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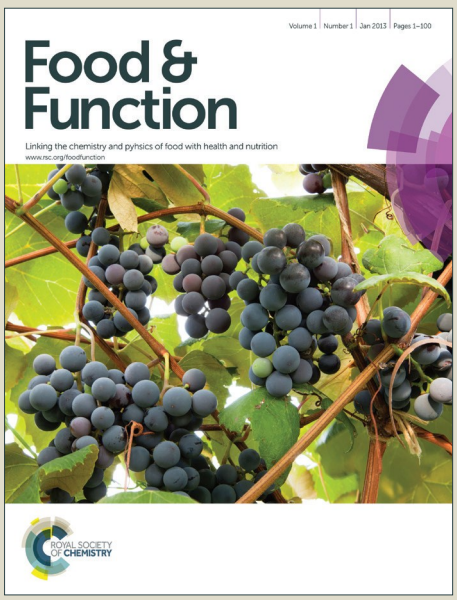

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Relevant pH and lipase for *in vitro* models of gastric digestion

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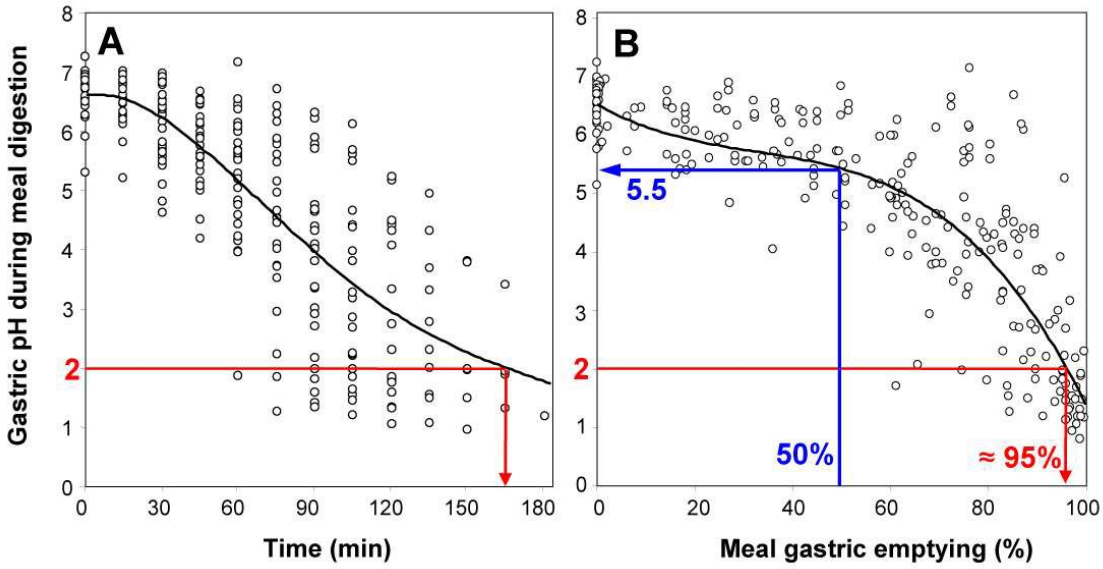

Expressing gastric pH as a function of gastric emptying instead of time makes it possible to reduce the inter-individual variability and highlight the pH values that are the most relevant for testing meal digestion in the stomach.

Abstract

The development of *in vitro* digestion models relies on the availability of *in vivo* data such as digestive enzyme levels and pH values recorded in the course of meal digestion. The variations of these parameters along the GI tract are important for designing dynamic digestion models but also static models for which the choice of representative conditions of gastric and intestinal conditions is critical. Simulating gastric digestion with a static model and a single set of parameters is particularly challenging because the variations in pH and enzyme concentration occurring in the stomach are much broader than those occurring in the small intestine. A review of the literature on this topic reveals that most models of gastric digestion use very low pH values that are not representative of fed conditions. This is illustrated here by showing the variations in gastric pH as a function of meal gastric emptying instead of time. This representation highlights the pH values that are the most relevant for testing meal digestion in the stomach. Gastric lipolysis is still largely ignored or performed with microbial lipases. *In vivo* data on gastric lipase and lipolysis have however been collected in humans and dogs during test meals. The biochemical characterization of gastric lipase has shown that this enzyme is rather unique among lipases: (i) stability and activity in the pH range 2 to 7 with an optimum at pH 4-5.4; (ii) high tensioactivity that allows resistance to bile salts and penetration into phospholipid layers covering TAG droplets; (iii) *sn*-3 stereospecificity for TAG hydrolysis; and (iv) resistance to pepsin. Most of these properties have been known for more than two decades and should provide a rational basis for replacement of gastric lipase by other lipases when gastric lipase is not available.

Introduction

There is a growing interest for *in vitro* digestion models because they are useful tools for studying food bioaccessibility, digestibility and bioavailability without, or before, performing animal and human studies [1]. This interest is not limited to food research: *in vitro* digestion models find important applications in pharmaceutical research for preclinical studies of oral drug bioavailability [2]. These models are critical for establishing good *in vitro-in vivo* correlations when drug dispersion and dissolution are highly dependent on digestive processes, as experienced with poorly water soluble drugs and lipid-based formulations for instance [3].

The development of *in vitro* digestion models relies on the availability of *in vivo* data such as digestive enzyme secretion and levels, pH values and their dynamic variations along the GI tract. A first level of confidence in the available data lies in their mode of collection. It is very important for instance that enzyme outputs/secretions have been measured in the course of meal digestion because meal components have a stabilizing effect: by providing their respective targets (lipids, proteins, carbohydrates) to digestive enzymes (lipases, proteases, amylases), they limit the probability of autolysis by proteases; by buffering gastric contents, these components also prevent enzyme denaturation at low pH and futher proteolysis by pepsin. These processes have been well described in the case of gastric lipase which is rapidly degraded under fasting conditions or pharmacological stimulation of its secretion [4, 5]. Highly variable concentrations of gastric lipase in fasting human gastric juice have thus been reported [6]. Degradation of pancreatic enzymes is even more pronounced [7] and researchers involved in the purification and characterization of pancreatic enzymes from pancreatic juice and pancreas have always taken great care of avoiding protease activation leading to autolysis and poor stability of these sources of enzymes [8, 9]. Storage conditions of

samples before analysis are also critical. Proteolysis is often promoted upon freezing/thawing process in the absence of protease inhibitors or other agents protecting enzymes from degradation such as bile salts and meal components [10, 11]. Once these potential artifacts have been discarded, relevant data can be extracted from the literature and used for designing both dynamic and static digestion models, the latter being the most widely used because of their simplicity, lower cost and higher throughput [1].

A major difficulty with static models is however the selection of a single set of parameters (pH, enzyme concentration, meal dilution, mixing) supposed to be representative of digestion at a given location within the GI tract. This difficulty is mainly encountered with intragastric digestion where variations in pH and enzyme concentration are much broader than those occurring in the small intestine [12-14]. Reproducing the two main functions of the stomach is also challenging: its higher part, the fundus, has a major contribution to gastric acid and digestive enzyme (pepsin, gastric lipase) secretion, whereas the lower part, the antrum, generates mechanical forces to mix, disrupt and transport gastric content by peristaltic movements[15, 16 17]. This has led to the development of many intragastric digestion models with different parameter sets [1, 18-24]. We will not discuss here the attempts to reproduce gastric mixing and fluid flow and the use of pepsin, which have been covered in previous articles and reviews [16, 25-27], and will focus on the choice of pH and lipase for mimicking gastric conditions. In a majority of gastric digestion models, low acidic pH values (1.4-3) are used but their relevance for meal digestion is questionable. Gastric lipase, the first enzyme of the GI tract involved in fat digestion [14, 28], is often replaced by other lipases from microbial origin without demonstration of their equivalence. The biochemical properties and specificity of gastric lipase, such *sn*-3 stereospecificity for triglyceride (TAG) hydrolysis [29-35], are however unique among known lipases. These various points will be illustrated based on data collected

in humans and dogs during test meals. The aim of this review is to provide reliable data on gastric pH and lipase to be used for designing *in vitro* digestion models.

