Pre-duodenal lipid digestion of emulsions: Relevance, colloidal aspects and mechanistic insight

Marcos R. INFANTES-GARCIA[1], Sarah H.E. VERKEMPINCK[1], Fréderic CARRIÉRE[2], Marc E. HENDRICKX[1], Tara GRAUWET[1]

[1] Laboratory of Food Technology and Leuven Food Science and Nutrition Research Centre (LFoRCe), Department of Microbial and Molecular Systems (M2S), KU Leuven, Kasteelpark Arenberg 22, PB 2457, 3001 Leuven, Belgium

[2] CNRS, Aix-Marseille Université, Bioénergétique et Ingénierie des Protéines, UMR 7281, 31 Chemin Joseph Aiguier, 13402 Marseille cedex 9, France

Abstract

The digestion of lipids in the human body has several health and nutritional implications. Lipid digestion is an interfacial phenomenon meaning that water-soluble lipases need to first adsorb to the oil-water interface before enzymatic conversions can start. The digestion of lipids mainly occurs on colloidal structures dispersed in water, such as oil-in-water (o/w) emulsions, which can be designed during food processing or structured during digestion. From a food design perspective, different *in vitro* studies have demonstrated that the kinetics of lipid digestion can be influenced by emulsion properties. However, most of these studies have been performed with pancreatic enzymes to simulate lipolysis in the small intestine. Only few studies have dealt with lipid digestion in the gastric phase and its subsequent impact on intestinal lipolysis. In this aspect, this review compiles information on the physiological aspects of gastric lipid digestion. In addition, it deals with colloidal and interfacial aspects starting from emulsion design factors and how they evolve during *in vitro* digestion. Finally, molecular mechanisms describing gastric lipolysis are discussed.

Keywords

Lipid digestion, gastric lipase; gastric digestion, interfacial characterization, molecular mechanism

1. Introduction

Lipids in foods consist of a diverse group of hydrophobic compounds such as fatty acids (FAs), triglycerides (TAGs), sterols, phospholipids and other non-polar and polar molecules (Akoh & Min, 2002). Among these, TAGs constitute the major group, representing more than 95% in refined oils and 99% in animal fats (Singh et al., 2009). Regarding their chemical structure, TAGs are organized as a glycerol backbone esterified to three FAs. The positions to which the FAs are attached to glycerol are usually identified by their stereospecific number *(sn)*. According to the Fischer projection of the glycerol backbone, *sn*-1, *sn*-2 and *sn*-3 refer to the carbon atoms at the top, middle and bottom positions of glycerol, the FAs at positions *sn*-1 and *sn*-3 being bound to primary alcohols and the FA at position *sn*-2 to a secondary alcohol (Figure 1) . The most abundant FAs in foods have a chain length ranging between 14 and 20 carbon units, nevertheless some short and medium chain length FA (4 to 12 carbons) are found in specific foods like milk fat, palm kernel or coconut oil. A particularity of milk TAGs is that short and medium chain FAs are specifically found at the *sn*-3 position of the glycerol backbone (Christie & Clapperton, 1982; Helander & Olivecrona, 1970; Otterby, Ramsey, & Wise, 1964). Palmitic (C16:0) and oleic acid (C18:1) are usually the main FAs found in TAGs whatever their origin. The essential polyunsaturated FAs (PUFAs) linoleic acid (C18:2 n-6) and linolenic acid (C18:3 n-3) are however majorly present in vegetable oil TAGs (Miao, S., Wang, P., Su, Z., & Zhang, 2014). These PUFAs are essential for a balanced nutritional status. The FA chain length, degree of unsaturation and the stereospecific position inside the glycerol molecule determine TAGs physicochemical properties, and consequently the digestibility of TAGs (Guo, Ye, Bellissimo, Singh, & Rousseau, 2017).

Lipids, naturally present in foods, added during food formulation and/or transformed during processing, have important roles in edible products. The first function associated with lipids is the sensory aspect, i.e. by solubilizing aroma compounds or creating textures in specific products (e.g. ice-cream). Another property of lipids is related to their capacity to form certain structures. For example, they can be dispersed

in an aqueous phase to form emulsions (Belitz, Grosch, & Schieberle, 2009). Apart from the sensory and structural roles, the nutritional functionality is very important since lipid consumption may have beneficial or detrimental implications. Some benefits of consuming lipids are the *(i)* provision of energy through non-essential FAs, such as palmitic or oleic acid; *(ii)* essential PUFAs like linoleic, linolenic (ALA) acids and long chain PUFAs like eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, which contribute to cellular organization, hormones synthesis, essential metabolic transport and are precursors of many anti-inflammatory or pro-inflammatory compounds (Meynier & Genot, 2017). In addition, lipids *(iii)* act as carriers for micronutrients and functional phytochemicals such as vitamin D and E, carotenoids and phytosterols. A detrimental aspect of lipids is related to their overconsumption. Lipid overconsumption may lead to a higher risk of developing cardiovascular diseases, diabetes and other metabolic-related disorders (Yaqoob, 2013). As explained above, the presence of lipids in foods have several nutritional implications. Consequently, the understanding of the physiological processes occurring during the digestion of lipids is of major importance (van Aken, 2010). Most studies have focused on understanding the lipolysis phenomena in the small intestine since lipid hydrolysis majorly takes place in that compartment (30-60%) (Armand, 2007). However, pre-duodenal lipolysis (10-25%) has also a key role in this matter Unfortunately, it has been sometimes overlooked or simply not considered, especially in *in vitro* studies dealing with food structure-function. In this aspect, the aim of this review is to highlight the importance of pre-duodenal lipolysis and revise the scientific studies dealing with the colloidal nature of gastric lipid digestion performed until today.

2. Why is lipolysis different from other nutrients digestion?

As opposed to other macronutrients, which digestion and transport takes place in the continuous aqueous phase of the digestive tract, lipids are not water soluble. Lipid digestion occurs at the lipid-water interface, and it is an interfacial phenomenon. The water-soluble lipases need to access their substrate by adsorbing

onto the surface of the lipid droplets to start the hydrolysis of TAGs (Wilde & Chu, 2011). O/w emulsions consist of two immiscible liquid phases with one referred to as oil droplets dispersed within the other referred to as the continuous phase (Guo et al., 2017).

