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Digestion of Food Ingredients and Food Using an *In Vitro* Model Integrating Intestinal Mucosal Enzymes

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Abstract

This study focuses on the development of an *in vitro* digestion model simulating oral, gastric and small intestinal fluids, applicable to the digestion of all three macronutrients, carbohydrates, proteins and lipids. To that aim, the effect of integrating intestinal mucosal enzymes in the small intestinal phase of the digestion reaction was investigated, together with that of other parameters including pepsin and pancreatin concentration, and pH of the small intestinal phase. Individual carbohydrate and protein ingredients for which digestive properties *in vivo* are generally understood (*i.e.* common corn starch, whey protein isolate) were used as reference substrates to validate the model and, at the end of development, the model was applied to evaluate the digestion of a reference lipid ingredient (*i.e.* olive oil) and of all three macronutrients present in a whole food system. Carbohydrate, protein and lipid hydrolysis was monitored, respectively, by quantitation of glucose, free amino groups and free fatty acids released at different times of digestion. The results demonstrate that including intestinal mucosal enzymes in the intestinal phase of digestion *in vitro* allows efficient digestion of starch and other carbohydrates into final product glucose and it also influences protein hydrolysis. Digestion profiles consistent with published *in vitro* and *in vivo* data support the validity of the developed method as an advanced tool for screening digestion of all three macronutrients whether presented alone or in a whole food system, all in a single digestion reaction.

Keywords

In Vitro Model, Carbohydrate, Protein, Lipid, Digestion, Intestinal

1. Introduction

Development of new food ingredients and food systems is an essential aspect of innovation in the food industry and, as an integral part of the development process, a profound chemical and rheological characterization of the new food ingredient or system is required. In addition, understanding of the digestive properties also becomes critical to the development of those new ingredients and food. Further, there is an increasing interest in the design of food structures to manipulate rate of digestion, as an approach to impact satiety and/or control location of nutrient delivery along the gastrointestinal tract [1]. A number of research articles have now demonstrated that matrix, structure, ingredient interactions and processing of foods can have a critical impact on digestion [1] [2] [3]. *In vivo* digestion screening tests using animal or human subjects is economically hindered, time consuming and, in the case of ingredients under development, unsafe. An alternative to *in vivo* digestion are *in vitro* digestion models which, while not without limitations, are widely accepted tools in the fields of food/feed science and nutrition to screen digestion properties prior to *in vivo* studies [4].

Digestion is a highly complex physiological process which includes multiple phases (mouth, stomach, small and large intestine), each of them characterized by specific solutions of unique composition (enzymes, organic and inorganic components), distinct pH and physicochemical/mechanical processes. That level of complexity is very difficult to completely reproduce *in vitro* and models are normally adapted to integrate the aspects of digestion deemed to be most relevant for a specific scientific question. Without doubt, dynamic models, capable of adjusting conditions throughout the digestion test [5] [6] [7] [8], have the ability to mimic a larger set of parameters than static models. Still, static models have an economical and large throughput advantage.

Historically, a number of static *in vitro* digestion models varying within a wide range of conditions have been developed [9] [10], often focused on a particular application and dedicated to studying one single type of macronutrient. For example, starch digestibility *in vitro* is typically evaluated by the technique of Englyst [11] which simulates gastric and small intestinal digestion, the later including porcine pancreatic α-amylase and fungal amyloglucosidase as digestive enzymes. This technique evaluates starch digestibility by determining, through glucose analysis, three fractions: a rapidly digested (20 min), a slowly digested (20 - 120 min) and a resistant to digestion (not digested within 120 min) fractions. The *in vitro* analysis of lipid digestion, on the other hand, is often performed using a pH stat method, including a small intestinal digestion with pancreatic enzymes and for which the amount of neutralizing alkali is widely accepted to be equivalent to the fatty acids produced [12] [13]. Also a pH stat method is commonly accepted to evaluate *in vitro* digestion of proteins [14]. Inherent to the dissimilarities among these traditional methods is the inability to simultaneously evaluate the digestion *in vitro* all three macronutrients: carbohydrates, proteins and lipids.

Advancements in this respect are seen in studies by [15] reporting the development of an *in vitro* digestion model that can be used to evaluate the decomposition of both proteins and lipids in milk, and also by [16] proposing a standard protocol for *in vitro* digestion of macronutrients and demonstrating predicted digestibility of starch in mouth, of protein in stomach and of lipid in the small intestine. More recently, a static *in vitro* digestion model [17] proposes harmonized conditions of digestion based on most recent knowledge from human determinations. While these newer models more precisely simulate many of the physiological conditions, there is recognition of the lack of a final step of small intestinal digestion [15] [17], during which disaccharidases and aminopeptidase enzymes of the brush border membrane (BBM) of the enterocytes (*i.e.* intestinal mucosal enzymes) work to complete carbohydrate and protein digestion [18]. This final step is key to providing nutrients readily for absorption across the epithelial cells. Particularly for proteins, publications [19] [20] provide evidence of the impact of BBM's enzymes on *in vitro* digestibility of dietary milk proteins. To our knowledge, no evidence exists on the utilization of intestinal mucosal enzymes as an integral part of a modern static *in vitro* model of digestion of carbohydrates and whole food systems.

The aim of this study was to develop a static *in vitro* digestion model that integrates conditions for simultaneously simulating carbohydrate, protein and lipid hydrolysis of foods as a tool to screen rate and extent of digestion of macronutrients as individual ingredients and in a final food product. To that effect, integrating intestinal mucosal enzyme extracts was of essence and we focused on evaluating and optimizing amount of intestinal mucosal enzyme extract needed to be incorporated into the digestion reactions that would result in physiologically relevant digestion, primarily for carbohydrates, in addition to evaluating their impact on both carbohydrate and protein hydrolysis, upon modifying other conditions of digestion, namely pepsin, pancreatin concentrations, and pH of small intestinal solution. Selected conditions were applied to digestion of olive oil and to a whole food system. Validation of the method was performed by testing selected reference substrates of known/expected digestibility properties and outcomes based on published human, animal and/or other *in vitro* data.

2. Materials and Methods

2.1. Chemicals and Reagents

$CaCl_2 \cdot 2H_2O$ and HCl from EMD Chemicals; KSCN and Urea from JT Baker; KCl, $MgCl_2$ (hexahydrate), Na_2SO_4, NaCl, NaH_2PO_4 (monohydrate), $NaHCO_3$, KH_2PO_4, NH_4Cl, NaOH from BDH; bile (B-8381), Gastric Lipase (L8525, from *Candida rugosa*, 1 MU/mg protein), Pancreatic lipase (L3126), mucin (M1778), pancreatin (M1778), pepsin from porcine gastric mucosa (P7000), human salivary α-amylase (A-0521, 2.5 kU), Rat Intestinal acetone Powder (RIP) (I 1630), Palatinose (P2007), TCA (T6399), Thimerosal (T5125), Betulin (B9757) from Sigma; Guar gum from Spectrum; Uric acid from Alfa Aesar; $MgSO_4 \cdot 7H_2O$ from

Mallinckrodt; Pyridin (P/N 25104) and BSTFA (P/N 38834) from Pierce Biotechnology; TLC Reference Standard (Olein mixture 18-6A) from Nu-Check Prep., Inc; Common Corn Starch (Cargill 3420), Whey Protein Isolate (BiPro, Davisco Foods); Extra Virgin Olive Oil (Bella).