Overview of *in vitro* digestion models including a gastric phase

A literature survey with Web of Science™ shows an exponential increase in the number of publications with "*in vitro* digestion model" as topic over the last years. Among these publications (>6,000), we selected and analyzed 340 articles giving detailed data on the type of model used (static vs. dynamic), the number of phase involved (oral, gastric, duodenal/small intestinal, colonic) and key parameters like pH values and source of digestive enzymes. Static models are the most predominant (95%) and a gastric phase is present in 84% of these models.

The pH values and the source of lipase, if any, used in static models and gastric phase are highly variable (Table 1). The pH of the gastric phase varies between 1 and 5.5 and *in vitro* gastric digestion assays can be classified in three groups: pH<2 (21%), pH 2 (53%) and pH>2 (26%). The majority of *in vitro* gastric digestion experiments are therefore performed at a very low pH supposed to reflect gastric conditions.

Contrary to pH and pepsin, the use of a lipase during the gastric phase is clearly neglected in most publications. Indeed, only 5 % of publications on static digestion models use a lipase to take into account intragastric lipolysis, against 39% with dynamic digestion models. Different sources of lipases can be identified but most published works use human gastric juice and commercially available microbial lipases from *Rhizopus oryzae* and *Aspergillus niger*. Lipases from *Candida rugosa*, *Pseudomonas fluorescens*, *Rhizomucor miehei* [36] and *Burkholderia cepacia* [37] are also used in a few studies. The use of gastric lipase purified from human gastric juice or other sources (animal tissue extract, recombinant lipase) is still limited to laboratories producing this enzyme. Lipase concentrations/activities are

highly variable and depend on the source of lipase. With gastric lipase, the choice of lipase concentration/activity is usually based on *in vivo* data [38, 39], the mean levels found in human gastric juice (around 100-110 µg/mL, which is equivalent to 120-130 U/mL using tributyrin as reference substrate [5, 28]) and meal to gastric juice volume ratio. For instance, a concentration of 17 µg/mL gastric lipase is chosen to simulated the gastric conditions at 50% gastric emptying when gastric juice is diluted 6-fold by the meal [38]. In the case of microbial lipase, some digestion assays are performed with 3,300 µg/mL of *Aspergillus niger* lipase with a specific activity of around 12 U/mg (i.e., 39 U/mL) while some other assays are performed with 200-250 µg/mL of *Rhizopus oryzae* lipase with a specific activity of 80 U/mg (i.e., 20 U/mL) [40-43]. Variable levels of the same lipase can also be found [40-42, 44, 45]. For instance, the mass concentration of *Rhizopus oryzae* lipase varies from 200 µg/mL to 2,700 µg/mL (10-fold), while activity level varies from 17.14 U/mL to 110 U/mL (6.5-fold) [42-45]. It is therefore difficult to compare these digestion assays supposed to mimic gastric digestion. It is even more difficult to find a rational basis for the choice of lipase mass concentration and activity in the absence of any publication comparing these microbial lipases with gastric lipase and gastric juice under *in vitro* digestion conditions. The definition of lipase units (U) is also a major concern because the activities of the lipase sources are often measured using specific assay conditions for each lipase and units cannot be compared.

Variations in gastric pH during meal digestion

The pH of gastric contents and its variations during a meal have been measured on various occasions in healthy volunteers using samples collected by naso-gastric tubes [13, 28, 46-48]. Gastric pH is not a constant parameter and its value changes continuously during digestion. After meal ingestion, gastric pH raises from 1.0-1.5 (basal fasting conditions) to 5-7 depending on the type of meal and its buffering capacity. It then decreases due to meal

dilution by gastric acid secretion before returning to basal conditions after around 3 hours. Gastric emptying also contributes to this pH decrease by removing the meal components from the gastric contents and reducing their buffering effects. These pH variations with time are shown in Figure 1A and 1C for liquid and solid-liquid test meals, respectively. Large inter-individual variations are usually observed that result in a great dispersion of pH values plotted as a function of time. With a solid-liquid meal for instance, the pH of gastric contents at 60-min after meal ingestion can range between 6 and 1.5 (Figure 1C). It is therefore very difficult to define the gastric pH after 1 hour of digestion. The main cause of this variability is gastric emptying and the residence time of the meal in the stomach that varies with the type of food ingested. The rate of gastric emptying is known to be one of the main factors of variability of postprandial events [49, 50], including pH variations, with significant inter- and intra-individual variations in healthy volunteers [51]. When meal gastric emptying is measured using a non-absorbable marker like PEG 4000, the gastric pH variations can be plotted as a function of gastric empting instead of time (Figures 1B and 1D). This non-conventional representation reduces the inter-individual variability and gives a new picture of pH variations in gastric contents during meal digestion [12, 28]. It thus appears that gastric pH remains rather high and decreases at a slow rate until 50-60% of the meal is emptied from the stomach (Figures 1B and 1D). With a liquid test meal, the gastric pH is still found between in pH 4 and 7 at 50% gastric emptying with a mean value of 5.5 (Figure 1B). With a solid meal, the mean gastric pH at 50% gastric emptying is 4.6 (Figure 1D). It is worth noticing that these values are found within the pH range [4-5.4] of optimum activity of gastric lipase on long chain triglycerides [52-54].

If we consider the pH value used for the gastric phase in 55% of static *in vitro* digestion models, namely pH 2, this pH value corresponds to 95% of meal gastric emptying (Figures

1B and D). This pH is therefore close to that of fasting conditions and is not representative of conditions where most of the meal is still present inside the stomach.

Variations in gastric lipase levels during meal digestion

Gastric enzyme concentrations are also affected by the rate of gastric emptying. Many investigations have been performed to find the relationship between gastric emptying and regulatory processes induced by hormones or neurotransmitters like enzyme secretions [55-57], as well as physical properties of test meals [58-62]. These correlations have usually been established on a time basis. In the case of human gastric lipase (HGL), however, the enzyme levels in gastric contents can be expressed both as a function of time (Figures 2A and 2C) and gastric emptying (Figures 2B and 2D). As previously shown with gastric pH (Figure 1), plotting HGL concentration as a function of gastric emptying instead of time allows reducing the inter-individual variability. After ingestion of the test meal, the HGL concentration in the gastric content is low because of the high dilution of basal gastric juice by the meal. Then the level of secretion increases because of the stimulation by the meal and at 50% gastric emptying HGL concentration is around 15-20 µg/mL. A drastic increase in the HGL concentration is observed after 70-80% gastric emptying. When meal gastric emptying is completed, lipase concentration reaches the level found in basal conditions, i.e. in pure gastric juice. The mean concentration of HGL in gastric juice is 108 ± 52 µg/mL, which corresponds to 130 ± 62 U/ml when HGL activity is measured with tributyrin as substrate and shows a specific activity of 1,200 U per mg of enzyme [5]. The total amount of HGL secreted during a meal is around 20 mg [13, 28, 48, 63]. Like gastric pH, HGL concentration undergoes large variations during a meal and it is not obvious to select a representative value to simulate the intragastric process of lipolysis with *in vitro* static models. In the *in vitro* digestion studies with a static model and using gastric lipase, an enzyme concentration corresponding to 50 %

gastric emptying (16-17 µg/mL) was usually chosen [38, 63-68], together with a pH value of 5.5. This pH value corresponds to the mean pH of gastric contents at 50% gastric emptying (liquid meal) and is also close to the pH (5.4) at which the optimum activity of HGL is measured using Intralipid, a soybean oil emulsion [52].

Unique biochemical properties of gastric lipase

Gastric lipase secretion, like those of gastric acid and pepsinogen, is stimulated by the gastrointestinal hormone gastrin [5, 69-71], but others signals such as stomach motion, choligernic stimuli [4] and test meals [28] also trigger gastric lipase secretion [72-75]. As a result, an appreciable digestion of fat by HGL occurs in the stomach where 10 to 25% of dietary TAG acyl chains can be released [13, 28, 63]. This significant contribution to fat digestion in the gastric environment is due to specific properties of HGL that can be considered as an extremophilic enzyme [76].