The general process of lipid digestion is shown in Figure 2 and can be divided in: *(i)* (de)structuring phenomena, referring to lipid emulsification when they are ingested as bulk oils or aggregation/destabilization phenomena occurring during digestion; *(ii)* biochemical reactions, consisting of the enzymatic hydrolysis of TAGs by gastric and pancreatic lipases; and *(iii)* micellar solubilization of lipolysis products, free fatty acids (FFAs) and monoglycerides (MAGs), by bile salts to make them absorbable in the small intestine after transport to the surface of enterocytes (Singh, Ye, & Horne, 2009). The phenomena described can simultaneously occur during food digestion. For instance, (de)structuring of food emulsions, with the occurrence of some coalescence and changes in particle size distributions, and enzymatic lipid hydrolysis by gastric lipase can take place at the same time in the gastric compartment. This has been well shown in the course of milk fat digestion, both *in vitro* (De Oliveira et al., 2016) and *in vivo* in newborns (De Oliveira et al., 2017). This process can even lead to the disruption of emulsions with some fat creaming in the stomach as observed by MRI (Hussein et al., 2015; Steingoetter et al., 2015). In the following sections, a focus on the lipid digestion phenomena will be given before food reaches the small intestine.

3. Lipid digestion in the mouth: a truth or a myth?

Lipids can be ingested as a bulk (e.g. olive oil), dispersed in food like meat fat or as emulsions. The latter mainly includes oil-in-water (o/w) emulsions (e.g. mayonnaise, sauces, dressings or dairy products) and to a lesser extent water-in-oil (w/o) emulsions (e.g. margarine or butter). Next to this, food sources naturally rich in lipids, such as salmon, oilseeds, and some dairy products, contain TAGs embedded in specific colloidal structures, cells (i. e. adipocytes from adipose tissues) and organelles (lipid droplets or

96 oleosomes found in adipocytes and seeds). For instance, lipid droplets in animal tissues and oleosomes in
97 seeds are surrounded by a membrane made of a polar lipid monolayer in which proteins are embedded.
98 The native milk fat globule is surrounded by three-layer of phospholipids, other polar lipids and proteins
99 resulting from the secretion of lipid droplets by exocytosis (Mu & Høy, 2004).

100 During mastication and even in the gastric phase, bulk lipids and w/o emulsions are structured into coarse
101 o/w emulsions (McClements, Decker, & Park, 2008). Dilution of the latter ones with saliva and gastric
102 juices and the presence of surface-active compounds cause the structuring into o/w emulsions. Despite
103 the short residence time in the oral cavity (0.5-2 min), the dilution effect of saliva and the mechanical
104 forces initiate this structuring process (Singh et al., 2009).

105 Additionally, some authors reported that the perception of certain FAs in the oral cavity (CD36 receptors)
106 might trigger physiological responses such as satiety induction (Lai, Chua, Gill, & Brownlee, 2019). It has
107 been shown in mice that FA interaction with CD36 at the oral level triggers the secretion of CCK and thus
108 can promote digestive functions like pancreatic enzyme secretion, leading to the concept of “fat taste”
109 (Gaillard et al., 2008; Khan & Besnard, 2009). This oral sensitivity for FAs is relevant in the context of food
110 overconsumption, obesity, and obesity-related diseases (Kulkarni & Mattes, 2014; Pepino, Love-Gregory,
111 Klein, & Abumrad, 2012). This overconsumption is partially caused by a reduced FA taste sensitivity due
112 to habitual high-lipid diets. In other words, high-fat diets may increase the taste thresholds of certain FAs
113 related to satiety mechanisms (Chevrot et al., 2014). As a consequence, satiety regulation is attenuated,
114 resulting in higher amounts of ingested high-lipid food (D. Liu et al., 2016). More insights about the role
115 of fat detection in the oral cavity are still needed (Brignot & Feron, 2019).

116 In the recent years, the discovery of the CD36 receptor in the mouth (“fat taste”) has provoked a new
117 interest into the possibility that a human lingual lipase could exist. This was also motivated by the proven
118 detection of lipolytic activity in mice and rats lingual tissue (Hervé Moreau, Gargouri, Lecat, Junien, &

Verger, 1988) However, these results observed in small rodents have been extrapolated to humans on an extremely weak basis (Bakala N'Goma, Amara, Dridi, Jannin, & Carrière, 2011). To date, there is no solid proof of human lingual lipase activity. Only traces of lipase activity can be detected in the human tongue and saliva. This activity most likely has a microbial cell desquamation origin. Indeed, the levels of activity reported (µmoles FFA released/h/g of tissue (Hamosh, 2020b, 2020a)) could be obtained after homogenization of any tissue, while activity levels >1000 µmoles FFA released/min/g of tissue can be measured in biopsy specimens of human gastric mucosa. To date, preduodenal lipases have been identified in several species, mainly associated with a single tissue where both the mRNA, the protein and a significant lipase activity were unambiguously observed, e.g., lingual (mice and rats), pharyngeal (calf, lamb, sheep), or gastric (rabbit, dog, human) tissues (Bakala N'Goma et al., 2011).

The fact that the lipase inhibitor Orlistat could decreased the detection of TAGs versus FFA in the mouth of obese subjects has also contributed to the renewal of the human lingual lipase myth (Pepino et al., 2012). It is however impossible from the reported study to estimate the lipolysis level involved and compare it to gastric or intestinal levels. After the discovery of CD36 in the mouth, several research groups have tried to associate this FA receptor with some lipolysis occurring in the mouth and due to a lingual lipase. Nevertheless, a lipase might not be needed. Indeed, low levels of FFA can be already present in foods, especially after maturation and cooking, at a level that can be detected by CD36.

A different scenario appears for intragastric lipolysis. In humans, a gastric lipase has been identified and extracted from human gastric mucosa and juices, exhibiting very high activity levels as discussed in the following section (Moreau et al., 1988; Tiruppathi & Balasubramanian, 1982). Similar to pepsinogen, it is produced by chief cells of the fundic mucosa (Moreau et al., 1989) from which the mRNA was isolated for cloning (Bodmer et al., 1987). It is the product of the LIPF gene that codes for human gastric lipase (HGL; see UniProtKB/Swiss-Prot: P07098).