2.2. Description of the Model

The *in vitro* model developed to digest ingredients and foods simulates the mouth, stomach, and small intestine fluids and was adapted from [21]. The digestive solution compositions they reported for a fed state were initially followed except that glucose, glucuronic acid, glucosamine hydrochloride and bovine serum albumin were not included in our model. In addition, *Candida rugosa* Lipase (CrL) and RIP-enzyme extract were introduced into the gastric and duodenal solutions, respectively. Thus, the initial composition of the digestive solutions simulating each of the digestive compartments was as follows. Saliva cocktail: 12 mM KCl, 2.1 mM KSCN, 5.1 mM NaCl, 7.4 mM NaH_2PO_4, 20.2 mM $NaHCO_3$, 4 mM Na_2SO_4, 3.36 mM urea,15 mg/L uric acid, 25 mg/L mucin, 15 mg human α-amylase (2.5 kU)/L; pH was adjusted to 6.8. Gastric cocktail: 11 mM KCl, 47.1 mM NaCl, 2.2 mM NaH_2PO_4, 1.4 mM urea, 3.6 mM $CaCl_2$, 78.7 mM HCl, 5.5 mM NH_4Cl, 0.2 g/L guar gum, 3 g/L mucin, 2.5 g pepsin (1170 kU)/L, 15 mg *Candida rugosa* Lipase (CrL) (600 kU)/L; pH was adjusted to 1.7 ± 0.2. Duodenal cocktail: 7.6 mM KCl, 120 mM NaCl, 0.5 mM KH_2PO_4, 41.6 mM $NaHCO_3$, 1.7 mM urea, 2.2 mM HCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 9 g pancreatin (31.5 Trypsin kU)/L, 1.5 g pancreatic lipase (150 kU)/L, dialyzed and lyophilized RIP-enzyme extract (8.5 - 140 IU)/L (equivalent to about 3 - 57 g/L inclusion dose); pH was adjusted to 8 ± 0.2. Bile cocktail: 5 mM KCl, 90 mM NaCl, 69 mM $NaHCO_3$, 4.2 mM urea, 1.8 mM HCl, 2 mM $CaCl_2$, 30 g bile/L; pH was adjusted to 8.2 ± 0.2. Mixed inorganic reagents were prepared as stock solutions and kept at 4˚C. Organic reagents and enzymes were added freshly to adequate volume aliquots of the stock solutions, prior to each experiment. Working pH in final duodenal-bile mix was 7.2 ± 0.2.

At the end of development, digestive solutions differed from initial composition as follows. Gastric cocktail: inclusion of 11 times lower concentration of pepsin (0.225 g (105 kU)/L). Duodenal cocktail: pancreatin concentration was reduced to 1 g (3.5 Trypsin kU)/L, 34 IU/L (about 14 g) RIP-enzyme extract intestinal mucosal enzyme extract, pH adjusted to 6.5 ± 0.2. Bile cocktail: pH adjusted to 6.7 ± 0.2. Final pH in gastric phase of digestion and small intestinal mix was 3.5 ± 0.2 and 6.3 ± 0.2, respectively.

Per reaction, 20 - 200 mg of reference materials or test ingredients (carbohydrate, protein, or lipid) are weighted into 15 ml conical test tubes. Substrate inclusion is standardized per nutrient of interest, thus maintaining constant ratio substrate/enzyme which, depending on the nutrient concentration, can result in variable inclusion weights. Digestion starts by mixing reference substrate or test ingredient with 1 ml of pre-warmed saliva cocktail (5 U α-amylase) in a 15 ml

conical test tube followed, after 5 min, by addition of 2 ml pre-warmed gastric cocktail (211 U pepsin, 1200 U CrL). Working gastric pH is 3.5 ± 0.2, representative of half emptying time *in vivo* [22]. After 2 h incubation at 37˚C with constant head-over-heels rotation, 0.3 ml of pre-warmed 0.2 M sodium phosphate buffer pH 6.5 are added to raise the pH of the saliva-gastric solution, followed by addition of 3 ml of a mixture of pre-warmed duodenal (7 U trypsin from pancreatin, 300 U pancreatic lipase, 70 mIU RIP-enzyme extract) and bile solutions (ratio 2:1) thus initiating the small intestinal phase of digestion. Thimerosal (19 mM solution) was added to final concentration of 10 ml/L to prevent bacterial growth. Working pH for small intestinal digestion was 6.3 ± 0.2, representative pH of the unstirred water layer that bathes the brush border [18]. Mixtures were incubated at 37˚C under constant head-over-heels rotation. Samples (0.33 ml) were taken recurrently at different time points during gastric (0, 2 h) and small intestine (0.5, 1, 2, 3, 4 h) phases of digestion. Gastric samples were neutralized with 5 M NaOH. All samples were centrifuged at 13,000 rpm and 4˚C for 3 min and rapidly frozen at −20˚C. This recurring sampling method was standard for carbohydrate and protein testing but was modified to a so called "one tube per sample and per time" method when testing lipid ingredient, thus eliminating errors inherent when aliquoting from heterogeneous liquid/oil phase digestive solutions.

Each substrate, digested in triplicate, and duplicate blanks containing all the solutions but no substrate were run under the same incubation and mixing conditions. Digestion of carbohydrates, proteins or lipids was monitored, respectively, by determining glucose, free amino groups or free fatty acids released into the digestive solutions, by the methods indicated below.

2.3. Preparation of Crude Intestinal Mucosal Enzyme Extract from Rat Intestinal Acetone Powder

Rat Intestinal acetone Powder (RIP) was used as a source of small intestinal disaccharidases (*i.e.* maltase-glucoamylase, sucrase-isomaltase) [23] [24] and amino peptidases [25]. RIP (10 g) was suspended in 200 ml of cold 0.1 M Phosphate buffer (pH 6.0) and stirred on a magnetic stirrer for 3 h at 4˚C, followed by centrifugation at 10,000 rpm for 10 min at 4˚C. The supernatant was further filtered on pre-folded filter paper #588 (Whatman Schleicher & Schuell) and dialyzed against 0.1 M Phosphate buffer (pH 6.0) for 24 h at 4˚C during which time buffer was replaced once. Dialyzed solution was lyophilized and stored frozen at −20˚C for up to 3 months. The activity of the crude intestinal mucosal enzyme extract was standardized based on the sucrase-isomaltase activity which was determined using isomaltulose (palatinose) as substrate. Briefly, 0.06 g of crude enzyme extract, 5.4 ml of 0.1 M Phosphate buffer (pH 6.0) and 0.6 ml of 29 µM isomaltulose substrate were incubated at 37˚C in a shaking water bath for 30 min. The amount of liberated glucose was determined using the glucose oxidase-peroxidase assay, as described below. One unit of activity is defined as the amount of enzyme that released 1 µmol of glucose per minute under the conditions described.

