 g of Na_2SO_4 powder to remove excess moisture from samples. Finally, an aliquot of the sample is pipetted into gas chromatography (GC) vial for analysis. Samples are analysed with gas chromatography coupled with flame ionisation detector (GC-FID). Determination of fatty acid composition was carried out in triplicates from both ISO and B&D extracts.

4.3.2.5. Determination of protein content

Kjeldahl method was used to determine protein content of food samples. Into the digestion tubes 0.5-2 g (±0.001 g) samples were measured and 2 mL 5% cupric sulphate solution, 1 g potassium sulphate and 20 mL sulphuric acid (98%) was added, and mixed. Tubes were placed into Gerhart Kjeldaterm and heated to 370 °C in increments and kept it for 2 hours. After cooling 40 mL distilled water was added and distillation was carried out in Gerhart Vapodest 45s. Nitrogen content was back titrated with 0.05 M sulphuric acid. pH was recorded and plotted as a function of the volume of solvent (mL). From the volume needed to reach pH 4.65 nitrogen content was calculated (%). Appropriate conversion factor was used to calculate the crude protein content of the samples (baked carp, baked beef: 6.25, cream, sour cream, sour cream analogue: 6.38, cooked pasta: 5.83). Protein content determination was carried out in triplicates.

4.3.3. Digestion simulations

Digestion simulations were made according to the Infogest protocol v1.0 ("PL"; using amylase, pepsin and pancreatin; (Minekus et al. 2014) and Infogest v2.0 ("GL+PL"; using amylase, rabbit gastric lipase (RGE) and pancreatin; (Brodkorb et al. 2019).

Simulated stock electrolyte solutions (SSF – simulated salivary fluid, SGF – simulated gastric fluid, SIF – simulated intestinal fluid) were made beforehand, and pH was adjusted to 7 (SSF), 3 (SGF) and 7 (SIF), respectively. Enzyme activities were measured via methods described in Brodkorb et al., and enzyme stock-solutions were made right before use. Pancreatin cannot be dissolved in SIF therefore a suspension protocol was used where pancreatin powder was mixed with SIF for 10 s with a handmixer and for 5 min in ultra-sonication water bath, then separated by centrifugation (3700 g, 5 min, 4 °C), and supernatant was used in digestion experiments (Sousa et al. 2023). Required volume of 6 M hydrochloric acid (HCl) and 1 M sodium hydroxide (NaOH) – to keep constant pH – was determined before each digestion experiment with a "pH test" using the same amounts of samples and solvents, only without enzymes.

All digestion experiments were conducted in triplicates. Blank digestions were also made for each triplicate using 5 g (±0.001 g) of distilled water (lipid digestibility experiments) or 5 g (±0.001 g) of protein free biscuits (protein digestibility experiments) used as sample.

In addition of lipid and protein digestibility test of various foods, lipase inhibition with co-digestion of the test matrices with bioactive rich foods was carried out. Positive control for lipase inhibition studies were made with the addition of Orlistat (a lipase inhibitory drug) where 40 µL of 0.5 M Orlistat solution in DMSO was added to fully inhibit activity of lipases.

For the digestion simulation, varying amount of sample is accurately measured into pre-weighed 50-mL centrifuge tube. For oral phase, 3.5 mL of SSF (tempered to 37 °C), 25 μL of 0.3 M $CaCl_2$, 0.5 mL of amylase stock-solution (1500 U/mL in SSF) and 0.975 mL of distilled water was added. Homogenized samples are incubated in an overhead shaker (Heidolph Reax 2) fitted inside a preheated drying cabinet (Memmert UNE300) for 2 min at 37 °C. In the gastric phase, 6.4 mL of SGF (tempered to 37 °C), 5 μL of 0.3 M $CaCl_2$, required volume of 6 M HCl, 1.6 mL of pepsin stock-solution (25000 U/mL in SGF) and 1.945 mL of water was added, and the mixture was incubated in the overhead shaker at 37 °C for 2 h. For the small intestine phase, 8.5 mL of SIF (tempered to 37 °C), 40 μL of 0.3 M $CaCl_2$, 2.5 mL of bile extract solution (160 mM in SIF, tempered to 37 °C), 5 mL of pancreatin supernatant (800 U/mL in SIF), required volume of 1 M NaOH and 3.86 mL of water was added, and mixture was incubated in the overhead shaker at 37 °C another 2 h. In the case of the Infogest "GL+PL" digestion, 1.6 mL of rabbit gastric extract (RGE, 750 U/mL in SGF) was added in the gastric phase and no pepsin was added. Addition of pepsin was omitted in experiments where RGE was added based on the appropriate pepsin activity of RGE to substitute porcine pepsin activity. After small intestinal digestion phase was completed, weight of digests was measured and sampled according to analytical purposes. Enzyme activity was inhibited with the addition of organic solvents after sampling as a part of the further sample preparation.

4.3.3.1. Single food digestions

Test foods were chosen with high fat and/or protein content. First, digestion experiments were carried out to test and validate the method for assessment of bioaccessible fatty acid content. Then, lipid and/or protein digestibility of these foods were defined i) to determine lipid and/or protein digestibility of test foods, ii) to use as control in lipase inhibitory experiments with bioactive rich foods, iii) to use as control in co-digestion experiments with other foods with high fat and/or protein content.

Single product digestions of BC were carried out with 5, 4, 1, 0.5 g (±0.001 g) samples. From BB 0.9 g (±0.001 g), from C 0.5 g (±0.001 g), from SC and SCA 1 g (±0.001 g), and CP 4 g (±0.001 g) sample were digested. All samples below 5 g were diluted with distilled water to reach proper sample size according to consensus (5 g).

4.3.3.2. Co-digestions – Lipase inhibitory studies with bioactive rich foods

These types of experiments focused on revealing the effects of simultaneous consumption of high fat foods and foods with proven lipase inhibitory effects on lipid digestibility of said high fat food.

Effect of rosemary was tested on baked carp lipids. Rosemary spiced baked carp (1±0.001 g) was digested alone. Effect of grape seed powder and black tea brew was tested on cream, and baked beef. First, dose-dependency tests were done where GSP was added to 0.5 g (±0.001 g) cream at three levels, 5, 10, and 15 w/w% and BTB was added to 0.5 g (±0.001 g) cream at three levels, at 1:1, 1:2 and 1:3 (cream : BTB) ratios. Levels were chosen based on recommended intake. GSP was added around the typical concentrations as suggested for this food supplement to mix with foods and BTB was added to cream in ratios that would be during consumption of English tea. Further experiments, with different substrate, were adjusted to fat content of cream (150 mg) thus to 0.9 g (±0.001 g) of baked beef the lowest effective level of GSP (5 w/w%) and BTB (1:2) were added.

4.3.3.3. Co-digestion of foods – interplay between lipid and protein digestion

Two sets of co-digestion experiments were conducted on revealing the interplay between fat and protein digestion, with the addition of different sources of fat to protein containing meals. To reveal how co-consumption changes the lipid digestibility of the high fat toppings (sour cream and sour cream analogue) "sour cream pasta" experiment was designed. Co-digestions were made using 4 g (±0.001 g) CP and either 1 g (±0.001 g) of SC or 1 g (±0.001 g) of SCA for this purpose.

Since in these experiments the addition of different type of fats seemed to impact protein digestibility next experiment was designed to see the how some edible oils affect protein digestibility of cooked pasta. Eleven types of oils were chosen, namely, sunflower oil, MCT oil, pumpkinseed oil, walnut oil, hemp oil, olive oil, linseed oil, coconut oil, sesame oil, grape seed oil, rapeseed oil and were added to 4 g (±0.001 g) of CP at 5 w/w% in co-digestion simulations.

4.3.4. Assessment of fatty acid-specific lipolysis – bioaccessible fatty acid content

Bioaccessible fatty acid (FA) content and composition were measured according to Tormási and Abrankó, 2021. All lipid components were extracted using B&D method (Bligh and Dyer, 1959). From digests, 5 mL samples were taken and transferred to pre-weighed 50-mL tubes. Weight of the aliquot is recorded for later calculations. Chloroform – 6 mL, intended for fat extraction – was immediately added to quench enzymatic digestion processes. Before fat extraction, 250 µL of C19:0 TAG internal standard (ISTD) solution (1 mg/mL in $CHCl_3$) was added to the samples. Then, 12.5 mL methanol was added, and vortexed for 2 min using Benchmark Benchmixer XL. Then 6.25 mL of chloroform was added, followed by 30 s mixing. Finally, after the addition of 6.25 mL distilled water, sample was vortexed again for 30 s. The formed two-phase solution was centrifuged at 3700 g for 20 min. After phase separation, from the lower phase (∑12.5 mL $CHCl_3$, containing fat components) 2x5 mL is pipetted into two round-bottom flasks and solvent was evaporated using a rotary evaporator.

One aliquot was methylated according to the ISO 12966-2:2017 standard's 'General method' to result the total fatty acid content (TFA method). After evaporation of the 5 mL chloroform, samples are methylated in the same round-bottom flask. First, boiling chips and 2 mL of 0.2 M sodium methoxide was added, mixed, and heated for 20 min on sand. Cooled samples were titrated in the present of phenolphthalein using 1 M sulfuric acid dissolved in methanol, in 100 µL portions until discoloration. Then another 200 µL acid was added in excess. Acidified samples were heated for another 5 min, then removed from heat and cooled under running water. Then sample was transferred into 15-mL screw top centrifuge tube, and flask washed with 4 mL saturated (40 g/100 mL) sodium chloride solution. Sample was homogenized before extraction of formed fatty acid methyl esters (FAMEs) by addition of 1 mL isooctane. Two-phase solution was separated by centrifuging at 3700 g for 10 min before transferring the upper phase into a GC vial.

The other 5-mL aliquot is trans methylated with the alkaline 'Rapid method' of the same ISO standard to obtain the esterified fatty acid content (EFA method). Total amount of dried fat extract in the bottom of the round-bottom flask was dissolved in 1 mL isooctane. Dissolved sample was completely removed (pipetted) into a 15-mL screw top centrifuge tube. To methylate esterified components, 100 µL of 2 M potassium hydroxide (dissolved in methanol) was added and vigorously vortexed for 1 minute. After the reaction, the sample became opaque, which was cleared after 2 minutes of resting. Then, 4 mL of saturated (40 g/100 mL) sodium chloride solution was added and vortexed. Two phase solution was centrifuged at 3700 g for 10 min before transferring the upper phase to another vial already containing 0.5 g of Na_2SO_4 powder to remove excess moisture from samples. Finally, an aliquot of the sample was pipetted into GC vial for analysis.

Total fat content (TFA) and esterified fat content (EFA) were analysed with GC-FID. The results were used to determine amount of free fatty acids (FFA, Eq. 3), as well as the release ratio of each fatty acids (RR, Eq. 4) and the bioaccessible fatty acid content in % (Eq. 5). Detailed description of the fatty acid-specific assessment method and the calculations is part of the Chapter *Results and Discussion*.

4.3.5. GC-FID method

Agilent 6890 GC-FID system equipped with Agilent 7683 autosampler was used. For separation, Phenomenex Zebron ZB-FAME (60 m, 0.25 mm, 0.20 µm) column with cyanopropyl stationary phase and hydrogen gas (1.2 mL/min) mobile phase was used. Inlet temperature was 250 °C, detector temperature was 260 °C. A split ratio of 50:1 and 1 µL injection volume was used. Temperature program started from 100 °C, which was kept constant for 3 minutes. Then column

was heated at 20°C/min to reach 166 °C, where kept for 5 minutes. Then heated to 180 °C, at 1 °C/min, finally to 240 °C at 10 °C/min, where kept for 3 minutes.

Calibration mix was prepared from a Supelco 37 component FAME mixture and each calibration level was spiked at 100 µg/mL with methyl nonadecanoate (C19:0 ME) ISTD (1 mg/mL dissolved in isooctane). Four-point calibration was made at 0, 30, 60, and 120 µg/mL nominal concentration for C16:0, at 0, 20, 40, 80 µg/mL levels for C4:0; C6:0; C8:0; C10:0; C12:0; C14:0; C18:0; C18:1n-9c, C18:3n-6c; C18:3n-3c; C20:0; C20:3n-6c; C20:4n-6c; C20:3n-3c; C22:0 and at 0, 10, 20, 40 µg/mL levels for C11:0; C13:0; C14:1n-5c; C15:0; C15:1n-5c; C16:1n-7c; C:17:0; C17:1n-7c; C18:1n-9t, C18:2n-6t, C18:2n-6c; C20:1n-9c; C20:2n-6c; C21:0; C22:1n-9c; C20:5n-3c; C22:2n 6c; C23:0; C24:0; C24:1n-9c; C22:6n-3c. In the actual calibration table, the exact concentration of each analyte was set. C4:0 was excluded from the set of analytes due to overlap with solvent peak. Chromatogram of FAME mixture with internal standard and detailed list of analytes, trivial names and retention times and resolution are shown in *Appendices (Figure A1 and Table A1)*.

4.3.6. *In vitro* protein digestibility (IVPD%)

After digestion simulation 2 mL samples were taken into 15-mL centrifuge tubes (weight measured). Bioaccessible protein content was isolated with 80% methanol precipitation (Polson et al. 2003), therefore 8 mL of methanol was added to reach 80 V/V% ratio of methanol : water. Tubes were mixed then were kept on -20 °C for 1 h to fully precipitate intact proteins. Pellets (P) were separated with centrifugation (3700 *g*, 20 min, 4 °C). The supernatants (SN) were used to determine amount of free amino (NH_2) groups in bioaccessible fraction (free NH_2%) and *in vitro* protein digestibility (IVPD%) based on free amino acid content after acidic hydrolysis of the bioaccessible fraction with two methods, i) based on total amino groups after hydrolysis with OPA method, ii) based on total amino acid content after hydrolysis with UHPLC-UV method after AQC (6-aminoquinoly-N-hydroxysuccinimidyl carbamate) derivatization.

4.3.6.1. Based on free-, and total amino group content

Amount of free amino (NH_2) groups was measured from SN without further sample preparation with OPA method (Nielsen et al. 2001). From SN 20 µL was added to 980 µL OPA reagent (1M borax, 6 M OPA, 3.5 M sodium dodecyl sulphate, 5.7 M dithiothreitol in water) and stirred for 5 s. Reaction time was 2 min each time then absorbance was measured at 335 nm (Thermo Electron Corporation Nicolet evolution 300). Free NH_2% was calculated according to Eq. 1. Total nitrogen content measured with Kjeldahl method was used as total protein content for calculations.

$$Free\ NH_2\% = \frac{[NH_2(MeOH) - NH_2(blank)] * \frac{100\ g}{digested\ sample\ size\ [g]}}{Protein\ content\ of\ product\ [\frac{g}{100g}]}$$

Equation 1: Calculation for free NH_2 content [%]. NH_2 (MeOH): amount of free amino nitrogen in serine equivalent in methanolic supernatant of sample digest, NH_2 (blank): amount of free amino nitrogen in serine equivalent in methanolic supernatant of blank digesta, protein content of the product: total nitrogen content of product in crude protein equivalent [g/100 g].

IVPD% based on total amino group content was measured from the SN after acidic hydrolysis ((AOAC.2018.06; Jaudzems et al. 2019) with the OPA method with minor modifications. Into a 1.5-mL chromatographic vial 50 µL SN was measured. Solvent was evaporated under nitrogen flow and samples were resolved in 260 µL of distilled water, 120 µL of 0.2 M NaOH (containing 0.1% dithiodipropionic acid), 120 µL of 0.2 M HCl, 500 µL of cc. HCl. Vials were capped after nitrogen rinse, mixed, and kept on 110 °C for 15 h (Memmert UNE500). From hydrolysed samples 20 µL was added to 580 µL OPA reagent and stirred for 5 s. Reaction time was 10 min each time then absorbance was measured at 335 nm (Thermo Electron Corporation Nicolet evolution 300). IVPD% was calculated according to Eq. 2. Total nitrogen content measured with Kjeldahl method was used as total protein content for calculations.

$$IVPD\% = \frac{[NH_2(MeOH, hydrolysed) - NH_2(blank, hydrolysed)] * \frac{100\ g}{digested\ sample\ size\ [g]}}{Protein\ content\ of\ product\ [\frac{g}{100g}]}$$

Equation 2: Calculation for *in vitro* protein digestibility [%]. NH_2 (MeOH, hydrolysed): amount of free amino nitrogen in serine equivalent in hydrolysed methanolic supernatant of sample digesta, NH_2 (blank, hydrolysed): amount of free amino nitrogen in serine equivalent in hydrolysed methanolic supernatant of blank digesta, protein content of the product: total nitrogen content of product in crude protein equivalent [g/100 g].

4.3.6.2. Based on free-, and total amino acid content

Bioaccessible protein content was also determined based on free-, and total amino acid content of the digesta after the isolation of bioaccessible protein content with 80% methanol precipitation (Polson et al. 2003). End digests were sampled (2 mL) and precipitated with 8 mL methanol to reach 80 V/V% ratio and kept for 1 h at -20 °C. Pellets were separated from supernatant by centrifugation (3700 *g*, 20 min, 4 °C). Supernatant was transferred into a clean tube.

For amino acid measurement 100-1000 µL from supernatant was transferred into a 1.5 mL vial and was evaporated under nitrogen gas. For determination of free amino acid content, the evaporated sample were resolved into 500 µL borate buffer (pH = 8.51), filtered (22 µm HPLC filter) and were derivatized. For derivatization 10 µL sample was added to 70 µL borate buffer then 20 µL Waters AccQTag reagent (AQC; 6-aminoquinoly-N-hydroxysuccinimidyl carbamate) was added and mixed. After 1 min rest on room temperature the mixture was incubated for 10 min at 55 °C then filtered (22 µm HPLC filter). For separation Waters Acquity UPLC H-Class instrument was used equipped with AccQ UPLC BEH C18 2.1x100 mm, 1.7 mm column (column temperature: 43 °C; injected volume: 10 µL; flow rate: 0.7 mL/min). For detection PDA detector on 260 nm was used. Quality and quantity evaluation was made with amino acid standards. To determine the total amount of amino acids in the bioaccessible fraction before derivatization samples were hydrolysed in a Milestone ETHOS One microwave oven thus evaporated samples were resolved in 6 M HCl (with 1% phenol content). Two types of hydrolysis were necessary in order to analyse all amino acids. Heat profile of hydrolysis were: i) general method: 10 °C/min to 180 °C, 20 min incubation, and cooling, and ii) method for tryptophan determination: 10 °C/min to 180 °C and subsequent cooling. After hydrolysis sample preparation continued with derivatization (see above).

Amino acid analysis (digestion and UHPLC-UV measurement) was carried by Éva Lengyel-Kónya, Mária Berki and Rita Tömösközi-Farkas which is kindly acknowledged.

4.3.7. Additional methods

4.3.7.1. Structural analysis

Microscopic images were taken after chemical digestion to assess changes during the gastric phase. Samples were measured into 50-mL centrifuge tubes as in the enzymatic digestion simulations and both products, single product digests and co-digested products were evaluated. Chemical digestion was as follows; 4 mL simulated SSF, 25 µL of 0.2 M $CaCl_2$ and 975 µL of water was added, mixed, and tempered for 2 minutes at 37 °C. Then 8 mL of SGF and 5 µL of 0.2 $CaCl_2$ were added, and the pH was lowered to 3 with 6 M HCl. Acidic digesta was tempered for 20 minutes at 37°C in an overhead shaker then pH was readjusted to 7 with 2 M $NaHCO_3$. Products were diluted at the same degree (to the same end volume) as the chemical digests with water. Coomassie Blue (CB, 0.2% CB, 7.5% acetic acid, 50% ethanol), and Nile Red (NR, 0.1 w/v% in acetone) were used to dye proteins and lipids, respectively. From chemical digests and diluted products, 1 mL aliquot was taken then 200 µL of CB and 10 µL of NR were added and mixed. After five minutes 5 µL was transferred to a microscopic glass plate and cover slip was placed onto. Images were

taken with Olympus BX41 (40x lens). Proteins were studied under normal light conditions and lipids were excited with fluorescent excitation: 510-550 nm (emission: 590 nm).

4.3.7.2. Characterization of polyphenolic compounds of rosemary

Rosemary spice was powdered with kitchen grinder (Sencor) and was sieved with 150 μm mesh sieve. From the prepared powder 0.5 g was accurately weighed (±0.001 g) into a 15-mL centrifuge tube and 10 mL distilled water was added. Extraction was aided with ultrasonication (30 min) at 37 °C. After extraction solid phase was separated with centrifugation (3700 *g*, 20 min) and upper layer was filtered (0.22 μm PTFE, syringe filter) and UHPLC-Ultivo ESI-MS/MS analysis was carried out with a Phenomenex Kinetex EVO C18 column (30 °C; 100 x 2.1 mm, 2.6 μm) and gradient elution (0.1 V/V% formic acid in water and acetonitrile; 0.5 mL/min). Mass spectrometry analysis was performed in negative ion mode by dMRM scanning except for chrysin. Matrix matched calibration was used for quantification. List of analytes: cyanidin-glucoside (kuromanin), 4-hydroxi-benzoic acid, cyanidin-rutinoside (keracyanin), catechin, caffeic acid, syringic acid, epicatechin, vanillin, syring-aldehyde, p-coumaric acid, ferulic acid, rutin, sinapic acid, quercetin-glucoside, genistin, conifer-aldehyde, sinap-aldehyde, daidzein, luteolin, genistein (aglycone), apigenin, kaempferol, isorhamnetin.

Polyphenol analysis was carried by Kata Nagy which is kindly acknowledged.

4.3.8. Statistics

Pairwise comparison was carried out in Microsoft Excel using Student's t test. Significance was recognized at $p<0.05$. Comparison of multiple samples was performed using Anova test in Microsoft Excel, however where significant difference was found ($p<0.05$), Tukey's post hoc test was performed using IBM SPSS Statistics 25. Equality of variances were tested with Levene's test ($p>0.05$). To evaluate dependency of factors Pearson correlation was used in Microsoft Excel. Correlation was acknowledged as high if correlation coefficient was under -0.8 or above +0.8 ($r<-0.8$ and $r>0.8$). Comparison of different analytical methods were made by using Bland-Altman plots (Bland and Altman, 1986).

5. Results and Discussion

I. METHOD DEVELOPMENT

The first challenge of my work was to design, test and validate analytical methods for determining extent of lipid and protein digestibility in complex foods. First, method created for assessment of lipid digestibility, then method used for evaluation of protein digestibility is described. In addition, specific questions on routine practice of the Infogest digestion simulation method are clarified.

5.1. Harmonized protocol to assess fatty acid-specific lipolysis of foods

In this first part the considerations and steps of method development for evaluating fatty acid-specific lipid digestibility are discussed.

5.1.1. Determination of bioaccessible fatty acid content

The main goal of method design was to create an analytical protocol that is linked to the widely used *in vitro* digestion simulation – the Infogest method – and that gives information of the content and the composition of the bioaccessible lipid fraction.

The lipid content of foods is mostly comprised of triacylglycerols which during human digestion hydrolyse into diacylglycerides, monoacylglycerides and free fatty acids by the action of the two main lipase enzymes, gastric lipase, and pancreatic lipase (Armand 2007; Benito-Gallo et al. 2015) (Figure 6).

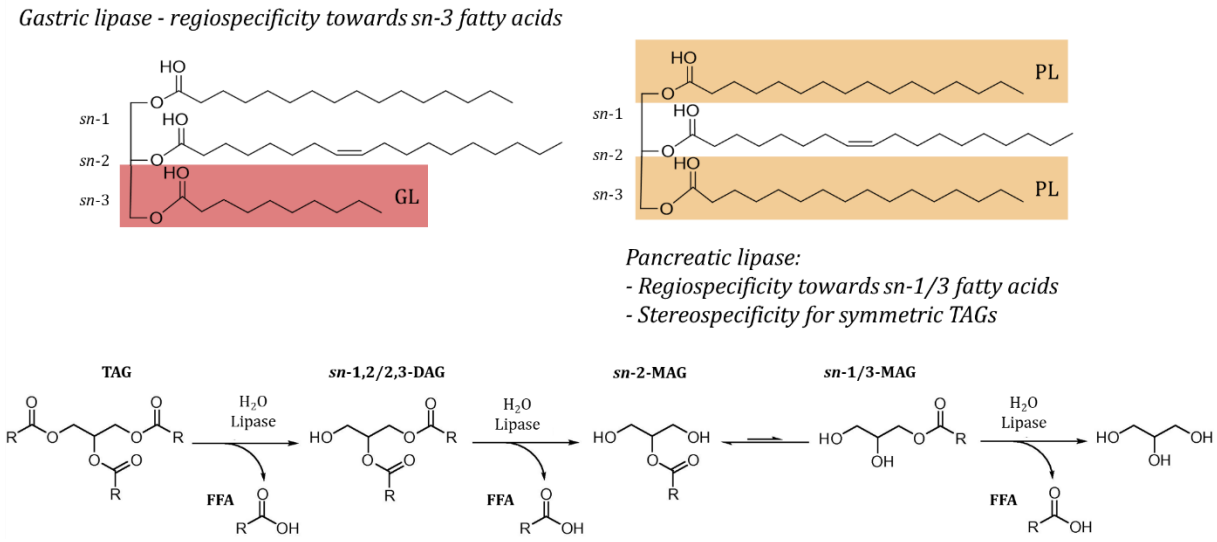

Figure 6. Specificity of lipases (gastric lipase (GL) and pancreatic lipase (PL), and the lipolysis of triacylglycerols (TAGs) *(Original image).*

In the small intestine, free fatty acids and monoacylglycerides could go through the intestinal wall by passive and active transport mechanisms (Ockner and Manning 1974; Mansbach and Gorelick 2007; Iqbal and Hussain 2009). Since free fatty acids are the end products of the hydrolysis reaction, lipid digestibility is usually evaluated by determination of the amount of these molecules in the small intestine. The most frequently used technique to determine bioaccessibility of lipids – the pH-stat method (Li and McClements 2010; Jannin et al. 2015; Mat et al. 2016; Ji et al. 2019) – is capable to determine the amount of free fatty acids released during the digestion of certain foods. However, this method cannot provide fatty acid-specific information. In addition, lot of the research using this technique focuses on describing the lipolysis kinetics. Despite the popular use of the pH stat method, it cannot be applied to evaluate the composition of the released FFAs only the summarised, gross amount of them.

My goal was to create a systematic and routine way to determine both the quantity and the quality of the bioaccessible lipid fraction at the same time. In addition, the developed method contains built in quality control points which serves simultaneous verification of the results. During method development there were a few key questions that are needed to be addressed to establish the final protocol.

5.1.2. Fat extraction method

After the *in vitro* digestion simulation, the bioaccessible free fatty acid content of foods could be determined from the small intestinal fluid (digesta). The digesta is an aqueous suspension of undigested food materials, various products of the enzymatic hydrolysis (peptides, amino acids, lipid species, simple and complex sugars), added digestive enzymes and ions. Since the water content is high, lipid compounds must be extracted from this matrix before the quality and quantity of fatty acids could be evaluated. One of the most used methods to extract lipids from aqueous environment is the Bligh and Dyer method (B&D; Bligh and Dyer, 1959). Recently it has been used to analyse digestion and absorption rate of dietary lipids from edible oils (Ye et al. 2019) and to assess the degree of lipolysis from milk fat (Teng et al. 2019). The method is based on the phase separation of the chloroform-methanol-water three component mixture, where the lipid components initially suspended in the sample with high water content are transported into the chloroform phase in a two-step extraction. Advantages of this method is that it does not require extensive volumes of organic solvents, it is fast, and all lipid components, i.e., TAGs, DAGs, MAGs and FFAs, are simultaneously extractable.

To accept the B&D method as the fat extraction method from the small intestinal digesta, I compared the fat content obtained with this method with the values measured by the relevant reference fat extraction method used for foods of the same class. To test the performance of the

B&D method, the total fat content of the tested food samples (baked carp, baked beef, cream, sour cream, sour cream analogue) was determined after B&D extraction via gravimetric method (extracts were dried at 103°C until constant mass) and the results were compared with the standard method used to asses fat content of each matrix (baked carp and baked beef: ISO 1444:2000; cream, sour cream and sour cream analogue: ISO 2450:2008) applying extraction with organic solvents, i.e. petroleum ether and diethyl ether. Beforehand moisture content of each sample was determined since solvent ratio of B&D extraction is adjusted to moisture content of the extracted sample (data not shown). Measurements were carried out in triplicates, and results were compared with statistical methods (Student's t test). Results and p values are shown in Table 1.

Table 1: Results of total fat determination of used matrices: baked carp, baked beef, cream, sour cream, and sour cream analogue. Total fat content from relevant ISO methods (baked carp, baked beef: ISO 1444:2000; cream, sour cream, and sour cream analogue: ISO 2450:2008) and from gravimetric weight measurement of Bligh and Dyer extracts are given in g fat/ 100 g sample in average ± deviation (RSD) format. Results from statistical evaluation (p value of Student's t test; n=3) are also given.