4. How important is lipid digestion in the stomach?

After mastication, the food bolus is swallowed into the esophagus to be transported towards the stomach. In this compartment, physicochemical conditions change compared to the oral cavity. The food bolus is mixed with gastric juices containing hydrochloric acid, mucins, electrolytes and enzymes forming the chyme (Ramsay & Carr, 2011). The transit time of food in the gastric compartment can vary from 15 min (e.g. for water) up to 4 h (e.g. for a highly structured solid meal) (Sensoy, 2021). In case of the intragastric pH, values for a healthy human are reported to range between 1 and 3 under fasted conditions due to basal hydrochloric acid secretion. After food is administered, the pH value increases to 5-7 within the first 5 minutes depending on the food ingested, and then progressively decreases to values below 3 over a period of 2-4 hours (Sams et al., 2016). It is important to note that at half gastric emptying time, the intragastric pH is closed to 5-5.5 (Sams et al., 2016), what is not extremely acid and fits with the optimum pH for HGL activity on long chain TAGs *in vitro* (pH 5.4; see Gargouri et al. (1986)). In addition, surface active compounds are normally liberated from the food matrix causing a decrease in the chyme surface tension magnitude. These surface-active compounds may structure bulk lipids in an emulsified form (Diakidou, Vertzoni, Dressman, & Reppas, 2009). They can also favor the stability and activity of HGL that can be irreversibly inactivated at high surface tension such as the pure oil-water interface (Gargouri et al., 1986). Bile salts also contribute to lowering interfacial tension and favor HGL activity. Based on their surface activity, bile salts (normally absent or at low levels in gastric contents) and proteins like serum albumin are used in the optimized assays for measuring HGL activity *in vitro* (Gargouri et al., 1986; Grundy et al., 2021).

4.1 Human gastric lipase: structure and function of a peculiar enzyme

Under the conditions indicated above, pre-duodenal lipolysis occurs due to the action of HGL. TAGs hydrolysis in the stomach, estimated from the pyloric outputs of lipolysis products, accounts for a

significant hydrolysis level depending on the type of meal ingested (Carrière et al., 2001; Carrière et al., 1993; Carrière et al., 2005). These levels are percent of the FAs initially present in meal TAGs. They correspond mainly to the conversion of dietary TAGs into diglycerides (DAGs) and FFAs. Higher levels reaching 40% are sometimes reported in the literature (Martine Armand, 2007; Golding & Wooster, 2010), but they usually correspond to individual time points in the late stage of gastric digestion and not to the overall lipolysis occurring in the stomach. In fact, gastric lipolysis cannot exceed high levels because gastric lipase becomes inhibited by the lipolysis products, i.e. long chain FAs, accumulating at the oil-water interface in the absence of acceptors like bile salt micelles in the water phase (Gargouri et al., 1986). This inhibition is suppressed when gastric lipase mixed with the chyme reaches the upper small intestine, where this lipase can still contribute a significant level of lipolysis (Carrière et al., 1993), especially in the pathological situation of exocrine pancreatic insufficiency (EPI) (Carrière et al., 2005).

HGL can be considered as an extremophilic enzyme since it remains stable above pH 1 (Ville et al., 2002). This lipase consists of a 43 kDa polypeptide of 379 amino acids. HGL is also highly N-glycosylated, which increases its molecular mass to around 50 kDa (Bodmer et al., 1987; Roussel et al., 1999). Similarly to other lipases, the access to the HGL active site is controlled by an amphiphilic lid domain (Roussel et al., 1999, 2002). Polar residues of the lid domain are exposed in the closed conformation, what makes this globular protein highly soluble in water, while the lid opening conformation exposes the hydrophobic residues forming a large hydrophobic ring. This ring surrounds the active site and provides an interfacial recognition site (Sams et al., 2016).

Regarding HGL properties, it is an acid-stable enzyme with an optimum stability range between pH 2 and 7 (Ville et al., 2002) and an optimum activity at pH 5.4 on long chain TAGs (Gargouri et al., 1986) This enzyme is resistant to pepsin hydrolysis and bile salts displacement (Singh et al., 2009; Ville et al., 2002). HGL is able to hydrolyze the ester bonds at all 3 positions in the glycerol moiety (Carriere et al., 1991; Tiruppathi & Balasubramanian, 1982), however the affinity for all positions is not the same. It

189 preferentially hydrolyzes the ester bond at the sn-3 position of TAGs (Rogalska, Ransac, & Vergers, 1990).
190 On the contrary to pancreatic lipase, HGL also presents some activity over the sn-2 position, which can
191 eventually lead to the formation of some sn-1(3)-MAG and glycerol in the gastric phase (Bakala-N'Goma
192 et al., 2022). Further information on the gastric lipolysis mechanism is given in Section 4.5. The HGL stereo-
193 preference for the sn-3 position is of major importance for infant nutrition. This is because the readily
194 absorbable short- and medium chain FAs in breastmilk are located at this position (Sams et al., 2016). This
195 enzyme has no FA preference based on the chain length (Gargouri et al., 1986) or unsaturation level;
196 however, long-chain FAs form a strong adsorbed layer at the interface inhibiting HGL activity by physical
197 entrapment (Pafumi et al., 2002). This is not observed with short and medium chain FAs which are partly
198 soluble in water (Gargouri et al., 1986). A human *in vivo* study revealed that the mean HGL basal activity
199 ranges between 15.6 and 64 U/mL (tributyrin units/stomach aspirate volume) for an empty stomach
200 (Armand et al., 1995), but activates in gastric secretion stimulated by pentagastrin can reach higher levels
201 (Ville et al., 2002). On average, the activity of pure human gastric juice is 100-120 U/mL, while postprandial
202 HGL activities in gastric contents range between 10 and 120 U/mL (Sams et al., 2016). This activity depends
203 on the ingested lipid amount: high-lipid diets can significantly increase HGL activity (Armand et al., 1995).
204 Furthermore, HGL is not inhibited by bile salt and it does not require a cofactor like colipase for pancreatic
205 lipase (Sams et al., 2016). In addition, HGL is considered as one the most surface-active lipases, with a
206 great capacity to penetrate adsorbed (multi)layers at high surface pressures compared to pancreatic
207 lipase. This is typically the case with phospholipid monolayers and emulsions stabilized by phospholipids
208 like Intralipidand milk (Bénarouche et al., 2017).This means HGL can hydrolyze lipids even in the presence
209 of adsorbed emulsifier multilayers, but it can be physically entrapped in long-chain FFAs clusters at the
210 droplet surface (Pafumi et al., 2002) or blocked by viscoelastic biopolymer adsorption layers made of
211 methylated nanocrystalline cellulose (Scheuble et al., 2016). Furthermore, high surface-active compounds
212 such as tweens are inhibitors of gastric lipase (Gargouri et al., 1986) and can inhibit *in vitro* the gastric

213 lipolysis of tween-stabilized emulsions (Couëdelo et al., 2015), probably because they prevent its
214 adsorption to the oil-water interface. This latter aspect will be more elaborated in Section 4.4 (interfacial
215 studies on gastric lipid digestion).