2.4. Determination of Glucose Content

The glucose content of the samples was determined by an enzymatic (GOPO)—colorimetric reaction using Stanbio glucose Liquicolor reagent (Stanbio Laboratories). Briefly, 10 µl of sample or glucose standard (0 - 4 mg/mL) were mixed with 1 ml glucose reagent and incubated at room temperature for 30 min. Absorbance was read at 500 nm (Synergy HT, Biotek). Results were expressed as percentage of total starch equivalent conversion to glucose.

2.5. Determination of Free Amino Groups

Free amino groups content was determined by TNBS (2,4,6-trinitrobenzene sulfonic acid; Picrylsulfonic Acid) method after protein precipitation with trichloroacetic acid (TCA) [26]. Briefly, 55% TCA solution was added to digesta samples at 1/10 (v/v) ratio and incubated at 4˚C for at least one hour. Samples were centrifuged at 13,000 rpm and 4˚C for 5 min and aliquots of clean supernatants were diluted 1/10 with 2.5% borate buffer pH 9.5. For reactions, 50 µl of diluted samples or 50 µl of Leucine standard solutions (0 - 6 mM) were mixed with 1 ml of 2.5% borate reagent and 20 µl of 0.5% TNBS, and incubated at room temperature for 30 min. Reactions were stopped by adding 0.5 ml of 1 M NaH_2PO_4 and absorbance was read at 420 nm. Degree of hydrolysis was expressed as % of free amino groups released from total, where total is the total number of amino groups per protein equivalent, calculated from the amount of protein included in the digestion reaction divided by the average molecular weight of amino acids (125 g/mole for most proteins) making a total number of peptide bonds per kg of protein of about 8 g equivalents [27] [28]. At each time point, the free amino group content measured in incubated blanks containing digestive juices and enzymes but no substrate, was deducted from that of test samples.

2.6. Determination of Free Fatty Acids (FFA)

FFAs were analyzed by Gas Chromatography (GC, Agilent 6890 Plus) using Flame Ionization Detection (FID). Digestion samples were acidified with 100 µl of concentrated HCl and total lipids extracted with 6 ml of Diethyl methane. The tubes were vigorously shaken and centrifuged at 1500 rpm for 5 min at room temperature. A glass pipette was used to recover 5 ml of the upper phase and transfer into clean tubes. Solvent was evaporated to dryness under Nitrogen flow. Lipids were dissolved in 5 ml of toluene containing internal standard Betulin (0.8 mg/ml) and a 90 µl aliquot of each sample was transferred to amber GC vials and mixed with 0.5 ml of Pyridine and 1 ml of BSTFA. An external standard, reference mixture containing 25% of each oleic acid, monoolein, diolein and triolein, was also dissolved in 5 ml of toluene containing the internal standard and treated as indicated for the samples. Separation was performed on a DBTM-5HT capillary column (15 m × 0.25 mm i.d. × 0.1 µm df) using Hydrogen as gas carrier at a flow rate of 40 ml/min and inlet pressure of 6.7 psi (at 110˚C). The temperature was programmed as follows: 110˚C initial, hold 0.2

min, 30˚C/min to 140˚C, 10˚C/min to 340˚C, hold 10 min, with 1 min equilibration time. Run time was 33 min and injection volume 1 µl. The FID temperature was 370˚C. Empower™ software was used to generate calibration curves from inputting weights (external and internal standards). Results were expressed as FFA released on a w/w percentage.

2.7. Determination of Trypsin Activity

Trypsin activity of pancreatin was measured as indicated in [17], with modifications. Briefly, three concentrations of pancreatin (0.5, 0.25, 0.1 mg/ml) were prepared in double distilled cold water and stirred for 10 min. For each assay, 2.6 ml of 46 mM sodium phosphate buffer containing 11.5 mM $CaCl_2$ (pH 8.1), 0.3 ml of a 10 mM TAME (p-Toluene-Sulfonyl-L-arginine methyl ester) solution in double distilled water were pipetted into quartz cuvettes, mixed by inversion and incubated at room temperature in a spectrophotometer for 5 min. Then, for each concentration tested, 0.1 ml of pancreatin solution was added and absorbance at 247 nm recorded every minute for at least 10 min. The slope (ΔA247) was determined from the initial linear portion of the curve. For blank assays, no enzyme was added and absorbance was also recorded every minute for at least 10 min and the slope was then determined. Calculation of enzyme activity was performed using the following formula:

$$\text{Units / mg} = \left[\left(\Delta\text{A247 test} - \Delta\text{A247 blank}\right) * 1000 * 3\right] / \left(540 * \text{X}\right)$$

where: ΔA247 is the slope (unit absorbance/minute) determined from the initial linear portion of the curve, 3 is volume of reaction mix (ml), 540 is the molar extinction coefficient of TAME at 247 nm, and X is the quantity (mg) of pancreatin in the final reaction mixture.

2.8. Electrophoresis

SDS-PAGE was performed using a Mini-Protean® Tetra Cell (Bio-Rad), according to manufacturer's instructions. Digestion samples and blanks (containing digestive solutions with enzymes and bile but no substrate) were reconstituted taking into account dilution during the gastric and small intestinal phases and solubilized in 2x Laemmli sample buffer (#161-0737, Bio-Rad). After addition of ß-mercaptoethanol (2%), samples were boiled for 5 min. Equal volume of samples were loaded onto Mini-Protean®TGX gels (#456-9034, Bio-Rad). Bio-Rad Precision Plus Protein Western C standard (#161-0376) was also run in the gels. Proteins were stained with Coomassie Safe (Bio-Rad Laboratories) and destained in water. Gels were imaged with a Li-Cor Odyssey infrared imager.

3. Results

3.1. Impact of Intestinal Mucosal Enzyme Extract (RIP-Enzyme Extract) Concentration on Starch Digestion

Concentration of intestinal mucosal enzyme extracts was optimized primarily

for starch digestion, macronutrient for which disaccharidases present in these extracts are required to achieve complete conversion to final product glucose. Common native corn starch, a material for which digestibility properties are commonly described in the literature, was selected as initial test substrate. *In vitro* digestion was evaluated under the initial conditions described in Materials and Methods with varying amounts of RIP-enzyme extract, ranging from 0 - 270 mIU of sucrase-isomaltase activity per reaction, added to the small intestinal solution. As shown in Figure 1, starch hydrolysis, as measured by final product glucose release, was not observed in the gastric phase of digestion while, in the small intestinal phase, starch digestion directly increased with the amount of RIP-enzyme extract. Thus, after 4 h of small intestinal phase less than 20% of starch (14.2% ± 0.5%) was converted to glucose in the absence of RIP-enzyme extract and around 35% digestion (34.7% ± 1.8%) was observed in the presence of the lowest amount of RIP-enzyme extract tested (17 mIU). Extent of starch digestion was enhanced up to 53.7% ± 1.0% in the presence of 34 mIU RIP-enzyme extract and close to 80% glucose release (77.7% ± 2.1%) was measured at the end of the intestinal phase of digestion in the presence of 70 mIU RIP-enzyme extract. Higher than 90% glucose was released at the end of small intestinal phase when including 140 or 270 mIU RIP-enzyme extract.