Food sample	**Fat content [g/100 g food product]**		**t test**
	ISO method	Bligh & Dyer method	(p value)
Baked carp	14.05 ± 0.78 (0.06)	14.44 ± 0.30 (0.03)	0.292
Baked beef	16.07 ± 0.34 (0.02)	14.76 ± 0.13 (0.01)	0.092
Cream	29.51 ± 0.25 (0.01)	29.18 ± 0.27 (0.01)	0.183
Sour cream	18.47 ± 0.72 (0.02)	18.91 ± 0.75 (0.03)	0.505
Sour cream analogue	18.62 ± 4.08 (0.22)	17.86 ± 0.59 (0.03)	0.777

According to these results the fat content extracted with the B&D method is in accordance with the fat content obtained from the ISO extraction methods. Although amount of extractable fat content from baked beef with B&D method is relatively lower than from the other matrices, i.e., recovery is only 92% (compared to the ISO method), it is not statistically significant from the value determined with the ISO protocol. Conclusively, the Bligh & Dyer method is applicable instead of ISO standards in total fat evaluation, as the results indicate that all the fat content could be extracted from various matrices thus this method is appropriate to determine total fat content of foods.

Since the ultimate goal is the evaluation of digestibility at the fatty acid level, the B&D extracted fats' fatty acid composition should be also the same as the ISO extracted fats' fatty acid composition. In addition to the determination of the total fat content, fatty acid profiling from these fats were also carried out. Fatty acid profile of B&D extracted, and ISO method extracted fats are shown in the *Appendices Table A2A, A2B.*

Based on the data showed in Table A2A and A2B, fatty acid-specific data gathered after the two extraction methods only differ in a few fatty acids and the difference of the mentioned fatty acids are below 3%. Besides the comparison of actual data, the results were compared using Bland-Altman plot (Figure 7). Bland-Altman plots give the average of the measurement data as a function of the difference of the measurement data to help determine if there is proportional bias.

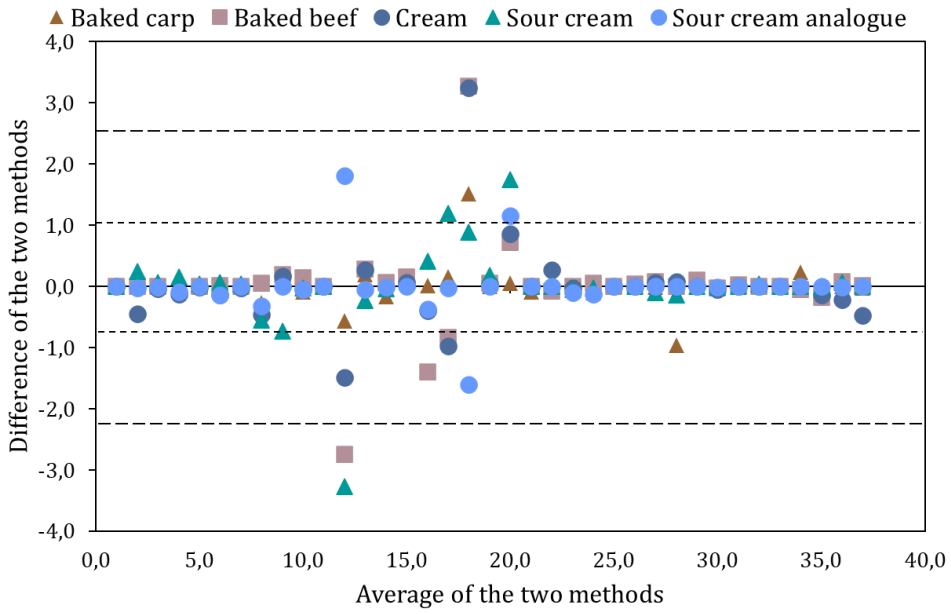

Figure 7. Bland-Altman plot comparing the fatty acid profile of food products (baked carp, baked beef, cream, sour cream, sour cream analogue) extracted with relating ISO method and Bligh & Dyer method. Dashed lines show the average±(1.96*STDEV).

Figure 7 shows that the difference between fatty acid composition data of the test foods gathered form the two methods (ISO and B&D) is close to zero. There are only a few points that are outliers meaning that these points are out of the limit line (large dashed lines).

Based on the results shown in the Tables 1, S2A and S2B and the comparison made with the Bland-Altman plot (Figure 7), there is no difference between either the fat content or the fatty acid composition of the tested foods measured with either method. So, the results confirm that the Bligh & Dyer method shows no fatty acid-specific bias and is applicable to use instead of the tested ISO methods.

5.1.3. Gravimetric determination of total fat content from digesta

As a proposed quality assurance step, gravimetric determination of total fat content from small intestinal digesta was also considered. The applicability of the measurement was tested on the baked carp meal sample. After *in vitro* digestion, moisture content of small intestinal digesta was

determined with drying at 103 °C until constant mass, since solvent ratio of B&D extraction is adjusted considering moisture content of the extractable sample (96.27±0.003 g/100 g; n=6). Fat content of digesta was extracted with the B&D method and non-polar (chloroform) phase containing lipid components was dried. After drying, mass of total lipids was calculated (6.14±0.65 g/100 g baked carp). A significant difference between total fat content of the sample (with ISO and B&D methods) and total fat content of the digesta was found (Student's t test was $p<0.001$). Loss of weight during drying was most probably due to evaporation or degradation of compounds with lower boiling points, i.e., short-chain free fatty acids, and short-chain fatty acid containing MAGs, and DAGs. Since the digestibility of baked carp was high (more than 50%) the amount of free fatty acids might be responsible for the loss. Conclusively, from the small intestinal digesta the total fat content could not be determined with gravimetric method.

5.1.4. Determination of fatty acid release

In the human gastrointestinal system, both MAG and free fatty acids are capable of passing through the epithelium of the small intestine with passive or active transport mechanisms. Although providing information on digested lipids at the fatty acid level, would give a more realistic insight of the lipid digestibility of foods. Nevertheless, simplification is usually made due to practical reasons, i.e., lack of appropriate analytical methods. Lipid digestion is mainly monitored by amount of free fatty acids released during the digestion simulation (Mat et al. 2016). My aim was to overcome this problem and develop an appropriate analytical method that can be used on a routine basis to provide fatty acid-specific information on digested lipids.

In general, fatty acids are separated and identified by gas chromatography and detected with flame ionization detector (Aarak et al. 2014). Fatty acids however could not be analysed with GC in their native form thus before separation derivatization should be carried out. The most common derivatives being fatty acid methyl esters (FAMEs) there are several methods on (trans) methylation of lipid compounds depending on the studied food product, evaluated components, in-house routines, and availability of standard methods (Swackhamer et al. 2019). However, these protocols frequently neglect free fatty acids during determination of FAMEs since foods mainly composed of esterified fatty acids (TAGs). The most used protocols are based on the alkaline trans methylation where esterified fatty acids are transformed to FAMEs, but free fatty acids are saponified and excluded (ignored) during further analysis. Direct methylation of free fatty acids is possible though the reaction uses highly toxic reagents, and most laboratories prefer to avoid it. This method was not considered during method development. Alternatively, amounts of individual free fatty acids could be determined by subtracting the value of the individual fatty acid in

esterified form (TAGs, DAGs, MAGs) from total value of the same individual fatty acid (TAGs, DAGs, MAGs and FFAs) as suggested previously by Zhu and colleagues (Eq. 3; (Zhu et al. 2013).

To determine total amount of fatty acids (TFA method) and amount of esterified fatty acids (EFA method) two methods from the ISO 12966-2:2017 standard were chosen. Determination of TFA is a two-step reaction. First, esterified fatty acids are trans methylated in alkaline media (0.2 M NaOH in methanol) where free fatty acids are saponified, and secondly the saponified fatty acids are methylated in acidic environment (0.2 M H_2SO_4 in methanol). During the reaction Ca-soaps formed during digestion are also methylated (Torcello-Gómez et al. 2018), i.e., become a part of the bioaccessible fatty acid pool, thus increasing the apparent digestibility. To determine the amount of esterified fatty acids a second method is used. Esterified fatty acids are trans methylated in one-step i.e. alkaline environment (0.2 M KOH in methanol) where free fatty acids form soaps.

After the fatty acid-specific determination of TFA and EFA fractions derivatized with the two methods is completed, lipid digestibility scores could be determined i.e., bioaccessibility (release ratio; RR) of each individual fatty acid (Eq. 4) and amount of bioaccessible fatty acids (Eq. 5) could be determined.

$$FFA_i = TFA_i - EFA_i \ (Eq. 3)$$

$$Bioaccessibility\ of\ FA_i / Release\ ratio = \frac{FFA_i}{TFA_i} \ (Eq. 4)$$

$$Bioaccessible\ FA\ content\ [\%] = \left(\frac{\sum FFA_i}{\sum TFA_i}\right) \times 100 \ (Eq. 5)$$

Equation 3-5: TFA_i is the total (free and esterified) amount of an individual FAs in the small intestinal digesta, EFA_i is the amount of the same FA present in esterified (undigested) form, FFA_i is the amount of the same individual free FA in the small intestinal digesta, $\sum FFA_i$ is the sum of the amount of each individual FFAs and $\sum TFA_i$ is the sum of the amount of each individual FA in the small intestinal digesta.

Additionally, this method also could be applicable to determine the initial free fatty acid content of food products. These FFAs that are originally in the food sample (e.g., food additives) are metabolised the same ways as FFAs liberated from TAGs (Mortensen et al. 2017). However fat content of foods is mostly composed of TAGs, free fatty acids in the sample before digestion are added to and included in the bioaccessible free fatty acid value. Thus, the method described above could also be a way to assess free fatty acid content of food products.

The above described method is applicable to determine the total fatty acid content (TFA) of the extracted fat from the digesta which could be used to determine fatty acid composition of the food product and the bioaccessible fatty acids (based on Eq. 3) as well. After B&D extraction of all lipid compounds from the digesta the fatty acid composition was determined with the TFA method. This fatty acid composition was compared with the fatty acid composition of the ISO method extracted fat's profile using the Bland-Altman plot (Figure 8).

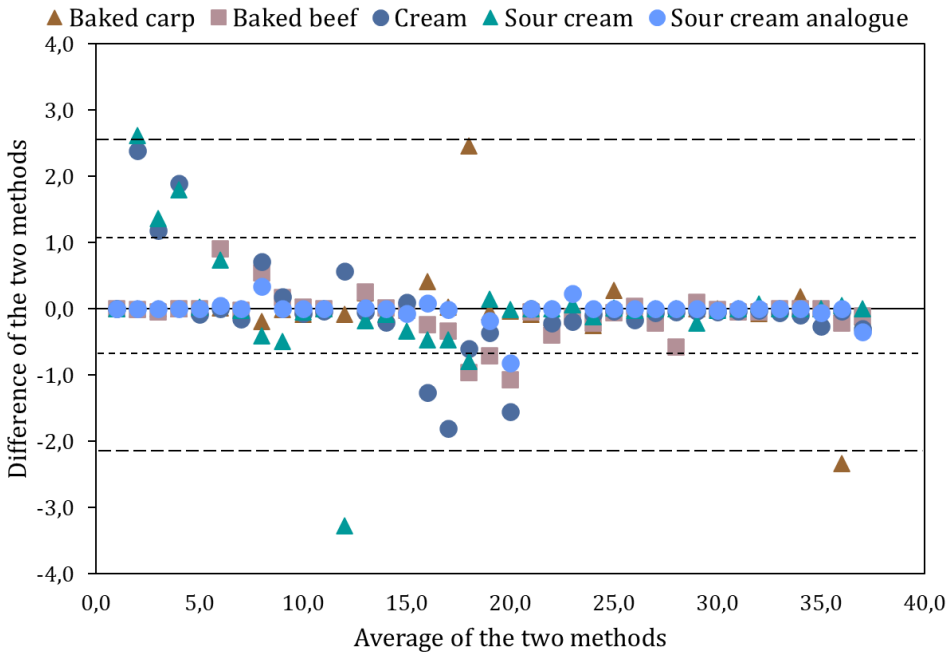

Figure 8. Bland-Altman plot comparing the fatty acid profile of food products (baked carp, baked beef, cream, sour cream, sour cream analogue) extracted with relating ISO method and the total fatty acid profile of the digesta extracted with the Bligh & Dyer method. Dashed lines show the average±(1.96*STDEV).

The plot comparing the fatty acid profile of the test foods and their digesta's show that B&D extraction and the TFA derivatization method have no proportional bias to the ISO extracted fatty acid profile. There could be a slight deviation of the determination of the fatty acids present in a smaller concentration however this does not affect overall fatty acid profiling.

5.1.5. Internal Standardization

Protocols for determination of fatty acid methyl esters always include one or two internal standards. The primary purpose of standards is to correct any differences in sensitivity experienced during calibration and sample measurement. Therefore, ISTDs usually added after sample preparation before analysis. However, with this approach, losses occurred during sample preparation and extraction are not accounted for. Since the method to evaluate bioaccessible free

fatty acids include multiple steps parallel to each other, it is necessary to track sample losses caused by extraction or different reaction efficiency. To correct all steps of sample preparation (i.e., fat extraction, derivatization, analysis), the internal standard is added right after sampling the small intestinal digesta and before extraction of lipid compounds.

In order to understand, which ISTD is the most appropriate, some considerations should be made. It should be considered that after extraction of all lipid components, the samples are derivatized by two methods. One reacts with all (esterified and free) fatty acids (TFA) and the other involves only esterified fatty acids (EFA). Most methods generally use FAMEs as internal standards (Ye et al., 2019), which is considered appropriate to monitor extraction efficiencies, however these compounds do not react in either derivatization method (TFA and EFA), thus could not correct for reaction efficiency of the derivatization. Another option could be free fatty acids nevertheless these components do not react in the EFA method thus should be excluded. Finally, TAGs have been appointed as appropriate internals standards. TAGs are extracted with all lipid components and react in both derivatization methods thus could correct every step of sample preparation.

Another question was the to select an appropriate TAG. Since internal standards are chosen to cover most of the analytes it should be eluted in the middle range of the chromatographic run. In addition, it is a requirement that the analysed sample should not contain the chosen internal standard. Considering all the conditions, glyceryl trinonadecanoate (C19:0 TAG) was chosen. Nonadecanoic acid is a suitable option since this fatty acid does not occur in any natural foods of food products (Lohninger et al. 1988). It also elutes in the middle range of the chromatogram, representing short-, medium-, and long chained fatty acids as well. One hindsight of using this fatty acid as an additional compound into the samples is that special effort needed to be made for the acceptable separation from linoleic acid (C18:2n-6c). So, temperature profile of gas chromatographic separation was tailored so these two FAMEs in the internal standard spiked samples could be distinguished.

5.1.6. Recovery

To test the chosen internal standard's applicability, and the yield of the chosen derivatization methods, a recovery test was carried out. Two additional compounds that are usually not found in foods were chosen for this purpose. One is glyceryl triheptadecanoate (C17:0 TAG), representing esterified fatty acids, and heptadecanoic acid (C17:0 FFA), representing free fatty acids liberated during digestion of triacylglycerols. The use of both compounds allows to test the recovery of both derivatization methods, i.e., total fatty acid method and esterified fatty acid method. The compound C17:0 TAG being an esterified fatty acid transformed in both methods, however C17:0 FFA only converts into FAMEs in the two-step derivatization method used for

determination of total fatty acid amount. In the method used to determine amount of esterified fatty acids C17:0 FFA turns into soaps thus in is not detected as FAMEs. The recovery experiment was designed to comprehensively validate the proposed method to accurately determine both EFA and FFA species in the small intestinal digesta. This step is crucial since sum of EFAs and FFAs (i.e., TFA) and FFAs are used to quantitate bioaccessibility of sum of fatty acids (Eq. 3) and of individual fatty acids (Eq. 2) as well. In addition, with the use of these two compounds effectiveness of the fat extraction method towards EFAs and FFAs are also could be evaluated assuring the extraction methods applicability to all lipid species.

In this experiment, four digests were used. Namely, blank digest (using 5 g water), baked carp digest (1 g homogenized sample), baked beef digest (0.9 g homogenized sample), and cream digest (0.5 g homogenized cream). Appropriate amounts of samples were suspended in distilled water to reach 5 g sample weight before in vitro digestion simulation was carried out according to Infogest protocol. From each digesta, 5 mL homogenized samples were taken and 250 µL of 1 mg/mL C17:0 FFA (in $CHCl_3$) and 250 µL of 1 mg/mL C17:0 TAG (in $CHCl_3$) as well as 250 µL of 1 mg/mL C19:0 TAG (in $CHCl_3$) was added to each sample then B&D extraction was carried out with addition of the appropriate volumes of chloroform, methanol, and water. At the end of extraction, all standards and lipid compounds were dissolved in the 12.5 mL of chloroform. From this, two times 5 mL were transferred into two 50-mL round bottom flasks and TFA method on one and EFA method on the other was executed. The 5 mL sample from the chloroform phase contained 100 µg of all three standards. With TFA method all three standards reacted and transformed into FAMEs, giving 100 µg of C19:0 ME and 200 µg of C17:0 ME, i.e., 100 µg from C17:0 TAG and 100 µg from C17:0 FFA. In the EFA method only TAG standards turned into FAMEs, hence only 100 µg of C19:0 ME and 100 µg of C17:0 ME could be recovered in the sample. Results of recovery experiment are shown in Table 2.

Table 2: Results of the recovery experiment. Concentration of heptadecanoic methyl ester in μg/mL in samples spiked with the same volumes of C17:0 TAG and C17:0 FFA, derivatized with TFA and EFA methods (taken from ISO 12966-2:2017 as detailed). Samples were also spiked with C19:0 TAG at 100 μg/mL and calibration solutions were spiked with C19:0 ME at 100 μg/mL as well. Values are given in average ± deviation (RSD) format.

Test food	Concentration of C17:0 ME [μg/mL]			
	TFA method	Recovery	EFA method	Recovery
Blank digesta	157.4 ± 23.5 (0.15)	78.7%	77.9 ± 1.8 (0.02)	77.9%
Baked carp digesta	185.7 ± 23.2 (0.13)	92.8%	97.1 ± 15.9 (0.16)	97.1%
Baked beef digesta	193.0 ± 14.1 (0.07)	96.5%	97.9 ± 17.3 (0.18)	97.9%
Cream digesta	188.8 ± 2.3 (0.01)	94.4%	90.1 ± 3.6 (0.04)	90.1%

As presumed in the samples spiked with C17:0 TAG and C17:0 FFA twice the amount of C17:0 ME were detectable after the TFA method was conducted than when the EFA method was the choice of derivatization method. Recovery from the real sample digests was high, between 90-98%. There was no significant difference between the recovered amounts of C17:0 ME with either the TFA method ($p= 0.985$) and the EFA method ($p= 0.223$). Recovery was the lowest from the blank digesta only 78-79% although due to the extensive sample preparation this recovery is acceptable as well. In spite of the fact the analysis was made from the small intestinal digesta, where the sample is homogenized in high volume of different solutions, i.e., digestion simulation fluids and enzyme solutions, these results show that there is no matrix effect or the applied ISTD could correct for it in the designed method. Additionally, this experiment showed that the fat extraction method chosen especially for the purpose to extract lipid compounds from aqueous solutions does not show any bias to either lipid species (EFA and FFA). Conclusively the method applying the B&D extraction and C19:0 TAG as an ISTD is suitable for accurate determination of both FFAs and EFAs from the small intestinal digesta. Using this method, bioaccessibility of foods could be determined on a fatty acid-specific way.

5.1.7. The final harmonized protocol to evaluate lipid bioaccessibility of foods

After all quality assurance steps of method development were clarified, the final protocol was set up (Tormási and Abrankó, 2021). The outline of the protocol is shown on the Figure 9.

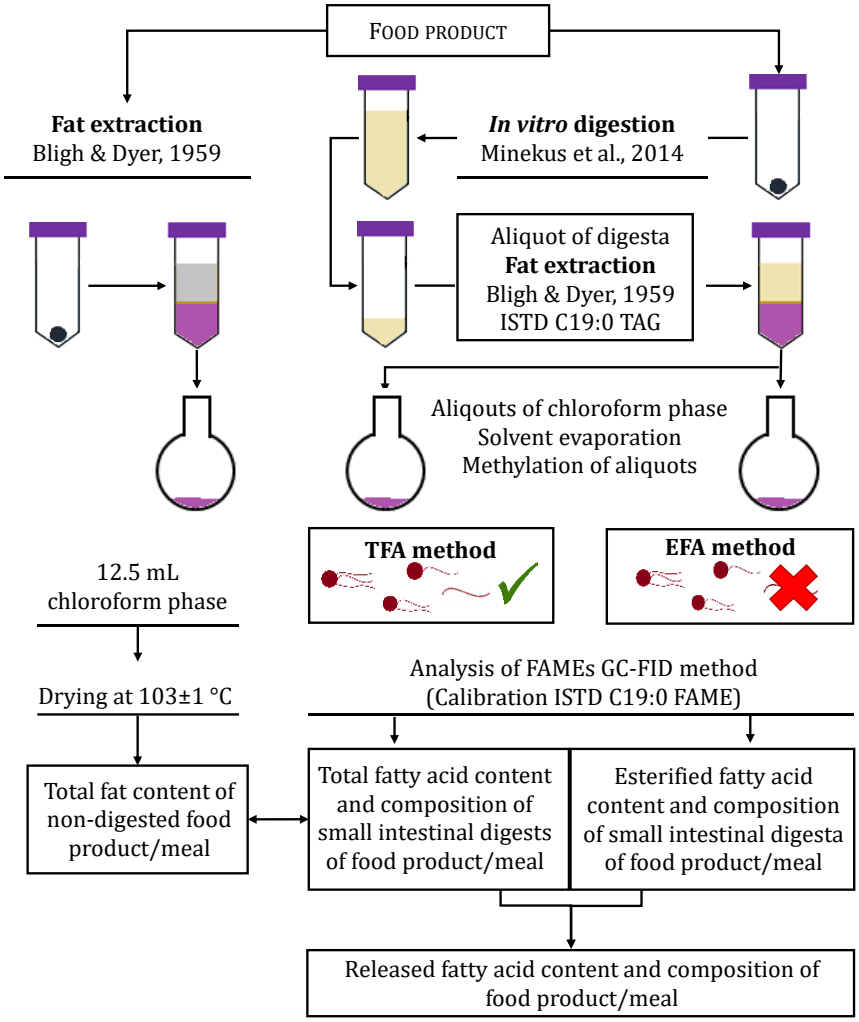

Figure 9. Protocol outline for the harmonized sample preparation of *in vitro* digesta for the assessment of free fatty acid release in food samples along with the protocol for fat content determination of the same food sample *(Original image).*

In the proposed routine workflow, fat content of the tested food could/should be determined after fat extraction with the Bligh & Dyer method – if ISO stated standard method is not routinely used or unavailable. After *in vitro* digestion simulation – according to the Infogest protocol – is carried out, all lipid content is extracted from the small intestinal digesta with the Bligh & Dyer method. During extraction, polar components are separated from the non-polar components, which are transferred into the chloroform phase already spiked with internal standard (C19:0 TAG). From the total volume of chloroform (12.5 mL), two times 5 mL aliquots (containing 100 µg C19:0 TAG) are taken for derivatization, one with the total fatty acid method and the other with the esterified fatty acid method (ISO 12966-2:2017). For separation of FAMEs gas chromatography is used. Qualitative and quantitative analysis is based on a four-level calibration (0, 10, 20, 40 µg/mL approximate concentration) with a FAME mixture composed of 37 FAMEs spiked at 100 µg/mL at each level with C19:0 ME. Fatty acid content and composition from TFA

method derivatized aliquot should be compared with B&D extracted fatty acid content and composition of the food sample as a built-in quality control step. Bioaccessible fatty acid content and individual release ratio of fatty acids are gotten after subtracting EFA results from TFA results.

5.2. Standardized method for evaluating protein digestibility of foods

During my PhD studies, I had the opportunity to take part in an international ring trial on standardization of a protein digestibility assessment method based on the Infogest digestion simulation method, called "*In vitro digestion protocol for the analysis of protein digestibility and in vitro DIAAS in dairy products*". Since I have already been working on a protein digestibility assessment method my work focused on bringing the two alternative methods together and to expand the use of the method for non-dairy products. This part focuses on the questions taken into consideration.

5.2.1. Overview of the standardized method

The proposed method for the protein digestibility analysis gives an option to descaling the original Infogest digestion simulation. In this approach, instead of the 5 g food sample (containing 200 mg protein) using 1 g food sample (containing 40 mg of protein) is recommended, therefore end volume of the digesta will be 8 mL instead of 40 mL. Logical reasoning behind the decreased weight of sample used will be understood in the further sample preparation steps for protein content analysis. After the digestion simulation, intact protein content of the small intestinal digesta – with 8 mL end volume – is isolated in one step, with the addition of methanol, thus no sample loss could occur. After methanolic precipitation, the supernatant – containing bioaccessible protein fraction – and the pellet – containing undigested proteins – could be separated. Afterwards, both phases should be hydrolysed to amino acids then the protein content could be determined with several methods as i) the Kjeldahl method, ii) the OPA method, or iii) based on amino acid content. In the protocol, the *in vitro* protein digestibility is determined according to Eq. 6.

$$In\ vitro\ protein\ digestibility\ [\%] = \frac{F_S - C_S}{(F_S - C_S) + max(0; F_p - C_p)} * 100$$

Equation 6: *In vitro* protein digestibility (IVPD%) is calculated from the protein contents of the i) food supernatant (F_s), ii) the food pellet (F_p), iii) the control supernatant (C_s), iv) the control pellet (C_p). Control food is a protein free biscuit used to correct with autolysis of digestive enzymes.

5.2.2. Necessity of control food digestion

In my previous digestion experiments a blank digesta was always added next to the food samples. Blank digesta is treated equally to all other samples, thus lipid content from added reagents and enzyme solutions could be determined and subtracted from the total lipid content including the lipid content of the food samples. In evaluation of lipid digestibility, distilled water is used as blank. This approach is sufficient in lipid digestion experiments since water does not contain lipid molecules and all lipid molecules from different sources could be recovered in the fatty acid determination methods even if some of them are hydrolysed during the simulation.

However, the use of water as blank sample is not sufficient when protein digestibility is assessed. Enzymes used to digest the foods are added protein molecules. Some of these enzymes are proteolytic enzymes which have the goal to cleave other protein molecules creating peptides and free amino acids. If there is no other substrate available these enzymes tend to cleave each other which is called autolysis. The peptides and amino acids released during autolysis of enzymes create higher apparent background than the enzymes realistically would give. The higher background will result lower calculated protein digestibility. To avoid the error caused by autolysis of enzymes, a protein free biscuit is used as a buffer or dummy substrate in the blank sample. The protein free biscuit is generally used during *in vivo* experiments as well (Moughan et al. 2005).

5.2.3. Hydrolysis before determination of bioaccessible protein content

The Infogest method is a time-, and enzyme-concentration-limited (so-called static) simulation method mimicking human digestion conditions. After digestion simulation, the small intestinal digesta contains free amino acids, various lengths of peptides and even intact – undigested – proteins. The precipitation method separates the larger protein molecules from the fully or partially digested proteins. Thus, the amount of amino acids and peptides in the methanolic solution is considered the bioaccessible fraction. The validation of this isolation method, i.e., the comparison of the results of this approach with *in vivo* data is recently published (Sousa et al. 2023). Direct measurement from the extracted phase (supernatant) could give an approximate value on protein digestibility however without further hydrolysis of remaining peptides, these molecules are counted as a single amino acid in most widely used protein determination methods (except Kjeldahl method which includes a further digestion step). Therefore, an additional hydrolysis step is required before protein content determination in methods relying on detecting free amino groups such as the OPA method or on derivatization of free amino acids such as the AQC derivatization method.

The protein hydrolysis could be done by several methods. Usually, acidic hydrolysis of proteins is done with 6 M HCl for 20-24 h at 110 °C. One disadvantage of this method is that it is not

applicable to determine tryptophan content, i.e., during acidic hydrolysis tryptophan gets decomposed (Fountoulakis and Lahm 1998). My first goal was to find an appropriate method which could be used to determine all amino acids. Tested hydrolysis method include hydrolysis in methane sulfonic acid (Chiou and Wang 1988), basic hydrolysis – for only tryptophan – in sodium hydroxide (Zhang et al. 2009), hydrolysis in mixed media (in acidic and basic solvents; AOAC 2018.06; (Jaudzems et al. 2019) and microwave-assisted acidic hydrolysis (see *Materials and Methods* chapter *4.3.6. In vitro protein digestibility*). Showing details of test results of this comparison is beyond the scope of this thesis. However, after careful consideration – and in accordance with the ring trail protocol – the latter two method was chosen. In my further experiments AOAC method is used when OPA measurements were carried out and microwave-assisted acidic hydrolysis is used when amino acid profiling is done from the supernatants.