216 **4.2 Relevance of gastric lipolysis in humans**

217 Diverse *in vivo* implications may be triggered by gastric lipid digestion. Gastric lipolysis accounts for 10 to
218 25 % of gastrointestinal lipolysis of dietary TAGs (Carrière et al., 2001; Carrière et al., 1993) and can thus
219 contributes to up to one fourth of total TAGs digestion. In patients with severe EPI, such as in chronic
220 pancreatitis, this contribution can even reach one third in the absence of pancreatic lipase (Carrière et al.,
221 2005). It is therefore important for people suffering of EPI, who present a low secretion of pancreatic
222 enzymes including lipase (Layer & Keller, 2005) and a 3 to 4-fold increase in gastric lipase secretion
223 (Carrière et al., 2005). The contribution of gastric lipase explains why severe EPI patients are still able to
224 digest some fat. Gastric lipase is also essential for infant lipid digestion since their pancreas is not yet fully
225 developed at birth, resulting in a relatively low secretion of pancreatic lipase, while on the contrary, their
226 HGL expression is already fully mature (Bourlieu et al., 2014; Lindquist & Hernell, 2010). In preterm infants,
227 HGL concentration gastric contents and intragastric lipolysis levels are similar to those found in adults
228 (Roman et al., 2007). It has also been shown *in vitro* that gastric lipolysis triggers the action of pancreatic
229 lipase on emulsions covered by phospholipids like Intralipid (Gargouri et al., 1986) and milk (Bernback,
230 Blackberg, & Hernell, 1990; Bernbäck, Bläckberg, & Hernell, 1989). The release of FFAs by HGL promotes
231 changes in the interfacial properties and can favor the interfacial adsorption of the pancreatic lipase-
232 colipase complex (Dahim & Brockman, 1998; Smaby, Muderhwa, & Brockman, 1994). Some hormones
233 secreted in the stomach and related to appetite and satiety regulation have been linked to the ingestion
234 of lipids. Among these hormones, ghrelin, leptin (Hynes & Jones, 2001) and gastrin-releasing peptide
235 (Jönsson et al., 2006) can be found. However, no studies have been found on the effect of lipid structural
236 properties on the release of these hormones in the gastric phase. The release of these hormones might

be linked to the lipolysis rate, but more research is needed to prove this relation. Ghrelin is an orexigenic gastric peptide produced by X-cells in the gastric corpus that initiates food uptake (Cummings & Shannon, 2003) but also growth hormone release (Kojima et al., 1999). Ghrelin needs to be acylated by octanoic acid (C8:0) to be functional and this octanoylation is specifically ensured by ghrelin-O-acyltransferase (GOAT) (Al Massadi, Tschöp, & Tong, 2011). C8:0 partly comes from the diet (milk, MCT) and is directly used for the acyl-modification of ghrelin (Nishi et al., 2005; Nishi, Mifune, & Kojima, 2012). Since medium chain FAs (MCFA) can be directly absorbed in the stomach (<C12) (Lemarié et al., 2016), gastric lipase may therefore play an important role in the release of C8:0 for ghrelin acylation in the stomach. However, a study in rats showed no short-term effect of trioctanoin consumption and subsequent increase of C8:0 in gastric tissue on plasma octanoylated ghrelin concentration (Lemarié et al., 2016).

Intragastric lipolysis by HGL may also trigger the secretion of the hormone cholecystokinin (CCK) because CCK is produced by I-cells in the proximal small intestine and its secretion is mainly stimulated by the LCFA released in the upper part of the duodenum (Hildebrand et al., 1998). CCK is a master-regulator of digestion in the upper small intestine (Dockray, 2014) where it matches delivery of nutrient to the capacity for nutrient digestion. This is achieved by triggering bile and pancreatic secretion thereby delivering bile salts and digestive enzymes to the intestine for food digestion. The link between LCFA and CCK release is ensured by the LCFA receptors. Among them CD36 is considered as the main contributor (Sundaresan et al., 2013). More studies are required to confirm the contribution of gastric lipolysis to the acylation/activation of ghrelin and release of CCK, what would further support the role of gastric lipolysis in the kick-off of lipid digestion.

The preferential cleavage of the *sn*-1 and 3 positions by gastrointestinal lipases (Mitchell et al., 2008) results in the generation of FFAs and sn-2-MAGs that can be directly absorbed by small intestine enterocytes, where they are reassembled into TAGs. The regio- and stereo-specificity of gastric lipase has, however, some other nutritional implications. We have seen above that its *sn*-3 stereopreference

allows the fast release of SCFA and MCFA from milk and therefore their gastric absorption, what results in a fast uptake of energy. Because it can also cleave the ester bond at the *sn*-2 position of TAGs, gastric lipase can generate *sn*-1,3-DAG and then *sn*-1(3)-MAGs that are converted into glycerol. In that case, *sn*-2-MAG is not generated and this can impact the pathway by which TAGs are resynthesized in the enterocytes (Bakala-N'Goma et al., 2022). This property of gastric lipase challenges the dogma according to which the FA at the *sn*-2 position of TAGs is preserved in the absorbed *sn*-2-MAGs (Summers et al., 1999). It fits, however, with the observation by Couedelo et al. that FAs at position *sn*-2 in dietary TAGs can be significantly reshuffled in plasma TAGs (Couědelo et al., 2012). It also fits with the early findings on the balance between the *sn*-2-MAG and Glycerol-3-phosphate (G3P) pathways for TAG synthesis in the enterocytes (Mattson & Volpenhein, 1964).