3.2. Effect of Varying Gastric and Duodenal Conditions in the Presence of a Given Concentration of Intestinal Mucosal Enzyme Extract on Starch Digestion

While maximum starch digestibility was demonstrated for the two highest

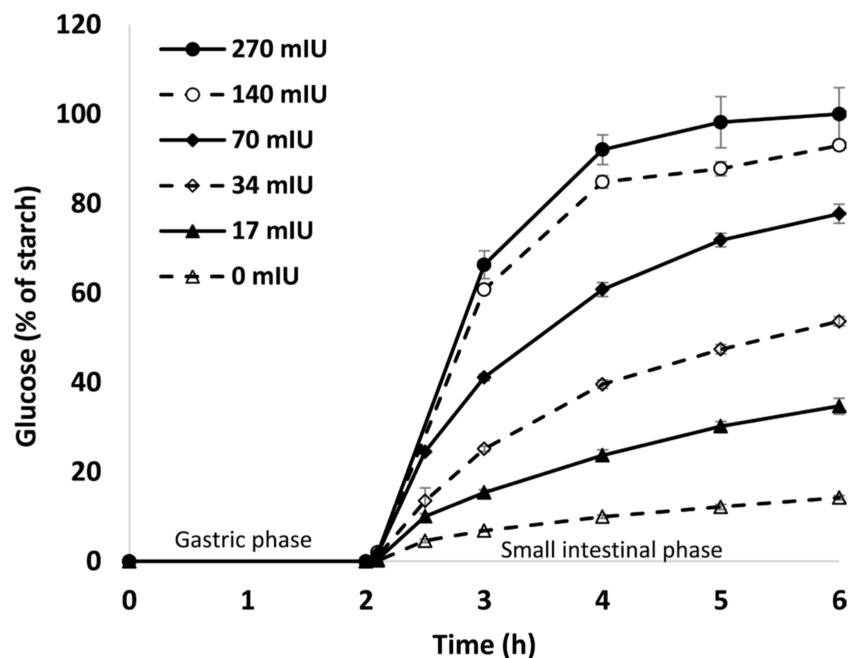

Figure 1. *In vitro* digestion of corn starch under initial method conditions, in the absence and in the presence of different inclusion doses of intestinal mucosal extracts (RIP-enzyme extract) (n = 3).

concentrations of RIP-enzyme extract tested (140 and 270 mIU IM activity), an amount of RIP-enzyme extract resulting in less than maximum hydrolysis of starch (70 mIU) was selected to evaluate the effect of modifying other digestion conditions in the small intestinal phase. We targeted pH and pancreatin inclusion. It is known that mucosal disaccharidases exhibit an optimum pH of 6.0 - 6.5 [24] [29] and we hypothesized that lowering the small intestinal pH and/or reducing potential endogenous proteolytic activity by lowering pancreatin inclusion could result in higher disaccharidase activity and digestibility of starch. Thus, pancreatin was tested at 7 Trypsin U per digestion reaction (9 times lower concentration than original conditions), and pH was reduced by one unit in the final small intestinal mix, from pH 7.2 ± 0.2 to pH 6.3 ± 0.2. The concentration of pepsin in the gastric phase was also tested at a 11x lower concentration (211 U per digestion reaction) and this modification, while not expected to impact starch digestion, was evaluated to be consistent with protein digestion testing conditions (protein digestion section below).

As shown in **Figure 2**, in the presence of 70 mIU of RIP-enzyme extract, maintaining the original intestinal pH (pH 7.2 ± 0.2) and lowering the amount of pepsin and pancreatin did not have an impact on the glucose release from corn starch compared to higher (original) inclusion of those digestive enzymes. However, lowering the intestinal pH by about one unit (pH 6.3 ± 0.2), resulted in faster and more extended hydrolysis of corn starch, despite the lower inclusion of pancreatin. Under these conditions (70 mIU RIP-enzyme extract, 211 U pepsin, 7 U trypsin activity from pancreatin, per reaction, and intestinal pH 6.3 ± 0.2), hydrolysis was comparable to that previously observed with original concentration of pepsin and pancreatin and 3 and 4 times higher amounts of RIP enzyme extract (140 and 270 mIU, respectively) (shown in **Figure 1**), with about 95% total glucose released after 4 h of small intestinal digestion. From these results, 211 U pepsin in the gastric phase, 70 mIU of RIP-enzyme extract (corresponding to about 15 g/L duodenal solution), and 7 trypsin U from pancreatin, respectively, in the small intestinal phase, and pH 6.3 ± 0.2 in the final small intestinal mix, were selected as adequate conditions for digestion of corn starch and utilized for further experiments.

3.3. Evaluation of the Effect of RIP Enzyme Extract and Varying Gastric and Duodenal Conditions on the *in Vitro* Digestion of Proteins

Intestinal mucosa is not only a source of disaccharidases but also aminopeptidase enzymes. As such, the effect of the inclusion dose of RIP enzyme extract optimized for carbohydrate digestion (70 mIU) was also evaluated on protein digestion. Whey Protein Isolate (WPI) was utilized as reference material. Consistent with the different conditions tested for carbohydrate digestion, whey protein digestion was also evaluated under high (original at start of method development) and low, factor of 11 and 9, pepsin and pancreatin concentrations, respectively, and high (7.2 ± 0.2) and low (6.3 ± 0.2) pH in the small intestinal phase.

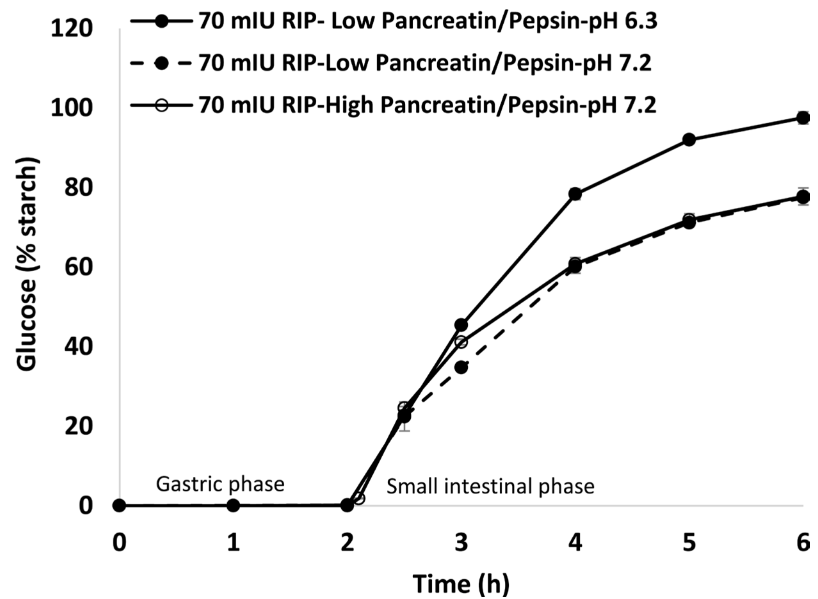

Figure 2. *In vitro* digestion of corn starch in the presence of intestinal mucosal extract (RIP-enzyme extract) and modified method conditions (pepsin and pancreatin inclusion and small intestinal pH) (n = 3).