HGL is a triacylglycerol hydrolase (EC 3.1.1.3) consisting of a 379-amino acid polypeptide of 43 kDa [77] with an α/β hydrolase fold [78] Native HGL purified from human gastric juice is highly N-glycosylated (15 % w/w), which results in a global molecular mass of 50 kDa and the existence of four major isoforms with isoelectric point ranging from 6.8 to 7.4 [79]. Like other lipases, it is a serine hydrolase with a catalytic triad (Ser153-His353-Asp324) and an oxyanion hole (Gln154, Leu67), and the access to its active site is controlled by an amphiphilic lid domain [78, 80, 81]. Whereas the first X-ray structure of HGL (Protein Databank ID: 1HGL) was obtained with the lid in the closed conformation [78] (Figure 3A and C), the crystallization of recombinant dog gastric lipase (rDGL) covalently inhibited by a phosphonate inhibitor allowed to solve a second 3D structure of a gastric lipase with the lid in the open conformation (Protein Databank ID: 1K8Q)[80]. The conformational changes occurring upon lid opening in HGL could be deduced from these two structure since DGL[82]

and HGL[77] share 86 % amino acid sequence identity (Figure 3B and D). Figure 3 shows how the opening of the amphiphilic lid gives access to the active site. While lid polar residues are exposed in the closed conformation, hydrophobic residues become exposed upon lid opening and form a large hydrophobic ring surrounding the active site entrance and constituting the interfacial recognition site (IRS).

HGL is highly stable in acidic conditions and in gastric juice at pH values ranging from 2 to 7, especially between pH 3 and 5 with half-inactivation times > 24 hours [5]. Its stability decreases below pH 2 and above pH 7 with half-inactivation times of 43 ± 9 min at pH 1 and 24 ± 18 min at pH 8 [5]. HGL shows a maximum activity at pH 5-5.4 on long chain TAG [52], whereas most lipases show optimum activity at pH 7 or above. This particular property of gastric lipase does not fit with the known mechanism of action of serine hydrolases with a catalytic triad and a histidine residue involved in the charge relay system [83]. Indeed, the nucleophilic character of the serine residue depends on the ionization of histidine and is normally enhanced at pH values above the histidine pKa (6.5). The 3D structures of HGL and rDGL did not reveal any specific features or charged residues in the vicinity of the catalytic triad that could suggest a lower pKa for the catalytic histidine residue His353. Moreover, it was shown that gastric lipase acts on soluble substrate (vinyl butyrate) with an optimum activity above pH 7, which suggests that gastric lipase is able to hydrolyze ester bonds via the classical mechanism of serine hydrolases [84]. The optimum activity of gastric lipase is however shifted towards lower pH values when the concentration of vinyl butyrate exceeds the solubility limit and an oil-in-water phase appears [84]. Under these conditions, gastric lipase must first bind at the lipid-water interface before the insoluble substrate is hydrolyzed, which suggests that the lipase adsorption preferentially occurs at low pH. Experiments performed with long chain TAG emulsions confirmed that gastric lipase binds optimally to the oil-water interface at low pH values [84]. To study the effects of the pH on the adsorption step

independently from substrate hydrolysis, gastric lipase adsorption on solid hydrophobic surfaces was monitored by total internal reflection fluorescence, as well as using a quartz crystal microbalance. Both techniques showed a pH-dependent and reversible gastric lipase adsorption process, which was optimum at pH 5 [84]. Similar results were obtained when the pH-dependent adsorption of gastric lipase at the lipid-water interface was studied with phospholipid monomolecular films [85]. The optimum activity of gastric lipase at acidic pH can therefore be explained by a better adsorption of the enzyme at the lipid-water interface at low pH and the fact that the lipase adsorption is the rate limiting step in the overall process of lipolysis [84].

In the stomach, HGL mainly converts TAGs into diglycerides (DAG) and free fatty acids (FFA)[28] but *in vitro*, gastric lipase is able to cleave the three ester bonds of TAGs under conditions optimized to reach high hydrolysis levels [53]. The limited action of HGL in gastric contents is due to its inhibition by the lipolysis reaction products, long chain FFAs, that accumulate at the lipid-water interface in the absence of acceptors [52, 86], and also to the decrease in gastric pH below optimum values for HGL activity. Once it is emptied in the duodenum with the chyme, HGL finds more favourable conditions for its activity and can still contribute to fat digestion as shown in chronic pancreatitis patients with no pancreatic lipase [13].

HGL shows a high activity on TAG and DAG substrates forming oil-in-water emulsions, and much lower activity on monoglycerides (MAG) [87, 88]. Gastric lipase can also hydrolyze mono- and di-esters of polyethyleneglycols (PEG) with various chain length [87, 88], but has no activity on phospholipids and cholesterol esters [53]. HGL shows its highest activity on tributyrin and this short chain substrate has been used for developing a sensitive standard assay of gastric lipase [28, 52]. Nevertheless, the rates of short, medium and long acyl chain TAG hydrolysis by gastric lipase are found in the same order of magnitude [52, 53]. Whatever the

substrate, optimum activity of gastric lipase is always measured in the pH range 4-6 [52, 53, 87-89], except when the substrate is partly soluble like vinyl butyrate and is used at a concentration below the solubility limit [84].

Another specificity of HGL is the fact that this lipase is active in the presence of physiological (micellar) concentration of by bile salts[52, 90] whereas most lipases are inhibited and human pancreatic lipase (HPL) requires a specific protein cofactor to display its activity under these conditions [91] (Figure 4A). Bile salts are strong surfactants that compete with lipases for adsorption at the lipid-water interface and thus can prevent the enzyme from reaching its substrate. HPL counteracts these effects by forming a stochiometric complex with colipase that anchors the lipase-colipase complex at the interface[91, 92]. HGL is a more tensioactive enzyme than HPL and does not require a cofactor for adsorption at the lipid-water interface in the presence of micellar concentrations of bile salts[74], probably because of its amphiphilic structure (Figure 3). The high tensioactivity of gastric lipase has been shown by adsorption studies onto monomolecular films (Figure 4B). The critical surface pressure for HGL penetration into a monolayer of egg phosphatidylcholine (PC) spread at the air-water interface is 23-25 mN/m [93, 94] whereas that of HPL is 15-18 mN/m [91, 93]. HGL is thus as tensioactive as the HPL-colipase complex (1:1 mol/mol) that penetrates into egg PC monolayer with a critical surface pressure of insertion of 27 mN/m [93]. This property of HGL is important for its biological function: HGL can act in the duodenum where it was found to be active an stable[13, 28]; HGL can initiate the lipolysis of TAG emulsions covered by phospholipids, which are resistant to pancreatic lipase[94]. Gastric lipase thus plays an essential role in the lipolysis of milk fat globules [39, 95] and triggers the activity of pancreatic lipase [94, 96].

Although gastric lipase can hydrolyze the three ester bonds of TAG molecules, this enzyme has however a preference for hydrolyzing the ester bond at the *sn*-3 position of TAGs[29-33, 35, 97]. This stereopreference of gastric lipase has been demonstrated using synthetic

chiral TAG[97], enantiomeric glyceride analogs[31] and prochiral triglycerides like triolein[30]. In the latter case, the *sn*-3 stereopreference of gastric lipase gives rise to a transient enantiomeric excess of 1,2-*sn*-diolein in the course of TAG hydrolysis (Figure 5A) [33], that can be used as a tracer of gastric lipase activity *in vivo*[32]. Indeed, a large enantiomeric excess (35-65%) of 1,2-*sn*-diolein was observed in gastric contents of dogs which received a test meal containing triolein as the sole source of fat (Figure 5B) [32]. The enantiomeric excess of 1,2-*sn*-diolein is only transient and decreases with the hydrolysis level because 1,2-*sn*-diolein is further hydrolyzed and converted into 2-sn-monoolein (Figure 5A). The relative specificity constants of TAG conversion into 1,2-*sn*-DAG (or 2,3-*sn*-DAG) and 1,2-*sn*-DAG (or 2,3-*sn*-DAG) conversion into 2-*sn*-MAG have been determined for rDGL and some other lipases (Table 4). These specificity constants allow the quantitative comparison of the stereoselectivity fingerprints for various lipases acting the prochiral triolein substrate [34]. While rDGL preferentially forms 1,2-*sn*-DAG from TAG and then preferentially hydrolyzes 1,2-*sn*-DAG versus 2,3-*sn*-DAG, the lipase from *Rhizomucor miehei* shows a reverse stereospecificity (Table 4).