4.3 Emulsion design factors and their effect on gastric lipolysis

This section discusses the impact of emulsion design factors on gastric lipid digestion. These design factors refer to emulsion structural properties such as droplet size, interfacial composition, lipid phase composition, and others, which can be affected during product formulation and/or processing (Figure 3). This part is not intended as an exhaustive review but compiles some representative studies in which diverse emulsion properties were tested under gastric conditions. These gastric conditions have been mainly simulated using *in vitro* experiments due to the advantages these methods offer such as flexibility, high throughput, no ethical constraints and others. These *in vitro* studies often employed relevant substitutes of HGL, such as lipases extracted from rabbit stomachs (Capolino et al., 2011) or recombinant dog gastric lipase (rDGL) expressed in transgenic plants (Williams et al., 2012). This is because obtaining HGL involves ethical constraints. In these last years, recombinant HGL has become commercially available, however *in vitro* simulations turn into quite expensive experiments when included. In other *in vitro* studies, more available microbial lipases (e.g. from *Aspergillus niger*) have been employed, although their activity and specificity are significantly different from those of gastric lipases (Sams et al., 2016).

4.3.1 Droplet size

An important emulsion structural property is the size of the oil droplets. Many authors agree in stating that oil droplet size directly impacts the rate of lipid digestion. In this aspect, the seminal study of Benzonana & Desnuelle (1965) showed for the first time that the rate of lipolysis by pancreatic lipase depends on the droplet size and the specific surface available for lipase adsorption. This means that the rate of lipolysis increases when the droplet size decreases, and *vice* versa. This emulsion design factor has been widely studied under *in vitro* small intestinal conditions. For instance, recent studies have confirmed the inverse relation between droplet size and the rate and extent of small intestinal lipolysis (L. Liang, Zhang, Wang, Jin, & McClements, 2018a; Salvia-Trujillo et al., 2017). However, this is also true for gastric lipolysis, as shown by Bourlieu et al. when comparing the *in vitro* digestion by purified rabbit gastric lipase (RGL) of native milk fat globules of 4 µm and processed milk with a size distribution centered on 0.2–0.6 µm. Lipolysis of the processed milk (4.70 ± 0.20 to 6.34 ± 0.05 µmol of FFA released per min) was much faster than that of the native milk (0.25 ± 0.36 µmol of FFA released per min) (Bourlieu et al., 2015). In the study of Infantes-Garcia et al. (2020), o/w emulsions containing 5% triolein, and stabilized with 1% sodium taurodeoxycholate were homogenized with different pressures resulting in diverse initial droplet sizes (0.58 µm, 1.82 µm, and 4.00 µm). These emulsions were subjected to static *in vitro* gastric digestion at pH 5.5 and lipase activity of 20 U/mL. In this research, rabbit gastric extract (RGE) was employed as a physiological relevant source of RGL and pepsin. Diverse lipolysis products including diolein and monoolein regioisomers were quantified using a HPLC-charged aerosol detector method. It was shown that the final extent and initial rate of lipid digestion had an inverse relation with respect to the droplet size of emulsions during digestion (Figure 4). The relation between lipolysis rate and emulsion droplet size is logic since the latter determines the interfacial load of adsorbed gastric lipase. However, one would not expect that the final extent of digestion is also dependent on the emulsion droplet size. This phenomenon may be explained by the parallel effect of product inhibition and available interfacial surface. Product

309 inhibition usually takes place in the gastric phase due to the lack of bile, so lipolysis products cannot be
310 removed and accumulate at the interface entrapping gastric lipase (mainly by oleic acid) as previously
311 demonstrated (Pafumi et al., 2002). Consequently, product inhibition is reached earlier for larger droplets
312 since a lower concentration of FFA is needed to cover the interfacial area with these clusters.

313 In other *in vitro* studies, o/w emulsions presenting the same formulation, but different droplet sizes were
314 also subjected to simulated gastric digestion using diverse physiological conditions. For instance, Pafumi
315 and colleagues prepared three low-fat emulsions stabilized by phospholipids with average particle sizes
316 of 0.7, 2, and 15 µm. These emulsions were subjected to *in vitro* static gastric digestion at pH 5.4 and
317 enzyme activity of 2.5 U gastric lipase/µmol triolein (substrate) (Pafumi et al., 2002). In this case, HGL
318 purified from human gastric juices was employed. Similar trends in lipolysis kinetics (inverse relation
319 between rate/extent and droplet size) were obtained as compared to the study of Infantes-Garcia et al.
320 (2020). The final lipolysis extents found here laid between 10 and 40%. In other studies, milk phospholipid-
321 based emulsions (Luo et al., 2019) and milk fat globules (Bourlieu et al., 2015) with different droplet size
322 were digested in a pH-stat device. This device was programmed to establish 4 subsequent pH stages (from
323 6.5 until 4) of diverse duration to simulate infant gastric digestion. In the first study, the lipase produced
324 by *Rhizopus oryzae* (~43 U/mL gastric mixture) was employed (Luo et al., 2019), while in the second study
325 gastric lipase (26 U/mL gastric mixture) purified from rabbit stomach was included (Bourlieu et al., 2015).
326 Here again the trend was slower and less extended lipolysis kinetics for larger droplets.

327 Similar findings with respect to the effect of oil droplet size on gastric and small intestinal lipid digestion
328 were also found during an *in vivo* study. For instance, a clinical trial showed that an emulsion with a small
329 average droplet size of 0.7 µm infused in healthy volunteers resulted in a faster and more extended lipid
330 hydrolysis compared to a larger-sized emulsion of 10 µm (36.5% vs 15.8% lipolysis extent) (Armand et al.,
331 1999). In the clinical trial mentioned above, the two emulsions presenting a different initial oil droplet size

were stabilized by both proteins and lecithin, which probably provided a relatively good stability along the gastric phase, so the observed lipolysis kinetics could be attributed mainly to differences in droplet size.

4.3.2 Interfacial composition

A second relevant emulsion design property is the composition of the interface (McClements, 2018). This refers to the surface-active compounds added during emulsion preparation to kinetically stabilize the o/w interface. Without these stabilizing agents, physical destabilization of oil droplets would occur leading to an eventual phase separation. Different surface-active compounds can be employed in emulsions such as surfactants (e.g. lecithin or Tween 80) and amphiphilic biopolymers (e.g. diary proteins or gum Arabic) (Berton-Carabin, Sagis, & Schroën, 2018). As explained in section 2, lipid digestion is a phenomenon taking place at the o/w interface. After the emulsion is ingested, the interfacial properties of the compounds adsorbed to the interface can affect lipid digestion by: *(i)* impacting the emulsion physical stability, *(ii)* influencing the competitive adsorption between the surface-active compounds and lipases, and *(iii)* being potentially digested. The relation between the interfacial composition and the above phenomena linked to lipolysis in the gastric phase is described in the following paragraphs.