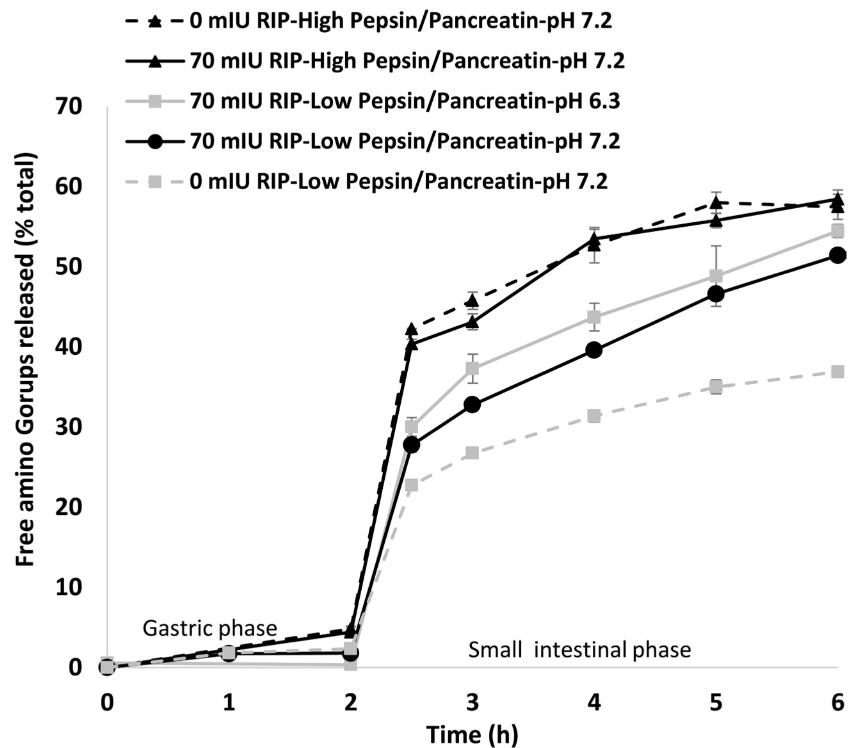

Figure 3. *In vitro* digestion of Whey Protein Isolate (WPI) (n = 3): in the presence and absence of intestinal mucosal enzymes (RIP-enzyme extract), under initial and modified method conditions.

As it can be observed in Figure 3, at the conditions tested at the start of method development (*i.e.* 2.3 kU pepsin, 63 trypsin U from pancreatin, per digestion reaction, and high intestinal pH 7.2 ± 0.2), about 5% free amino group re-

lease was detected at the end of 2 h gastric digestion. This rapidly increased up to about 40% free amino groups within 30 min of small intestinal digestion, reaching 57.5% ± 1.6% at the end of 4 h small intestinal phase. Addition of RIP-enzyme extract did not appear to influence the *in vitro* digestion of whey protein isolate and similar digestion, 58.4% ± 1.2% of amino groups release after 4 h of small intestinal digestion, was observed in the presence and in the absence of RIP-enzyme extract. In contrast, when lower concentrations of pepsin (211 U) and pancreatin (7 Trypsin U) were present, respectively 11× and 9× reduction from original inclusion dose, addition of RIP-enzyme extract positively impacted whey protein digestion. Thus, about 15% higher amino groups release was measured after 4h small intestinal phase when RIP-enzyme extract was included in the digestion reaction (51.4% ± 0.5%) compared to reactions lacking RIP-enzyme extract (36.9% ± 0.7%). Under the lower digestive enzymes (pepsin and pancreatin) conditions, lowering the pH of the small intestinal phase from 7.2 ± 0.2 to about 6.3 ± 0.2 did not result in appreciable differences. While about 10% higher free amino group release was obtained at the early stages of the intestinal phase under the conditions tested at the start of method development (40.4% ± 0.6% vs 30.0% ± 1.1% after 30 min), the fact that the extent of digestion, 58.4% ± 1.2% free amino groups, was comparable to that observed with the modified conditions selected for carbohydrate digestion (70 mIU RIP, 211 U pepsin and 7 U trypsin activity (from pancreatin), per digestion reaction, and intestinal pH 6.3 ± 0.2) (54.5% ± 0.8%), supports the adequacy of adopting the later conditions for protein digestion as well.

The digestion of whey protein was also monitored by SDS-PAGE as seen in Figure 4. Under the selected conditions for carbohydrate digestion, β-Lactoglobulin, the most abundant component in undigested whey protein isolate product (lane 5) was resistant to the action of pepsin (lane 6) but was rapidly digested by the enzymes present in the simulated intestinal mix (lanes 7-9). α-lactalbumin component was partially digested after 2 h of gastric digestion (lane 6) and was practically undetected after 30 min of intestinal digestion (lane 7). In general, there was a clear shift from high molecular weight components present in the undigested material (lane 5) to lower molecular weight bands after 2h of pepsin digestion (lane 6). After 4 h of small intestinal digestion (lane 9) SDS-PAGE profile is comparable to that of blank (lane 3) in which the visible bands correspond to the proteins present in the digestive solutions, this clearly demonstrating the extensive hydrolysis of whey protein components.

3.4. Reproducibility of Digestion: RIP Enzyme Extracted from Different Batches

To evaluate the reproducibility of digestion under the final conditions of the model, namely for corn starch and whey protein isolate, macronutrients for which intestinal mucosal enzymes are relevant, ingredients were submitted to the digestibility protocol using 3 different batches of RIP enzyme extracts. The average digestibility, standard deviation and % Coefficient of Variation (CV) of