The stereospecificity of gastric lipase is important for its biological function: HGL is the first lipase involved in the digestion of milk fat and its *sn*-3 stereopreference allows the release in the stomach of short and medium chain fatty acids that are specifically found at the *sn*-3 position of milk TAGs[98-100]. This was shown for instance in gastric samples collected from premature infants who received mother milk [101]. Although caprylic acid (C8:0) is found at low levels in human milk (0.07±0.01% wt/wt of total milk fatty acids in this study[101]; Figure 6A), the proportion of C8:0 in FFA found in gastric contents after 90 min of digestion was 50-fold higher (3.48±1.53% wt/wt of total FFA; Figure 6B) than the proportion of C8:0 in human milk fatty acids, indicating a preferential release of the fatty acids at the *sn*-3 position of milk TAGs by HGL. Fatty acids with chain length up to C12 can be absorbed in

the stomach[102, 103] and the action of gastric lipase therefore allows an early uptake of short and medium chain fatty acids from milk. This could be a faster route to provide energy via fatty acids and supplementation of meals with medium chain triglycerides (MCT) is based on these findings. Nevertheless, the gastric absorption of medium chain fatty acids like C8:0 may be more important for the regulation of appetite through the acylation of the gastrointestinal hormone ghrelin. Ghrelin, produced by X-cells in the gastric corpus initiates food uptake [104] but also growth hormone release [110]. Ghrelin needs to be acylated by C8:0 to be functional and this octanoylation is specifically ensured by ghrelin-O-acyltransferase (GOAT)[105]. C8:0 partly comes from the diet (milk, MCT) and is directly used for the acyl-modification of ghrelin[106, 107]. It is therefore assumed that gastric lipase plays an important role in the release of C8:0 for ghrelin acylation in the stomach. The mechanism by which C8:0 is absorbed and then used for ghrelin octanoylation has however not been studied in detail so far.

A last and important property of gastric lipase is its resistance to pepsin. No degradation of HGL by pepsin is observed in gastric juice at pH values ranging from 2 to 7, i.e. the pH range in which HGL is highly stable and preserves its enzyme activity. A degradation of HGL by pepsin is only observed at pH 1 and below, and it occurs after HGL is first denatured by gastric acid [5]. Therefore, the 3D structure of native HGL does not provide cleavage sites to pepsin. The removal of N-glycosylation sites in HGL (Asn15, Asn80, Asn252 and Asn308) by site-directed mutagenesis has revealed that N-glycosylation plays a role in the resistance to pepsin hydrolysis [108].

Replacement of gastric lipase by other lipases

In summary, gastric lipase combines biochemical properties that make this enzyme rather unique among lipases: (i) stability and activity in the pH range 2 to 7 with an optimum around pH 5; (ii) high tensioactivity that allows resistance to bile salts and penetration into

phospholipid layers covering TAG droplets; (iii) *sn*-3 stereospecificity for TAG hydrolysis; and (iv) resistance to pepsin. Most of these properties have been known for more than two decades and should provide a rational basis for replacement of gastric lipase by other lipases when gastric lipase is not available.

Among the microbial lipases that have been often used for *in vitro* gastric digestion, only the acid-resistant fungal lipase from *Aspergillus niger* has an optimum activity at acidic pH from 5 to 6.5, is active down to pH 2.5 and is resistant to pepsin (Table 4) [109]. It is however inhibited by bile salts [110] , which indicates that it is a less tensioactive enzyme than gastric lipase, and its stereospecificity in TAG hydrolysis is unknown.

The *sn*-3 strereopreference of gastric lipase appears to be important for milk fat digestion in early life and probably for the digestion dairy products like cheese in adults. Only a few lipases have been shown to display *sn*-3 stereospecificity. Using prochiral triolein as substrate, several lipases have been ranked based on the enantiomeric excess of 1,2-*sn*-diolein vs. 2,3-*sn*-diolein (*sn*-3 stereopreference) or 2,3-*sn*-diolein vs. 1,2-*sn*-diolein (*sn*-1 stereopreference) measured at low TAG hydrolysis levels (3-6 %; Table 3) [29, 111]. Apart from gastric lipases, *sn*-3 stereopreference in TAG hydrolysis was reported for the plant lipase from *Carica papaya* latex (CPL) [111], dog pancreatic lipase, fungal lipases from *Fusarium solani* and *Penicillium simplicissimum*, and bacterial lipases from *Chromobacterium viscosum* and *Pseudomonas glumae* [29]. Only *Fusarium solani* cutinase was however found to display a high *sn*-3 stereospecificity like gastric lipases (Table 3). Among the *sn*-3 lipases, only CPL was tested under test meal conditions *in vitro*. CPL shows an optimum activity and stability at pH 6-7, in the presence and absence of bile, but its stability at pH below 5 is very low[112]. It is resistant to many proteases like papain[113], but its resistance to pepsin was not tested so far. Among the microbial lipases used for *in vitro* gastric digestion, lipases from *Rhizopus oryzae* (same as *Rhizopus arrhizus*), *Candida rugosa*, *Pseudomonas fluorescens* and *Rhizomucor*

miehei are all *sn*-1 stereospecifc enzymes (Table 2) [29, 33, 34] and therefore display a reversed stereospecificity compared to gastric lipase. The lipase from *Aspergillus niger* displays a 1,3-*sn*-regioselectivity in TAG hydrolysis but its stereospecificity towards *sn*-1 or *sn*-3 position was not demonstrated to our knowledge. One article reports *sn*-3 stereospecificity for a lipase from *Aspergillus niger* NCIM 1207 strain but careful reading reveals that *sn*-3 stereospecificity cannot be deduced from the thin layer chromatography analysis of triolein hydrolysis presented in this article [114]. Indeed, the authors identified a band with the same retention time as 1,2-diolein used as reference standard, and concluded that the ester bond at *sn*-3 position of triolein was cleaved by *Aspergillus niger* lipase, but 1,2-diolein and 2,3-diolein have the same retention time and cannot be distinguished using this separation method [114].

Other preduodenal lipases from the acid lipase gene family [115, 116] and various mammalian species might be used for replacing human gastric lipase. Depending upon the species investigated [117, 118], preduodenal lipase is synthesized and secreted either by the von Ebner glands of the posterior area of the tongue (lingual lipase in small rodents) or the oro-pharyngeal tissues (pregastric or pharyngeal lipase in young ruminants) or in the gastric mucosa (gastric lipase in omnivores and monogastric herbivors) (Figure 7 and Table 5). Whatever the species, preduodenal lipase is produced from a gene orthologous to the gene of HGL in humans (LIPF) [77, 115, 116, 119, 120]. The pregastric lipase of ruminants is found in young animals (calf, kid goat, lamb) during the lactation period and it is responsible for the hydrolysis of milk fat globules in the abomasum (stomach) [121-123]. In addition to this physiological role, pregastric lipases of ruminants have been used in the dairy industry for various applications: enhancing the flavour of cheeses, accelerating cheese ripening, manufacturing cheese-like products and lipolysing butterfat and cream [123-125]. Each type of pregastric lipase, depending upon the animal species, gives rise to its own characteristic

flavour profile due to the fatty acid and/or regio- and stereoselectivity of the corresponding enzyme [126, 127]. Commercial pregastric lipases are available from various suppliers (DSM for Capalase® K and Capalase® KL lipases; CHR Hansen for Lipase Kid-Goat ST20, Lipase Calf 57 LFU, Spice IT™ AC and Spice IT™ AG; DuPont Danisco, Clerici-Sacco) in the form of liquid extracts, pastes and vacuum or freeze dried powders. Studies on pregastric lipases have often been carried out with these crude enzyme preparations [128]. Only few kinetic and biochemical studies have been performed with the enzymes purified from either the epiglottis / gullet, the GI tract region bordered anteriorly by the vallate papillae of the tongue and posteriorly by the pharyngeal end of the oesophagus [129-133] or complete rennet paste [134]. In this later case, it is not always clear from the literature whether the enzyme characterized and named pregastric esterase is identical to the acid pregastric lipase. Like gastric lipases, the pregastric lipases of ruminants show an optimum activity at low pH (4 for lamb and 5-6 for calf) and their activity can be measured in the presence of bile salts [117, 130]. These enzymes may therefore be used for *in vitro* digestion studies and their abundance (Table 5) in animals present in the human food chain suggests the possibility to source these enzymes as by-products of meat production. One limitation for the use of pregastric lipases of ruminants might be however their resistance to acid conditions. Indeed, these lipases are less resistant to acid denaturation than gastric lipase with a pH threshold for enzyme inactivation at around 3.5 while gastric and lingual lipases remain stable down pH 1.5 in general [117]. One article reports the use of calf pregastic lipase in an *in vitro* model of gastric digestion but the enzyme stability was found to be low and the reproducibility of results was impaired [135].