The emulsion physical stability is perhaps the most obvious phenomenon affected by the interfacial composition. This phenomenon influences the surface area available for lipase adsorption and subsequently lipolysis kinetics (McClements, 2018). The physical stability effect of emulsions stabilized by surface-active agents of different chemical nature on small intestinal lipid digestion has been demonstrated in multiple *in vitro* studies (M. R. Infantes-Garcia, Verkempinck, Gonzalez-Fuentes, Hendrickx, & Grauwet, 2021; L. Liang, Zhang, Wang, Jin, & McClements, 2018b; L. Liu, Pan, Zhang, Zhang, & Li, 2020; Verkempinck et al., 2018; Wang, Lin, Ye, Han, & Singh, 2019). In the gastric phase, emulsion physical stability certainly impacts lipolysis kinetics as well. Infantes-Garcia et al. (2020) observed that an o/w emulsion stabilized by the purified bile salt sodium taurodeoxycholate presented very low physical stability under *in vitro* static gastric conditions (pH 3 and rabbit gastric lipase activity 60 U/mL) (M. R.

Infantes-Garcia et al., 2021). The oil droplet size increased from 0.9 to ~50 µm after 2 hours of gastric digestion indicating some coalescence. This provoked a limited lipid hydrolysis (only during the first 5 min of digestion), linked to the early emulsion instability.

A second phenomenon is the emulsion interfacial composition which can modulate the competitive adsorption between the initially present surface-active compounds and gastric lipase (Berton-Carabin et al., 2018). This competitive adsorption phenomenon determines the partition of lipases between the aqueous phase and interface. The higher the surface activity of the initially present emulsifier is, the lower the amount of adsorbed lipase present at the interface. Therefore, the affinity of emulsifiers for the interface could modulate gastric lipase adsorption. An important aspect to consider is that these surface-active compounds can both be derived from emulsifiers added during emulsion preparation (Scheuble et al., 2016; Sonesson, Elofsson, Brismar, & Callisen, 2006), as well as from surface-active agents endogenously produced via digestion of the lipid phase (e.g. MAGs or FAs) (Reis et al., 2008). For instance, it was demonstrated that lipid digestion was drastically reduced during *in vitro* gastric digestion due to the limited gastric lipase adsorption onto an interfacial layer composed of the strong surfactant, Tween 80 (TW80) (Couëdelo et al., 2015; M. R. Infantes-Garcia et al., 2021; M.R. Infantes-Garcia, Verkempinck, Saadi, Hendrickx, & Grauwet, 2022).

A third phenomenon affected by the interfacial composition is the potential hydrolysis of the interfacial layer (Singh et al., 2009). Emulsions stabilized by proteins, digestible carbohydrates (e.g. modified starches), and/or digestible lipids (e.g. lecithin, MAGs and DAGs) are prone to digestion. In the gastric phase, the activity of pepsin towards adsorbed proteins to the oil-water interface certainly impacts the physical stability of emulsions. This is based on the different stabilizing capacities of the hydrolysis products which in fact depends on the emulsifier nature and the cleavage extent reached during digestion (Ye, Wang, Lin, Han, & Singh, 2020). This phenomenon was observed in a reported study which subjected a soy protein isolate stabilized emulsion to *in vitro* static digestion (M. R. Infantes-Garcia et al., 2021). In

the first minutes of gastric digestion, conformational changes in proteins caused flocculation which increased the particle size and retarded lipid digestion as compared to other emulsions. Afterwards, flocculation degree slightly decreased due to soy protein hydrolysis by pepsin, 'releasing' flocculated oil droplets and allowing lipid digestion. This hydrolysis most likely took place both in the aqueous phase and interface, which changed the interfacial composition of oil droplets. Interfacial hydrolysis of proteins has been reported before by other researchers (Maldonado-Valderrama, Terriza, Torcello-Gómez, & Cabrerizo-Vílchez, 2013; Maldonado-Valderrama, 2019; Maldonado-Valderrama, Wilde, Mulholland, & Morris, 2012). The peptides produced by soy protein degradation were probably still capable to stabilize the emulsion after hydrolysis as reported before for soy proteins (Nishinari, Fang, Guo, & Phillips, 2014).

In some cases, a mixture of emulsifiers can be employed to prepare emulsion with the aim of obtaining a tailored lipid digestion. This was the case in a study in which TW80 and citrus pectin (CP) were employed at different ratios to design emulsion with mixed interfaces (M.R. Infantes-Garcia et al., 2022). An interfacial load analysis confirmed that both emulsifiers were present at the interface at diverse proportions as a function of TW80 concentration. Emulsions prepared only with TW80 or CP presented two significantly different lipolysis behaviors during *in vitro* gastric digestion (M. R. Infantes-Garcia et al., 2021). One the one side, the CP emulsion presented a relatively good physical stability and reached a very high lipolysis extent. On the other side, the TW80 emulsion had an excellent stability but negligible lipid digestion. By mixing these emulsifiers, the physical stability of the new emulsions was improved as compared to the CP emulsion. In addition, lipolysis rate constants and extents were lower for increasing TW80 concentrations as observed in Figure 5. This study demonstrated that it is possible to obtain tailored lipolysis in the gastric phase by mixing stabilizing agents with different lipolysis behavior. More insights into the interfacial changes occurring with these emulsions is given in Section 4.4.

4.3.3 Lipid phase composition

The chemical nature of lipids is also an important emulsion design factor affecting lipid digestion. Diverse *in vitro* studies demonstrated that mainly the chain length affects lipid digestion kinetics in the small intestinal phase. Regarding this lipid compositional property, the lipolysis kinetics will be faster and more extensive for shorter chain lengths (Liang et al., 2016; Zou et al., 2016). This behavior is related to the fact that most lipases display a higher specific activity on short and medium chain TAGs than on long chain TAGs. In addition, short- and MCFAs (<12 carbons) have some solubility in water. They quickly move to the surrounding aqueous media after cleavage from a TAG molecule, allowing access of lipases to the remaining TAG molecules, on the contrary to insoluble long chain FAs(>12 carbons) that accumulate at the oil-water interface restricting the access of lipases to further hydrolyze the substrate. Therefore, the lipolysis kinetics can be slowed down or even completely inhibited. The effect of FA chain length on the lipolysis kinetics was studied in an *in vitro* experiment using purified HGL, in which a tributyrin emulsion resulted in faster kinetics compared to a soybean oil emulsion (Gargouri, Pieroni, Riviere, et al., 1986).