5.2.4. Applicability of sampling

In the standard protocol, after completing the digestion simulation, the isolation of bioaccessible protein fraction is carried out using a one-step methanolic precipitation. It means, the analyst completely consumes and sacrifices the total amount of sample (digesta) for a single analysis, i.e., determination of protein bioaccessibility. The one-step isolation of bioaccessible protein fraction is a safe and reproducible option when only protein digestibility of a certain food product is in question. With this approach however, the total volume of the digesta is required and consumed for the analysis of one type of analyte. If digestibility of other components with different characteristics should be simultaneously determined, this approach is not beneficial. In my experiments, the goal was to develop a protocol, that enables the simultaneous determination of the bioaccessibility of multiple macronutrients, thus there was a need to find different ways to separate bioaccessible protein fraction.

To resolve this matter, I wanted to try isolation of bioaccessible protein fraction after sampling aliquots of the digesta. In this way, multiple samples could be taken from the small intestinal digesta of a single digestion, and from the different aliquots, different analytes could be measured. Since there was no data on the fitness for purpose of such sub-sampling approach regarding protein content determination, a validation protocol of this sampling technique was designed and performed.

Digestion experiments were carried out on a certified reference material (NIST 3252; protein drink mix) containing known amount of protein with known amino acid composition. First, digestion simulation was made according to the ring trial protocol, i.e., 1 g sample (containing 40 mg of protein) digested to 8 mL end volume, separated with 32 mL methanol in one step. Results of this experiment were compared to the second digestion simulation using 5 g sample (containing

200 mg protein) digested to 40 mL end volume. From this 40 mL digesta, 5 mL aliquots were taken after homogenization, then 25 mL methanol was added to isolate the bioaccessible fraction. After isolation, samples were treated in the same manner. After addition of methanol tubes were stored at –20 °C for 1 h for complete precipitation. Supernatant was separated from pellet with centrifugation (3700 *g*, 4 °C, 20 min), and amino acid content of both fractions were determined after microwave-assisted acidic hydrolysation. Comparing the results of the two digestion simulations (Table 3) give information on the reproducibility of the digestion simulation with different sample sizes and on the applicability of the sampling of the digesta.

Table 3: Protein content of the pellets and supernatants separated with one-step precipitation and separated after sampling with methanolic precipitation, also sum of pellet and supernatant is given. Protein content is calculated from amino acid content [mg/ 100 g sample]. Values are given in average ± deviation (RSD) format. *In vitro* protein digestibility (IVPD%) is calculated according to Eq. 6 either from pellet + supernatant (P+SN) or according to Eq. 7 from only the supernatant (SN).

Protein content [mg/100 g sample]	**One-step precipitation (40 mg protein)**	**Precipitation after sampling (200 mg protein)**	**t test (p value)**
Pellet	6.98 ± 1.21 (0.17)	42.25 ± 2.76 (0.07)	-
Supernatant	33.92 ± 1.67 (0.05)	172.17 ± 13.80 (0.08)	-
Sum	40.90 ± 0.59 (0.01)	214.42 ± 14.85 (0.07)	-
IVPD% (P+SN)	82.9 ± 3.2 (0.04)	80.3 ± 1.4 (0.02)	0.195
IVPD% (SN)	84.8 ± 4.2 (0.05)	86.1 ± 6.9 (0.08)	0.707

As the Table 3 shows, the yield of both versions is adequate (102-107%). In both digestion simulations, the sum of the protein content – the pellets and the supernatants – gives the amount of protein that was introduced to the simulation thus there was no substantial sample loss with either method. Additionally, there was no significant difference between the ratio of SN and P weights of each tubes (p= 0.165). The *in vitro* protein digestibility (IVPD%) was also calculated according to Eq. 6 from the protein content of the pellet and the supernatant. There was no significant difference between the calculated IVPD% of the two methods. According to the results the sampling method is considered applicable instead of the one-step precipitation and the estimated IVPD% is the same with either sample preparation method. Hereafter the determination of protein digestibility is done from the sampled digesta.

5.2.5. Calculation of *in vitro* protein digestibility

If the *in vitro* protein digestibility is assessed from the aliquots taken from the digesta, another question arises linked to the calculation given in the ring trial protocol. In Eq. 6 IVPD% is calculated from the protein content of the pellet and the supernatant of the food sample and of the blank cookie. However, the weight of the formed pellet after precipitation and centrifugation is rather small. In the one-step precipitation it is around 20-30 mg for the food samples and 300-500 mg for the blank sample. When the small intestinal digesta is sampled, the aliquot will contain a small portion of the digesta therefore after precipitation pellet weights will be smaller (~10 mg). Although determination of protein content (amino acid content) is possible from these small quantities of pellets, in routine practice, it introduces higher uncertainty to analytical results, thus it is not advantageous to do so. Another option for calculation of IVPD% is by only determining the protein content of the supernatant and relate it to the introduced amount of protein, as according to Eq. 7.

$$In\ vitro\ protein\ digestibility\ [\%] = \frac{F_S - C_S}{F} * 100$$

Equation 7: *In vitro* protein digestibility (IVPD%) is calculated from the protein contents of (i) the food supernatant (F_s), (ii) the control supernatant (C_s), (iii) the food weighed in (F). Control food is a protein free biscuit used to correct with autolysis of digestive enzymes.

In vitro protein digestibility is also calculated according to the Eq. 7 and shown in Table 3. Although the Eq. 7 calculated digestibility is higher than the Eq. 6, calculated values these values do not differ from each other (t test, p= 0.707) and from the value given according to the standard protocol (t test, p= 0.341). The results are similar using either digestion methods or either calculation method, so it can be concluded that the alternative sampling and the calculation of IVPD% from the protein content of the supernatant gives the same results as the original – ring trial – protocol. The use of the sampling technique will give the opportunity to analyse more than one type of macronutrient from the same digesta, thus interplay between food components could be determined simultaneously, e.g., lipid and protein digestibility could be routinely and effectively assessed from the same digestion simulation.

5.2.6. Protein quality scores

Previously the importance of protein quality was noted in the Chapter *Literature overview*. Besides protein quantity, the protein quality of consumed foods is important. Deficiency of essential amino acids could cause serious health problems, an issue which could occur even if enough amount of

protein had been consumed (Bailey and Stein, 2019). In addition to assessment of overall protein digestibility, a more detailed – amino acid based – information could be given from the results obtained. As previously presented, the supernatant could be used to evaluate protein digestibility and after determination of amino acid composition of food and the supernatant the results could be used to calculate digestibility of individual amino acids. To further assure the suitability of the sampling method, comparison of the amino acid profile was performed between the supernatant of the two methods (Table 4).

Table 4: Amino acid content [g AA/100 g sample] of supernatants from the two versions of sample preparation techniques: from one-step precipitation and from precipitation after sampling. Amino acids are abbreviated in the three-letter form. Difference between values is given with t test, p values are shown. Values are given in average ± deviation (RSD) format.

Amino acids (three-letter abbreviation)	Amino acid content [g AA/100 g sample]		t test (p value)
	One-step precipitation	Precipitation after sampling	
His	1.5±0.12 (0.08)	1.3±0.24 (0.18)	0.376
Ser	2.8±0.12 (0.04)	3.0±0.18 (0.06)	0.193
Arg	3.1±0.17 (0.05)	3.1±0.22 (0.07)	0.917
Gly	1.5±0.08 (0.05)	1.7±0.17 (0.10)	0.139
Asp	4.6±0.32 (0.07)	5.1±0.36 (0.07)	0.081
Glu	8.6±0.52 (0.06)	8.8±0.51 (0.06)	0.711
Thr	3.1±0.15 (0.05)	3.1±0.18 (0.06)	0.965
Ala	2.3±0.14 (0.06)	2.7±0.16 (0.06)	0.010
Pro	4.4±0.16 (0.04)	4.3±0.18 (0.04)	0.441
Cys	0.2±0.02 (0.13)	0.2±0.03 (0.17)	0.285
Lys	4.6±0.34 (0.07)	5.0±0.29 (0.06)	0.120
Tyr	2.6±0.13 (0.05)	2.6±0.19 (0.07)	0.738
Met	1.3±0.08 (0.06)	1.1±0.09 (0.08)	0.032
Val	3.8±0.19 (0.05)	3.9±0.20 (0.05)	0.794
Ile	3.6±0.15 (0.04)	3.4±0.17 (0.05)	0.199
Leu	5.6±0.25 (0.04)	5.4±0.26 (0.05)	0.225
Phe	2.8±0.12 (0.04)	2.6±0.19 (0.07)	0.170
Trp	0.4±0.04 (0.09)	0.4±0.03 (0.07)	0.052

Amino acid analysis (digestion and UHPLC-UV measurement) was carried by Éva Lengyel-Kónya, Mária Berki and Rita Tömösközi-Farkas which is kindly acknowledged.

As the Table 4 shows there are only two amino acids that differ (at p= 0.05 significance level) between the results: alanine (Ala) and methionine (Met). Alanine is not an essential amino acid thus overestimation do not affect essential amino acid accessibility. Methionine is part of the sulphur-containing amino acids (SAA) with cysteine. These two amino acids are known for being

difficult to determine since during sample preparation derivatives could be formed (Fountoulakis and Lahm, 1998). Although the difference is shown to be significant, it is not higher than with other amino acids, e.g., phenylalanine or isoleucine. The applicability of the sampling method is also proven by the comparison of the amino acid profiles gotten from the two methods by using the Bland-Altman plot (Figure 10).

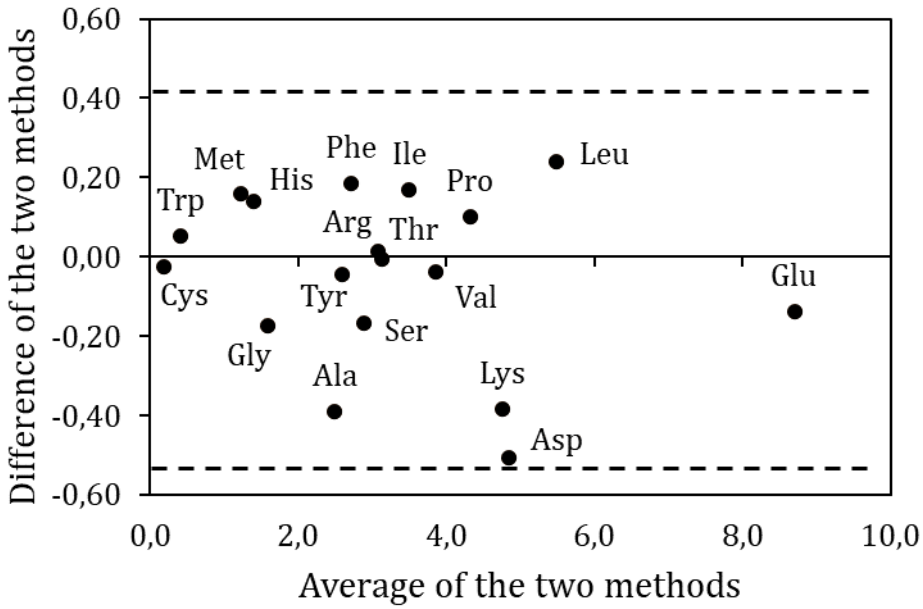

Figure 10. Bland-Altman plot comparing the amino acid profile of the reference material after *in vitro* digestion and one-step precipitation and *in vitro* digestion and precipitation after sampling. Dashed lines show the average±(1.96*STDEV).

The comparison shows no proportional bias between the two methods therefore the precipitation after sampling could be used instead of one-step precipitation when *in vitro* digestibility is at question.

From the amino acid specific results protein quality indicators could be calculated, i.e., *proxy*-PDCAAS and *in vitro* DIAAS, accordingly to Eq. 8-9, respectively. Calculated protein quality scores are shown in Table 5.

$$proxy - PDCAAS = MIN\left[\frac{mg\ AA_i/g\ product\ protein}{mg\ AA_i/g\ reference\ protein}\right] \times IVPD\%$$

Equation 8: AA_i, is the concentration in mg/g protein of the i^{th} essential amino acid of the product or in the reference protein (FAO et al. 2007), IVPD%: the protein digestibility determined by Infogest *in vitro* digestion simulation.

$$in\ vitro\ DIAAS\ = MIN\left[\frac{\frac{mg\ BAA_i/g\ product}{mg\ AA_i/g\ product} \times\ mg\ AA_i/g\ product\ protein}{mg\ AA_i/g\ reference\ protein}\right]$$

Equation 9: AA_i, is the amount of each amino acids, BAA_i, is the bioaccessible amount of each amino acids. Reference protein composition is given by FAO for three age groups (FAO, 2013).

Table 5: Protein quality scores of the test matrix (CRM NIST 3252 protein drink mix) calculated according to Eq. 8 for *proxy*-PDCAAS and Eq. 9 for *in vitro* DIAAS. Scores are given in "value (limiting amino acid)" format.

Sample preparation method	***proxy*-PDCAAS**		
	Preschool child (2-5 year)	Schoolchild (10-12 year)	Adult
One-step precipitation	46 (Trp)	56 (Trp)	100 (Trp)
Precipitation after sampling	47 (Trp)	57 (Trp)	100 (Trp)

Sample preparation method	***in vitro* DIAAS**		
	Infant (0-6 month)	Child (6-36 month)	Older child, adolescent, adult
One-step precipitation	39 (Trp)	79 (Trp)	96 (SAA)
Precipitation after sampling	35 (Trp)	69 (Trp)	87 (SAA)

As Table 5 shows between the two sample preparation methods in the *proxy*-PDCAAS values there is no difference however minor deviation in *in vitro* DIAAS values is noticed. *Proxy*-PDCAAS is not affected by the inaccuracies of the amino acid measurement from the digesta shown in Table 5 and since overall digestibility did not differ these values are in accordance with each other. In contrast DIAAS is calculated from the amino acid profile of the digesta. The shown minor differences in amino acid values could cause more pronounced difference in the DIAAS value. For example, in the second age group tryptophan (Trp) is the limiting amino acid with 79 and 69 value according to the results from the two sample preparation methods. The amount of Trp in the digests are 449 mg/100 g sample and 395 mg/100 g sample, respectively,

which is less than 50 mg difference and was not significantly different according to the t test seen in Table 4. As the results show even this minor deviation could cause more distinct calculated the *in vitro* DIAAS values therefore more caution and accuracy are needed in amino acid profiling of the digesta.

Considering all the results presented, the modified protocol is applicable to determine *in vitro* protein digestibility from the supernatant after methanolic precipitation of the sampling the digesta. From the results protein quality indicators – *proxy*-PDCAAS and *in vitro* DIAAS – could be calculated. It can also be concluded that it is not the differences in the sampling protocol that solely responsible for the observed differences in *in vitro* DIAAS values obtained by the application of the two protocols.

5.3. Practical aspects of the Infogest protocol

During my work, the method of choice for the digestion simulation was the Infogest method (Minekus et al. 2014; Brodkorb et al. 2019). Advantages and disadvantages of using a static *in vitro* protocol have been detailed. Even though the use of *in vitro* protocols is questioned from one point of view – dynamic nature of digestive tract is neglected – use of them could be preferred in special circumstances – beginning of heath effect-based product development. During my work, the Infogest method have been used to simulate the human digestive processes before determination of lipid and protein digestibility of animal products without known data on specific digestibility. Although the source of the method provides quite detailed instructions, there were some aspects that had been considered when using it in routine practice and when designing analytical methods based on the protocol.

5.3.1. Sample weight

As the protocol states, sample weight measured in for digestion simulation should be "5 g with the thickness similar to mustard" which must be achieved by grinding and/or suspending the food in water. However, there is an option for reducing sample weight based on consistency and depending on further sample preparation (as seen in protein digestibility method) there is no information on compositional details of the digested foods.

In my early experiments strictly following the "5 g sample weight" rule, the results showed unbelievably low lipid digestibility of the analysed food sample. Therefore, to determine the causes of this effect several digestion experiments with different sample weights were conducted using baked carp meal as a test food, as an example. Extent of lipolysis based on bioaccessible fatty acid content of baked carp meal with different sample sizes is shown in Figure 11.

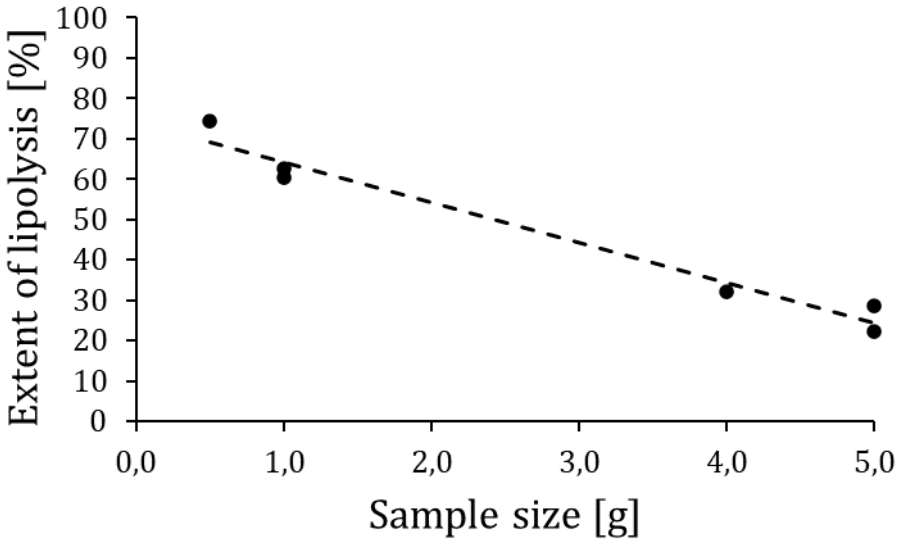

Figure 11. Extent of lipolysis [%] measured after *in vitro* digestion simulation of different sample sizes of baked carp meal.

As the Figure 11 shows that the bioaccessible fatty acid content depends on the weight of the sample introduced to the Infogest digestion simulation. A negative correlation is noticed between the two factors (Pearson correlation coefficient: r = -0.82) therefore with the increase in the sample size the measured lipolysis deceases.

It seems that although the "5 g sample weight" approach is straightforward, this instruction does not directly applicable in all situations. This might be stemmed back to enzyme-substrate concentrations applied in the static Infogest method. Each enzyme has a unique concentration limit above which effective activity could not be reached. In food samples with high fat content, this limit is quickly reached, and hydrolysis of TAGs stops before completion. Therefore, additional information on lipid content of the digestible sample is needed when determining the mass of the sample. This information was provided in the ring trial protocol when it is stated that the enzyme-substrate ratio of the Infogest method was designed to realistically mimic human conditions, which means enzymes added to the simulation could efficiently hydrolyse 200 mg of protein and 250 mg of lipids in a digestion simulation with 5 g sample weight. Although this guideline is necessary, it still leaves room to vary sample weight greatly.

Additionally, sample weight could be harmonized with the analytical method for limiting further freedom of variants. Therefore, it was necessary to determine the sampling volume at which the fat content of the digesta taken could be extracted, derivatised and analysed with sufficient efficiency and accuracy. Sampling adjustments were made with 5 g digested samples, and volumes

0.5 mL and 5 mL were taken from the homogenized digesta. When comparing the results of the samples measured after the same sample preparation, difference is observed in case of the 5 mL sample volumes (p= 0.034). To understand the causes of the difference, total fatty acid content of the two sampling was compared. The total fatty acid results obtained with different sampling of 5 g digests are shown in Table 6. Theoretical TFA means the calculated fatty acid content based on the sample weight and sampling volume without additional correction with methyl ester side groups.

Table 6: Total fatty acid content of 5 g baked carp meal digesta in µg/mL unit. Theoretical and measured values are given with two different sampling volumes. Yield [%] of total fatty acid determination is calculated from the two values [%].

Sample	**Baked carp meal**	
Sample weight [g]	5	5
Sampling volume [mL]	0.5	5
Volume of isooctane [mL]	1	10
Theoretical TFA in aliquot [µg/mL]	8935.9 ± 312.85 (0.04)	9199.8 ± 226.39 (0.02)
Measured TFA in aliquot [µg/mL]	8158.2 ± 345.68 (0.04)	6585 ± 465.8 (0.07)
Yield [%]	91.3 ± 3.87 (0.04)	71.6 ± 5.02 (0.07)

The amount of total fatty acid in the 5 mL small intestine digest of 5 g baked carp is about 90 mg, which after extraction is split into two (45 mg) must be taken up in a larger amount of solvent (10 mL isooctane instead of 1 mL) to achieve the appropriate dilution. The inhomogeneity of the distribution of this small amount of FAMEs in the larger volume of solvent (45 mg fat/10 mL isooctane) may cause the lower yield (71%) shown in Table 6. Due to extensive dilution and sample transferring inaccuracies the correction by the internal standard is not enough to appropriately assess lipid content of digesta. However, in the other cases, the fat content of the sample from the small intestine digest is only 10-20 mg (10-10 mg for each derivatization method), which can be analysed without further dilution thus the yield of the measurements is higher (91%) i.e., no losses could occur due to excessive sample transfer. Differences in the total fatty acid content, due to different yields, may cause apparent differences in digestibility. Thus, based on the fat digestibility determined in several measurement set-ups, I conclude that the fat content of the initial sample for digestion simulation should not exceed 150 mg to appropriately determine the bioaccessible fatty acid content with the above detailed method.

According to my calculations based on bioaccessible fatty acid content measured with different sample masses, the digestion simulation of a sample with 250 mg fat content should result the

same digestibility value as the digestion simulation of a sample with 100-400 mg fat content (within the 10% confidence). However above 250 mg, the enzymatic digestion is hindered therefore the fat content of the sample must be between 100-250 mg when analysing fat digestibility of foods. Note that during determination of bioaccessible fat content it is recommended to keep the fat content of the digestion simulation under 150 mg.

During digestibility assessment of various compounds, it is important to keep in mind the physiological relevance of the simulation method. However, using the right analytical tool is also key. Overload of digestive enzymes and subsequent analytical measurements will cause the measured digestibility to be apparently lower. Therefore, sample weight needs to be normalized to the assessed analyte, e.g., lipid content or protein content. In addition, when effects of certain bioactive compounds, different matrices or structure is in question digestion experiments need to be carried out in the same settings to be comparable.

5.3.2. Use of multiple lipolytic enzymes

When looking at the digestion of lipids there are two main lipolytic enzymes should be mentioned, i.e., gastric lipase, produced by the stomach wall, and pancreatic lipase secreted into the duodenum produced by the pancreatin. The two enzymes have different activity and preference towards triacylglycerols. Gastric lipase accounts for approximately 5-40% of all-over lipid digestion (Armand 2007), pre-digesting high fat foods before they can enter the small intestine. In the first protocol (Minekus et al., 2014; v1.0, “PL”) there is a note on the necessity of the use of gastric lipase. However, without commercially available source it was omitted in the first version. In the improved version of the method (Brodkorb et al., 2019; v2.0, “GL+PL”) this remark is further emphasised. Here, detailed reasoning behind the use of gastric lipase is given, i.e., it contributes to overall digestion of TAGs (10% with solid foods, 25% with emulsified liquid samples), and it triggers the subsequent action of pancreatic lipase on substrates that might be poorly digested by pancreatic lipase alone e.g., milk fat droplets and lecithin-stabilized TAG emulsions (Gargouri et al. 1986). Since my main focus was on lipid digestibility this aspect needed to be addressed. Therefore, when analysing lipid digestibility of foods, mechanistic approach was applied using both methods (PL and GL+PL) for digestion simulation comparing the effect exposed on gastric and pancreatic lipase separately on a fatty acid basis. Later, when effect of certain food components on lipases activity was evaluated, wherever a significant reduction in pancreatic lipase activity was found, further experiments were carried out, to assess whether gastric lipase was also affected or not.

II. APPLICATIONS

5.4. Single food digestions

5.4.1. Fatty acid-specific digestibility of test foods

In my experiments, digestibility of chosen test foods, baked carp, baked beef, cream, sour cream, and sour cream analogue was first determined in so-called single food digestion experiments. Single food digestion in this context means that even if a food being tested is typically not consumed on its own, but rather co-consumed (paired) with other commodities as a part of a complete meal, in a single food digestion experiment, the food being tested is intentionally subjected for digestion alone.

The bioaccessible FA content was measured after both versions of the Infogest consensus protocol. To simulate lipid digestion, Infogest v1.0 (hereafter abbreviated as "PL") uses only pancreatin containing pancreatic lipase, whilst Infogest v2.0 (hereafter abbreviated as "GL+PL") highlights the importance of gastric lipid digestion and implements rabbit gastric extract (a source of gastric lipase) in the gastric phase of the method. Results from co-digestion simulations with the addition of bioactive rich foods and with food pairing were compared to these initial (single food digestion) results. In this first section, total and bioaccessible fatty acid content of chosen test foods are presented, the role of gastric lipase and differences caused by structural variation is discussed. *Supplementary Table A3* shows all data (total fatty acid content, free fatty acid content, and release ratio) of test matrices.

5.4.1.1. Baked carp

The total fatty acid (TFA) composition of the baked carp test matrix was evaluated from baked carp digesta. The detailed FA composition is presented in Table A3 is in accordance with the results presented in Table A2A and A2B as shown on Figure 8. Relevant FAs (above 1 w/w% of carp meal) in this sample were oleic acid (C18:1n-9c: 52.8 ± 0.3%), palmitic acid (C16:0, 18.4 ± 0.2%), linoleic acid (C18:2n-6c, 9.0 ± 0.1%), palmitoleic acid (C16:1n-7c; 7.1 ± 0.01%), stearic acid (C18:0, 5.9 ± 0.1%), gondoic acid (C20:1n-9c: 2.7 ± 0.1%), and α-linolenic acid (C18:3n-3c, 1.1 ± 0.1%). The sum of these FAs gives 97.0% of all FAs detected in the sample.

The released FA content was measured via the two versions of the Infogest consensus protocol and FA-specific release ratio of individual FAs were also determined (Table A3 and Figure 12). The total released FA content (calculated according to Eq. 3) was 62.8 ± 1.5% and 72.3 ± 0.9% for the PL and GL+PL protocols, respectively. This observed 9.5% increase in the total released FA content correlates with data from the literature on the effect of gastric lipase, i.e., adds 5-40%

to overall lipolysis. Release ratio of relevant fatty acids after each digestion protocol is shown on Figure 12.

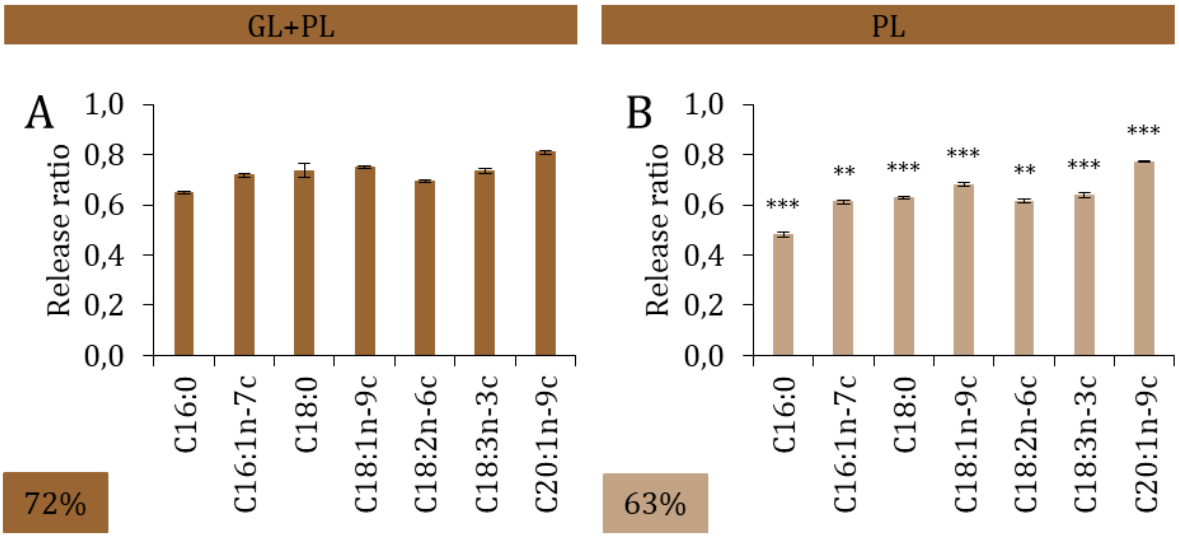

Figure 12. Release ratio of individual fatty acids after Infogest v2.0 (A; GL+PL) digestion and Infogest v1.0 (B; PL) digestions of relevant fatty acids (>1 w/w%) of baked carp meal. Percentages show the bioaccessible fatty acid content. Asterisk show significant difference between release of fatty acids after the two methods (*p<0.05; **p<0.01; ***p<0.001).