Production of gastric lipase

Gastric lipase has been obtained and characterized from various sources because it is a candidate for pancreatic enzyme replacement therapy (PERT) in patients with pancreatic

exocrine insufficiency (PEI) [76, 136, 137]. Most advances on the biochemical characterization of gastric lipase have been obtained from the enzyme (HGL) purified from human gastric juice [138] or stomachs from various species like rabbit [89] and dogs [53]. Although purified HGL has been tested *in vitro* under test meal conditions and compared to human gastric juice [38, 63, 139], the use of such enzyme is limited by the availability of gastric juice and ethical considerations. The production of recombinant gastric lipase as an alternative and sustainable source of enzyme was explored since the 1980s. An active recombinant human gastric lipase (rHGL) was found to be produced in the yeasts *Saccharomyces cerivisae* [77, 140] and *Schizosaccharomyces pombe* [141, 142], in Cos-7 and human embryonal kidney (HEK-293) cells [123, 124], in insect cells [120, 121], as well as *Nicotiana benthamiana* leaves using a transient expression system [143]. Yeast and insect cell systems were not suitable however for an industrial production since the lipase either remained stacked to the yeast cell wall [76, 120] or produced at low levels (< 40 mg/L of culture) [140, 144, 145]. Recombinant dog gastric lipase (rDGL) was also produced in insect cells using a baculovirus expression vector (35 mg/L of culture) [146]. Production using baculovirus-infected insect cells was however not continuous and led to a protein with lower glycosylation compared to native gastric lipase and poor resistance to pepsin [108]. In addition to high production costs, the biochemical properties of rHGL produced in insect cells preclude its used for *in vitro* digestion in the presence of pepsin. rHGL produced in tobacco leaves appeared to be highly resistant to pepsin and reasonable production yields could be envisioned [143]. Production yield in Cos-7 and HEK-293 cells were not documented [147, 148] but producing a recombinant protein in these cells is usually more expensive than in yeast, fungi or bacteria.

Whatever the application of recombinant gastric lipase in PERT or *in vitro* digestion, the enzyme should be produced at a low cost, similar to that of porcine pancreatic extracts. This was achieved with the production of a recombinant dog gastric lipase (rDGL) in

transgenic plants, in which therapeutic proteins can be produced at reasonable cost (US $ 5.90-43 per g) compared the same proteins produced in mammalian cells (US $ 300-3,000 per g) [149]. DGL was selected because it was the lipase showing the highest lipolytic activity on long chain TAGs at low pH levels, with an optimum activity at pH 4 [53]. The cDNA encoding DGL [150] was used to transform first tobacco [151, 152] and then corn [80, 149]. rDGL was produced at a high levels in both tobacco leaves and corn seeds (approx. 1 g/kg of corn; Personnal communication from Dr Dominique Mison, Meristem Therapeutics SA, Clermont-Ferrand, France). The production and clinical development of rDGL was however seriously impaired by concerns about GMO and transgenic plants in France and Meristem Therapeutics SA stopped its activities in 2008. Although rDGL is no more in production today, a few batches have been available for *in vitro* digestion studies. rDGL was thus used for testing the gastric lipolysis of lipid-based drug delivery systems [64, 87, 88, 153, 154], of citric acid esters of mono- and diglycerides (CITREM) and CITREM-containing infant formula/emulsions [67] and various emulsions of flaxseed oil [68].

Another possibility to obtain gastric lipase is the purification from animal sources since animal and human gastric lipases share similar biochemical properties [53, 89]. The production of native gastric lipase from animal stomachs was investigated by the pharmaceutical industry (Jouveinal Laboratories, Fresnes, France) and CNRS in the 1980s, in order to produce a drug product similar to porcine pancreatic extracts for the treatment of PEI [136]. After a screening of preduodenal lipases in different mammals [117], the rabbit was identified as one of the species with the highest level of lipase in gastric tissues, in relation to its size (Figure 7 and Table 5). Since rabbit is present in human food chain, it could be used as an avaiable source of gastric lipase as by-product of meat production. Protocols to prepare rabbit gastric extract (RGE) [136] and further purified rabbit gastric lipase (RGL) [89] were implemented. The clinical development of RGE as a drug from animal origin was later stopped when prion diseases

potentially transmissible from animal to humans were discovered but, nevertheless, the feasibility of RGE production was established at that time. There is today a new interest in RGE for *in vitro* digestion experiments, particularly because it contains both gastric lipase and pepsin and can be used as a global source of gastric enzymes, like porcine pancreatic extracts for pancreatic enzymes. A combination of RGE and porcine pancreatic extracts was tested in a two-step static digestion model and the *in vitro* gastrointestinal lipolysis of test meal TAG was found to be similar to the lipolysis obtained with either human gastric and pancreatic juices or purified HGL and HPL [65]. More recently, purified RGL and pepsin were used for testing the gastric lipolysis and proteolysis of different milk formula under premature infants conditions [39].

Conclusion

The development of physiologically relevant *in vitro* models of gastric digestion requires that the gastric phase of lipolysis and the role of gastric lipase are taken into account. Gastric lipolysis is not only significant and triggers further action of pancreatic enzymes, but gastric lipolysis and proteolysis processes are tightly linked. The data collected in this review should help researchers in designing *in vitro* gastric digestion models with relevant pH values and lipase. So far, none of the microbial lipases used to replace gastric lipase displays the biochemical properties of this unique enzyme and their use should not be recommended. If one has to select single gastric conditions for static digestion model, the use of pH conditions prevailing in the stomach at 50% gastric emptying (pH 5-5.5) makes more sense than using low pH values corresponding to fasting conditions or to low amount of food into the stomach. This will mean however a major adaptation of the current practice, often consisting in a first incubation at low pH (gastric phase; mostly pH 2) before rising the pH to 6-7 for the intestinal phase of digestion. Under these conditions, gastric lipolysis, if any, is certainly extremely low

and its impact on overall digestion and intestinal events may not be seen. These drastic changes in pH conditions may also affect the stability and structure of food and emulsions, in a manner that does exist *in vivo*, and thus may create artifacts. Reconsidering the gastric phase of *in vitro* digestion will certainly imply to re-evaluate the data previously obtained with static intestinal digestion models.

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Figure legends

Fig. 1: *Variations in gastric pH during test meal digestion in healthy volunteers.* Panel A: pH variation as a function of time during a liquid test meal (256 values from 30 individual experiments [28, 48, 63]). Curve-fitting equation obtained by polynomial regression: y=6.5905+0.0061x-0.0008x^2+6×10^{-6}x^3-10^{-8}x^4, R^2=0.6456; Panel B: same pH values plotted as a function of meal gastric emptying (%).Curve-fitting equation obtained by polynomial regression: y=6.6731-0.0484x+0.0011x^2-10^{-5}x^3, R^2=0.7412; Panel C: pH variation as a function of time during a solid-liquid test meal (545 values from 53 individual experiments [13, 63]). Curve-fitting equation obtained by polynomial regression: y=5.672+0.002x-0.0013x^2+10^{-5}x^3-5×10^{-8}x^4,+7×10^{-11}x^5, R^2=0.6923; Panel D: same pH values plotted as a function of meal gastric emptying (%). Curve-fitting equation obtained by polynomial regression: y=5,7531-0.0245x+0.0002x^2-4×10^{-6}x^3, R^2=0.6506.