4.4 Interfacial studies under *in vitro* gastric conditions

In situ interfacial studies are of great importance because they can reveal the interactions between emulsifiers adsorbed to the interface, bile salts and lipases. In this aspect, the majority of lipid digestion studies have characterized interfacial changes during the small intestinal phase since this is the main compartment for lipid digestion (Aguilera-Garrido, del Castillo-Santaella, Galisteo-González, José Gálvez-Ruiz, & Maldonado-Valderrama, 2021; Corstens, Osorio Caltenco, de Vries, Schroën, & Berton-Carabin, 2017; Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009; J. Maldonado-Valderrama et al., 2013; Wei et al., 2020). Only few studies have attempted to evaluate the interfacial changes occurring during gastric lipolysis since substitutes for HGL have become available only very recently.

In a previous research, interfaces composed of β-lactoglobulin (β-lg) and/or methylated nanocrystalline cellulose (metNCC) were employed to investigate their interfacial dynamics after being subjected to gastric digestion (pH 5) (Scheuble et al., 2016). In this case, rDGL was employed as a relevant substitute of HGL (lipase concentration 1.33 µg/mL). Diverse interfacial techniques such as pendant drop, interfacial shear and dilatational rheology, and neutron reflectometry were combined to elucidate interactions between emulsifiers and gastric lipase. On the one hand, interesting findings were related to the (almost) fully displaced β-lg by gastric lipase from the interface. This protein presented a lower surface activity compared to the enzyme which allowed gastric lipase adsorption. On the other hand, metNCC formed a compact thick layer which prevented gastric lipase adsorption. A similar scenario occurred when metMCC and β-lg formed a composite layer, which was highly resistant to gastric lipase displacement. The polymer metNCC was incorporated at the interface as a Pickering particle. Particles are known to have very high desorption energies, which is the reason behind its non-displacement by gastric lipase (Sarkar & Dickinson, 2020). Therefore, this interesting study demonstrated that the type of surface-active compound adsorbed to the interface can modulate the adsorption of gastric lipase, and thus tailor the lipid digestion process.

As already discussed in Section 4.3.2, in the study of Infantes-Garcia et al., CP and TW80 were mixed to obtain emulsions with both emulsifiers adsorbed to the interface (Infantes-Garcia et al., 2022). These emulsions exhibited complementary functionalities in terms of emulsion physical stability and lipid digestion kinetics during *in vitro* gastric digestion. More specifically, it was possible to tailor the rate and extent of lipolysis by designing emulsions with a fixed bulk concentration of CP (1% w/w) and variable bulk TW80 concentration (0.00625-0.1 % w/w) (Infantes-Garcia et al., 2022). These findings generated more questions about the actual interfacial phenomenon taking place during *in vitro* digestion. It was hypothesized that competitive adsorption between emulsifiers and gastric lipase was the driving force behind the modulation of lipolysis kinetics. For that reason, a modified pendant drop technique (OCTOPUS system, Universidad de Granada) was employed to investigate the interfacial dynamics occurring during

gastric digestion (pH 3) in presence of rabbit gastric lipase (60 U/mL)) (Infantes-Garcia et al., 2022). Interfacial tension kinetics and interfacial rheology of four different types of interfacial layers (bulk composition: 1% CP, 1% TW80, 1% CP+0.00625% TW80, and 1% CP+0.1 % TW80) were monitored during gastric digestion, as shown in Figure 6. Two different interfacial tension kinetics were observed when single layers of CP (fast decrease in tension) or TW80 (no change in tension) were subjected to *in vitro* gastric digestion, which was correlated with the lipolysis kinetics of their correspondent emulsions. This kinetics indicated that the CP film did not restrict the access of gastric lipase, while the TW80 layer blocked lipase adsorption preventing lipolysis. In case of CP and TW80 mixed interfacial layers, intermediate kinetics of interfacial tension were detected as function of TW80 concentration. This research proved that competitive adsorption (e.g. orogenic displacement) played an important role on lipid digestion of emulsions stabilized by CP and/or TW80. This is an indication that interfacial design can permit tailored lipolysis kinetics of emulsions (Infantes-Garcia et al., 2022).

4.5 *In vitro* gastric lipolysis mechanism

It is very interesting to elucidate the (bio-)chemical reactions taking place in the digestive tract. When describing an enzymatic reaction, the first mechanistic approach one would consider are the Michaelis-Menten kinetics modelling approaches. However, the application of this type of kinetics is not straightforward since the Michaelis-Menten model is established relying on the boundary condition that both the enzyme and substrate are in the same phase. Lipases are water-soluble, interfacial enzymes which diffuse through the aqueous environment of the gastrointestinal tract, while the water-insoluble substrate, the lipid, is located at the oil-water interface. Therefore, the reaction rate constants estimated using this model as such would not be meaningful (Aloulou et al., 2006; Reis et al., 2009). In the last decades, diverse kinetic models derived from the Michaelis-Menten equation have been developed for interfacial enzymes. The simplest one is the Verger-De Haas' model that involves an adsorption step prior

471 to a Michaelis-Menten model in a two-dimensional space depending on substrate surface concentration
472 (Verger & de Haas, 1973).

473 The lipid digestion process also involves several consecutive or parallel reactions that can also be
474 described from a chemical perspective at a molecular level (Mitchell, Rodriguez, Carrière, Baratti, &
475 Krieger, 2008; Mitchell, Rodriguez, Carrière, & Krieger, 2008). Verkempinck et al. (2019) proposed a proof
476 of concept by using the multi-response modeling technique to describe the chemical reactions of *in vitro*
477 lipid digestion in the small intestinal phase. This modeling technique consists of multiple steps: *(i)* propose
478 a chemical reaction scheme, *(ii)* transform this scheme into differential equations assuming first order
479 kinetics, and *(iii)* solve the differential equations. The construction of the small intestinal lipolysis multi-
480 response model was based on *(i)* the evaluation of 28 different *in vitro* data sets, *(ii)* the proposition of
481 interrelated reaction networks, and *(iii)* the application of an iterative approach. This proposed model
482 gave more insights than the empirical case-by-case models because it simultaneously described multiple
483 responses that are interrelated within a reaction network. This was the first proof that multi-response
484 modeling can be applied to elucidate the mechanism of multi-response digestion phenomena, such as *in*
485 *vitro* lipolysis reactions in the small intestinal phase.