It can be concluded that independent of the applied PL or GL+PL protocol, FA-specific significant differences (ANOVA, p<0.001) can be seen in the RR of different FAs (Figure 12 and Table A3). Based on the results obtained from the comparison of protocol PL and GL+PL, the contribution of gastric lipase can be characterized. RR values of all FAs after digestion simulation of baked carp meal, varied between 0.45 and 0.77 for the PL protocol and 0.40 and 0.87 for GL+PL. It is noteworthy that the RR value of C16:0, the second most abundant FA in the baked carp meal, is remarkably below the total released FA content in both cases. The FA-specific analysis of the results shows that gastric lipase has a biased relative contribution to the liberation of FAs. Its contribution to the release of saturated FAs (SFA) was more than double (+27%) that of the unsaturated species (+12%). Our FA-specific comparison shows that the most significant difference in the RR was for C14:0 (+58%), C15:0 (+31%) and C16:0 (+35%). However, the increase of C14:0 and C15:0 has a minor role in the increase in total SFA. Conclusively, the increase in the RR, primarily of C16:0 and to lesser extent C18:0 (+18%), is most probably responsible for the majority of the observed change in the total amount of released SFAs due to their dominancy amongst FAs. A further important role of gastric lipase in relation to C16:0 can be postulated from our observations. The RR of C16:0 was among the lowest (RR = 0.48) ones

without gastric lipase, which showed one of the largest increases (~35%) associated to GL (RR = 0.65). Moreover, it is worth mentioning that the observed RR change of C18:1n-9c (oleic acid) caused by the addition of GL is below the overall average increase (only +9%); however, being the most abundant FA in the baked carp meal, its contribution to the total released FA pool is of importance (Table A3). The GL induced increase in the release of SFAs and the dispreference towards C18:1n-9c may be of nutritional significance (EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA), 2010; Figueiredo et al., 2017).

5.4.1.2. Baked beef

The TFA composition of the baked beef test matrix was evaluated, relevant FAs (above 1 w/w%) in this sample were oleic acid (C18:1n-9c: 44.9 ± 0.02%), palmitic acid (C16:0, 26.8 ± 0.06%), stearic acid (C18:0, 14.9 ± 0.08%), palmitoleic acid (C16:1n-7c; 5.0± 0.02%), myristic acid (C14:0: 2.6 ± 0.01%) and linoleic acid (C18:2n-6c, 2.1 ± 0.02%). The detailed FA composition is presented in Table A3 is in accordance with the results presented in Table A2A and A2B.

After *in vitro* digestion simulation of baked beef, total FA release and individual RR of FAs were also determined. Interestingly, there was no difference between total digestibility determined after Infogest PL and GL+PL. Overall FA release was 67.7 ± 2.5% and 67.2 ± 1.6%, respectively (p= 0.742). In contrast, between individual RRs of FAs there were some differences (Figure 13).

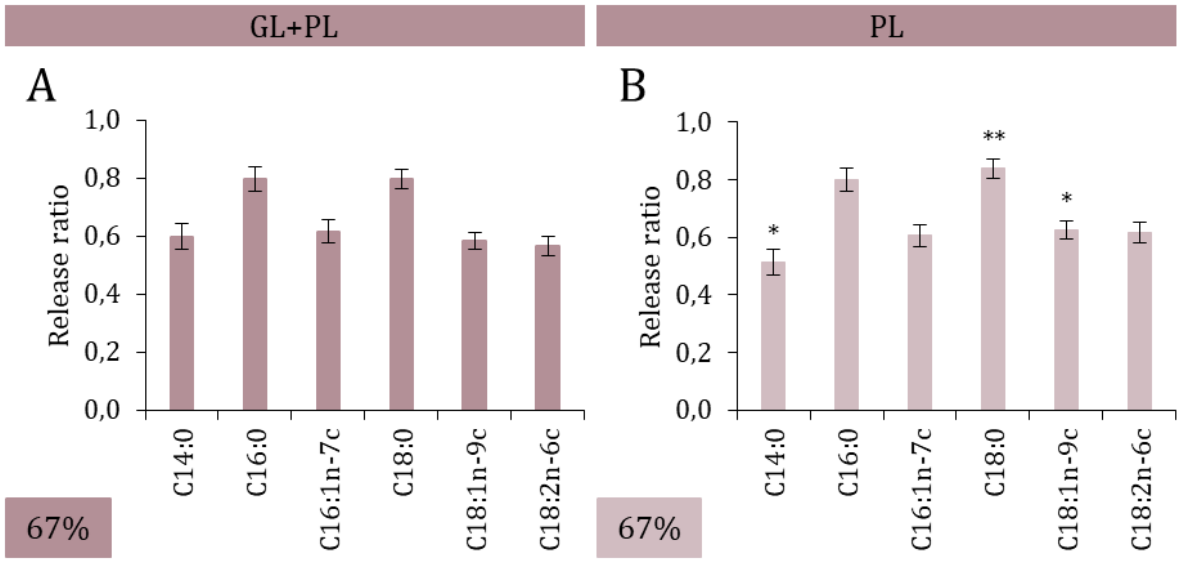

Figure 13. Release ratio of individual fatty acids after Infogest v2.0 (A; GL+PL) digestion and Infogest v1.0 (B; PL) digestions of relevant fatty acids (>1 w/w%) of baked beef meal. Percentages show the bioaccessible fatty acid content. Asterisk show significant difference between release of fatty acids after the two methods (*p<0.05; **p<0.01; ***p<0.001).

With the addition of gastric lipase RR of C14:0 increased from 0.51 to 0.60 and RR of C18:0 and C18:2n-6c decrease slightly from 0.84 and 0.62 to 0.80 and 0.56, respectively. The minor change in the FA composition of the digesta caused by the difference in the RR of these FAs evens out, thus no change is detectable in the overall digestibility of baked beef. In both cases FA composition of digesta differed from FA composition of the baked beef meal. Relative amount of C14:0; C16:1n-7c, C18:1n-9c and C18:2n-6c decreased and C16:0 and C18:0 increased, thus SFA content of digesta also increased by 13.7%. Similarly, Hur and colleagues looked into lipid digestibility of beef and found that after *in vitro* digestion, the composition of digesta differed from initial FA composition of beef (Hur et al. 2009). Therefore, in could be concluded that although GL has minor effect in digesting beef fat, lipid digestibility of baked beef meal is a relevant factor in nutrient accessibility of it.

5.4.1.3. Cream

As seen previously, the TFA content of cream also was determined from the small intestinal digesta (Table A3) as well and were compared with the data from the ISO and B&D extracted samples shown in Table A2A and A2B. Most abundant FA in cream was palmitic acid (C16:0: 33.3 ± 0.3%), oleic acid (C18:1n-9c: 24.5 ± 0.3%), stearic acid (C18:0: 11.9 ± 0.2%), myristic acid (C14:0: 11.2 ± 0.1%). It also contains several FAs under 5 w/w% such as linoleic acid (C18:2n-6c: 4.1 ± 0.1%), lauric acid (C12:0: 2.8 ± 0.2%), elaidic acid (C18:1n-9t: 2.4 ± 0.3%), palmitoleic acid (C16:1n-7c: 2.2 ± 03%), capric acid (C10:0: 1.3 ± 0.3%), and pentadecanoic acid (C15:0: 1.3 ± 0.02%).

After *in vitro* digestion simulation with both versions of the Infogest protocol, free fatty acid content and release ratio of each individual FA was determined (Table A3 and Figure 14).

Total lipid digestibility based on fatty acid release of cream determined with GL+PL method was 77.1 ± 5.0% (RSD: 0.07). Fatty acid-specific results were determined according to Eq. 2. The release ratio of individual fatty acids showed little variation between 0.70 and 0.82. Only one FA (C6:0) deviated from the rest, which showed relatively low digestibility (RR= 0.45) compared to the other FAs. These results show that in realistic digestion conditions i.e., when both lipolytic enzymes are added, most fatty acids are released in a similar extent from cream matrix.

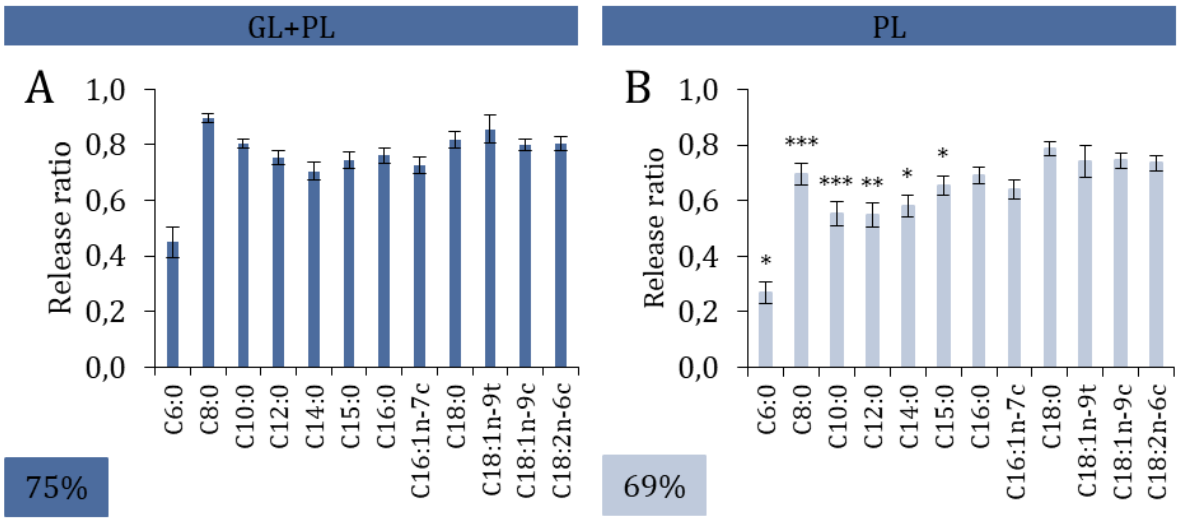

Figure 14. Release ratio of individual fatty acids after Infogest v2.0 (A; GL+PL) digestion and Infogest v1.0 (B; PL) digestions of relevant fatty acids (>1 w/w%) of cream. Percentages show the bioaccessible fatty acid content. Asterisk show significant difference between release of fatty acids after the two methods (*p<0.05; **p<0.01; ***p<0.001).

To gain more insight to digestion mechanism of lipolysis, digestibility of lipids was also measured after *in vitro* digestion simulation with PL digestion. Compared to the other version of the Infogest method, overall digestibility decreased by 11% to 69.2 ± 6.1% if only pancreatic lipase were added to the digestion simulation (PL). It is in accordance with the consensus on gastric lipase contribution i.e., pre-digestion by gastric lipase adds between 5-40% to overall digestion. The difference between RR of individual FAs were more pronounced. Small and medium chained FAs (SCFAs and MCFAs) showed RR from 0.55 to 0.65 and longer chained FAs (LCFAs) showed RR from 0.69 to 0.73 (Figure 14). Significant difference between RR values of PL and GL+PL were only between C10:0, C12:0, C14:0 and C15:0 (Table A3). Due to this shift in RR values of small and medium chained FAs – with the addition of GL – SFA content of bioaccessible pool was affected which increased by 14%. Based on these results, it can be concluded that addition of gastric lipase mostly affected the release of small and medium chain FAs and consequently the SFA level increased in the bioaccessible fraction of digested cream compared to just using pancreatic lipase to digest lipids.

5.4.1.4. Sour cream

Milk fat composition of sour cream resembles to FA composition of cream however minor differences were measurable probably due to source of milk (Michalski et al. 2013). Compared to cream chosen sour cream product contained more myristoleic acid (C14:1n-5c: +0.3%), palmitic

acid (C16:0: +4.6%), and less linoleic acid (C18:2n-6c: –2.2%). Detailed data is shown in Table A2A, A2B and A3.

After *in vitro* digestion simulation, bioaccessible FA content of sour cream was 61.1 ± 3.9% (GL+PL). This value decreased by 16% without the addition of gastric lipase to 52.6 ± 3.7% (Figure 15).

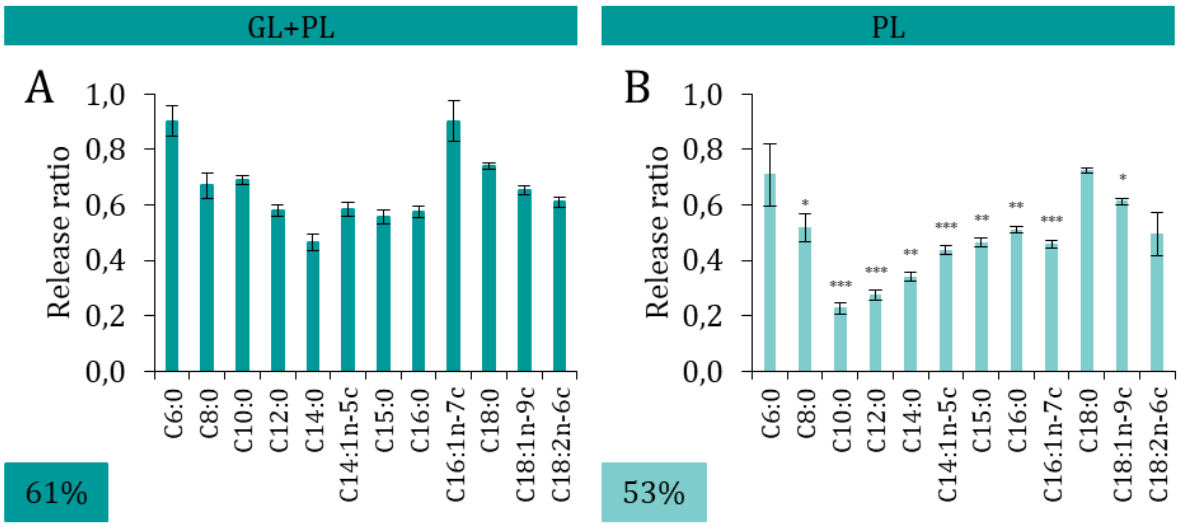

Figure 15. Release ratio of individual fatty acids after Infogest v2.0 (A; GL+PL) digestion and Infogest v1.0 (B; PL) digestions of relevant fatty acids (>1 w/w%) of sour cream. Percentages show the bioaccessible fatty acid content. Asterisk show significant difference between release of fatty acids after the two methods (*p<0.05; **p<0.01; ***p<0.001).

Fatty acid-specific lipolysis was also determined, and it was shown that gastric pre-digestion also affected the RR of each FAs. The RR varied between 0.29 and 0.72 after PL digestion and between 0.47 and 0.90 after GL+PL digestion. Presence of gastric lipase somewhat evened out the digestibility of the FAs however, the lipid digestion of sour cream was not able to reach higher levels. The increase of the release ratio for several FAs (from C8:0 to C16:1n-7c) were significant (p<0.05). Moreover, for some FAs such as C10:0, C12:0, and C16:1n-7c the release was much higher than for the others. Compared to the average increase (+20-30% increase), the release of C10:0 tripled and C12:0 and C16:1n-5c doubled when GL was also used to aid digestion.

5.4.1.5. Sour cream analogue

The sour cream analogue chosen for analysis contains five fatty acids above 1 w/w%, namely palmitic acid (C16:0: 42.5 ± 0.12%), oleic acid (C18:1n-9c: 41.6 ± 0.25%), linoleic acid (C18:2n-6c: 9.3 ± 0.07%), stearic acid (C18:0: 5.3 ± 0.28%), and myristic acid (C14:0:

1.3 ± 0.01%). This fatty acid content is in harmony with the FA composition of palm oil (Mancini et al. 2015).

After *in vitro* digestion (GL+PL), 66.2 ± 2.5% of total fatty acid content was liberated (Figure 16). FA-specific release was between 0.52-0.83, the lowest being linoleic acid (C18:2n-6c) and the highest being stearic acid (C18:0) (Table A3 and Figure 16).

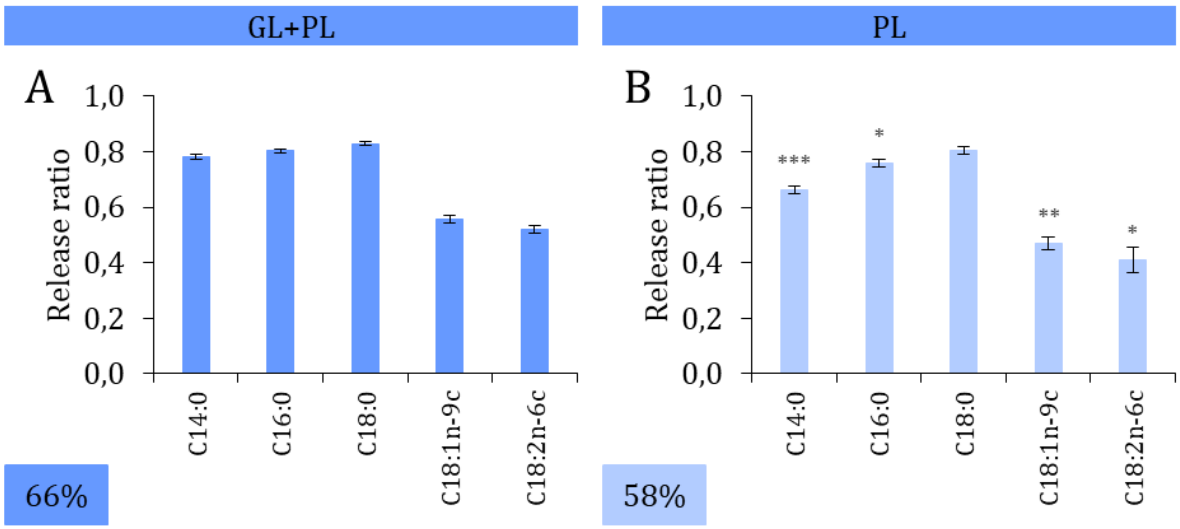

Figure 16. Release ratio of individual fatty acids after Infogest v2.0 (A; GL+PL) digestion and Infogest v1.0 (B; PL) digestions of relevant fatty acids (>1 w/w%) of sour cream analogue. Percentages show the bioaccessible fatty acid content. Asterisk show significant difference between release of fatty acids after the two methods (*p<0.05; **p<0.01; ***p<0.001).

If only pancreatin (pancreatic lipase) was used in the simulation (PL), the overall digestibility was lower by 13%, 58.5 ± 4.5%. When the release ratio of individual fatty acids was also examined, three fatty acids (out of the five main ones) showed a greater increase in release ratio when GL+PL simultaneously was used than the others. These are C14:0 (18%), C18:1n-9c (18%) and C18:2n-6c (27%). It is noteworthy that from the two main FAs in sour cream analogue (C16:0 (43 w/w%) and C18:1n-9c (42 w/w%) only release of the unsaturated FA (USFA) was affected by the action of GL. However, since the release ratio of C16:0 was initially higher than that of C18:1n-9c the significant increase by the effect of GL was not sufficient to cause a change in the SFA/USFA ratio of the bioaccessible pool. Therefore, slightly higher levels of SFA were measured in the digesta of SCA that of USFA.

5.4.2. Conclusions on lipid digestibility based on single food digestions

The results obtained from the determination of lipid digestibility of the studied test foods clearly demonstrate that assessment of lipolysis at the level of individual FAs has importance in nutritional studies, e.g., the health benefits and risks of SFAs and mono- or polyunsaturated FAs (MUFAs and PUFAs) should be evaluated separately. The results distinctively show that the FA profile of non-digested food products and the released FA content and composition of the digests provided by the two versions of the Infogest consensus protocol can be significantly different (Table A3 and Figures 12-16). In addition, it is showed that individual FAs occurring in a food or food product might show different RRs. There are several factors which could be related to such changes e.g., TAG composition, matrix effects, or the specific substrate preference of pancreatic or gastric lipase toward different FAs (Meynier and Genot, 2017). In this chapter these effects are described in detail with examples based on the test matrices shown previously.

5.4.2.1. Role of gastric lipase

Lipid content of food products are mainly comprised of triacylglycerol (TAGs). During digestion, triacylglycerols are cleaved via enzymatic hydrolysis. In addition to released free fatty acids, various residual products (diacylglycerides and monoacylglycerides) are also formed during TAG digestion. Gastric lipase (GL) and pancreatic lipase (PL) are the two main enzymes guiding breakdown of lipids of foods; however, these enzymes have slightly different substrate preferences. Gastric lipase is a *sn*-3 specific lipase (Carrière et al. 1997; Mitchell et al. 2008; Infantes-Garcia et al. 2020) whereas pancreatic lipase shows the similar selectivity towards the hydrolysis at positions *sn*-1 and *sn*-3 of the TAG (Golding and Wooster, 2010; Bakala-N'Goma et al. 2022) provided that *sn*-1 and *sn*-3 FA side chains are identical (Benito-Gallo et al. 2015). Although gastric lipase only contributes to approximately 5-40% of overall lipid digestion it also serves as a catalyst for further lipid digestion, i.e., pre-digestion and emulsification enhances latter pancreatic lipase activity (Armand 2007). By that logic, gastric lipase action results in an overall increase of bioaccessible fatty acid content and increased release of *sn*-3 fatty acids especially when the TAGs are not symmetric, i.e., FAs that *sn*-1 and *sn*-3 positions on the glycerol backbone are not the same.

In the previous chapter it was shown that release of individual FAs is different from the test foods and it is dependent on the food matrices and the enzymes used for digestion simulation. Additionally, experiments conducted using both lipolytic enzymes (gastric and pancreatic lipase, GL+PL) compared to using only pancreatic lipase (PL) showed that gastric lipase has a significant role in ameliorating and improving the release of certain fatty acids.

Based on their fat source and lipid composition, the five test foods could be group as meat products (baked carp and baked beef containing muscle fat), dairy products (cream and sour cream containing milk fat) and plant-based products (sour cream analogue containing palm oil). From these sources milk fat is the one with the most interesting FA composition. Milk fat (lipid source of cream and sour cream) contains a diverse variety of FAs containing short chain fatty acids (SCFA), medium chain fatty acids (MCFA), and long chain fatty acids (LCFA). The high degree of FA diversity measured in cream and sour cream (i.e., milk fat) necessarily reflects in the types of TAGs as well. A mixture of TAGs with heterogeneous composition is characteristic of milk fat (Omar et al. 2017). Short (C4-C8) and medium (C10-C14) length FAs are mainly located at the *sn*-3 and *sn*-2 positions of TAGs next to MCFAs and LCFAs. Therefore, milk fat TAGs are considered to contain the most "asymmetric" TAGs among animal fats (Christie and Clapperton, 1982).

In contrast, the FA profile of beef fat and sour cream analogue are much simpler than that of milk fat. Both fat sources contain only a few FAs (six and five, respectively) from which only one FA (C14:0) is a MCFA whereas others are LCFAs. Consequently, the structure of most beef and most SCA TAGs are more symmetric than of cream and sour cream TAGs (Christie et al. 1991; Smith et al. 1998). Baked carp provides a bridge between the two. Although it contains a few FAs in larger quantities, it also contains medium chain FAs and several long unsaturated FAs.

Results of GL+PL and PL digestion simulations showed on Figure 17 (cream and sour cream) and Figure 18 (baked carp, baked beef and sour cream analogue) grouped based on fat source and TAG structure.

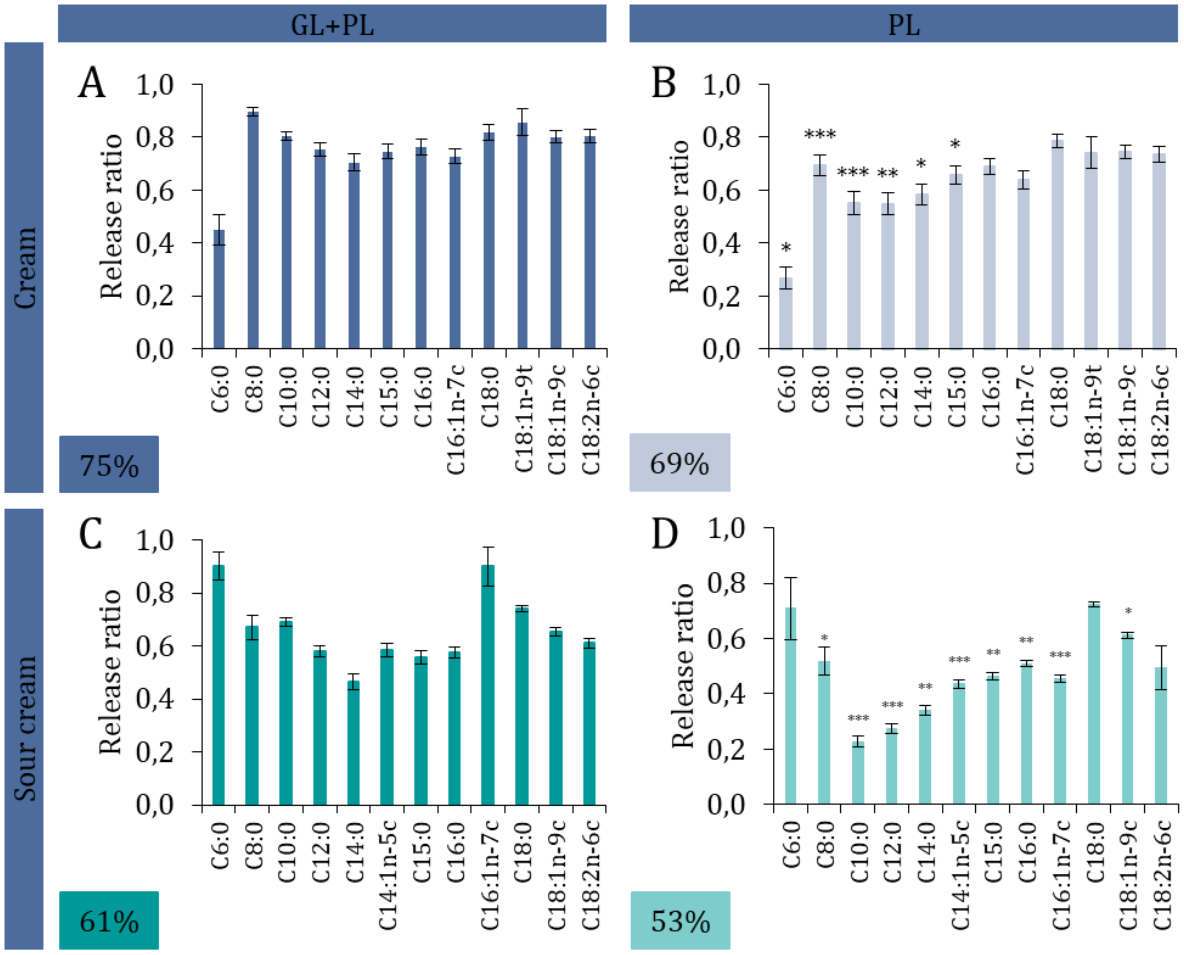

Figure 17. Bioaccessible fatty acid content of test foods (%; Eq. 5) and release ratios (Eq. 4) after Infogest digestion simulations (GL+PL and PL). A: Cream GL+PL, B: Cream PL; C: Sour cream GL+PL, D: Sour cream PL. Asterisk labels (*) show significant difference between release ratio of individual fatty acids after GL+PL and PL digestions (*p<0.05; **p<0.01; ***p<0.001).

Addition of GL increased lipid digestibility of both cream (+12%) and sour cream (+16%) compared to only PL digestions. Moreover, the increase manifested differently on SCFAs, MCFAs and on LCFAs. The release of SCFAs and MCFAs were markedly higher for both test foods than for LCFAs (Figure 17). According to these results, the contribution of GL was most pronounced in the lipolysis of short (C4-C8) and medium (C10-C14) chain FAs. These FAs are mainly located in the *sn*-3 and *sn*-2 positions of the TAGs, most probably forming asymmetric TAGs in milk fat. The results also lend support to the assumption that that TAGs containing SCFA and MCFA are non-preferred substrate types for PL. Consequently, PL alone is rather ineffective in the digestion of TAGs containing short and medium chain FAs and GL is of key importance in the lipolysis of such asymmetric TAGs of milk fat. Findings of Benito-Gallo et al. also suggest that PL might

shows the same selectivity towards the hydrolysis at positions *sn*-1 and *sn*-3 of the TAG, provided that the FA side chains are identical (Benito-Gallo et al. 2015). This further supports the observations regarding the key role of GL in the digestion of asymmetric TAGs of milk fat.