Fig. 2: *Variation in gastric lipase concentration in gastric contents* ($[HGL]_g$) *during test meal digestion in healthy volunteers.* Panel A: $[HGL]_g$ variation as a function of time during a liquid test meal (256 values from 30 individual experiments [28, 48, 63]). Curve-fitting equation obtained by polynomial regression: y=4.041+0.1944x+0.0017x^2, R^2=0.471; Panel B: same $[HGL]_g$ values plotted as a function of meal gastric emptying (%).Curve-fitting equation obtained by polynomial regression: y=4.6117+1.2653x-0.0368x^2+0.0003x^3, R^2=0.5459; Panel C: $[HGL]_g$ variation as a function of time during a solid-liquid test meal (545 values from 53 individual experiments [13, 63]). Curve-fitting equation obtained by polynomial regression: y=1.2388+0.485x-0.0027x^2+10^{-5}x^3, R^2=0.3307; Panel D: same $[HGL]_g$ values plotted as a function of meal gastric emptying (%). Curve-fitting equation obtained by polynomial regression: y=0.9692+0.7024x-0.0165x^2+0.0002x^3, R^2=0.1918.

Fig.3: *Structure of human gastric lipase.* Panel A: Molecular surface representation of HGL 3D structure with the lid in the closed conformation obtained by X-ray crystallography (Protein Databank ID: 1HGL) [78]. All amino acid residues are shown in purple, except the lid colored in blue. The N-terminal end (1-9) of HGL is not defined in the electronic density and is only shown as Cα-tracing on the left side of HGL. The conformation of the open lid is shown as ribbon model with α-helices in cyan. Panel B: 3D model of HGL with the lid in the open conformation, built from HGL-rDGL sequence alignment and the known X-ray structure of open rDGL (Protein Databank ID: 1K8Q) [80]. The conformation of the closed lid is shown as ribbon model with α-helices in cyan. The active site serine residue (Ser153) is shown in red. Panels C and D: same views as A and B, respectively, but hydrophobic amino acids are colored in white and polar amino acids in yellow.

Fig.4: *Comparison of HGL and HPL interfacial properties.* Panel A: Effects of bile salts (NaTDC, sodium taurodeoxycholate) on the lipase activity of of HGL, HPL and HPL-colipase complex (2-fold molar excess of colipase); Adapted from [52, 74, 155]. Panel B: Adsorption/penetration of HGL, HPL and HPL-colipase complex (1:1 molar ratio) onto egg phosphatidylcholine monolayer spread at the air-water interface. Data for HGL are from [93, 145]: Data for HPL and HPL-colipase complex are from [91].

Fig.5: *Stereoselective hydrolysis of TAG by gastric lipase.* Panel A: Reaction scheme for the enzymatic conversion of TAG into DAG and their subsequent conversion into MAG by lipases. The $k_{\text{subscript}}$ symbols represent the specificity constants for the corresponding reactions. Panel B : DAG enantiomeric excess measured in gastric samples recovered from dog stomach in the course of test meal digestion. The liquid test meal contained only triolein as a prochiral triglyceride substrate. DAG enantiomeric excess ($ee_{1,2}$ %) was estimated using

the following equation: $ee_{1,2}$ % = 100×[1,2-*sn*-diolein - 2,3-*sn*-diolein]/[1,2-*sn*-diolein + 2,3-*sn*-diolein], taking into account the respective amounts of 1,2-*sn*-diolein (hydrolysis of *sn*-3 ester bond in triolein) and 2,3-*sn*-diolein (hydrolysis of *sn*-1 ester bond in triolein) in gastric samples, obtained after total lipid extraction, derivatization of DAG into diastereomeric carbamates and separation by HPLC. Adapted from [32].

Fig. 6: *Selective release of C8:0 fatty acid from mother milk fat by gastric lipase in the stomach of newborns.* Panel A: Typical separation by gas chromatography of human milk fatty acids. Panel B: Respective proportions of caprylic acid (C8:0) in human milk triglycerides (TAG) and free fatty acids recovered from gastric content of newborns 90 minutes after meal ingestion. Adapted from 106.

Fig.7: *Tissular localization of preduodenal lipase activities in different mammalian species.* Lipase activities were measured with the assay conditions optimized for gastric lipase and are expressed in units (U) per g of fresh tissue, with 1 U = 1 µmole of fatty acid released per minute. Adapted from [72, 117].

Table 1 : *pH values and lipase sources used in static in vitro digestion models.* These data were extracted from a literature survey of 340 articles published from 1967 to 2015 period and containing "*in vitro* digestion" in their title.

Digestion phase	*Parameter*	Value / origin	*% of total in vitro models*
Gastric phase	pH	<2	21
		2	53
		>2	26
	Lipase	no lipase	94.9
		human gastric juice	2.3
		Rhizopus oryzae lipase	0.9
		Aspergillus niger lipase	0.45
		purified gastric lipase	1
		unknown origin	0.45
Duodenal phase	pH	<6.5	12
		6.5 - 7.5	80
		>7.5	8
	Lipase	no lipase	9.6
		human duodenal juice	4.3
		pancreatin	70.9
		lipase from hog	10.4
		pancreatin + lipase from hog	3.9
		phospholipase A2	0.4
		cholesterol esterase	0.4

Table 2 : *Comparison of main biochemical properties of gastric and microbial lipases.* Adapted from [29, 111]. Enantiomeric excess of 1,2-*sn*-diolein (1,2-*ee*) or 2,3-*sn*-diolein (2,3-*ee*) were measured at 3% hydrolysis of triolein, except for *Carica papaya* lipase (2%)[111]. n.d., not documented. [a] Some names of microbrial species have changed and former names are indicated in parenthesis. [b] relative to TAG hydrolysis. [c] relative to long chain TAG hydrolysis. [c] since bile salts can be lipase activators at low concentration (<CMC) and lipase inhibitors at high concentration (>CMC) [156, 157], only the inhibition at bile salt concentration >CMC was considered here.

Lipase [a]	*Regioselectivity* [b]	*Stereospecificity* [b]	*Optimum pH* [c]	*Inhibition by bile salts* [d]	*Resistance to pepsin*
Gastric lipase	No [53]	*sn*-3 [30]	4-5.4 [52, 53, 89]	No [52, 74]	Yes [5]
Rhizopus oryzae (or *arrhizus*, or *delemar*)	1,3 [158]	*sn*-1 [29]	7-8 [159]	Yes [116, 117]	Yes [159]
Aspergillus niger	1,3 [160]	n.d.	5-6.5 [159, 161]	Yes [110]	Yes [113, 115]
Candida rugosa (or *cylindracea*)	No [162]	*sn*-1 [29]	5-8 [161, 163]	Yes [163] No [164]	No [165]
Pseudomonas fluorescens	1,3 [29]	*sn*-1 [29]	7-10 [166-170]	n.d.	n.d.
Rhizomucor miehei (or *Mucor miehei*)	1,3 [171]	*sn*-1 [29, 33]	7-8.2 [161]	n.d.	n.d.
Burkholderia cepacia (or *Pseudomonas cepacia*)	No [171]	*sn*-1 [172-174]	8 [175, 176]	n.d.	n.d.

Table 3 : *Stereoselectivity of various lipases determined with the prochiral triolein substrate.* Adapted from [29, 111]. Enantiomeric excess of 1,2-*sn*-diolein (1,2-*ee*) or 2,3-*sn*-diolein (2,3-*ee*) were measured at 3% hydrolysis of triolein, except for *Carica papaya* lipase (2%)[111]