486 The advanced multi-response technique was also applied recently to describe the gastric lipid digestion
487 reactions at a molecular level (Infantes-Garcia et al., 2021, 2020; M.R. Infantes-Garcia et al., 2022). An
488 important requisite to apply this approach is that the multiple analytes included in the modeling process
489 have a sufficiently high concentration following a time-dependent behavior. Therefore, a suitable
490 analytical technique is necessary to reliably quantify diverse lipolysis products (Infantes-Garcia,
491 Verkempinck, Guevara-Zambrano, Hendrickx, & Grauwet, 2021). In addition, several experimental points
492 should cover the most important lipid digestion stages (e.g. beginning of digestion) so an adequate fit and
493 parameter estimation could be obtained. This mechanistic model consisted of a set of seven interrelated
494 reactions of which five correspond to enzymatic hydrolysis and two are isomerization reactions (Figure 7).

Therefore, the hypotheses on which this model was based were proven through multi-response modeling. These hypotheses included: *(i)* the activity of gastric lipase towards all positions, what has been shown *in vitro* with various substrates, and *(ii)* the occurrence of isomerization reactions that may occur. The good fitting of the experimental data points confirmed the proposed hypotheses. In this way, the model improved the understanding of the gastric lipase specificity. Some enzymatic reactions indicated a gastric lipase regio-preference for the sn-1/3 position, however, activity over the sn-2 position was also possible as suggested by the complete release of FAs from TAGs (Carriere et al., 1991) and the observation of sn-1,3-DAGs in some experiments (Rogalska et al., 1990). An interesting feature of this mechanistic model is its robustness. This is because the model was first proposed using 2 experimental data sets generated under a gastric pH of 5.5 and lipase activity 20 U/mL. Then, it was validated with eight data sets produced at a gastric pH of 3 and lipase activity 60 U/mL. This work proposed for the first time a physiological relevant reaction scheme, which provided advanced insight into the lipolysis mechanisms under simulated gastric digestion conditions.

Another interesting finding of the developed multi-response model is the much higher rate of sn-1,3- DAG hydrolysis compared to sn-1,2/2,3-DAG (4th and 5th reaction in Figure 7). This finding can explain why sn-1,3-DAGs are usually not observed during *in vitro* and *in vivo* studies. Only sn-1,2/2,3-DAG were observed in most studies and reported, while Infantes-Garcia et al., 2020 have observed some low levels of sn-1,3-DAG in the course of triolein lipolysis. This was sufficient to consider the possibility that sn-1,3-DAG could be formed and further hydrolyzed. This aspect was recently highlighted by Bakala-N'Goma et al. (2022). In a previous model describing TAG lipolysis in the gastric phase, the production and cleavage of sn-1,3-DAG has been omitted considering the previously discussed aspects (Mitchell et al., 2008). In other words, based on the mechanistic model in Figure 7, gastric lipase can generate diverse intermediate products such as diolein monoolein isomers. However, gastric lipase affinity for these intermediate products was found to be different.

519 The multi-response modeling process employed in the above considered time as the only variable for both
520 digestive phases under static *in vitro* conditions. A challenge in this aspect would be how to deal with data
521 sets generated under dynamic *in vitro* conditions. This would create other independent variables apart
522 from time to be included in the modeling process (e.g. pH decrease in the gastric phase) (Le Feunteun et
523 al., 2021). As an additional research challenge, the estimated *k* values obtained through multi-response
524 modeling (primary modeling) could also be expressed as a function of emulsion properties. This secondary
525 modeling needs to be carefully thought through because some emulsions could exhibit more than one
526 property changing simultaneously during *in vitro* digestion (e.g. interfacial composition and droplet size
527 varying at the same time).

528 ## 5. Conclusions

529 The study of lipids consumption is of major importance because of the nutritional implications related to
530 their consumption. Additionally, the increasing interest of consumers for health-promoting and functional
531 foods has influenced the study of food emulsions and their fate after consumption. These studies have
532 mainly employed *in vitro* methods and focused on the small intestinal phase. However, the study of gastric
533 lipid digestion is also highly relevant due to its contribution to the overall extent of lipolysis, small
534 intestinal lipolysis promotion, the potential release of some satiety signals, and its importance for specific
535 population groups. From a food design perspective, the manipulation of the emulsion composition and
536 structure has been claimed to modulate the release of lipolysis products. Some studies on gastric lipolysis
537 have revealed that the lipid fate of emulsions can be modulated by playing with emulsion design factors.
538 This was specially evidenced when employing emulsions with mixed emulsifiers which could tailor the rate
539 and extent of lipolysis based on emulsion formulation and processing. In this aspect, some interfacial
540 studies also demonstrated the capacity of specific stabilizing agents to restrict gastric lipase adsorption,
541 thus explaining the lipolysis kinetics modulation observed with the emulsions. Finally, a mechanistic

insight from a molecular perspective has been proposed for gastric lipid digestion. This mechanism includes enzymatic and chemical reactions that may expand the current understanding of gastric lipase contribution to the lipid digestion phenomena. This mechanistic insight could be linked with emulsion design properties to further develop *in silico* models in which kinetic parameters are expressed as a function of emulsion properties. Next to this, more detailed mechanisms could be obtained with the support of analytical techniques that could identify and quantify stereoisomers derived from lipid digestion. Other research opportunities are linked to the study of lipid digestion in the gastric phase under dynamic conditions. The gradual intragastric pH drop combined with the progressive release of gastric juices (containing gastric lipase) could result in different emulsion physical stability and lipolysis kinetics as compared to static *in vitro* methods. In sum, recent *in vitro* studies on gastric lipolysis have confirmed the significant contribution of this kick-off step in the overall process of gastrointestinal lipolysis. They have deepened into the possibility of modulating gastric lipid digestion based on emulsion design and obtained mechanistic insights into this process. Thus targeting the gastric phase of lipid digestion may allow controlling the whole process and the subsequent intestinal absorption and physiological impact. This new approach involving emulsion design is supported by an independent approach using lipase inhibitors specific of gastric lipase (Point et al., 2016).

Corresponding authors

During submission process:

Marcos R. Infantes-Garcia. Laboratory of Food Technology, KU Leuven.

Email: marcos.infantesgarcia@gmail.com

During post-publication:

Tara Grauwet. Laboratory of Food Technology, KU Leuven.

Email: tara.grauwet@kuleuven.be

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Declaration of interests

The authors of this work declare no conflict of interests.

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