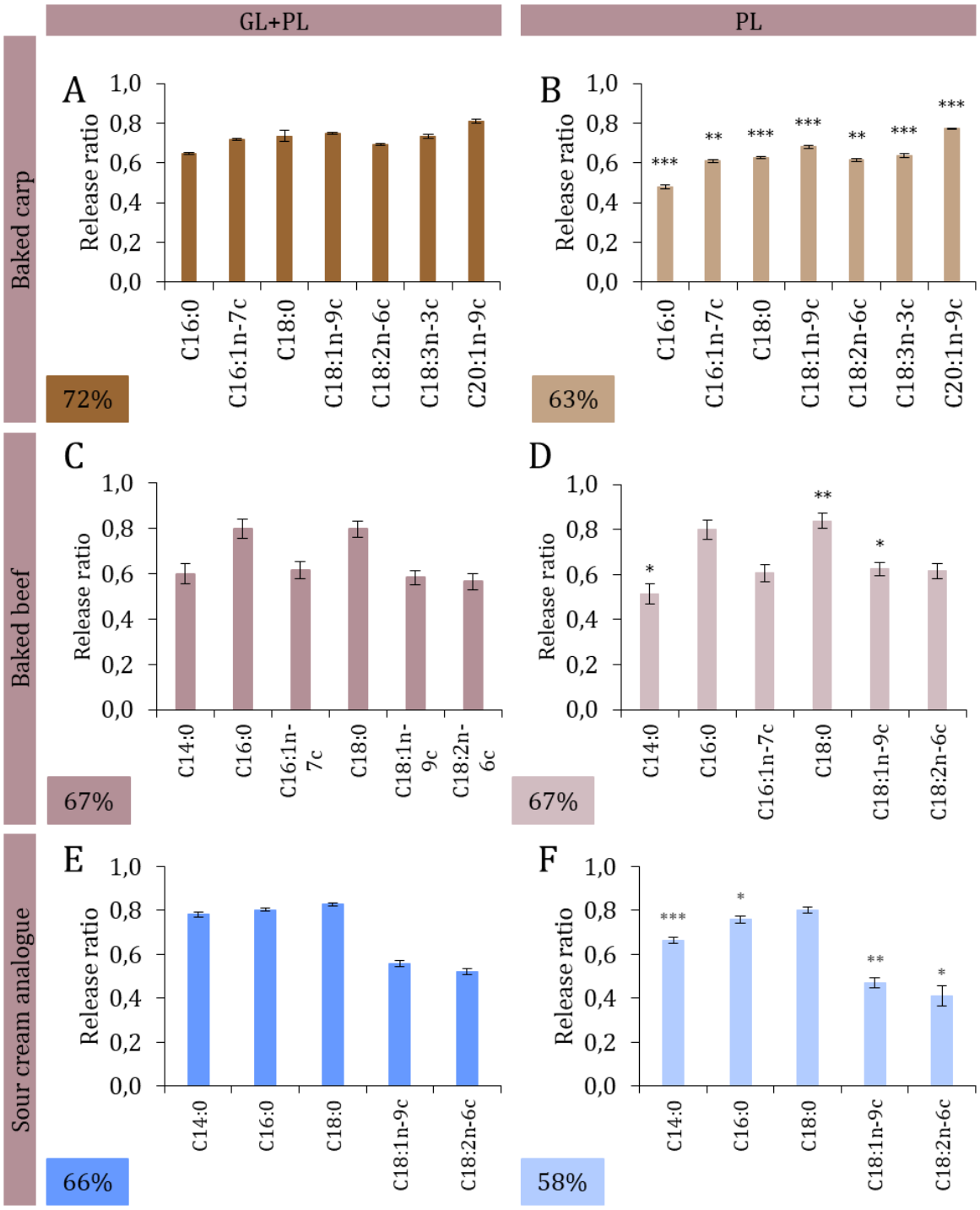

Figure 18. Bioaccessible fatty acid content of test foods (%; Eq. 5) and release ratios (Eq. 4) after Infogest digestion simulations (GL+PL and PL). A: Baked carp GL+PL, B: Baked carp PL; C: Baked beef GL+PL, D: Baked beef PL; E: Sour cream analogue GL+PL, F: Sour cream analogue PL. Asterisk labels (*) show significant difference between release ratio of individual fatty acids after GL+PL and PL digestions (*p<0.05; **p<0.01; ***p<0.001).

In comparison, Figure 18 shows the fatty acid-specific results after simulated digestion of the three other test foods; baked carp, baked beef and sour cream analogue. These test foods have less diverse FA composition therefore contain more symmetric TAGs and show markedly different lipid digestion characteristics. Looking at the release ratios of these three foods (baked carp, baked beef and sour cream analogue) it is clear that addition of gastric lipase did not affect the release patterns of the main FAs. Moreover, presence of GL during digestion simulation only modified extent of lipid digestion of two test foods out of three, namely baked carp (+15%) and sour cream analogue (+13%) however not baked beef.

Baked beef meal and sour cream analogue both are fat sources with a rather symmetric TAG composition. However, in baked carp there are MCFAs and several longer unsaturated FAs which could be present in asymmetric TAGs which might be a cause and explanation for the marked difference in overall lipid digestibility with the addition of GL. This logic might also be appropriate for the sour cream analogue since the two FAs of which's release ratio increased are C14:0 (an MCFA) and C18:2n-6c (an UFSA) which are most probably are a part of asymmetric TAGs (Christie et al. 1991). The reason behind the distinct behaviour of baked beef could be stemmed back to the lack of diversity of FAs and consequently of TAGs. The symmetric TAGs (containing similar FAs) are a preferred substrate for PL therefore the addition of GL did not resulted increase in overall digestibility.

The results of digestion simulations show that the TAGs containing SCFAs and MCFAs are non-preferred substrate types for PL and consequently, PL alone is rather ineffective in the digestion of such TAGs containing SCFAs and MCFAs. However, this specificity is not characteristic for GL. Thus, the presence of GL is resulted in the increased release of SCFAs and MCFAs (C6:0–C15:0), especially if these FAs are part of asymmetric TAGs, most probably bound to the glycerol in an outer (*sn*-1/3) position. This effect is more pronounced in milk fat containing products such as the analyzed cream and sour cream. The results also showed that when there is no marked diversity in FA composition and consequently heterogeneity in the TAG structure is limited, the contribution of GL could be negligible such as in the case of baked beef, where overall fat digestibility remained the same. However, the contribution of GL to fat digestibility might be more complex in other food commodities such as in the case of baked carp and sour cream analogue. For the lipid digestibility of these foods, the addition of GL caused simultaneous however somewhat negligible increase of release ratio the relevant FAs (in larger quantities) but the presence and marked RR increase of the few minor FAs caused distinct increase in the extent of lipid digestibility.

It has been shown that the ability of GL to cleave the PL-not preferred FAs from *sn*-3 position would have additional benefits. The TAGs that are ineffectively digested by PL – such as presumably asymmetric TAGs containing SCFAs and MCFAs in outer positions – would appear in pre-digested *sn*-1,2/2,3 DAG forms in the next (ileal) stage of digestion, which PL can now cope with more easily. Moreover, the FFAs produced during gastric digestion, due to their emulsifying ability can further improve PL's access to TAGs, thus improving the efficiency of fat digestion in the duodenum (Armand, 2007). This synergistic effect of the two enzymes is clearly demonstrated in lipid digestion of substrates with substantial quantity of SCFAs and MCFAs, such as milk fat.

5.4.2.2. Role of food matrix

During my experiments two fermented dairy products were evaluated for lipid digestibility; sour cream (SC), and sour cream analogue (SCA). In spite of their nutritional importance (Wang and Li, 2008; FAO, 2013; Astrup, 2014) as the potential benefits have come under question (Thorning et al. 2016; Gil and Ortega, 2019) the consumption of milk and dairy products is decreasing and moving away from the advised level in many countries. While researchers have recommended the reduction of total SFAs in the diet for the prevention of cardiovascular disease (CVD) (Sacks et al. 2017), the relationship between dairy fat and CVD is still unsettled. Moreover the fear of dairy products are amplified through to the evidence that by replacing dairy fat with fat source rich in polyunsaturated fatty acids, especially from plant-based foods may confer health benefits (Li et al. 2015). Modification of the fat composition or replacement of fat content by other fats of dairy products are common practices in the food industry (Izsó et al. 2020). Products in which milk fat is partially or wholly substituted by vegetable fats are defined as dairy analogues (FAO/WHO Codex Alimentarius). Sour cream is a popular, high fat dairy product used as topping or as base for sauces and creams. Sour cream is a fermented dairy product made from high fat cream after standardization, pasteurization, homogenization, and fermentation with the addition of bacterial culture. Its analogue most commonly made from skimmed milk mixed with vegetable fat and fermented by the bacterial culture.

When the SC (product) or the SCA (product) was subjected to digestion simulation alone (digestion of the product), the two products showed significantly different lipid digestibility. Lipids from sour cream (61.1 ± 3.9%, Figure 19A) showed lower FFA release than from its analogue (66.2 ± 2.5%, Figure 19C).

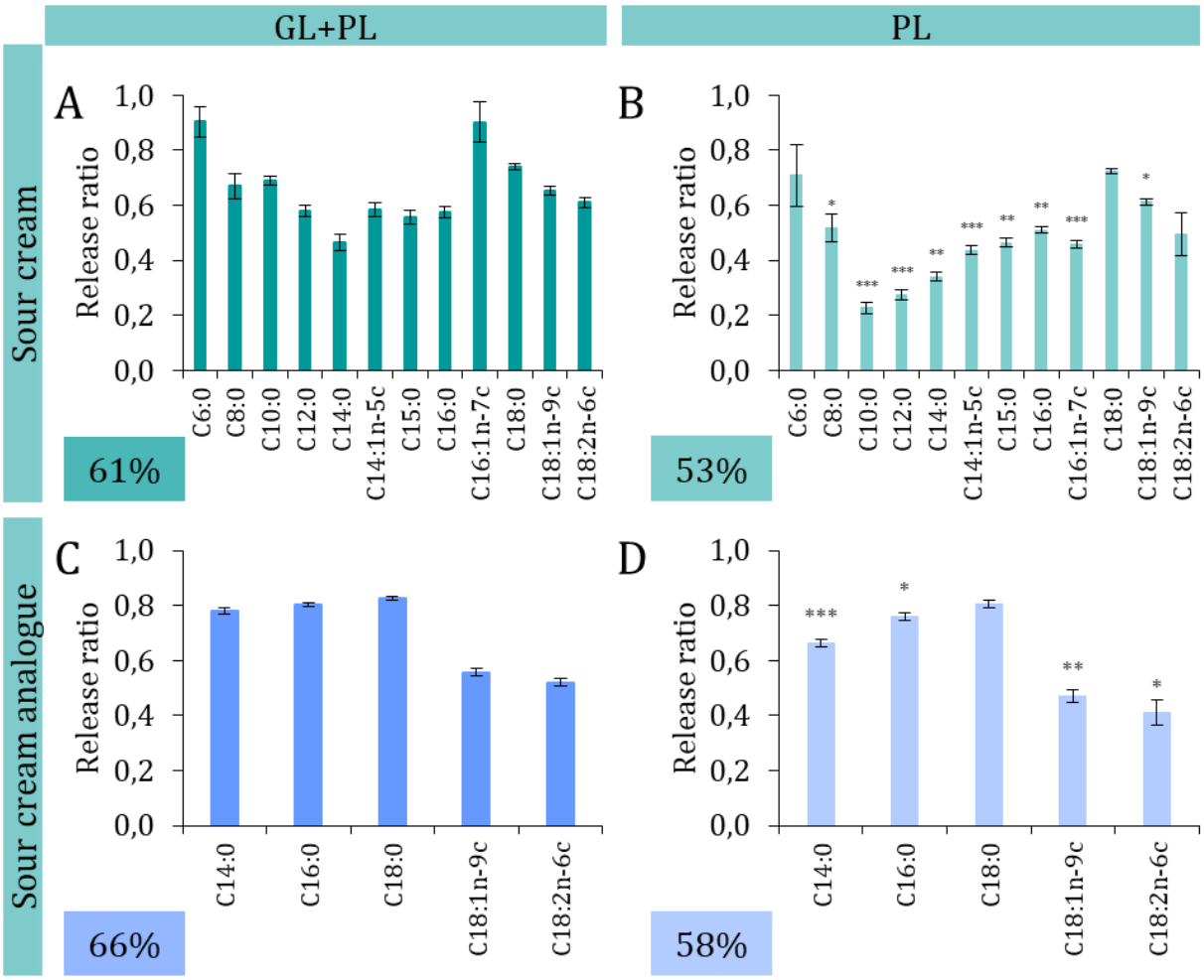

Figure 19. Release ratios of individual fatty acids after digestion simulation of the two high fat toppings. Digestion simulations were carried out using either both lipolytic enzymes (GL+PL) or only pancreatic lipase (PL). Percentages show extent of lipolysis calculated from free fatty acids released during digestion. A: sour cream GL+PL; B: sour cream PL; C: sour cream analogue GL+PL; D: sour cream analogue PL. Asterisk labels (*) show significant difference between release ratio of individual fatty acids after GL+PL and PL digestions (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

My previously showed results highlighted that the fatty acid profile of the lipid source is an important factor that may influence the behaviour of gastric lipase in a way that it could result in considerably different lipid digestibility. Since the two products have very different FA profile, the effect of this difference on gastric lipase activity during digestion was evaluated. The aim was to reveal if the distinct lipid digestibility of SC and SCA could be associated with the specific preference of gastric lipase.

The addition of gastric lipase significantly improved the digestibility of sour cream, represented in a 16% increase in extent of lipolysis (from PL: 52.6 ± 2.7% to GL+PL: 61.1 ± 3.9%; p= 0.001; Figure 19B). When evaluating the results at the fatty acid level, the increase of the release ratio for several FAs (from C8:0 to C16:1n-7c) were significant ($p<0.05$). Moreover, for some FAs such as C10:0, C12:0, and C16:1n-7c the release was much higher than for the others. Compared to the average increase (+20-30% increase), the release of C10:0 tripled and C12:0 and C16:1n-5c doubled when GL was also used to aid digestion.

In the case of the sour cream analogue, the addition of gastric lipase also improved the overall lipid digestibility, from 58.5 ± 4.5% to 66.2 ± 2.5% (13% increase in the presence of GL, Figure 19D). When the release ratio of individual fatty acids was also examined, three fatty acids (out of the five main ones) showed a greater increase in release than the others. These are C14:0 (18%), C18:1n-9c (18%) and C18:2n-6c (27%).

These results confirm my previous findings that gastric lipase plays a significant role in the release of short and medium chain fatty acids as well as long chain unsaturated fatty acids. However, since the addition of GL improved the overall lipid digestibility of both SC and SCA products (with a similar ratio) the difference in lipid digestibility between the two products cannot be solely explained by the specific preference of GL to short and medium chain FA containing triacylglycerols. To investigate the possible additional reasons for the lipolytic behaviour of SC and SCA, the lipid microstructure of the products and their non-enzymatic gastric digests was examined using a fluorescent microscopy (Figure 20).

The microscopic images show that the chemical changes during gastric digestion (i.e., pH drop to around 3.0) resulted in marked difference in the size of fat droplets (Figure 20C, 20D). This difference in droplet size was not obviously present in the two products (Figure 20A, 20B) before subjecting to gastric conditions. This observation suggests that the physical mixing and chemical environment simulating gastric conditions are responsible for the change in the droplet size of the SC product. This finding lends support to our assumption that the different behaviour of the two products in lipid digestibility can be associated to the observed structural change that occurs under gastric digestion.

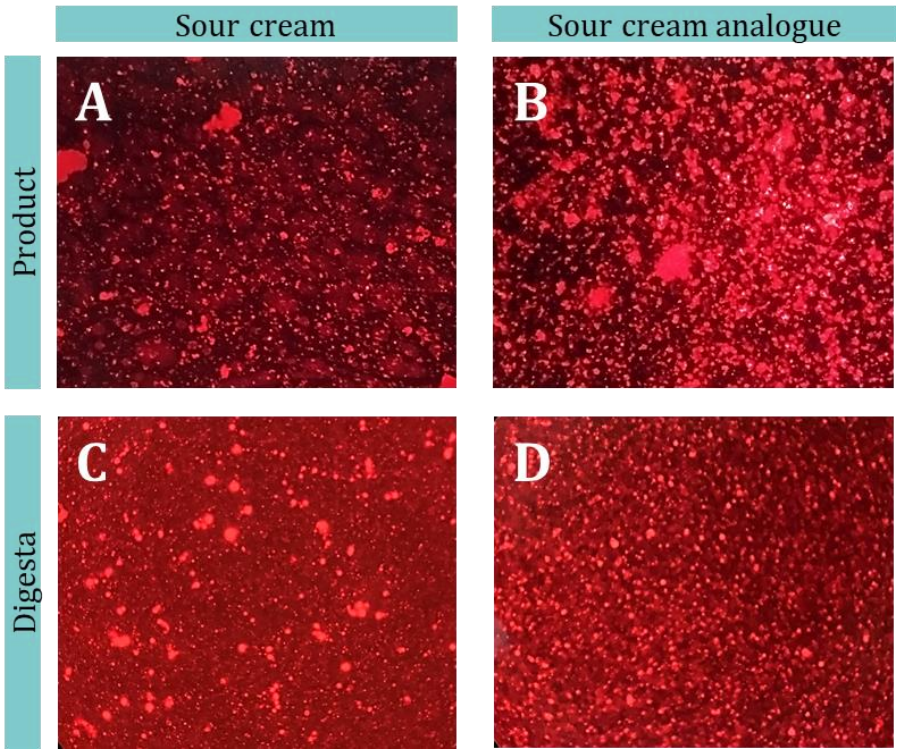

Figure 20. Microscopic images of fat droplets in the products and in non-enzymatic digests stained with Nile red dye. A: sour cream product; B: sour cream analogue product; C: sour cream chemical digesta; D: sour cream analogue chemical digesta.

The structure of the products is a result of several production step. The denatured whey and casein acid gel structure, formed during fermentation, surrounds lipid droplets in both products. In addition, SC contains milk fat, in which fat globules are covered with a membrane (milk fat globule membrane, MFGM), also containing various proteins. In the case of milk fat, the denatured surface proteins of the MFGM can be prone to protein-protein interactions with the protein gel matrix. Based on composition of the products there were two reasons suspected behind the deviation: i) different accessibility of lipid droplets during gastric digestion from the acid gel matrix, in which the lipid droplets are embedded in, or ii) different behaviour of the lipid droplets during digestion based on structural composition and emulsification properties.

Behaviour of acid gel structures made from milk proteins (i.e., cheese and yoghurt) were a topic of several studies before (*in vitro*: Acevedo-Fani et al. 2021; Qazi et al. 2021; *in vivo*: Barbé et al. 2014). It can be concluded from literature that during gastric digestion of the acid gel matrix, the bolus with an inherently lower pH – due to its acidic nature – does not require prolonged pH equilibration which promotes the activity of proteolytic enzymes. Consequent restructuring – because of casein aggregation – did not occur in the acid gel matrix under gastric

conditions because the pH is lower than required for inducing cleavage of the κ-casein and subsequent coagulation of the casein micelles (Qazi et al. 2021). Although some studies found evidence for micro-densification of the protein network associated with acidification. It was shown that in acid gels, the disintegration is still rapid in spite of the inconsistency of structural behaviour (Le Feunteun and Mariette, 2008; Floury et al. 2018). Based on rapid disintegration of the acid gels, which would result in a fast release of lipid droplets, the behaviour of the protein gel might be of lesser importance for particle size increase, which was observed only of the MFGM covered fat droplets of SC and not of the palm oil droplets of SCA.

It has been speculated that the MFGM has profound effects on the accessibility of the triacylglycerols for lipase-catalysed digestion (Patton and Keenan, 1975). This statement was also studied and confirmed by Ye at al., comparing lipid digestibility of native (raw) milk and recombined milk (skim milk homogenized with milk fat) showing that in fact milk fat have a higher rate of lipolysis when the surface is covered with casein and serum protein rather than with MFGM (Ye et al. 2010). In this study it was also revealed that the difference in lipid digestion behaviour is caused by the rapid flocculation of MGFM covered milk fat during early stages of digestion. During gastric digestion, the MFGM covered fat globules form larger particles via coalescence, from which triacylglycerols are released gradually (Ye et al. 2010). Moreover, multiple studies showed that the emulsion destabilization is continued during intestinal digestion due to further coalescence of the globules (Gallier et al. 2012; Giang et al. 2015). These findings suggest that milk fat-based emulsions are less stable when the fat globules are covered in the MFGM explaining our observed size increase in droplet size of MFGs of SC during gastric digestion. Increased size of fat globules are associated with decreased extent of lipid digestibility due to lesser binding sites for lipolytic activity (Ye, 2021).

In comparison, another research by Kuroiwa et al. showed that sodium caseinate is a good emulsifier of palm oil in water-in-oil emulsions obtaining monodisperse droplets (Kuroiwa et al. 2020). Therefore, in the presence of casein in the milk serum during SCA production could result in a more stable emulsion from palm oil than of milk fat. This hypothesis is in accordance with our findings, i.e., no remarkable increase in lipid droplet size was observed for SCA in gastric conditions as shown in Figure 20B and 20D. In addition, the external fat droplets added to skim milk make a similar structure and behaviour than of recombined milk (lipid droplets covered with casein) shown to have higher lipid digestibility due to increased lipase activity on a casein covered surface (Ye et al. 2010).

Fatty acid-specific digestibility results of SC strengthened the previous findings about the special preference of gastric lipase (GL) towards short and medium chained fatty acids (SCFAs and

MCFAs). It was also showed that in the case of these two products, the main reason behind different lipid digestibility lays in the surface properties of the lipid droplets not only in the FA composition. MFGs are prone to flocculation and aggregation resulting in increased globule size meanwhile decreasing lipid digestibility due to lesser available binding sites for lipolytic enzymes.

5.5. Co-digestions with bioactive rich foods

Besides assessing the lipid digestibility of different foods, the evaluation method described in previous chapter for fatty acid-specific lipid digestibility assessment is suitable for the determination of lipase inhibitory effects of food ingredients with high quantity of bioactive compounds. The next chapter will be focusing on evaluating the influence of bioactive containing foods on lipid digestibility. For further experiments foods were selected that have previously been shown to exhibit lipase inhibitory effect with *in vitro* enzyme activity studies. Chosen materials were rosemary (Slanc et al. 2009), grape seed powder (Moreno et al. 2003) and black tea (Sellami et al. 2017; Jamous et al. 2018). In addition, applicability of method for this purpose were tested with Orlistat as a positive control.

5.5.1. Indication of lipase inhibition with Orlistat

Orlistat is currently the only clinically approved drug for obesity management in Europe. The molecule inhibits gastric and pancreatic lipase activity, thus helping in the reduction of triacylglycerol bioaccessibility. This substance was chosen as a positive validation control to demonstrate the capability of the proposed method to indicate shifts in the bioaccessibility of TAGs in a real food sample as a result of treatment with a compound with proven lipase inhibition potential. For this purpose, *in vitro* digestion of the baked carp meal was amended with Orlistat and determination of the released FA content was carried out as the proposed protocol. The addition of 40 μL of 0.5 M Orlistat solution (in DMSO), resulting in a 0.5 mM concentration in the final small intestinal digesta, was performed at the beginning of the oral stage of the digestion protocol. The concentration of Orlistat solution added was chosen between the amount in the approved drug (Xenical ®) and the amount appropriate to stop all lipid activity as seen in the work of Carriere and colleagues (Carrière et al. 2001).

The results show a remarkable decrease in the released FA content of the Orlistat treated baked carp sample. The total released FA content was only 2.1 ± 0.9%. This result was further cross validated by determining the fat content (by weight) in the B&D chloroform extract of the digesta after evaporation and drying at 103±1 °C. It was found that the fat content in the extract was not significantly different from the sum of FAs measured by the TFA method (t test, p= 0.239). This

result also indirectly lends support to the previously described observation that when a high percentage of FFAs form during digestion, which tend to evaporate or degrade more easily during drying, the fat content measured by weight in the digested small intestinal fluid is not in agreement with the sum of FAs measured by the TFA method (see *Chapter 1.3. Gravimetric determination of total fat content from digesta)*.

5.5.2. Rosemary

Rosemary is frequently used as spice for meat and fish. In previous *in vitro* studies it was shown via enzyme assays that rosemary extract could exhibit pancreatic lipase inhibitory effect (Slanc et al. 2009; Bustanji et al. 2010). In the first experiment the effect of rosemary was assessed on lipid digestibility of baked carp. As mimicking a more realistic consumption situation raw herbs were added to carp fillets before baking (5 w/w% rosemary/raw weight of carp), and effect was assessed after digestion experiment of rosemary-spiced carp meal. Digestion experiment was carried out according to PL method, i.e., only pancreatic lipase was responsible for lipid digestion.

According to the results gathered from *in vitro* digestion simulations rosemary spice showed no influence on overall lipolysis of baked carp meal (t test $p= 0.557$). Moreover FA-specific evaluation revealed no effect on release of individual FAs (t test $p>0.05$).

To detect the lack of effect, a characterisation of aqueous rosemary extract was performed (*Polyphenol analysis was carried by Kata Nagy which is kindly acknowledged*). It was found that 97% of water-soluble compounds was rosmarinic acid. According to Bustanji and colleagues, rosmarinic acid showed some lipase inhibitory effect via *in vitro* enzyme assays however it produced the lowest activity amongst the four main component of rosemary, i.e., rosmarinic acid, chlorogenic acid, gallic acid caffeic acid (Bustanji et al. 2010). It was also discussed that although potential medicinal properties have been associated with rosmarinic acid lipase inhibitory effect is likely not one to be paired with it.

Based on these data, the first conclusion of the inhibition studies was that the results of the *in vitro* enzyme assays may not be relevant in a more realistic model, i.e., the Infogest model. In addition, investigation of organic extracts may not necessarily reflect the effect during conventional consumption (seasoning), but their use may optimise the effect in order to make food supplements.

5.5.3. Grape seed powder and black tea brew

Wine production is one of the most important agricultural activities in the world and causes the generation of a large number of by-products, including grape skins, -seeds, and -stems (Maicas and Mateo, 2020). Wine by-products have high potential as food ingredients, since they facilitate increased sustainability increase in the wine industry by reusing a product that is usually

considered waste (Ferrer-Gallego and Silva, 2022). Therefore, further utilization of by-products can be of great importance, and a great deal of research effort is being devoted to testing the putative beneficial effects of grape parts (Akaberi and Hosseinzadeh, 2016). Both grape seed (Moreno et al. 2003) and grape skin (Serea et al. 2022) extract showed potential pancreatic lipase inhibitory effect according to *in vitro* enzyme assays (Kurihara et al. 2003; Costamagna et al. 2016). Inhibitory effect is attributed to a variety of polyphenolic compounds, e.g., proanthocyanidins, found in solid parts of grape (seeds and skins; Saucier et al. 2001; Chedea et al. 2011; Das et al. 2020; Ramos-Pineda et al. 2020; Dwibedi et al. 2022).

Tea (*Camellia sinensis*) is one of the most frequently consumed beverages (Chantre and Lairon, 2002). The health benefits of tea have been widely studied, and these effects are closely related to the structure and composition of polyphenols (Zhang et al. 2019). Depending on level of fermentation there several types of tea, which all are associated with potent lipase inhibitory properties (white (Gondoin et al. 2010), oolong (Nakai et al. 2005), Pu-erh (Chen et al. 2018), green (Chantre and Lairon, 2002) and black (Glisan et al. 2017; Sellami et al. 2017).

Most of these studies focus on use of simple enzyme assays with model TAGs and the diversity in the TAG composition of consumed food commodities is often neglected. However, based on the encouraging results of the enzyme assays, the influence of grape seed powder (GSP) and black tea brew (BTB) was evaluated on lipid digestibility with a more complex *in vitro* digestion simulation using two foods with different FA composition: cream and baked beef.

First, effective treatment levels of GSP and BTB have been determined by performing dose-response experiments on cream as test food. Effective dosage was further tested on BB for evaluation of substrate-specific effects. During my experiments these two bioactive containing foods showed similar effects therefore they are discussed together.

5.5.3.1. Dose and substrate dependency

To evaluate the required effective dosage of the selected bioactive rich foods, first, co-digestion experiments with cream test food were conducted at three levels. In the case of GSP, it was tested in small quantities, namely in 5, 10 and 15 w/w% relative to the weight of cream to simulate realistic consumption behavior of the used food supplement (according to packaging instructions). As Figure 21A shows that significant decrease (by 12%) was observed at the first level (5 w/w% GSP) from 74.9 ± 2.0% to 65.9 ± 3.1%, and further addition of GSP did not result added decrease in lipid digestibility. This was noticed in both GL+PL and PL digestion simulations, i.e., the lowest tested level already resulted a significant effect in cream, however with only PL digestions the decrease was higher around 15% ($p = 0.017$). The observed decrease in lipolysis in PL digestions

indicates that as a result of inhibition, the dispreference of PL to digest TAGs containing SCFAs and MCFAs is presumably further enhanced. At the same time, the results of the GL+PL digestions suggest that GL is also inhibited and could not perform the lipolysis of TAGs containing SCFAs and MCFAs efficiently, i.e., they continue to reach the duodenum. Their lipolysis will not be completed in the small intestine either due to the dispreference shown by PL towards such substrates, and moreover, because this dispreference is enhanced as a result of the inhibition.