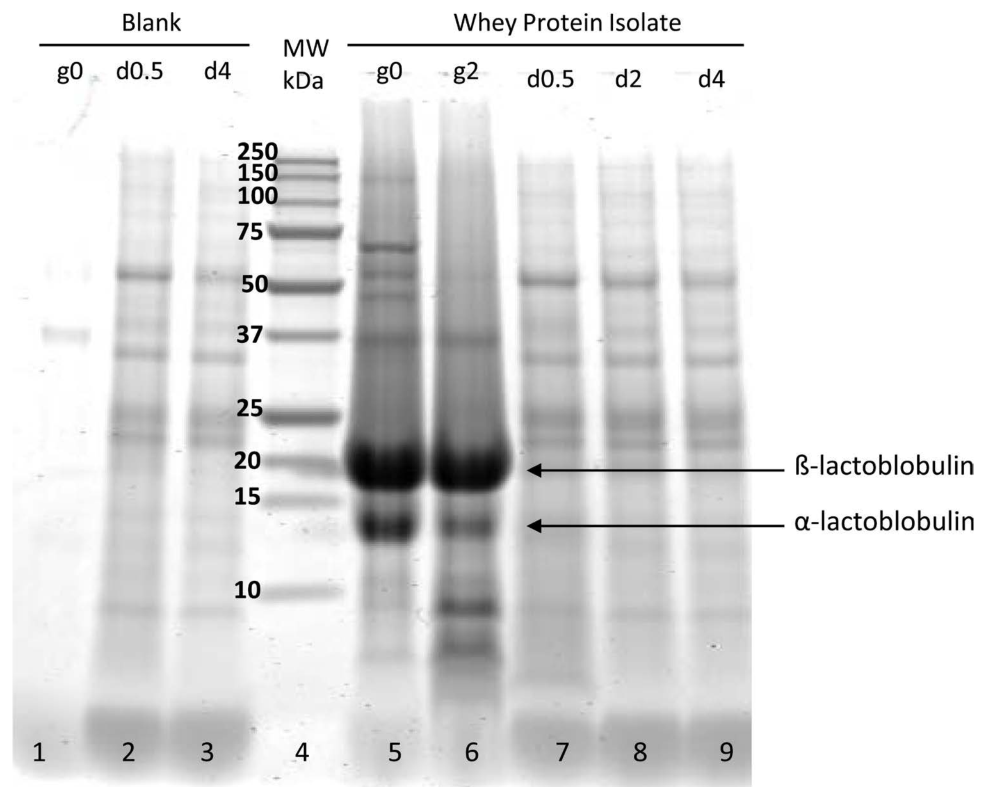

Figure 4. SDS-PAGE analysis of Whey Protein Isolate (WPI) and Blanks (digestive solutions and enzymes) at different gastric and small intestinal times of digestion *in vitro*. Lanes: (1) Blank at gastric time zero, (2) Blank at small intestinal time 0.5 h, (3) Blank at small intestinal time 4 h, (4) Protein Molecular Weight standard, (5) WPI at time zero of gastric digestion, (6) WPI after 2 h gastric phase, (7) WPI at 0.5 h into small intestinal digestion, (8) WPI at 2 h small intestinal digestion, (9) WPI after 4 h small intestinal digestion.

three independent experiments (n = 3 biological replicates of RIP enzyme extracts) where the ingredients were tested in triplicate (total n = 9 replicates), and at different times of digestion, is shown in **Table 1**. % CV of corn starch and whey protein isolate was lower than 8% at different times of digestion (0.5, 1, 2, 3 and 4 h small intestinal phase).

3.5. Lipid Olive Oil Digestion

As indicated in the Materials and Methods section, one tube per time per sample approach was used to minimize recovery losses due to the heterogeneity of the digestive solutions when testing lipids as single ingredients.

In vitro digestion of reference substrate olive oil was evaluated under the conditions selected for starch digestion and also adopted for protein digestion (70 mIU RIP-enzyme extract, 211 U pepsin and 7 U trypsin activity (from pancreatin), per digestion reaction, and intestinal pH 6.3 ± 0.2). Under these conditions, and in the absence of an adequate commercial mammalian gastric lipase, *Candida rugosa* lipase (CrL) was included in the gastric phase and tested at 1200 U per digestion reaction. As shown in **Figure 5**, 6.3% ± 0.1% FFA (w/w) were released

Table 1. Reproducibility of corn starch and whey protein isolate *in vitro* digestion[a].

Time (h)	Corn Starch			Whey Protein Isolate		
	Glucose (% of starch)	SD	% CV	Free Amino Groups (% of total)	SD	% CV
2.5	27.78	1.80	6.47	26.91	1.41	5.25
3	48.35	2.67	5.51	36.01	2.66	7.38
4	75.58	2.73	3.62	45.08	2.08	4.61
5	84.79	3.22	3.80	48.75	2.98	6.12
6	91.84	2.04	2.22	53.09	3.35	6.31

[a]Three independent experiments performed with 3 different batches of RIP enzyme extracts and samples tested in triplicate (*i.e.* 9 analytical samples per time point).

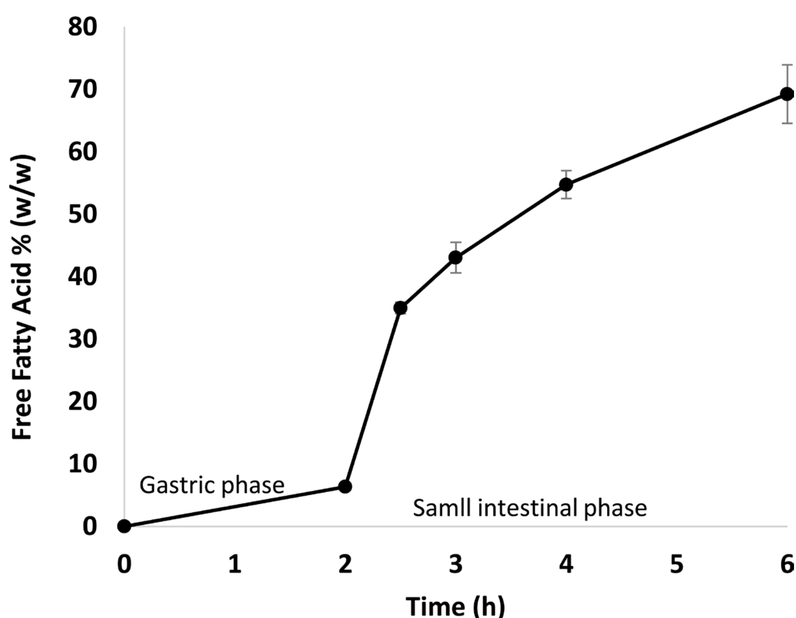

Figure 5. Total Free Fatty Acids (% w/w) released during *in vitro* gastric and small intestinal digestion of olive oil (n = 3).

after 2 h of gastric digestion. A rapid increase in FFA released, 34.9% ± 0.9% FFA (w/w), was observed within 30 min of small intestinal digestion and 69.2% ± 4.7% FFA (w/w) were detected at the end of 4 h small intestinal phase. Furthermore, about 20% TAG and less than 5% TAG (w/w) were measured, respectively, at 30 min and at the end of small intestinal digestion (data not shown).

3.6. *In Vitro* Digestion of a Whole Food System

A commercially available product, Ensure nutrition powder, was selected as an example of a complete and balanced food system from which digestibility of all 3 macronutrients could be evaluated in the developed *in vitro* model, all within the same digestion reaction. In order to obtain enough amount of sample to perform all the analytical tests required, the *in vitro* digestion was scaled up by a factor of 6. Thus, when 1 ml, 2 ml and 3 ml of saliva, gastric and small intestinal solutions, respectively, represent the volumes of a typical reaction for a single

ingredient, digestion of the whole food was performed utilizing 6 ml, 12 ml and 18 ml of the above solutions. Amount of food inclusion, based on nutrients present at the lowest concentration (protein and fat), was also scaled by a factor of 6 this resulting in 1.2 g of Ensure powder added to the reaction tubes. Nutrient composition of Ensure nutrition powder, based on manufacturer nutritional label, is 16% fat, 60% carbohydrate and 16% protein. Testing lipid digestibility on a whole food system did not require the "one tube per sample and per time" approach given the naturally emulsifying properties provided by the food matrix.

Digestion of all three macronutrients proceeded simultaneously and the curves of digestion are shown in Figure 6. For the carbohydrate component of the food system, 3.4% ± 1.2% of free glucose was detected at time zero of digestion and this amount was still present after 2 h of gastric phase; 34.0% ± 1.7% and 64.3% ± 2.2% glucose equivalent percent of the total carbohydrate was observed during the first 30 min and after 4 h of small intestinal digestion, respectively. Regarding the protein component, 1.2% ± 0.4% free amino groups were detected after 2 h of gastric digestion and 24.6% ± 1.1% after 30 min of small intestinal phase. At the end of digestion, 52.3% ± 2.2% free amino groups were measured. About 10% FFA (w/w) was detected after 2 h of gastric digestion and about 48.4% ± 0.5% FFA (w/w) were measured after 0.5 h small intestinal digestion, reaching after 4 h, 66.8% ± 1.2% FFA (w/w).