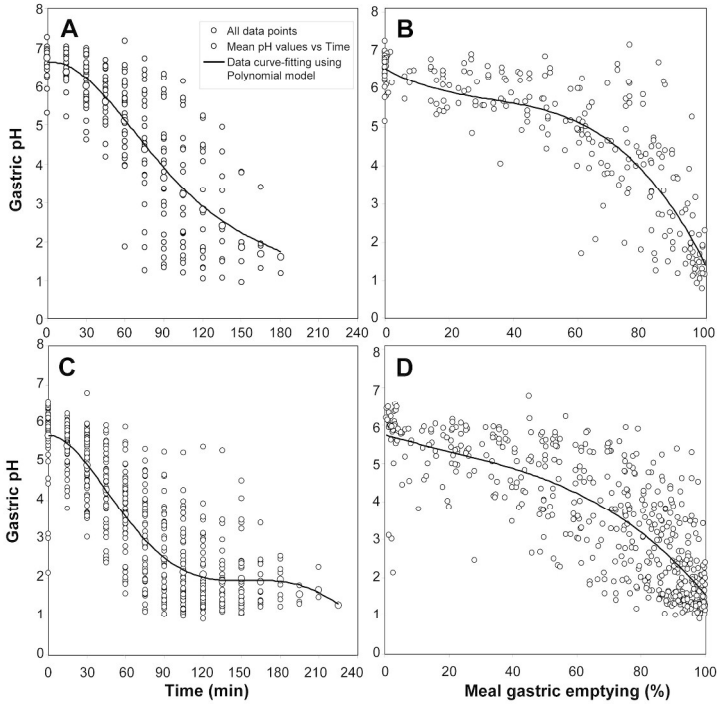
Lipases	*Stereopreference*	*1,2-ee (%)*	*2,3-ee (%)*
Dog gastric lipase	*sn*-3	76.2	-
Human gastric lipase	*sn*-3	73.0	-
Rabbit gastric lipase	*sn*-3	46.4	-
Candida antarctica A lipase	*sn*-1	-	18.5
Candida antarctica A lipase	*sn*-1	-	38.5
Carica papaya lipase	*sn*-3	17.0	-
Candida rugosa lipase	*sn*-1	-	18.5
Chromobacterium viscosum lipase	*sn*-3	21.5	-
Dog pancreatic lipase	*sn*-3	9.0	-
Fusarium solani cutinase	*sn*-3	71.9	-
Geotrichum candidum A lipase	*sn*-1	-	28.3
Geotrichum candidum B lipase	*sn*-1	-	24.8
Thermomyces lanuginosus lipase	*sn*-1	-	26.0
Rhizomucor miehei lipase	*sn*-1	-	88.6
Pseudomonas aeruginosa lipase	*sn*-1	-	15.8
Penicillium camemberti lipase	*sn*-1	-	64.7
Pseudomonas fluorescens lipase	*sn*-1	-	68.1
Pseudomonas glumae lipase	*sn*-3	25.0	-
Penicillium simplicissimum lipase	*sn*-3	30.5	-
Rhizopus arrhizus (*orizae*) lipase	*sn*-1	-	57.5

Table 4 : *Comparison of the stereoselectivity fingerprints for various lipases acting the prochiral triolein substrate.* The $k_{subscript}$ symbols $k_{+1,2}$, $k_{+2,3}$, $k_{-1,2}$ and $k_{-2,3}$ represent the relative specificity constants, with $k_{+2,3}$ (specificity constant for the removal of a fatty acid from the *sn*-1 position of the triacylglycerol) = 1. Subscrit definition: +1,2, relative to the reaction producing the 1,2-*sn*-DAG; +2,3, relative to the reaction producing the 2,3-*sn*-DAG; -1,2, relative to the reaction consuming the 1,2-*sn*-DAG; -2,3, relative to the reaction consuming the 2,3-*sn*-DAG. The specificity constant values listed in the Table are from [34].

Lipases	$k_{+1,2}$	$k_{+2,3}$	$k_{-1,2}$	$k_{-2,3}$
Dog gastric lipase	3.55	1.00	3.70	1.39
Dog pancreatic lipase	1.25	1.00	1.01	0.75
Porcine pancreatic lipase	1.00	1.00	1.74	1.70
Rhizomucor miehei lipase	0.16	1.00	0.15	2.16
Yarrowia lipolytica LIP2 lipase	1.08	1.00	1.93	1.69

Table 5 : *Tissular localization and amounts of preduodenal lipases in different mammalian species.* Lipase activities were measured with the assay conditions optimized for gastric lipase and are expressed in units (U), with 1 U = 1 µmole of fatty acid released per minute. Adapted from [117] for animal species and [72] for humans.

Species	*Localization*	*Lipase activity*	
		U/organ	U/kg body weight
Rat	Tongue	73	243
Mouse	Tongue	7	100
Calf	Pharynx/gullet	13,130	73
Lamb	Pharynx/gullet	3,000	55
Guinea pig	Stomach	432	864
Rabbit	Stomach	7,130	3,240
Dog	Stomach	13,590	230
Horse	Stomach	30,860	62
Hog	Stomach	5,185	52
Baboon	Stomach	1,315	88
Macaque	Stomach	2,110	210
Human	Stomach	164,000	2,340



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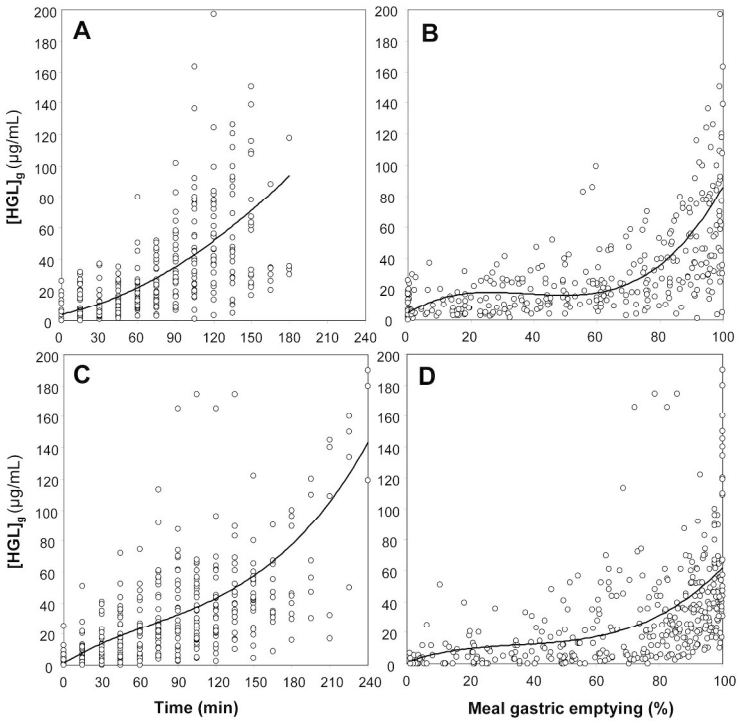

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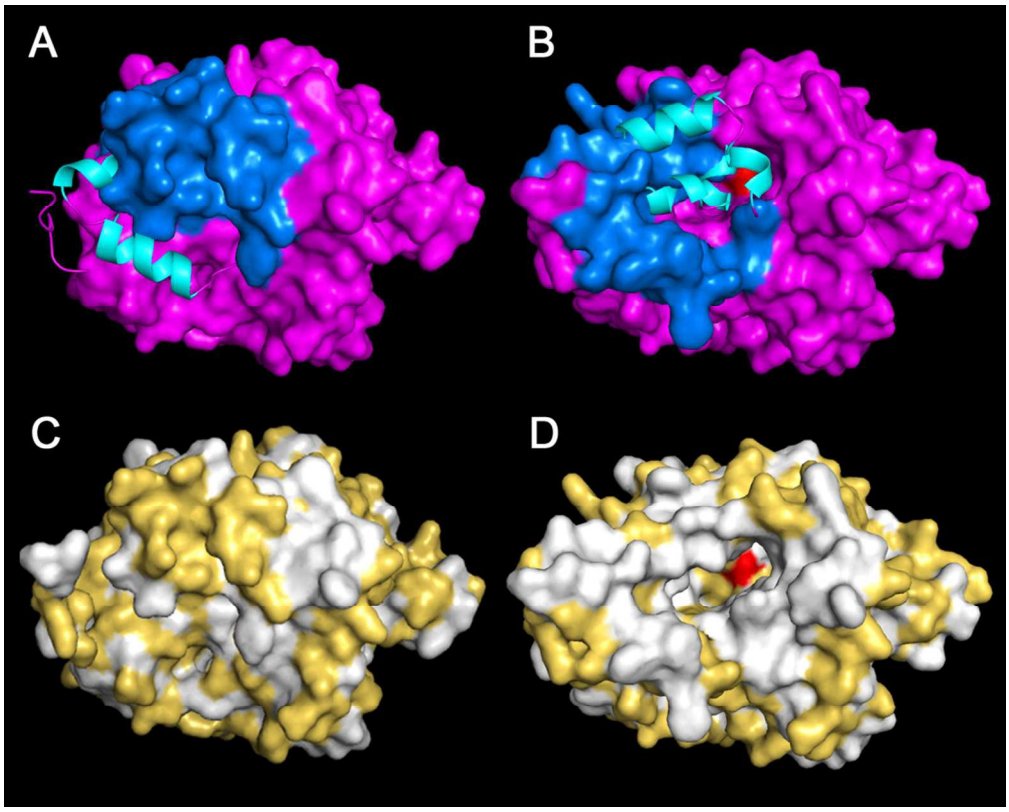