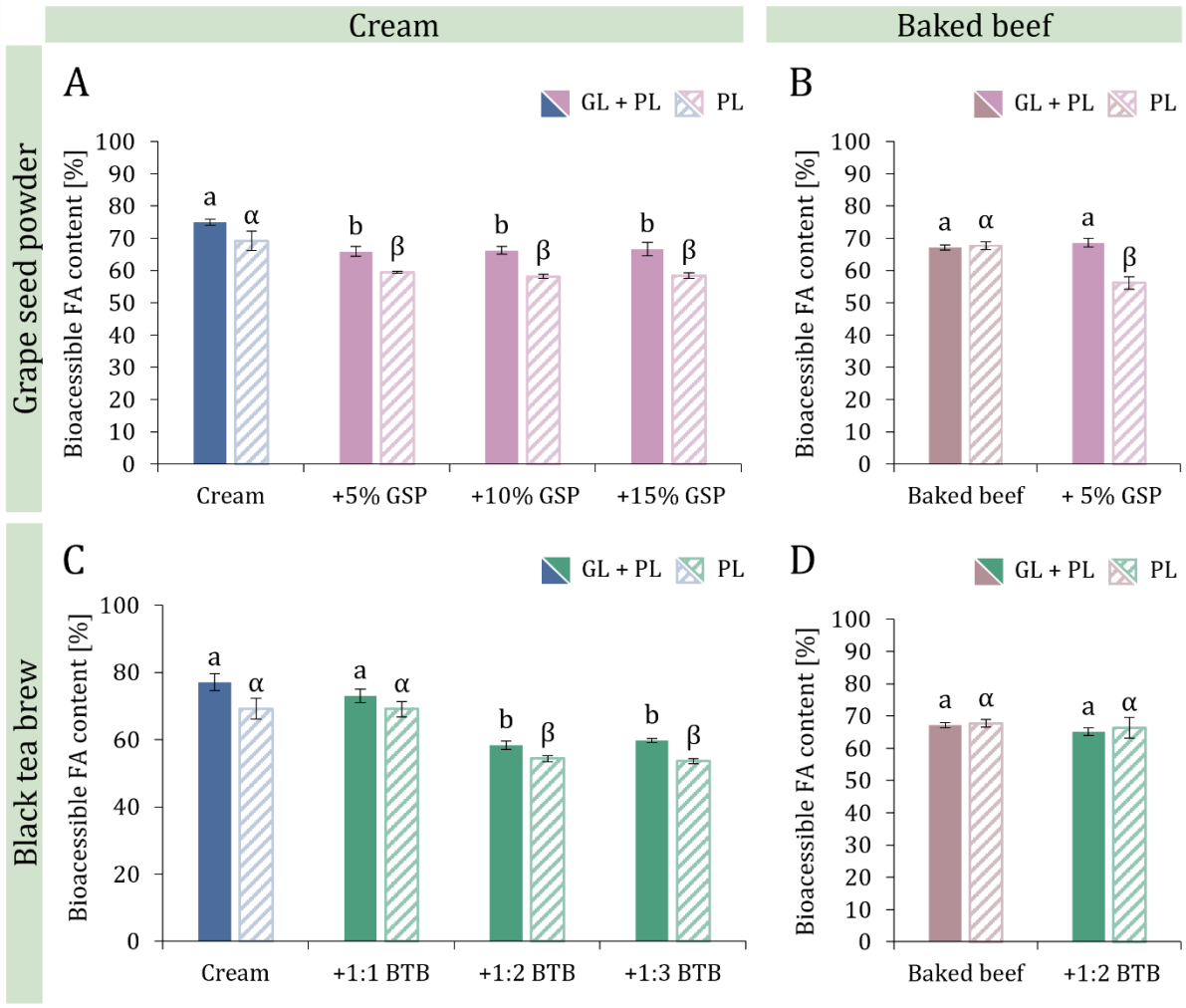

Figure 21. Dose dependency tests with cream test food (A, C) and effect of bioactive rich foods on baked beef (B, D) in the most effective ratio. A, B: effect of grape seed powder (GSP); C, D: effect of black tea brew (BTB). Significant difference within groups is marked with Latin letters (GL+PL) or with Greek letters (PL) ($p<0.05$).

The 5 w/w% GSP treatment was also tried on baked beef in further co-digestion experiments (Figure 21B). Interestingly, addition of GSP only resulted in decreased lipid digestibility by 12% ($p<0.001$) when the digestion simulation was carried out with PL protocol. In the GL+PL

digestion, no change was observed (p= 0.316). This shows that in the case of a less marked presence of TAGs containing SCFA and MCFA, the decrease in gross lipolysis is less significant.

The effect of BTB was also tested with cream test food first. Aqueous extract of a selected black tea cultivar with high tannin content was mixed with cream before digestion. In this case, cream to black tea brew weight ratios of 1:1, 1:2, and 1:3 were tested (Figure 21C) similarly to English tea consumption. Maximum inhibitory effect of BTB is reached at the 1:2 level (by 22%) from 74.9 ± 2.0% to 58.3 ± 2.5%, and further addition did not increase the effect. Observed degree of inhibition did not differ between the GL+PL and PL digestion simulations since 22% (p= 0.426) decrease in overall lipid digestibility was measured in both digestion simulations. Similarly to the experiments observed with GSP, no effect of BTB on overall lipid digestibility was observed with baked beef as a test food, when the same BTB to fat ratio was tested, which seemed effective for significant lipase inhibition in the case of the 1 cream: 2 tea experiment (Figure 21D). This result clearly suggests that differences in the fat source, i.e., the type of substrate for digestive lipase enzymes, has marked influence on manifested lipase inhibition characteristics of the same bioactive-rich food. Most probably, the difference in FA profile, TAG structure and other matrix specific characteristics of the consumed food not only influences lipid digestibility of fat from different foods, but also influences the lipase inhibitory effects of the same bioactive rich food when consumed together with foods containing different types of fat sources.

5.5.3.2. Fatty acid-specific effects: reduced digestibility of SCFAs and MCFAs

The results showed that the exerted lipase inhibitory effects of the tested GSP and BTB are substrate dependent, i.e., the appropriate level of bioactive addition to inhibit cream lipid digestion was not enough to reach the same level of inhibition for baked beef test food with identical lipid concentration. To further study the specific lipase inhibitory effects of the selected bioactive rich foods, FA-specific analysis was conducted (Figure 22 and 23).

In the case of GSP and BTB treatments, similar trend regarding the changes in FA profile could be observed for cream as a test food. Lipase inhibitory effects of the tested bioactive rich foods primarily affected the SCFAs and MCFAs. In GL+PL digestion simulations with GSP (Figure 22A) the release of SCFAs and MCFAs were reduced more drastically than the release of LCFAs. Namely, the digestibility of SCFAs and MCFAs (C8:0-C15:0) reduced in the range of 43-22% and the release of LCFAs were reduced between 5-18%, compared to the control digestions. In the case of BTB (Figure 23A) the inhibitory effect in digestion simulation with both enzymes were more evened out, since the reduction of the release of all FAs were between 16-50%, however the average reduction was higher for SCFAs and MCFAs with 5%. Moreover, the effect of both foods containing inhibitory bioactive compounds seems to be more pronounced on the pancreatic lipase

activity. After PL only digestions (Figure 22B and 23B) addition of both GSP and BTB resulted in a decreased release of SCFAs and MCFAs. With GSP RR of C10:0-C15:0 decreased by 46-32% compared to average 7% decrease of longer FAs, and with BTB RR of C6:0 and C10:0-C15:0 decreased by 52% and 51-25% compared to the average 19% of longer FAs.

In accordance with the previous observations, in experiments with baked beef test food, neither GSP nor BTB showed inhibitory effect on lipolysis. The reason for this could be explained by differences in the characteristics of fat sources. In BB, relative abundancy of SCFAs and MCFAs are negligible and consequently, asymmetric TAGs in which SCFAs and MCFAs are present are also negligible. Thus, the dispreference shown by PL towards such substrates as well as the reduced contribution of GL to pre-digest these substrates as a consequence of the inhibition does not result in the apparent decrease in overall digestibility of beef fat.

Interestingly one exception was found, the 5 w/w% addition of GSP to BB test food where the inhibitory properties on PL could be manifested when only PL was added (Figure 21B). The effect could be pointed to the difference between the two bioactive rich foods found in the extent of inhibition on PL, i.e., GSP had higher effect on only PL than GL+PL, whereas it was not observed for BTB. This effect was not uniform on all FAs (Figure 22D). The RR decrease was higher on the only MCFA (C14:0: 25%) and on the unsaturated FAs (C16:1n-7c: 27%, C18:1n-9c: 22% and C18:2n-6c: 29%) than on the other two, saturated FAs (C16:0: 13% and C18:0: 10%). This also highlights the specific dispreference of PL towards the asymmetric TAGs, since TAGs containing – most probably one or two – unsaturated FAs can also be considered as asymmetric type of TAGs.

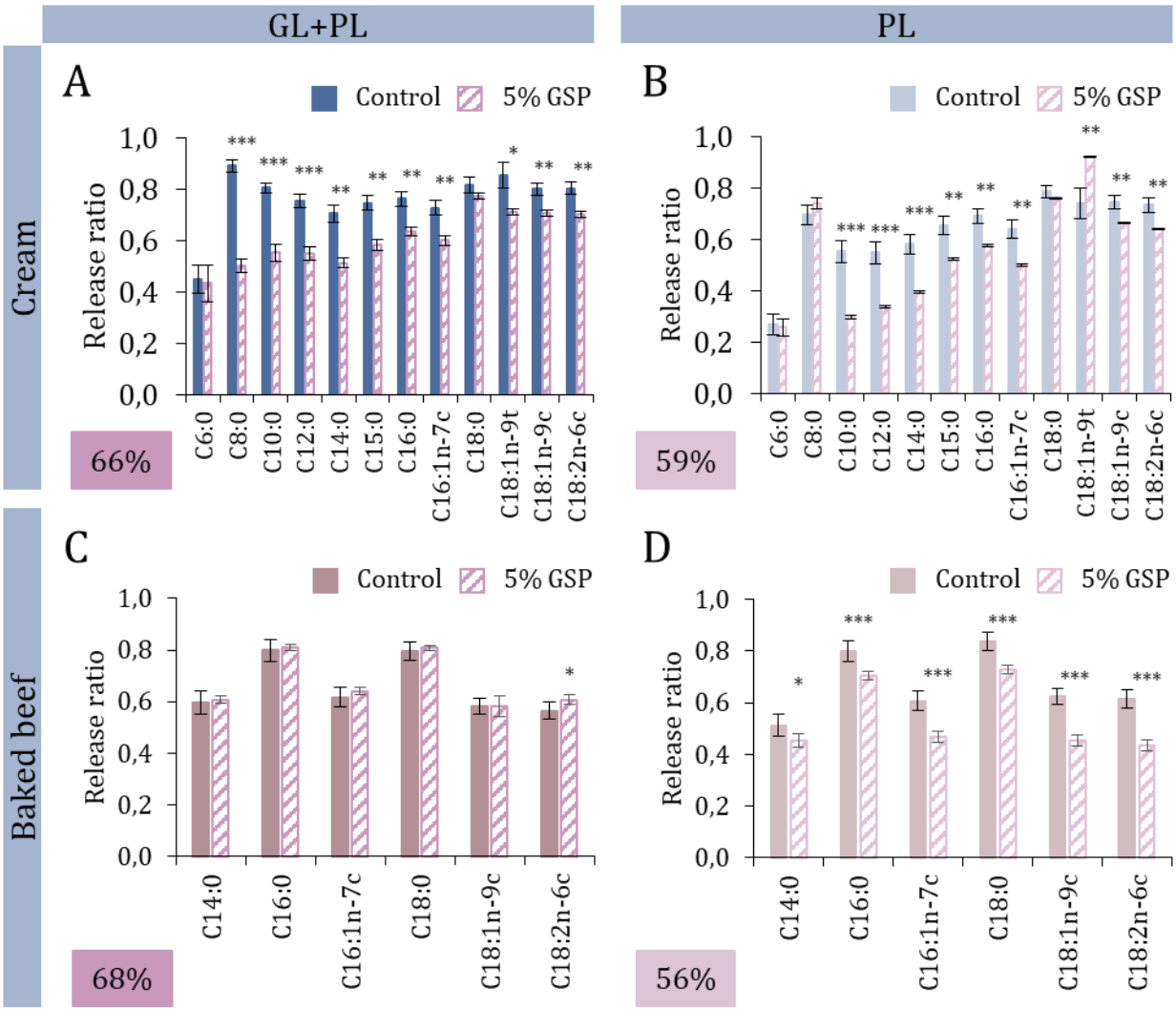

Figure 22. Effect of 5 w/w% of grape seed powder on release ratio of fatty acids. A, B: cream test food in digestion simulation with GL+PL and PL, respectively; C, D: baked beef test food in digestion simulation with GL+PL and PL, respectively. Percentages show extent of lipolysis calculated from free fatty acids released during digestion. Asterisk labels (*) show significant difference between release ratio of individual fatty acids after GL+PL and PL digestions (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

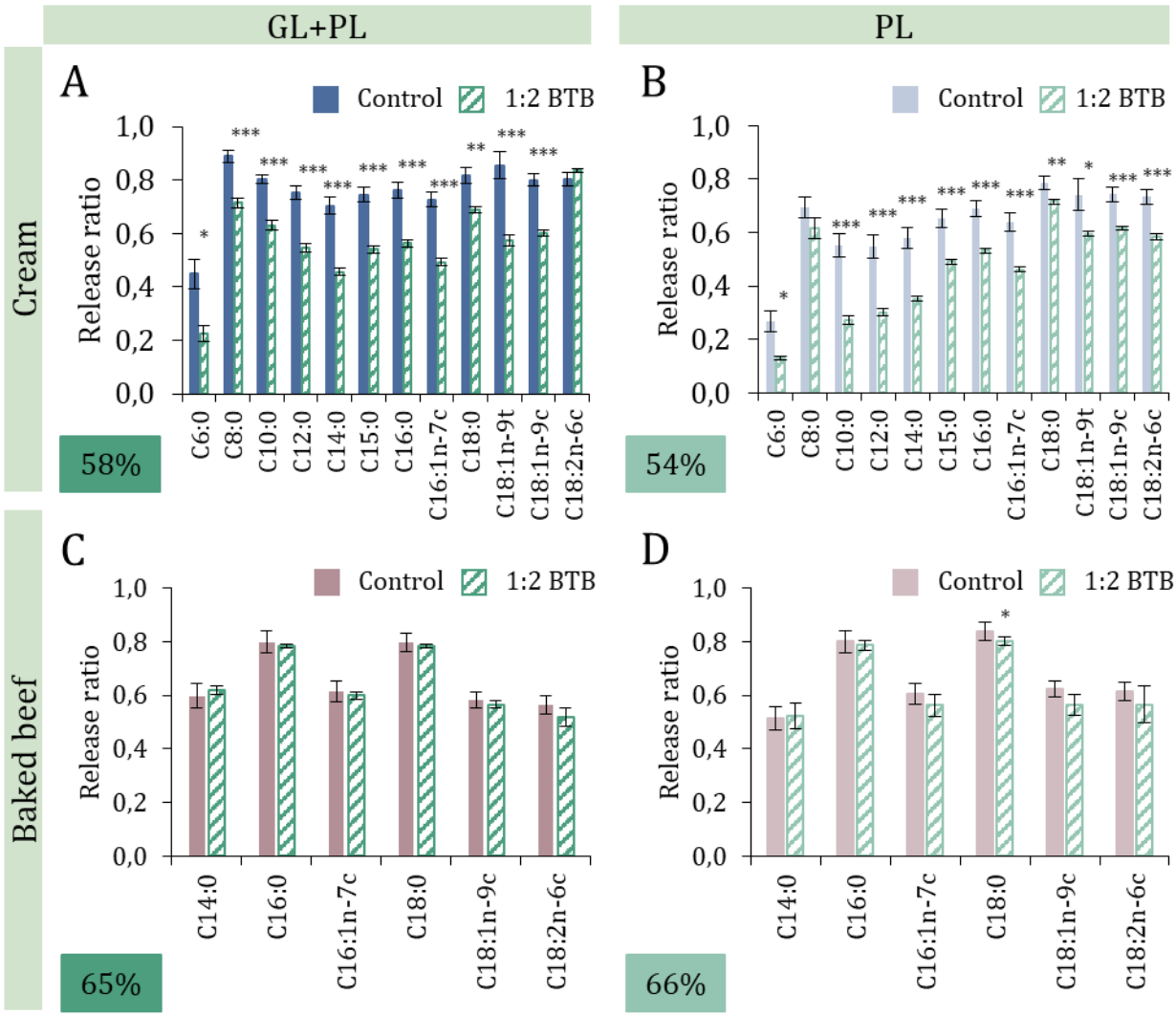

Figure 23. Effect of black tea brew (1 (cream/baked beef) :2 (BTB) ratio) on release ratio of fatty acids. A, B: cream test food in digestion simulation with GL+PL and PL, respectively; C, D: baked beef test food in digestion simulation with GL+PL and PL, respectively. Percentages show extent of lipolysis calculated from free fatty acids released during digestion. Asterisk labels (*) show significant difference between release ratio of individual fatty acids after GL+PL and PL digestions (*p<0.05; **p<0.01; ***p<0.001).

In conclusion to the digestion simulation experiments carried out with two selected test foods (cream and baked beef) having markedly different FA profiles, it is postulated that TAGs containing SCFAs and MCFAs are non-preferred substrate types for pancreatic lipase (PL) and consequently, PL alone is rather ineffective in the digestion of such TAGs containing SCFAs and MCFAs. However, this specificity is not characteristic for gastric lipase (GL). Thus, the presence of GL is resulted in the increased release of SCFAs and MCFAs (C6:0- C15:0). This effect is prominent in milk fat containing products such as the analyzed cream. Results also showed that

when there is no marked diversity in FA composition of the consumed fat source and consequently heterogeneity in the TAG structure is limited, the contribution of GL could be negligible such as in the case of baked beef. It is also concluded that the difference in FA profile, TAG structure and other matrix specific characteristics of the consumed food not only influences lipid digestibility of fat from different foods, but also influences the lipase inhibitory effects of the same bioactive rich food when consumed together with foods containing different types of fat sources.

The findings together suggest that lipase inhibitory effect of both GSP and BTB is primarily affects the lipolysis of short and medium chain FAs. The reason behind that can be explained by the hypothesis that the dispreference of PL to digest TAGs containing SCFAs and MCFAs is presumably further enhanced as a result of treatment with such bioactive-rich foods as GSP or BTB. At the same time, it is postulated that GL is also inhibited to some extent and could not perform the pre-digestion of SCFA- and MCFA-containing TAGs efficiently either. It means, undigested SCFA and MCFA containing TAGs can reach the duodenum, where their lipolysis will not be completed either due to the dispreference shown by PL towards such substrates, and on the other hand, because this dispreference is enhanced as a result of the PL inhibition.

Another interesting conclusion is that the two different bioactive-rich food, showed lipase-inhibiting effects with the similar characteristics. That is, both GSP and BTB had a significant effect in the case of cream, while they proved to be ineffective in inhibiting the breakdown of fat from baked beef. This finding highlights the importance of the characteristics of the fat source and the food matrix as key determinants in observed lipase inhibitory effect of a bioactive-rich food. This observation also suggests that the hypothesized effects of various foods rich in lipase-inhibiting bioactive components on gross lipolysis should be investigated under conditions that simulate the human digestion (ideally using both GL and PL enzymes) and these studies should involve experiments with different types of real foods, ideally with different characteristics that might influence their presumed effects.

5.6. Co-digestion of foods – interplay between lipid and protein digestion

As seen previously, lipid digestibility of high fat toppings (SC and SCA) differs based on microstructure influenced by lipid composition and surface properties of lipid droplets. Besides interfacial properties of the lipid droplets or presence of enzyme inhibitory components (de la Garza et al. 2011; Palanisamy et al. 2012), there are several factors that could affect digestibility of dietary fats, such as other nutrients of the food matrix (Dias et al. 2019; Hiolle et al. 2020; Guevara-Zambrano et al. 2022) and their structural changes during the several phases of digestion

(Mackie, 2017; Junejo et al. 2019; Mulet-Cabero et al. 2019). Since consumption of food products – especially the type of high fat toppings such as sour cream – usually happens as part of a meal, additional ingredients could modify digestibility behaviour of the products. Therefore, revealing and understanding the effect of co-consumption on the lipid digestibility, the two products SC and SCA were studied during co-digestion with cooked pasta (CP).

Cooked pasta was chosen as a low fat test product (<1% fat content) and an important element of western and Mediterranean diet (Simonato et al. 2015), usually consumed with simple toppings or sauces. Lipid digestibility of the two products was studied in co-consumption experiments, therefore both products were co-digested with cooked pasta to simulate a real meal. Lipid digestibility of the two toppings after co-digestion with cooked pasta was determined after digestion simulation using both lipolytic enzymes (GL+PL) or only pancreatic lipase (PL) based on fatty acid release. Bioaccessible FA content and release ratios after co-digestions were compared to the SC or SCA single food digestion results (detailed discussion in Chapter *1.2.2. Role of food matrix*) and are shown on Figure 24.

It was observed that lipid digestibility of SC in the pasta dish increased compared to the single food digestion however this was not the case for SCA where the extent of lipolysis did not change. The addition of cooked pasta increased the lipid digestibility of sour cream by 9% (GL+PL: $p= 0.021$; 61.1 ± 3.9% to 66.8 ± 3.2%; Figure 24A). However, this increase was only observed when both lipolytic enzymes were present. In the simulation with only PL, the addition of cooked pasta did not significantly ameliorate lipid digestibility (PL: $p= 0.565$; 52.6 ± 2.7% to 54.0 ± 4.8%; Figure 24B). When examining the release of fatty acids in GL+PL digestions, it can also be seen that the release of some fatty acids (C8:0-C14:1n-5c) increased more when pasta was added to aid the digestion of milk fat than others. Affected fatty acids are C8:0 (+20%), C10:0 (+22%), C12:0 (+28%), C14:0 (+15%) and C14:1n-5c (+11%). The release of these FAs has been previously linked to gastric lipase activity, therefore it seems that the addition of pasta specifically increases the efficiency (activity and/or accessibility) of gastric lipase, which has a preference towards digesting triacylglycerols with SCFAs and MCFAs.

For sour cream analogue, the co-digestion did not result in any change in lipid digestibility. There was no significant effect of adding cooked pasta in either GL+PL ($p= 0.454$; Figure 24C) nor PL ($p= 0.599$; Figure 24D). In addition, only the release ratio of C14:0 is increased by 5% in the digestion simulation with both digestive enzymes (GL+PL, Figure 24C). Since C14:0 is a MCFA, the increased release of this FA is also most probably linked with a slight improvement in GL activity or accessibility.

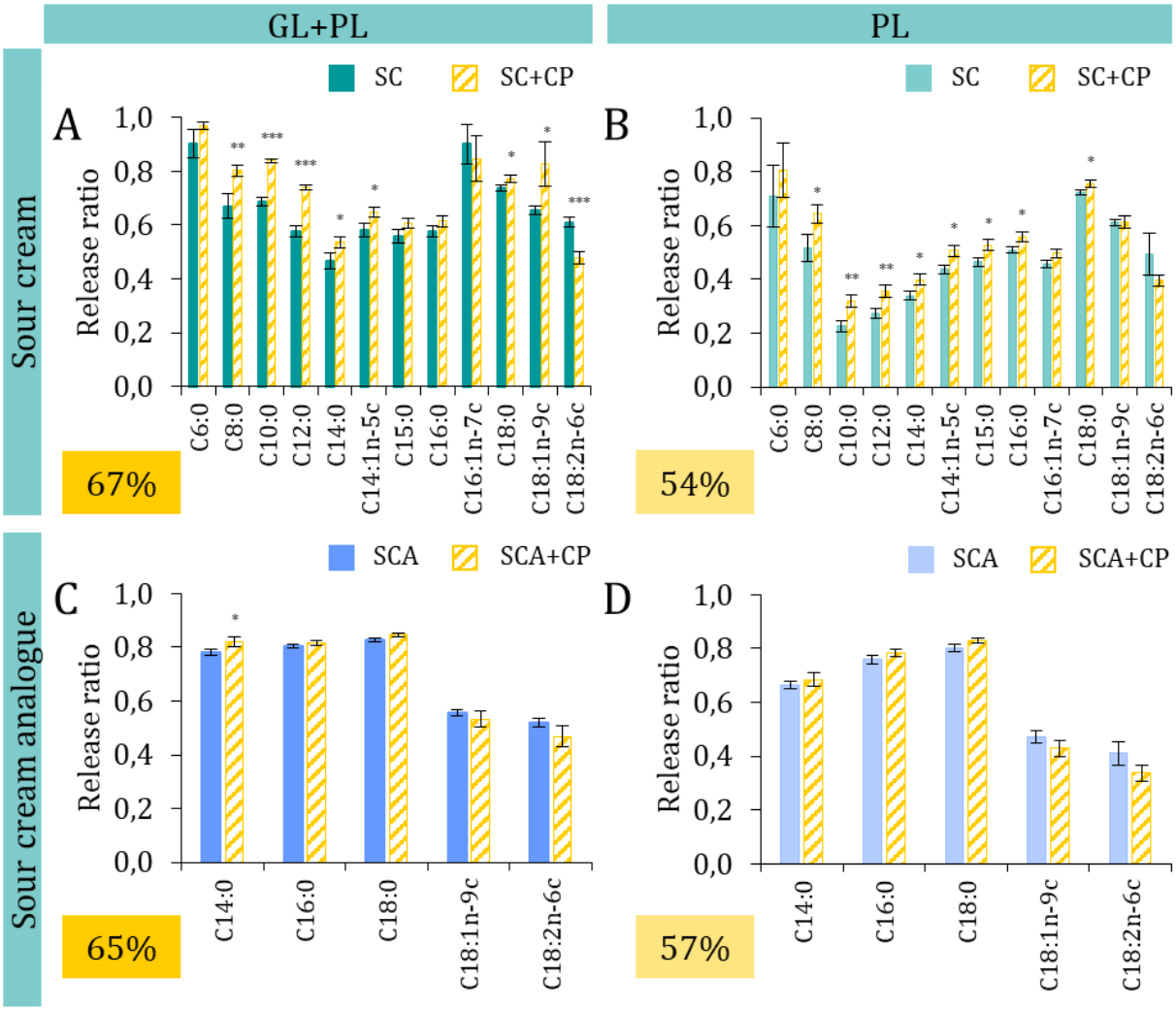

Figure 24. Release ratio of individual fatty acids after co-digestion simulation of high fat toppings (SC and SCA) with cooked pasta (CP). Digestion simulations were carried out using either both lipolytic enzymes (GL+PL) or only pancreatic lipase (PL). Percentages show extent of lipolysis calculated from free fatty acids released during digestion. A: SC+CP, GL+PL; B: SC+CP, PL; C: SCA+CP, GL+PL; D: SCA+CP, PL. Asterisk labels (*) show significant difference between release ratio of individual fatty acids after GL+PL and PL digestions (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

Fatty acid-specific results of both co-digestion experiments point to the fact that the addition of CP modifies the efficacy (activity and/or accessibility) of gastric lipase during gastric phase of digestion. To further investigate this issue, microscopic analysis of the non-enzymatic co-digests were also carried out for SC+CP and SCA+CP digestions. Lipid structure was dyed as before (with Nile red dye) and in addition, simultaneous staining with Coomassie blue was also applied to the

same samples. By this means the interplay of protein structures of pasta and lipid structure of SC or SCA could also be simultaneously studied. Results are presented in Figure 25.

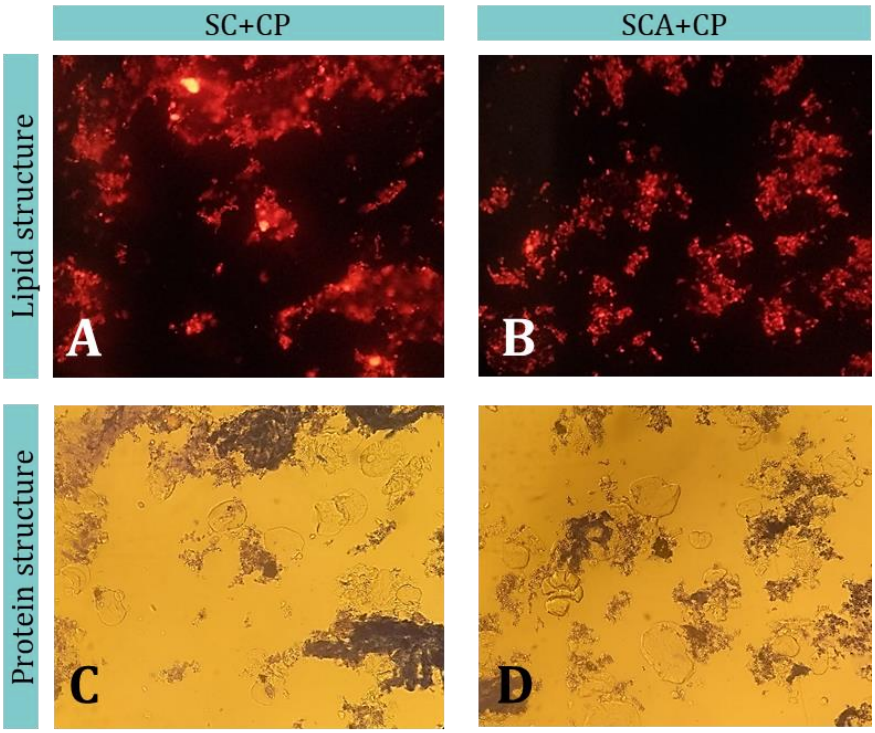

Figure 25. Microscopic images of non-enzymatic co-digests: sour cream and cooked pasta (SC+CP) and sour cream analogue and cooked pasta (SCA+CP). Pictures were taken from the same sample dyed with both dyes. Above (A, B) lipid structure dyed with Nile red, under (C, D) protein structure dyed with Coomassie blue.