4. Discussion

In this study, conditions of digestion *in vitro* were evaluated for achieving

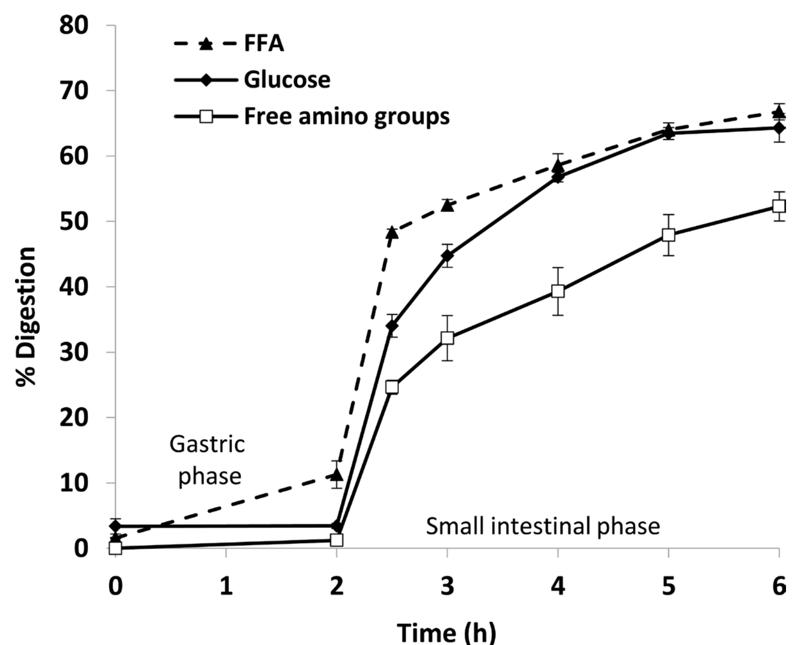

Figure 6. *In vitro* digestion of a whole food system Ensure Nutrition Powder (n = 3). % Digestion represents Glucose (% of starch), Free Amino Groups (% of total) and Free Fatty Acids% (w/w), for carbohydrate, protein and lipid components, respectively.

simultaneous hydrolysis of all three macronutrients (carbohydrate, protein and lipid) of foods, within physiological range. At this effect, intestinal mucosal enzymes were integrated in the digestion reactions, as they are a critical component for the final step of starch and protein hydrolysis which renders nutrients available for absorption across the epithelial cells [18]. Digestion of starches in humans initially relies on α-amylase (both salivary and pancreatic) which hydrolyzes internal α-1,4 linkages and releases maltose and α-limit dextrins. Those become substrate for the mucosal α-glucosidases, including maltase-glucoamylase and sucrase-isomaltase complexes, with ability to hydrolyze α-1,4, α-1,6, and α-1,3 linkages in di- and oligo-saccharides [18] [30]. Furthermore, work with recombinant human small intestinal maltase-glucoamylase [31] has shown that this disaccharidase also has capability to hydrolyze native starch granules. These authors also evaluated the impact of fungal Amyloglucosidase (AMG), enzyme typically included in *in vitro* models for carbohydrate digestion such as the extensively utilized Englyst method [11], on morphology of native starches of different botanical origins and compared it to that of human recombinant maltase-glucoamylase. Interestingly, remarkable differences on the morphology of starch granules were observed by scanning electron microscopy, demonstrating different susceptibilities of raw starches from different botanical sources to the two enzymes (human and fungal), thus strengthening the significance of including mammalian intestinal mucosal enzyme extracts versus fungal Amyloglucosidase when modeling digestion *in vitro*. Additional evidence amplifying the physiological relevance of including mammalian intestinal mucosal enzymes in *in vitro* digestion models, as it relates to proteins, comes from the fact that more than 20 major peptidases have already been identified in the small intestinal brush border membrane of humans and rats [32].

The initial *in vitro* conditions of digestion utilized in this study were based on the model described by [21], a model that has been extensively used as reference by others [15] [33]. From there, this study focused on evaluating and optimizing the amount of intestinal mucosal enzyme extract needed to be incorporated into digestion reactions that would result in approximate to physiological digestion, in addition to evaluating its impact upon modifying other conditions of digestion, namely pepsin and pancreatin concentrations, and pH of small intestinal solution.

Intestinal mucosal extracts include a complex mixture of enzymes which makes quantitation of all activities difficult. Carbohydrate digestion was prioritized for testing and thus, standardization of the extract was done on the basis of disaccharidase sucrase-isomaltase (palatinase) activity. At the initial conditions tested, which did not include intestinal mucosal extracts, less than 20% of starch was converted into glucose after 4 h of small intestinal digestion. Upon addition of intestinal mucosal enzymes, starch digestion consistently increased with increasing intestinal mucosal extract concentration up to 140 mIU (palatinase activity) inclusion, level at which digestibility of starch appeared to be maximized and doubling inclusion of intestinal mucosal enzyme extract did not result in

faster or more extended digestion. Two factors were hypothesized that could have a significant impact on the *in vitro* digestibility of starch in the presence of intestinal mucosal enzymes: pH in the small intestinal phase and inclusion of pancreatin. The pH of the small intestinal phase of these initial *in vitro* experiments was 7.2 ± 0.2 and it is known that mucosal disaccharidases exhibit an optimum pH of 6.0 - 6.5 [24] [29]. Pancreatin positively contributes to starch digestion by providing pancreatic *α*-amylase but a potential negative effect due to proteolytic action on intestinal mucosal enzymes could also be hypothesized. Thus, it appeared appropriate to evaluate the effect of lowering the pH in the final mix as well as pancreatin inclusion, utilizing a dose of small intestinal mucosal extract below that resulting in maximal hydrolysis. Results showed that, when small intestinal pH was maintained at 7.2 ± 0.2, lowering pancreatin inclusion by a factor of 9 decreased starch digestion. However, lowering the small intestinal pH to 6.2 ± 0.2 reversed this effect and, despite the lower concentration of pancreatin, starch digestion was increased to levels comparable to those observed with initial conditions including higher pancreatin levels and twice and four times higher inclusion of RIP-enzyme extract. This data, while not demonstrating whether inactivation of mucosal enzymes by proteolytic effect at high pancreatin inclusion level occurs, does demonstrate the importance of the small intestinal pH in the complex digestion reactions and supports a small intestinal pH of 6.2 ± 2, the inclusion of small intestinal mucosal enzyme extract at 70 mIU, and 9 times lower inclusion of pancreatin than in the original model, as adequate conditions for further experiments. Under these conditions, common corn starch was digested at about 25% - 30% after 30 min and around 80% after 2 h of small intestinal phase, the later representing 50% - 55% digestion occurring from 30 - 120 min. Hydrolysis reached more than 90% after 4 h of small intestinal digestion. These values are in good agreement with those observed for digestible starch [34], on the basis of the *in vitro* Englyst method, widely used for carbohydrate digestibility, showing 22.4%, 53.0% and 22.6% as percentages for rapidly digestible starch (RDS, 20 min), slowly digestible starch (SDS, 20 - 120 min) and resistant starch (RS, not digested at 120 min) from common maize starch. However, when the kinetics of enzymatic hydrolysis were followed beyond the standard 2 h digestion time, almost 100% digestibility of native maize starch was observed, this suggesting that the 2 h time point of digestion of the Englyst method is too short to accurately represent the resistant starch fraction of native starches. In addition, work comparing *in vitro* starch digestion using a modified Englyst model with *in vivo* portal glucose appearance in pigs [35], demonstrated that up to 54.4% of total portal glucose appearance takes place after 120 min of consumption of diets, from 120 - 480 min, thus providing additional support to the biological relevance of extended times beyond 2 h of small intestinal digestion for starch hydrolysis.