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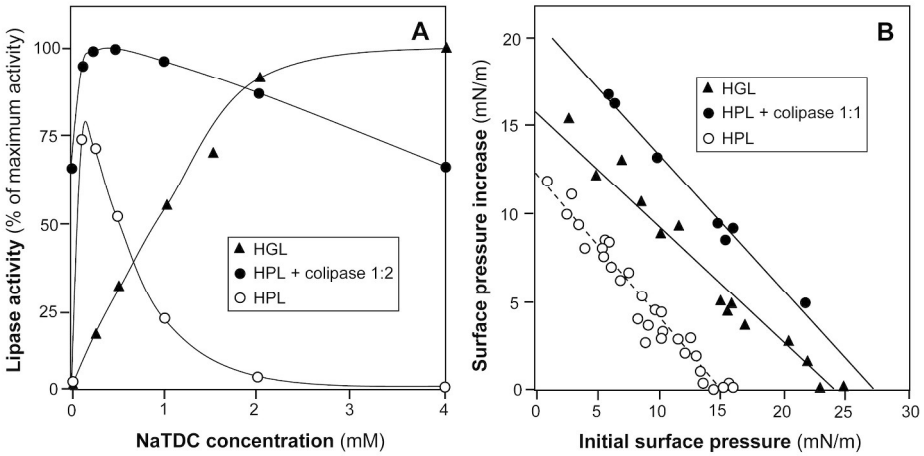

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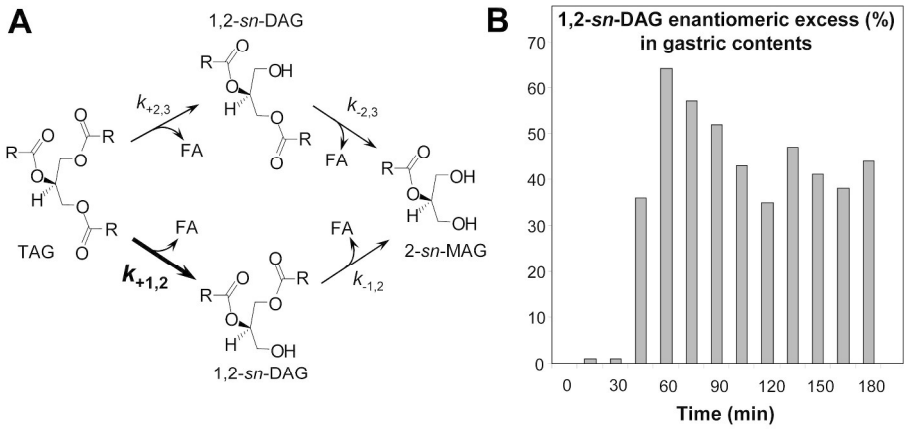

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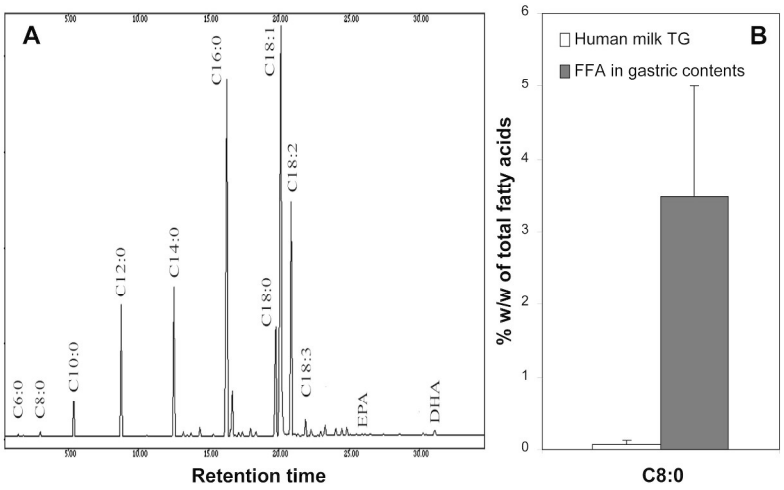
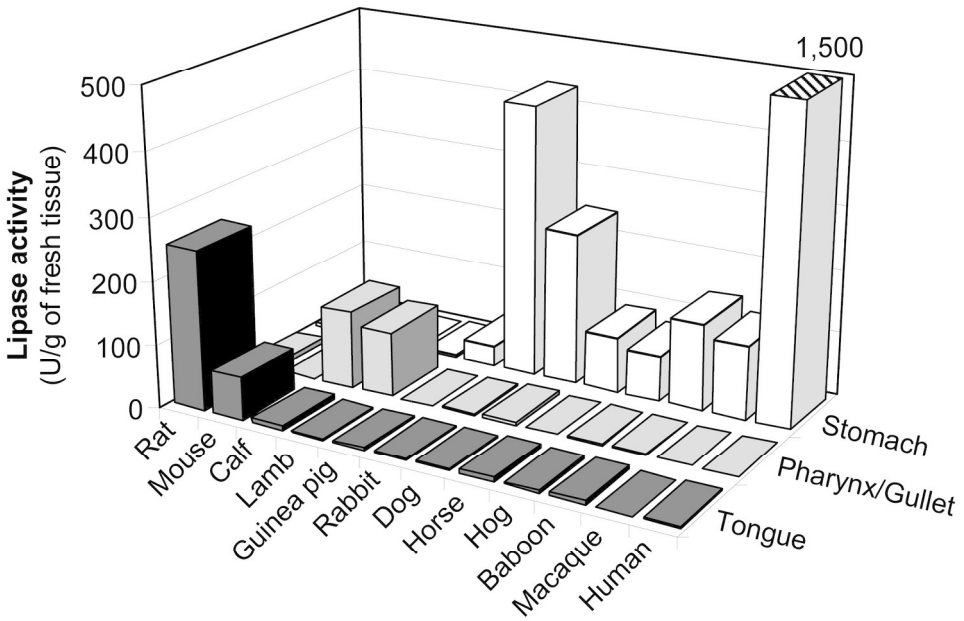
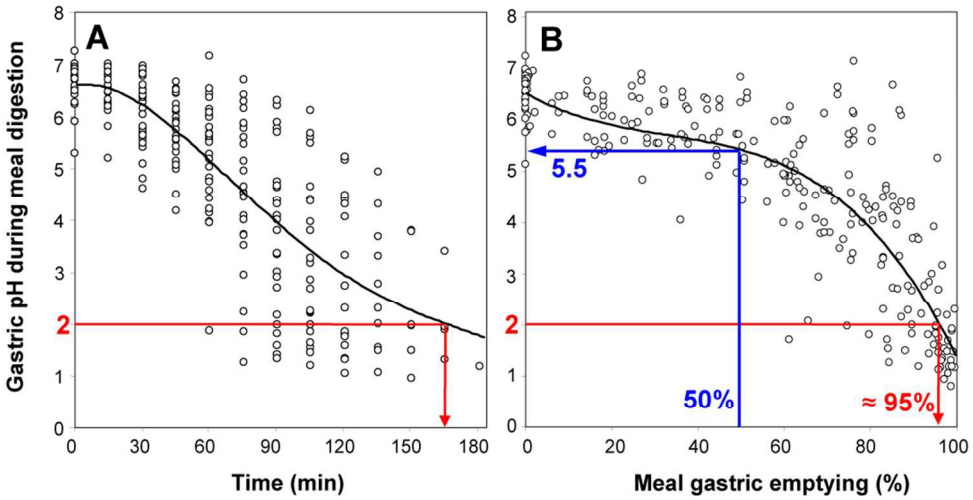


254x190mm (300 x 300 DPI)



178x120mm (300 x 300 DPI)



189x99mm (150 x 150 DPI)