As described before, milk fat globules are prone to flocculation and aggregation during gastric digestion results in increased size of lipid droplets (Ye et al. 2010; Gallier et al. 2012; Giang et al. 2015), and lower lipid digestibility due to decreased surface and hence binding site for lipolytic enzymes (Qazi et al. 2021). As shown in Figure 25, lipid droplets during gastric co-digestion behave differently than during gastric single food digestion (as seen on Figure 20). Both milk fat globules and palm oil droplets are arranged on the surface of available pasta protein structures. It is speculated that the structural change caused by the presence of pasta proteins prevents MFGs to flocculate and aggregate during gastric digestion (Figure 25A, 25C). This surface association of MFGs on pasta proteins may help avoiding association of MFG droplets. In other words, pasta proteins may have protective effect that prevents MFG droplet associations and thus droplet size increase. Consequently, without the increase in droplet size, lipid digestion is not hindered by the decreased surface area. It is also postulated that this reserved original MFG size during gastric

digestion enables GL to perform lipolysis as efficiently as in SCA. This latter hypothesis is supported by our findings, namely an observed significant increase of the GL-preferred FAs (C8:0; C10:0; C12:0) when SC is co-digested with CP (Figure 24A).

In the case of palm oil droplets, it was observed that lipid droplets are also organized on the surface of pasta protein similarly to MFGs (Figure 25B, 25D). However, on the contrary to MFGs, this surface association did not accompany with a change (reduction) in the droplet size of SCA lipids. Accordingly, no further improvement of lipid digestibility is expected and observed due to this structural rearrangement.

Based on fatty acid-specific evaluation and structural analysis, it can be concluded that the ameliorating effect during SC+CP co-digestion caused by the presence of pasta has happened in the gastric phase is twofold: i) pasta proteins inhibit structural disintegration of MFGs during gastric conditions that would result in coalescence and increased lipid droplet size. Thus, the reserved smaller globule size provides more efficient lipolysis for gastric lipase, and ii) the increased efficiency of gastric lipase (compared to single food digestion) manifested in the increased release of GL-preferred FAs (SCFAs and MCFAs).

5.7. Protein digestibility of test foods

5.7.1. Single food digestion for evaluation of protein digestibility

Some test foods with higher protein content (baked carp, baked beef) and cooked pasta were chosen for the purpose of protein digestibility determination. Protein content of the test foods were determined with Kjeldahl method (relevant conversion factor was used). After *in vitro* digestion simulation bioaccessible protein content of these foods were isolated with methanolic precipitation. According to Sousa et al., the supernatant obtained by this method contains the bioaccessible protein content, which could be absorbed in the small intestine (Sousa et al. 2023). The extract will contain smaller proteins with different degree of polymerization, i.e., amino acids, di- and tripeptides will be present. Determination of protein content in the supernatant was carried out in three ways: i) unhydrolysed supernatant based on free amino group with the OPA method, ii) hydrolysed supernatant based on free amino group with the OPA method, and iii) hydrolysed supernatant based on amino acids with AQC derivatization and HPLC-UV analysis. Measurement of the unhydrolysed supernatant will give information on the degree of digestion, additionally to the overall protein digestibility (IVPD%) obtained from the other two methods. Protein content (%) of test foods determined with Kjeldahl method and *in vitro* protein digestibility (%) measured with different methods described above are shown in Table 7.

Table 7: Protein content (%) of test foods: baked carp, baked beef and cooked pasta, determined with Kjeldahl method and *in vitro* protein digestibility (%) measured with different methods: unhydrolysed OPA (Eq. 1), hydrolysed OPA (Eq. 2) and hydrolysed AA (Eq. 7).

		Test food		
	Mode of determination	**Baked carp**	**Baked beef**	**Cooked pasta**
Protein content [%]	Kjeldahl method	22.3±0.3	29.9±0.6	4.9±0.2
***in vitro* Protein digestibility [%]**	Unhydrolysed OPA	36.8	33.1	21.7
	Hydrolysed OPA	n.a.	n.a.	54.2
	Hydrolysed AA	76.3	89.8	100.0

n.a. - not applicable

As seen in Table 7, protein digestibility results obtained with different methods are controversial. Moreover, hydrolysed OPA values (Hydrolysed OPA, see Chapter *2.3. Hydrolysis before determination of bioaccessible protein content*), could be derived only for cooked pasta test food. In the other two cases, most probably due to malfunction of analysis for such matrices, completely uninterpretable signals were obtained. It is also clear that the IVPD% calculated form hydrolysed OPA and hydrolysed amino acid results are different however these values should be the same. As noted before the hydrolysis method used to breakdown peptides in the bioaccessible fraction before OPA measurement is developed for dairy product with low fat content. None of the tested foods were dairy products and consequently did contain substantially higher amount of other components, i.e., animal product contained more than 10% of fat and cooked pasta contains around 20-30% of starch. These additional compounds could influence efficiency of protein hydrolysis.

One explanation for this could be the presence of fat in the meat matrix as shown by Ding et al. (Ding et al. 2022). It was concluded that in high-fat conditions, solubility of meat proteins decrease caused by the emulsification of amphiphilic proteins. As a result, these proteins are adsorbed at the oil-water interface, which reduces the level of protein dispersion in the water and consequently the protein digestibility is reduced. An effect which does not occur during digestion of the pasta due to the absence of fat content. In contrast, from the unhydrolysed OPA results it shows that the degree of hydrolysis is lower after cooked pasta digestion than digestion of meat products. This additional information tells that however digestibility of pasta proteins are higher the bioaccessible fraction contains a higher ratio of longer peptides than the bioaccessible fraction of the small intestinal digesta of the meat products.

It was concluded that OPA procedure in its current form is not suitable for the assessment of protein digestibility in the studied matrices. Therefore, analysis of AAs in the methanolic supernatant (bioaccessible protein fraction) seemed to be a straightforward alternative for this purpose. HPLC measurement of AAs (see Chapter *4.3.6.2. Based on free and total amino acid*

*conte*nt) was carried out after microwave digestion of the methanolic extract and the sum of AAs measured in the methanolic supernatant was used to calculate IVPD%. An additional advantage of this approach that availability of individual AAs enables the calculation protein quality attributes such as PDCAAS and DIAAS. In this study, both *in vitro proxy*-PDCAAS and *in vitro* DIAAS were calculated from bioaccessible amino acid content measured by HPLC. Calculation of these values were previously detailed (*in Chapter 2.6. Protein quality scores*). Results of *proxy*-PDCAAS and *in vitro* DIAAS values are given in Table 8.

Table 8: Protein quality scores: *proxy*-PDCAAS and *in vitro* DIAAS, calculated from amino acid-based evaluation of *in vitro* protein digestibility after simulated digestion, according to Eq. 8 for *proxy*-PDCAAS and Eq. 9 for *in vitro* DIAAS. Scores are given in "value (limiting amino acid)" format.

	proxy*-PDCAAS		
Test matrix	Preschool child (2-5 year)	Schoolchild (10-12 year)	Adult
Baked carp	69 (Leu)	87 (Ile)	100 (SAA)
Baked beef	55 (Trp)	68 (Trp)	100 (Trp)
Cooked pasta	30 (Lys)	39 (Lys)	100 (Lys)

	in vitro* DIAAS		
Test matrix	Infant (0-6 month)	Child (6-36 month)	Older child, adolescent, adult
Baked carp	50 (AAA)	90 (AAA)	103 (Leu)
Baked beef	40 (Trp)	80 (Trp)	103 (Trp)
Cooked pasta	17 (Trp)	27 (Lys)	32 (Lys)

**Calculated based on hydrolysed AA results. PDCAAS values above 100 were truncated.*

Results show that *proxy*-PDCAAS values could be misleading. In calculating these values only overall protein digestibility is considered and the AA specific digestibility of each AA is neglected. Hindered digestibility of amino acids caused by structural (e.g., denaturation during heat treatment), compositional (e.g., adsorption to fat molecules) or environmental (changes during digestion e.g., co-digestion with protease inhibitors) reasons are not taken into consideration.

Whereas *in vitro* DIAAS is a score that takes into account the varying digestibility of each AA. For example, the limiting amino acid of cooked pasta i.e., the one that would determine the utilization of all accessible amino acids is lysine in both cases. However, the *in vitro* DIAAS value is the third of the *proxy*-PDCAAS which means that the digestibility of lysine is very low. This example highlights that considering AA-specific digestibility, can be – at least in some cases – a crucial factor to correctly assess the nutritional value of a protein source.

5.7.2. Co-digestion of cooked pasta and different type of oils

In the previous experiment, addition of cooked pasta seemed to impact the lipid digestibility of high fat toppings containing milk fat and palm oil. The question arises that if cooked pasta could affect the release of fatty acids form different fat sources, could different fat sources (i.e., edible oils) modify protein digestibility of cooked pasta? To test this theory, experiment was designed to see the how edible oils affect protein digestibility of cooked pasta. Eleven types of oils were chosen, namely, sunflower oil (SFO), MCT oil (MCT), pumpkinseed oil (PSO), walnut oil (WO), hemp oil (HO), olive oil (OO), linseed oil (LO), coconut oil (CO), sesame oil (SO), grape seed oil (GSO), rape oil (RO). Fatty acid composition of oils was determined after derivatization with GC-FID (*Chapter 4.3.5. GC-FID method*). In the co-digestion simulations, oils were added CP at 5 w/w%. Bioaccessible protein content of small intestinal digesta was isolated with methanolic precipitation and supernatant was used to determine IVPD% before and after acidic hydrolysis (AOAC 2018.06) as shown before. Additionally amino acid-based results were gathered from the supernatant after microwave assisted hydrolysis. Protein digestibility of cooked pasta co-digested with the eleven types of edible oils are shown on Figure 26.

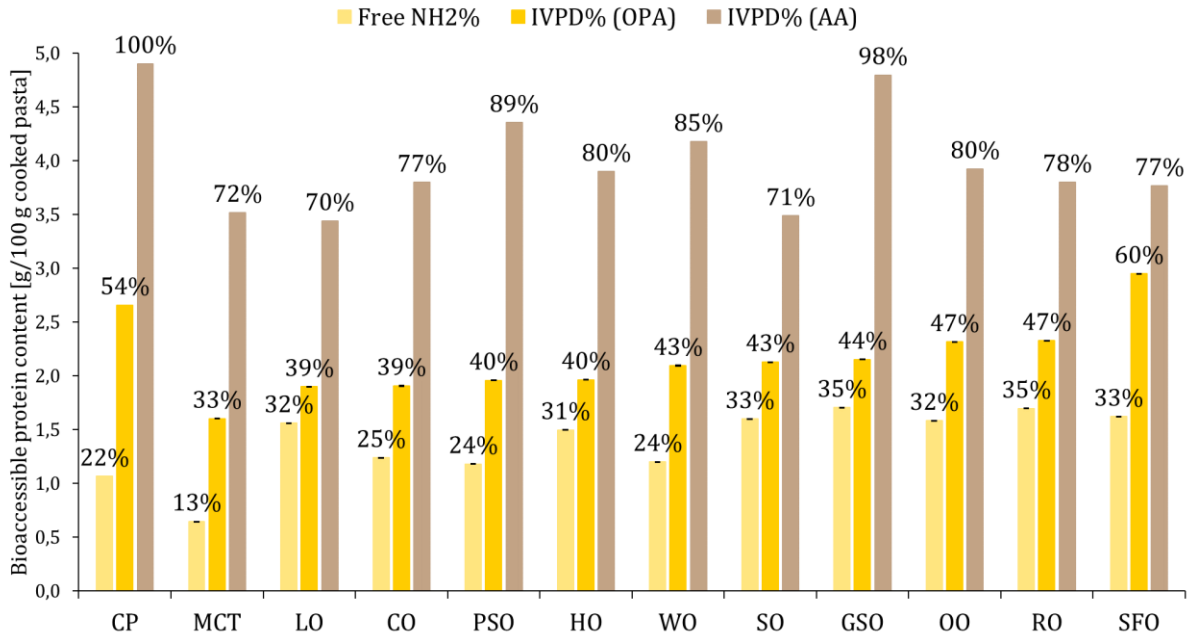

Figure 26. Bioaccessible protein content (g/100 g cooked pasta) of cooked pasta determined after *in vitro* digestion simulation. Bioaccessible protein content (g/100 g cooked pasta) after co-digestion with eleven types of edible oils are also shown. Percentages show values calculated according to Eq. 1 (Free NH2%), Eq. 2 (IVPD% (OPA)), and Eq. 7 (IVPD% (AA)). *Amino acid analysis (digestion and UHPLC-UV measurement) was carried by Éva Lengyel-Kónya, Mária Berki and Rita Tömösközi-Farkas which is kindly acknowledged.*

The main objective of these experiments was to determine how the addition of fats of different sources and with varying composition could affect protein digestibility of pasta proteins. Therefore, some indicators of lipid composition of these oils were created (USFA/SFA, MUFA/PUFA, C18:1/TFA). Protein digestibility was correlated with these indicators (Pearson correlation). Lipid composition indicators and correlation coefficients are shown in Table 9. Correlation matrix were created two times, i.e., one with and one without inclusion of MCT oil, since all indicators were 0 for this oil. However, results were generally not affected by the exception.

Table 9: A: Lipid composition indicators created form fatty acid profiles of the eleven examined edible oils and IVPD% results gathered after *in vitro* co-digestion experiments. B: Correlation matrix with and without the inclusion of MCT oil.

A: Lipid composition indicators and IVPD% results

Edible oils	Indicators			IVPD%		
	USFA/SFA	MUFA/PUFA	C18:1/TFA	Free NH_2 (%)	IVPD% (OPA)	IVPD% (AA)
MCT	0.00	0.00	0.00	13.2	32.7	71.7
LO	13.58	0.20	0.15	31.8	38.7	70.2
CO	0.08	4.31	0.06	25.2	38.9	77.5
PSO	6.09	0.46	0.27	24.1	39.9	88.8
HO	11.37	0.22	0.17	30.5	40.0	79.5
WO	11.82	0.25	0.18	24.5	42.7	85.2
SO	7.23	0.73	0.37	32.6	43.4	71.1
GSO	10.77	0.60	0.34	34.7	43.9	97.9
OO	6.70	10.19	0.79	32.3	47.2	80.0
RO	20.79	2.33	0.66	34.6	47.4	77.5
SFO	15.81	7.74	0.83	33.1	60.2	76.8

B: Correlation matrix (r values)

	with MCT			without MCT		
	USFA/SFA	MUFA/PUFA	C18:1/TFA	USFA/SFA	MUFA/PUFA	C18:1/TFA
Free NH_2 (%)	0.69	0.31	0.64	0.57	0.22	0.59
IVPD% (OPA)	0.61	0.66	0.89	0.48	0.64	0.86
IVPD% (AA)	0.07	-0.08	0.06	-0.10	-0.17	-0.08

Correlation coefficient (r) was only strong ($r<-0.8$ or $r>0.8$) in one case, i.e., when oleic acid content was compared to IVPD% (OPA) values (0.89). As already showed, this method (acid hydrolysed methanolic supernatant and determination of free amino groups with OPA method) was not always applicable or the results do not coincide with the sum of AA-specific digestibility. It was found that oleic acid could form complexes with proteins (Brinkmann et al. 2013)

Kalogianni et al. (2017) also showed that the complex formation could occur with various type of proteins after thermal denaturation. Thermal denaturation increases chance of protein-oleic acid interactions due to unfolding and opening of interaction sites. In the bioaccessible fraction, a part of the protein content is present as peptides, therefore interaction sites are already available to bind to oleic acid, which reaction might be amplified by the heat treatment during the hydrolysis process. Thus peptide-oleic acid complexes might form, which could hinder peptide hydrolysis towards amino acids. This hypothesis is in accordance with previous assumptions that acid hydrolysation is affected by the high fat content of the food (Dias et al. 2019). Finally, it was concluded that the obtained r= 0.86 correlation coefficient between the IVPD% (derived via OPA determination) and C18:1/TFA cannot be considered as a robust data. Most probably, the interference of lipids with the OPA procedure was manifested in the obtained correlation.

The correlation found between Free NH_2 (%) and lipid composition indicators was also higher (medium correlation was found; r= 0.64). Although this value seems to be applicable to compare protein digestibility it should be used after careful consideration. Consequently, the results of AAs determined by HPLC microwave assisted hydrolysis was considered as the only solid and valid data for IVPD% assessment in this research.

The evaluation of protein digestibility based on the AA-specific digestibility of cooked pasta showed that it is affected by fat addition however there is no clear correlation between lipid composition and IVPD%. Based on these results lipid composition specific interaction between edible oils and protein digestibility was not found in the co-digestion experiments it was confirmed that fat content of high-fat foods could affect determination of protein digestibility after *in vitro* digestion simulation.

6. Conclusions and recommendations

It was shown in this research that thorough consideration of food digestibility can be of great importance since nutrient accessibility can be significantly altered by several factors. These factors may include but not limited to ones such as the food matrix, structural changes occurring in the food before consumption and during digestion, the presence of substances capable of causing changes in enzyme activity, such as antinutritive and bioactive molecules. Food pairing, or co-consumption of one type of food with another is also considered as a relevant key factor. Nevertheless, recently only limited understanding and data is available on nutrient digestibility and even less on the parameters such as detailed above that may influence it. In order to deepen our understanding in the field of food-, and nutrition science, it is of fundamental importance that appropriate analytical methods, suitable for routine measurements should be available.

As a result of my work, I was able to develop a method suitable for the fatty acid-specific assessment of lipid digestibility, which has been harmonised with the Infogest digestion simulation method. The method uses simulated small intestinal fluid to determine the bioaccessible fatty acid content of foods using fat extraction, internal standardisation with glyceryl trinonadecanoate, standard derivatisation methods and GC-FID methods.

For the first time, lipid digestibility of baked carp meal, baked beef meal, cream, sour cream and sour cream test foods was determined. Moreover, detailed mechanistic studies (using selected lipolytic enzymes) were also carried out to unravel the role and specific contribution of lipolytic enzymes, i.e., gastric lipase and pancreatic lipase. Results showed that pancreatic lipase has a dispreference for digesting triacylglycerols containing short and medium chain fatty acids, which is not characteristic of gastric lipase. It is therefore concluded that gastric lipase plays an essential role in the lipolysis of foods containing high concentrations of these fatty acids, and the addition of gastric lipase is a necessity in lipolysis studies of said foods. In addition, comparison of FA-specific digestibility and structural analysis of sour cream and sour cream analogue showed that foods with similar food matrix but different lipid source behave differently during digestion as a result of the distinct surface properties of the different lipid droplets. It was also concluded that in such cases the main determinant of the extent of lipid digestibility is the lipid droplet size and the structural arrangement of lipids.

In addition to lipid digestibility assessment of various foods, effect of bioactive substances on lipid digestibility was also determined. Co-consumption of rosemary and baked carp meal showed that the constituents in the aqueous extract of rosemary spice released during *in vitro* digestion were not sufficient to cause a significant change in the lipid digestibility of baked carp. It was also

shown that grape seed powder – a dietary supplement – and black tea brew could both reduce the extent of lipid digestion of cream, but not of baked beef. The results of this experiment showed that the lipid characteristics of the consumed food not only influence the lipid digestibility, but also influence the lipase inhibitory effects of the same bioactive rich food when consumed together with foods containing different types of fat sources. Furthermore, the results suggest that the lipase inhibitory effect of both grape seed powder and black tea brew primarily affects the lipolysis of short and medium chain FAs due to the more pronounced inhibition on pancreatic lipase activity.

Besides co-consumption with bioactive rich foods co-consumption of multiple foods has relevance since some food products are usually eaten as a part of a meal. Lipid digestibility of sour cream and sour cream analogue was studied in co-consumption experiments with cooked pasta to simulate a real meal. The addition of pasta resulted in elevated lipid digestion of sour cream but not of sour cream analogue. Based on fatty acid-specific results and structural analysis it was showed that ameliorating effect during co-digestion of sour cream and cooked pasta is caused by the presence of pasta and specifically it is speculated that association of milk fat globules on the surface of pasta protein structures prevents MFG droplet aggregation and thus droplet size increase which caused the initial difference in lipid digestibility between the two products. It was also shown and confirmed with fatty acid-specific data that the structural effect is manifested in the gastric phase as a result of gastric chemical conditions.

These results all emphasise the relevance of obtaining information on the lipid digestibility of foods at the compositional level and of using these results to reveal the effects of factors inducing changes during the lipolysis of foods, i.e., enzyme preference and activity, bioactive substances and the effect of other nutrients. The high variability of the tested foods, bioactive-containing foods and meals highlighted the importance of fatty acid-specific assessment of lipid digestibility and could therefore serve as a basis for opening a way to collect systematic data on lipid digestibility of foods in different circumstances.

Next to development and application of a fatty acid-specific lipid digestibility method, part of my work was to design and validate the *in vitro* protein digestibility method proposed to quantify amino acid content of small intestinal digesta in order to make it appropriate for simultaneous determination of digestibility of multiple macronutrients. It was shown that by changing the sampling method from one-step precipitation to taking aliquots and isolating bioaccessible protein content, *in vitro* protein digestibility could be appropriately determined without bias, and remaining digesta could be used for further analysis of other nutrients. The modified method was used to assess protein digestibility of baked carp, baked beef and cooked pasta as well as to reveal the effect of edible oils on protein digestibility of cooked pasta. These results showed that acidic

hydrolysis of the bioaccessible protein content of foods with higher fat content might not be applicable or shows bias due to the interlinkages during heat treatment between remaining peptides and oleic acid. Therefore, there is still room for improvement to adequately characterise the digestibility of multiple nutrients from the same digesta, e.g., amino acid-based determination or de-fatting is recommended.

7. New scientific results

1) **I established and validated a new harmonised sampling and analytical protocol suitable for simultaneous determination of lipid and protein digestibility from the same Infogest *in vitro,* static digestion simulation.**
   - It was proven that total lipid content of a digesta, containing a mixture of hydrolysed and intact lipid species (TAG, DAG, MAG, FFA) cannot be determined after solvent evaporation and weight determination, since some of the lipid species formed during digestion are lost during evaporation.
   - It was concluded that a sample size containing not more than 150 mg lipids is to be used in the developed protocol.
   - I proved that the developed sampling method is a more effective substitute of the currently accepted standardized method, which sacrifices the entire sample for studying only one nutrient.

2) **Fatty acid-specific lipid digestibility results of cream, sour cream, and sour cream analogue as well as the prepared, ready-to-eat meal forms of baked carp (PDO from Akasztó) and baked beef were presented for the first time using the Infogest digestion simulation method and it was shown that TAGs containing short and medium chain fatty acids are non-preferred substrates for pancreatic lipase (PL) however this specificity is not characteristic for gastric lipase (GL).**
   - It was shown for the first time on the example of cream and sour cream that GL plays a key role in ameliorating the digestibility of short and medium chain fatty acids from milk fat.
   - It has been shown, on baked beef as an example, that for lipid sources without significant amounts of short- and medium chain fatty acids, the inclusion of GL does not result in an additional increase in lipid digestibility compared to PL-only digestions.

3) **Using fatty acid-specific lipid digestibility assessment with the Infogest *in vitro* digestion simulation and microscopic structural analysis, I proved that divergent droplet size formed during gastric digestion is a key determinant of the difference in the extent of lipolysis of sour cream and sour cream analogue (containing palm oil).**
   - It was shown that on the contrary to sour cream, where milk fat globules are naturally covered by milk fat globule membrane, palm oil droplets of sour cream analogue (without membrane) are not prone to flocculation and aggregation under the conditions

typical during gastric digestion, thus the original lipid droplet size is not increasing during gastric digestion.

4) ***In vitro*** **digestion simulation of baked carp meal with rosemary spice (5 g dried commercially available rosemary/100 g baked carp) showed no effect on the digestibility of baked carp lipids.**

5) **Using fatty acid-specific lipid digestibility assessment with the Infogest *in vitro* digestion simulation, I proved that co-consumption of grape seed powder and black tea brew can inhibit lipid digestibility in some, but not any types of food.**
   - Direct addition of GSP (Bock Hungary) in 5 w/w% concentration and black tea brew (Himalayan Spring FF 2022 No.601) to cream in 1 (cream):2 (tea) ratio is sufficient to significantly decrease extent of lipid digestion of cream, whereas it did not affected digestibility of baked beef lipids.
   - I have shown that GSP and BTB primarily affect the lipolysis of short- and medium-chain fatty acids, and thus may reduce the lipid digestibility of foods with triacylglycerols containing significant amounts of these fatty acids.

6) **I proved that both GSP and BTB have similar *in vitro* lipase inhibitory effects when consumed together with cream. Namely, GSP and BTB selectively decrease the release of short- and medium chain fatty acids, which indicates an inefficient pancreatic lipase function.**

7) **Using fatty acid-specific lipid digestibility assessment with the Infogest *in vitro* digestion simulation, I proved that co-consumption sour cream and sour cream analogue with cooked pasta, increased the extent of lipid digestion of sour cream but not sour cream analogue.**
   - It was shown that presence of pasta protein inhibits structural disintegration of milk fat globules during gastric conditions that would result in coalescence and increased lipid droplet size. Thus, the reserved smaller globule size provides more efficient lipolysis for gastric lipase, which manifested in the increased release of gastric lipase-preferred short- and medium chain fatty acids.

8) **Using amino acid-specific assessment of protein digestibility with the Infogest *in vitro* digestion simulation, I proved on the example of eleven different edible oil types that co-consumption of edible oils with cooked pasta generally reduces protein digestibility of pasta proteins.**
    - Correlation of fatty acid composition with the *in vitro* protein digestibility results proved that observed proteolysis-reducing effect of edible oils could not be directly stemmed back to their fatty acid composition.

In the above text, "Infogest in vitro digestion simulation method" means the currently accepted standardized method published in Brodkorb, A., Egger, L., Alminger, M. et al. INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat Protoc 14, 991–1014 (2019). https://doi.org/10.1038/s41596-018-0119-1 (2023.03.16)

8. Summary

The growing international consensus on the importance of considering bioavailability in nutritional assessment, and in the future, in food labelling, has led to an increase in studies focusing on the digestibility of foods. Although *in vivo* methods are still the primary means of obtaining data on the bioavailability of food constituents, it is generally accepted that there is a need for more ethically appropriate and methodologically sustainable ways of providing these data. One result of this trend is the Infogest consensus method, which can be used to simulate upper intestinal digestion of various food components, such as macro- and micronutrients. Although there is a standardised agreement on the parameters of the digestion simulation, there is still a high variability in the analytical methods and analytes studied to determine the bioaccessibility of food components. Furthermore, analytical methods to quantify digestible forms of nutrients are still lacking.

The digestibility and nutrient accessibility of foods depend on several factors, i.e., the food matrix, the effect of structural changes before and after consumption, the presence of substances capable of causing changes in enzyme activity, such as antinutritive and bioactive molecules. Consumption behaviour of foods that are not normally eaten as single foods are also a relevant factor. In addition to the need for methods capable of properly and quantitatively characterising the digestibility of nutrients, these methods should be capable of revealing the effects of the listed factors on the bioaccessibility of nutrients.

The main objectives on my work were focused on the development of methods for the fatty acid-specific determination of lipid digestibility and for the amino acid-based determination of protein digestibility and protein quality. In addition, there was an emphasis on the applicability of the proposed methods, on determination: i) of lipid digestibility of chosen test foods; ii) of the effects of bioactive substances on lipid digestibility; iii) of the effects of macronutrient interactions on lipid digestibility; iv) of protein digestibility of chosen test foods; v) of effects of lipids on protein digestibility.

As a result of my work, I was able to develop a method suitable for the fatty acid-specific assessment of lipid digestibility, which has been harmonised with the Infogest digestion simulation method. The method was tested on baked carp meal, baked beef meal, cream, sour cream and sour cream test foods in mechanistic experiments, using only selected lipolytic enzymes. These results showed that gastric lipase has a preference for triacylglycerols consisting of short and medium chain fatty acids which is not characteristic of pancreatic lipase. In addition, comparison of FA-specific digestibility and structural analysis of sour cream and sour cream analogue showed that foods with similar food matrix but rather different lipid source behave differently during

digestion and that in such cases the main determinant of the extent of lipid digestibility is the lipid droplet behaviour.

The analytical method was used to reveal the effects caused by foods containing bioactive substances and by the presence of other foods and food macronutrients. Co-consumption of rosemary and baked carp meal showed that the constituents in the aqueous extract of rosemary spice released during *in vitro* digestion were not sufficient to cause a significant change in the lipid digestibility of baked carp. It was also shown that grape seed powder and black tea brew could both reduce the extent of lipid digestion of cream, but not of baked beef. Furthermore, the results suggest that the lipase inhibitory effect of both grape seed powder and black tea brew primarily affects the activity of pancreatic lipase and therefore the reduction of the release of short and medium chain FAs. The results of this experiment also showed that the lipid characteristics of the consumed food not only influence the lipid digestibility, but also influence the lipase inhibitory effects of the bioactive containing foods.

Co-consumption of foods such as sour cream and sour cream analogue co-digested with cooked pasta showed that realistic consumption behaviour could modify lipid digestibility of foods as the addition of cooked pasta resulted in elevated lipid digestion of sour cream but not of sour cream analogue. Based on fatty acid-specific results and structural analysis it was showed that ameliorating effect during co-digestion of sour cream and cooked pasta is caused by the presence of pasta and the effect is manifested in the gastric phase by the aggregation protecting effect of pasta proteins.

The *in vitro* protein digestibility method was proposed to quantify bioaccessible amino acid content of small intestinal digesta meanwhile appropriate for simultaneous determination of digestibility of multiple macronutrients. It was shown that with the sampling method, precipitation of the aliquots to isolate bioaccessible protein content, could be used to appropriately determine *in vitro* protein digestibility, and the remaining digesta could be used for further analysis of other nutrients. The method was used to assess protein digestibility of baked carp, baked beef and cooked pasta as well as to reveal the effect of edible oils on protein digestibility of cooked pasta. These results showed addition of edible oils have reductive effect on protein digestibility of cooked pasta, however the effect is not systematic to the specific oils. In addition, it was shown that acidic hydrolysis might not be applicable to determine bioaccessible protein content of foods with higher fat content due to the interlinkages during heat treatment between remaining peptides and oleic acid.

The developed methods proved to be viable means of obtaining information on the lipid digestibility and protein digestibility of foods at the compositional level and of using these results

to reveal the effects of factors inducing changes during the lipolysis of foods, i.e., enzyme preference and activity, bioactive substances, and the effect of other nutrients. The high variability of the tested foods, bioactive-containing foods and meals highlighted the importance of fatty acid-specific assessment of lipid digestibility and could therefore serve as a basis for opening a way to collect systematic data on lipid digestibility of foods in different circumstances. In addition, the results of the protein digestibility study with cooked pasta and edible oils show that there is still room for improvement to adequately characterise the digestibility of multiple nutrients from the same digesta.

Appendices

A1: Bibliography

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A2: Additional information

Figure A1: Chromatograms of A) FAME mixture (calibration level 4, spiked with 100µg/mL C19ME), B) small intestinal digesta of baked carp meal: 1) *TFA method*, 2) *EFA method* (see text for details). Numbers on Figure correspond to peak numbers shown in *Table A2*.

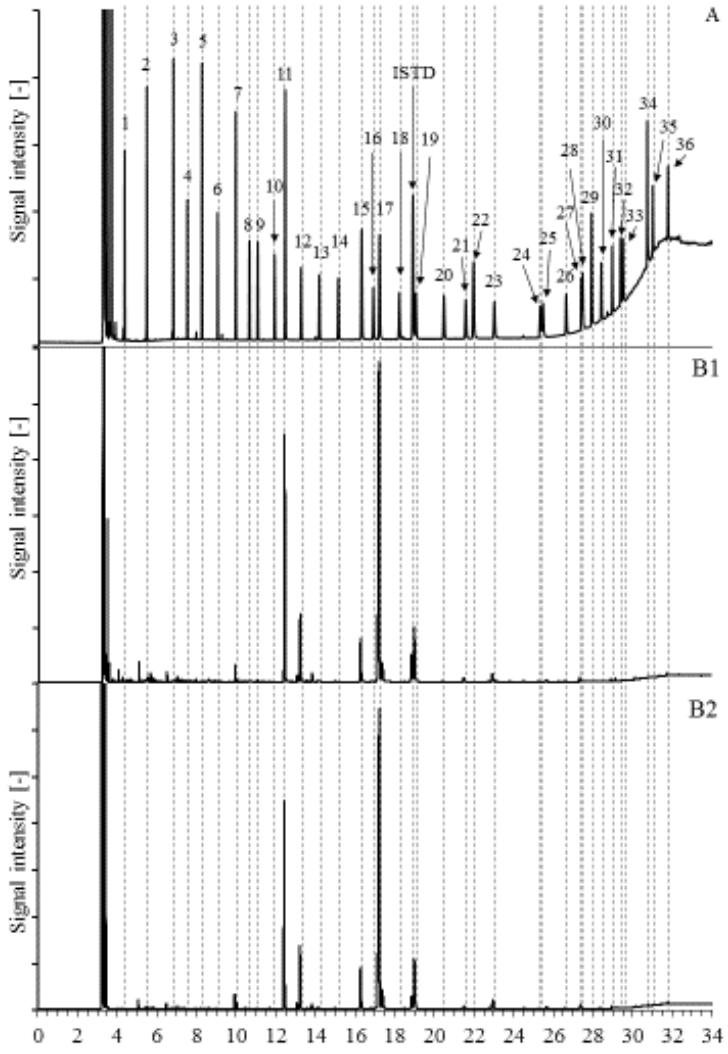

Table A1: Detailed list of analytes, including abbreviations, compound names, trivial names, retention times (R_t) [min] and resolution (R) [-].

Abbr.	Compound	Trivial name	R_t [min]	R [-]
C4:0	Methyl butanoate	Butyric acid	-	-
C6:0	Methyl hexanoate	Caproic acid	4.32	-
C8:0	Methyl octanoate	Caprylic acid	5.44	58.9
C10:0	Methyl decanoate	Capric acid	6.78	72.4
C11:0	Methyl undecanoate	Undecylic acid	7.49	38.4
C12:0	Methyl laurate	Lauric acid	8.22	37.7
C13:0	Methyl tridecanoate	Tridecylic acid	9.02	37.9
C14:0	Methyl myristate	Myristic acid	9.94	39.0
C14:1n-5c	(cis-9) Methyl myristoleate	Myristoleic acid	10.63	26.0
C15:0	Methyl pentadecanoate	Pentadecylic acid	11.05	14.4
C15:1n-5c	(Cis-10) Methyl pentadecenoate	Pentadecenoic Acid	11.89	27.0
C16:0	Methyl palmitate	Palmitic acid	12.43	15.8
C16:1n-7c	(Cis-9) Methyl palmitoleate	Palmitoleic acid	13.24	21.8
C17:0	Methyl heptadecanoate	Margaric acid	14.16	22.6
C17:1n-7c	(Cis-10) Methyl heptadecenoate	Heptadecenoic acid	15.12	22.1
C18:0	Methyl stearate	Strearic acid	16.30	24.8
C18:1n-9t	(Trans-9) Methyl octadecenoate	Elaidic acid	16.89	11.7
C18:1n-9c	(Cis-9) Methyl oleate	Oleic acid	17.20	6.0
C18:2n-6t	(all Trans-9,12) Methyl linoleaidate	Linolelaidic acid	18.20	18.4
C19:0 (ISTD)	Methyl nonadecanoate	Nonadecylic acid	18.89	12.1
C18:2n-6c	(all-Cis-9,12) Methyl linoleate	Linoleic acid	19.04	2.6
C18:3n-6c	(all-Cis-6,9,12) Methyl linolenate	γ-linolenic acid	20.45	23.8
C18:3n-3c	(all-Cis-9,12,15) Methyl linoleate	α-linolenic acid	21.56	17.6
C20:0	Methyl arachidate	Arachidic acid	21.95	5.9
C20:1n-9c	(Cis-11) Methyl eicosanoate	Gondoic acid	23.00	15.0
C20:2n-6c	(all-Cis-11,14) Methyl eicosadienoate	Eicosadienoic acid	25.33	31.8
C21:0	Methyl heneicosanoate	Heneicosylic acid	25.46	1.8
C20:3n-6c	(all-Cis-8,11,14) Methyl eicosatrienoate	dihomo-γ-linolenic acid	26.63	18.6
C20:4n-6c	(all-Cis-5,8,11,14) Methyl arachidonate	Arachidonic acid	27.40	15.8
C20:3n-3c	(all-Cis-11,14,17) Methyl eicosatrienoate	Dihomolinolenic	27.47	1.5
C22:0	Methyl behenate	Behenic acid	27.91	10.2
C22:1n-9c	(Cis-13) Methyl erucate	Erucic acid	28.41	11.6
C20:5n-3c	(all-Cis-5,8,11,14,17) Methyl eicosapentaenoate	Timnodonic acid	28.97	14.6
C22:2n-6c	(all-Cis-13,16) Methyl docosadienoate	Docosadienoic acid	29.38	11.7
C23:0	Methyl tricosanoate	Tricosylic acid	29.50	3.5
C24:0	Methyl lignocerate	Tricosylic acid	30.73	34.6
C24:1n-9c	(Cis-15) Methyl nervonate	Nervonic acid	31.02	8.2
C22:6n-3c	(all Cis-4,7,10,13,16,19) Methyl docosahexanoate	Cervonic acid	31.78	23.8

Table A2A: Results of fatty acid profiling I. ISO method extracted fat of baked carp, baked beef, cream, sour cream, and sour cream analogue. Total fat content of baked carp and baked beef was extracted with ISO 1444:2000 method, of cream, sour cream, and sour cream analogue with ISO 2450:200 method. Fatty acid profiling was carried out after trans methylation with "Rapid method" according to ISO 12966-2:2017. Amounts of fatty acids are given in g fat/ 100 g extracted fat (n=3).

Fatty acid composition [g/100 g fat] – Extraction with ISO method					
FAME	**Baked carp**	**Baked beef**	**Cream**	**Sour cream**	**Sour cream analogue**
C4:0	0.0	0.0	0.0	0.0	0.0
C6:0	0.0	0.0	2.5	2.6	0.0
C8:0	0.0	0.0	1.3	1.5	0.0
C10:0	0.0	0.0	3.4	3.4	0.0
C11:0	0.0	0.0	0.0	0.1	0.0
C12:0	0.0	0.0	3.9	3.8	0.2
C13:0	0.0	0.0	0.0	0.1	0.0
C14:0	0.7	2.6	12.1	11.7	0.9
C14:1n-5c	0.0	1.0	0.9	0.4	0.0
C15:0	0.0	0.4	1.2	1.2	0.0
C15:1n-5c	0.0	0.0	0.0	0.0	0.0
C16:0	18.4	26.8	33.5	32.4	43.8
C16:1n-7c	7.6	5.0	2.1	2.0	0.1
C17:0	0.0	0.9	0.5	0.6	0.1
C17:1n-7c	0.1	0.8	0.2	0.0	0.0
C18:0	5.6	14.9	10.8	10.8	4.6
C18:1n-9t	0.3	0.1	1.0	1.8	0.0
C18:1n-9c	53.8	44.9	23.5	23.3	39.7
C18:2n-6t	0.0	0.1	0.1	0.4	0.0
C18:2n-6c	8.8	2.0	2.4	3.5	10.4
C18:3n-6c	0.0	0.0	0.0	0.0	0.0
C18:3n-3c	0.9	0.0	0.4	0.0	0.0
C20:0	0.0	0.0	0.0	0.1	0.2
C20:1n-9c	2.3	0.1	0.0	0.0	0.0
C20:2n-6c	0.3	0.0	0.0	0.0	0.0
C21:0	0.0	0.0	0.0	0.0	0.0
C20:3n-6c	0.3	0.1	0.1	0.0	0.0
C20:4n-6c	0.0	0.0	0.1	0.0	0.0
C20:3n-3c	0.0	0.1	0.0	0.0	0.0
C22:0	0.0	0.0	0.0	0.0	0.0
C22:1n-9c	0.0	0.0	0.0	0.0	0.0
C20:5n-3c	0.3	0.0	0.0	0.1	0.0
C22:2n-6c	0.0	0.0	0.0	0.0	0.0
C23:0	0.2	0.0	0.0	0.0	0.0
C24:0	0.0	0.0	0.0	0.0	0.0
C24:1n-9c	0.0	0.1	0.0	0.1	0.0
C22:6n-3c	0.3	0.0	0.0	0.0	0.0

Table A2B: Results of fatty acid profiling II. Bligh and Dyer method extracted fat of baked carp, baked beef, cream, sour cream, and sour cream analogue. Total fat content of baked carp and baked beef was extracted with ISO 1444:2000 method, of cream, sour cream, and sour cream analogue with ISO 2450:2000 method. Fatty acid profiling was carried out after transmethylation with "Rapid method" according to ISO 12966-2:2017. Amounts of fatty acids are given in g fat/ 100 g extracted fat (n=3).

Fatty acid composition [g/100 g fat] – Extraction with Bligh & Dyer method					
FAME	**Baked carp**	**Baked beef**	**Cream**	**Sour cream**	**Sour cream analogue**
C4:0	0.0	0.0	0.0	0.0	0.0
C6:0	0.0	0.0	3.0	2.4	0.0
C8:0	0.0	0.0	1.3	1.4	0.0
C10:0	0.0	0.0	3.5	3.2	0.1
C11:0	0.0	0.0	0.0	0.0	0.0
C12:0	0.0	0.0	4.0	3.8	0.3
C13:0	0.0	0.0	0.0	0.1	0.0
C14:0	0.9	2.6	12.6	12.3	1.2
C14:1n-5c	0.0	0.8	0.8	1.2	0.0
C15:0	0.1	0.2	1.3	1.2	0.1
C15:1n-5c	0.0	0.0	0.0	0.0	0.0
C16:0	19.0	29.6	35.0	35.7	42.0
C16:1n-7c	7.4	4.7	1.8	2.3	0.2
C17:0	0.2	0.9	0.5	0.6	0.1
C17:1n-7c	0.0	0.6	0.1	0.0	0.0
C18:0	5.6	16.3	11.2	10.4	4.9
C18:1n-9t	0.1	0.9	2.0	0.7	0.0
C18:1n-9c	52.3	41.7	20.2	22.4	41.3
C18:2n-6t	0.0	0.0	0.0	0.2	0.0
C18:2n-6c	8.8	1.3	1.5	1.7	9.2
C18:3n-6c	0.1	0.0	0.0	0.0	0.0
C18:3n-3c	0.9	0.1	0.1	0.0	0.0
C20:0	0.0	0.0	0.0	0.1	0.3
C20:1n-9c	2.4	0.1	0.0	0.0	0.1
C20:2n-6c	0.3	0.0	0.0	0.0	0.0
C21:0	0.0	0.0	0.0	0.0	0.0
C20:3n-6c	0.3	0.0	0.0	0.1	0.0
C20:4n-6c	1.0	0.0	0.0	0.1	0.0
C20:3n-3c	0.0	0.0	0.0	0.0	0.0
C22:0	0.0	0.0	0.1	0.0	0.0
C22:1n-9c	0.0	0.0	0.0	0.0	0.0
C20:5n-3c	0.3	0.0	0.0	0.0	0.0
C22:2n-6c	0.0	0.0	0.0	0.0	0.0
C23:0	0.0	0.1	0.0	0.0	0.0
C24:0	0.0	0.2	0.1	0.0	0.0
C24:1n-9c	0.0	0.0	0.2	0.0	0.0
C22:6n-3c	0.3	0.0	0.5	0.0	0.0

Table A3: Total fatty acid content and free fatty acid content in mg/100 g cream (calculated according to Eq. 1) via Infogest v1.0 and v2.0 of the test matrices determined from small intestinal digesta. A: baked carp, B: baked beef, C: cream, D: sour cream, E: sour cream analogue. Contribution of the given FA in % is shown in parentheses. Release ratio (RR) of individual fatty acids was calculated according to Eq. 2 after Infogest v1.0 and v2.0.

FAs		Total Fatty Acid Content *		Free Fatty Acid Content (Infogest v1.0)		RR (Infogest v1.0)	Free Fatty Acid Content (Infogest v2.0)		RR (Infogest v2.0)
#	Abbr.	mg/100 g (%)	RSD	mg/100 g (%)	RSD		mg/100 g (%)	RSD	
A: Baked carp meal									
1	C14:0	77.8 ± 3.7 (0.8)	0.05	40.4 ± 1.1 (0.7)	0.03	**0.48 ± 0.06**	58.5 ± 3.5 (0.8)	0.06	**0.75 ± 0.03**
2	C15:0	10.2 ± 0.5 (0.1)	0.05	7 ± 0.4 (0.1)	0.05	**0.63 ± 0.07**	8.7 ± 1.1 (0.1)	0.13	**0.85 ± 0.08**
3	C16:0	1768.8 ± 86.4 (18.4)	0.05	867.7 ± 36.8 (14.5)	0.04	**0.48 ± 0.02**	1145.8 ± 55.1 (16.5)	0.05	**0.65 ± 0.01**
4	C16:1n-7c	685.8 ± 34.4 (7.1)	0.05	412.3 ± 15 (6.9)	0.04	**0.61 ± 0.02**	491.6 ± 24.3 (7.1)	0.05	**0.72 ± 0.01**
5	C17:0	11 ± 0.8 (0.1)	0.07	7.5 ± 0.6 (0.1)	0.07	**0.63 ± 0.09**	8.2 ± 1.1 (0.1)	0.13	**0.75 ± 0.07**
6	C18:0	566.3 ± 30.4 (5.9)	0.05	362.8 ± 25.5 (6)	0.07	**0.63 ± 0.01**	417.4 ± 44.9 (6)	0.11	**0.74 ± 0.06**
7	C18:1n-9c	5079 ± 266.6 (52.8)	0.05	3415.2 ± 160 (56.9)	0.05	**0.68 ± 0.01**	3799.9 ± 194.1 (54.6)	0.05	**0.75 ± 0.01**
8	C18:2n-6c	866.5 ± 45 (9)	0.05	525 ± 23.2 (8.7)	0.04	**0.61 ± 0.01**	600.7 ± 28.3 (8.6)	0.05	**0.69 ± 0.01**
9	C18:3n-6c	11.4 ± 0.6 (0.1)	0.05	7.3 ± 0.9 (0.1)	0.13	**0.64 ± 0.04**	10.2 ± 1.1 (0.1)	0.11	**0.89 ± 0.06**
10	C18:3n-3c	102.2 ± 5.9 (1.1)	0.06	65.2 ± 4.6 (1.1)	0.07	**0.64 ± 0.02**	75.1 ± 5 (1.1)	0.07	**0.73 ± 0.02**
11	C20:1n-9c	262.4 ± 16.5 (2.7)	0.06	206.1 ± 15 (3.4)	0.07	**0.77 ± 0.01**	212.5 ± 17 (3.1)	0.08	**0.81 ± 0.02**
12	C20:3n-6c	36.2 ± 1.9 (0.4)	0.05	25.3 ± 2.2 (0.4)	0.09	**0.67 ± 0.02**	26.6 ± 2.3 (0.4)	0.09	**0.73 ± 0.04**
13	C22:1n-9c	7.4 ± 0.6 (0.1)	0.08	6.9 ± 0.7 (0.1)	0.10	**0.91 ± 0.08**	5.7 ± 2.5 (0.1)	0.44	**0.76 ± 0.33**
14	C20:5n-3c	42.8 ± 2.2 (0.4)	0.05	25.7 ± 1.7 (0.3)	0.11	**0.58 ± 0.06**	31.7 ± 6.5 (0.5)	0.20	**0.74 ± 0.13**
15	C23:0	7.9 ± 1.5 (0.1)	0.19	6.4 ± 1.6 (0.1)	0.25	**0.74 ± 0.19**	6.8 ± 1 (0.1)	0.15	**0.87 ± 0.1**
16	C22:6n-3c	47.5 ± 2.8 (0.5)	0.06	24.1 ± 3.7 (0.4)	0.15	**0.45 ± 0.04**	19.2 ± 4.5 (0.3)	0.23	**0.40 ± 0.08**
B: Baked beef meal									
1	C14:0	361.7 ± 51.7 (2.6)	0.14	185.3 ± 22.2 (1.9)	0.12	**0.51 ± 0.06**	216.1 ± 10 (2.3)	0.05	**0.6 ± 0.03**
2	C14:1n-5c	132 ± 19.5 (1)	0.15	54.2 ± 10.4 (0.6)	0.19	**0.41 ± 0.08**	71.8 ± 7.2 (0.8)	0.10	**0.54 ± 0.05**
3	C15:0	53.5 ± 8 (0.4)	0.15	37.7 ± 6.5 (0.4)	0.17	**0.71 ± 0.12**	40.5 ± 4.3 (0.4)	0.11	**0.76 ± 0.08**
4	C16:0	3722 ± 530.7 (26.8)	0.14	2976 ± 96.6 (30.8)	0.03	**0.80 ± 0.03**	2970.6 ± 49.1 (31.7)	0.02	**0.80 ± 0.01**
5	C16:1n-7c	693 ± 99.7 (5)	0.14	420.3 ± 29.9 (4.3)	0.07	**0.61 ± 0.04**	427.1 ± 11.6 (4.6)	0.03	**0.62 ± 0.02**
6	C17:0	131.1 ± 20 (0.9)	0.15	107.3 ± 3.1 (1.1)	0.03	**0.82 ± 0.02**	104.3 ± 2.8 (1.1)	0.03	**0.80 ± 0.02**
7	C17:1n-7c	107.9 ± 15.5 (0.8)	0.14	67.9 ± 4 (0.7)	0.06	**0.63 ± 0.04**	67.6 ± 1.8 (0.7)	0.03	**0.63 ± 0.02**
8	C18:0	2073.9 ± 304.7 (14.9)	0.15	1738.7 ± 36.9 (18)	0.02	**0.84 ± 0.02**	1651.7 ± 24 (17.6)	0.01	**0.80 ± 0.01**
9	C18:1n-9c	6237.1 ± 902.3 (45)	0.14	3893.7 ± 237.8 (40.2)	0.06	**0.62 ± 0.04**	3638.8 ± 109 (38.8)	0.03	**0.58 ± 0.02**
10	C18:2n-6c	284.1 ± 43.9 (2)	0.15	174.8 ± 12 (1.8)	0.07	**0.62 ± 0.04**	160.4 ± 5.4 (1.7)	0.03	**0.56 ± 0.02**
C: Cream									
1	C10:0	966 ± 50 (3.4)	0.05	533.6 ± 85.7 (2.9)	0.16	**0.55 ± 0.09**	776.9 ± 33.7 (4.2)	0.04	**0.80 ± 0.03**
2	C12:0	1123 ± 55.9 (3.9)	0.05	615.1 ± 94.3 (3.3)	0.15	**0.55 ± 0.08**	846.8 ± 58 (4.6)	0.07	**0.75 ± 0.05**
3	C14:0	3481.2 ± 139.5 (12.1)	0.04	2024.6 ± 270.3 (11)	0.13	**0.58 ± 0.08**	2452.6 ± 229 (13.3)	0.09	**0.70 ± 0.07**
4	C15:0	345.8 ± 21.4 (1.2)	0.06	226.4 ± 23.8 (1.2)	0.11	**0.65 ± 0.07**	257.7 ± 19.7 (1.4)	0.08	**0.75 ± 0.06**
5	C16:0	9651.6 ± 366.7 (33.5)	0.04	6660 ± 592.3 (36.2)	0.09	**0.69 ± 0.06**	7355.5 ± 543.4 (39.9)	0.07	**0.76 ± 0.06**
6	C16:1n-7c	597.8 ± 31 (2.1)	0.05	382.5 ± 41.6 (2.1)	0.11	**0.64 ± 0.07**	434.4 ± 34.1 (2.4)	0.08	**0.73 ± 0.06**
7	C18:0	3107.4 ± 130.2 (10.8)	0.04	2443.3 ± 152.8 (13.3)	0.06	**0.79 ± 0.05**	2536.2 ± 183.4 (13.8)	0.07	**0.82 ± 0.06**
8	C18:1n-9c	6751 ± 260.9 (23.5)	0.04	5021.7 ± 362.9 (27.3)	0.07	**0.74 ± 0.05**	5399 ± 300.2 (29.3)	0.06	**0.80 ± 0.04**
9	C18:2n-6c	689.7 ± 11.4 (2.4)	0.02	506.5 ± 39.7 (2.8)	0.08	**0.73 ± 0.06**	554.4 ± 33.1 (3)	0.06	**0.80 ± 0.05**
D: Sour cream									
1	C10:0	416.8 ± 21.8 (3.4)	0.03	120.9 ± 31.3 (2)	0.26	**0.29 ± 0.08**	287.3 ± 13.4 (3.8)	0.05	**0.69 ± 0.04**
2	C12:0	493.4 ± 27.6 (4)	0.03	143.8 ± 32.1 (2.3)	0.22	**0.29 ± 0.06**	285.2 ± 20.7 (3.8)	0.07	**0.58 ± 0.04**
3	C14:0	1602.8 ± 94.8 (13)	0.03	519 ± 79 (8.4)	0.15	**0.32 ± 0.04**	746.2 ± 96 (9.9)	0.13	**0.47 ± 0.06**
4	C14:1n-5c	152.2 ± 8.4 (1.2)	0.03	66.8 ± 9.7 (1.1)	0.15	**0.44 ± 0.06**	88.7 ± 7.8 (1.2)	0.09	**0.58 ± 0.06**
5	C15:0	164.5 ± 9.6 (1.3)	0.03	75.9 ± 6.6 (1.2)	0.09	**0.46 ± 0.04**	91.6 ± 8.2 (1.2)	0.09	**0.56 ± 0.06**
6	C16:0	4688.1 ± 285 (37.9)	0.03	2327 ± 131.4 (37.8)	0.06	**0.50 ± 0.02**	2697.2 ± 196.2 (35.8)	0.07	**0.58 ± 0.04**
7	C16:1n-7c	300.1 ± 18.2 (2.4)	0.03	126.9 ± 16 (2.1)	0.13	**0.42 ± 0.06**	270.3 ± 43.8 (3.6)	0.16	**0.90 ± 0.14**
8	C18:0	1386.9 ± 84.2 (11.2)	0.03	997.6 ± 21.3 (16.2)	0.02	**0.72 ± 0.02**	1024.7 ± 32 (13.6)	0.03	**0.74 ± 0.02**
9	C18:1n-9c	2923 ± 176.4 (23.6)	0.03	1658.1 ± 133.2 (27)	0.08	**0.57 ± 0.04**	1908.9 ± 93.7 (25.3)	0.05	**0.65 ± 0.04**
10	C18:2n-6c	236 ± 11.6 (1.9)	0.02	112.5 ± 24.1 (1.8)	0.21	**0.48 ± 0.1**	143.9 ± 8.9 (1.9)	0.06	**0.61 ± 0.04**

FAs		Total Fatty Acid Content *		Free Fatty Acid Content (Infogest v1.0)		RR (Infogest v1.0)	Free Fatty Acid Content (Infogest v2.0)		RR (Infogest v2.0)
#	Abbr.	mg/100 g (%)	RSD	mg/100 g (%)	RSD		mg/100 g (%)	RSD	
E: Sour cream analogue									
1	C14:0	216.7 ± 11.2 (1.3)	0.01	127.5 ± 9.4 (1.2)	0.07	**0.59 ± 0.04**	169.3 ± 4.9 (1.5)	0.03	**0.78 ± 0.02**
2	C16:0	7068.2 ± 436.6 (42.5)	0.02	5584.9 ± 56.3 (53.2)	0.01	**0.79 ± 0.0**	5487.5 ± 472 (49.3)	0.09	**0.80 ± 0.02**
3	C18:0	878.3 ± 93.6 (5.3)	0.03	700.1 ± 18.9 (6.7)	0.03	**0.80 ± 0.02**	690.8 ± 92.5 (6.2)	0.13	**0.83 ± 0.02**
4	C18:1n-9c	6902.1 ± 419 (41.6)	0.02	3380.3 ± 89.8 (32.2)	0.03	**0.49 ± 0.02**	3945.3 ± 321.1 (35.5)	0.08	**0.56 ± 0.02**
5	C18:2n-6c	1547 ± 94 (9.3)	0.02	710.6 ± 18.6 (6.8)	0.03	**0.46 ± 0.02**	833.7 ± 91.3 (7.5)	0.11	**0.52 ± 0.02**

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