Similarly to starch digestion, intestinal mucosal enzymes play a significant role in protein digestion *in vivo*. Thus, the impact of intestinal mucosal enzyme

extract, small intestinal pH, and pepsin and pancreatin concentration on isolated whey protein digestion was also evaluated in this study. Under original conditions of digestion (*i.e.* high pepsin and pancreatin and small intestinal pH 7.2 ± 0.2), around 5% and 57% free amino groups were measured after 2 h of gastric phase and 4 h of small intestinal digestion, respectively, both in the absence and presence of intestinal mucosal extract at the concentration optimized for starch hydrolysis (70 mIU). Not unexpectedly, lowering pepsin and pancreatin inclusion by a factor of 11 and 9, respectively, resulted in about 1.5% free amino group release in the gastric phase and about 20% lower free amino groups released during the small intestinal phase lacking mucosal enzymes, compared to original conditions of digestion. However, addition of mucosal enzyme extract restored digestibility to comparable levels (around 55%) to those observed under original conditions of digestion, independently of the small intestinal pH (7.2 ± 0.2 or 6.3 ± 0.2). To our knowledge, neither an optimum pH nor a dose inclusion of intestinal mucosal enzymes that physiologically simulate food digestion *in vivo* have been reported in the literature, the complexity of the brush border membrane enzyme composition and distribution along the gastrointestinal tract, together with the variable pH from proximal to distal small intestine [32] [36] likely being major challenging factors. Thus, it was rational to determine the dose inclusion of intestinal mucosal extracts to reflect literature-reported digestive properties of reference starch substrates and to provide evidence that conditions optimized for starch digestion (70 m IU RIP, 211 U pepsin and 7 U trypsin activity from pancreatin, per digestion reaction, and intestinal pH 6.3 ± 0.2), were also adequate for protein and lipid digestion. For comparison, when converting the enzymatic activities into Units/g food, inclusion levels of pepsin and trypsin activity from pancreatin in our system were estimated to be, respectively, 2 and 6 times lower than the ones recommended in the standardized static *in vitro* digestion model which does not include intestinal mucosal enzymes [17].

Reference [20] observed 55% degree of hydrolysis of whey isolated protein after 1 h gastric plus 1 h duodenal digestion under conditions similar to the recently proposed harmonized model [17]. Interestingly, these authors also demonstrated about 16% further extension of whey protein isolate hydrolysis (71%), when the 1 h gastrointestinal digests were incubated with BBMs vesicles from pigs for additional 6 h. This value of milk protein digestibility in the presence of BBM vesicles is higher than that observed in our study (50% - 55% free amino groups) and could be explained by several factors. First, in our study, free amino group analysis of digested samples was performed on supernatants from TCA precipitation and a number of free amino groups from larger molecular weight peptides are removed in the precipitated fraction, this likely resulting in underestimation of free amino groups released from digestion and, hence, digestibility. Second, for each time point of digestion, we report free amino group values in digested samples after deducting those from blanks (digestive solutions and enzymes) that are incubated at 37˚C, thus correcting for proteolysis of the digestive

enzymes. Progressive auto-digestion of enzymes in intestinal digestive mixtures lacking any substrate and incubated at 37˚C for 6 h was recently evidenced [37]. While it is relevant to take into account the contribution of enzyme auto-digestion, it is also reasonable to assume that higher endogenous proteolysis will occur in the absence than in the presence of protein substrate in the digestive solutions, this resulting in overestimation of auto-proteolysis in blanks and, consequently, underestimation of protein hydrolysis.

Further evidence of proteolysis extension in this study was provided by SDS-PAGE protein profiles which demonstrated a more rapid hydrolysis of α-lactoglobulin component of whey protein isolate versus β-lactoglobulin in the gastric phase and extensive hydrolysis of both components at the end of 4 h small intestinal digestion. Similar hydrolysis profiles have been reported by others after *in vitro* digestion of milk proteins [38] [39]. Furthermore, tracking the *in vivo* release of bioactive peptides in the gut of pigs eating milk formulas [40] has shown similar protein profiles of hydrolysis of whey protein components: rapid hydrolysis of α-lactoglobulin and higher resistance of β-lactoglobulin at the duodenum site, both being fully hydrolyzed at the jejunal site.

Reference [22] reported that only 5% of static *in vitro* digestion models published between 1967 and 2015 included a lipase during the gastric phase. In our study, non-specific lipase from *Candida rugosa* was utilized as a surrogate of gastric lipase. Human gastric lipase is not commercially available and, while different biochemical properties are recognized [22] [41], a variety of fungal, microbial and mammalian enzymes have been used on a number of models that do mimic gastric lipolysis. A crude rabbit gastric extract was shown to result in similar rates of lipolysis of meal triacylglycerols compared to human digestive lipases recorded under the same *in vitro* conditions [42]. In addition, an assessment of the digestive properties of *Rhizopus oryzae*, gastric rabbit and recombinant human (rHGL) gastric lipases *in vitro* [43] showed differences in extent of digestion, fatty acids affinity and morphology changes of oil globules during digestion of infant formula among the three lipases and, based on expectations from previous *in vivo* data, rHGL was shown to be the best surrogate for *in vitro* models. Fungal lipase was included in the gastric phase of an *in vitro* digestion looking at the immunogenic proteins and peptides released from cooked pasta [3]. More recently, a study evaluating the impact of different experimental factors, reported addition of *Aspergillus niger* lipase to the gastric juice for *in vitro* digestion of fish lipid to obtain triglyceride hydrolysis levels close to that occuring *in vivo* [44].

It has been reported that lipase activity in the stomach may account for up to 10% and 25% of TAGs in solid and liquid meals, respectively [45] [46], with 95% overall efficiency of fat absorption in humans [47]. In our study, lipolysis of olive oil resulted in about 6% FFA (w/w) release after 2 h of gastric digestion and reached about 70% FFA (w/w) at the end of 4 h small intestinal digestion. These values, together with the very low recovery of TAG (less than 5% w/w) at the end of digestion, support high digestibility of the lipid oil under the final conditions

of the *in vitro* digestion model.

The suitability of the model to provide, in a single reaction, digestibility data on the three macronutrients present in a whole food system (Ensure nutrition powder) was also demonstrated in this study. According to the nutrition facts label, Ensure's carbohydrate fraction includes corn syrup, maltodextrin and sucrose. Digestion of both corn syrup and maltodextrin is expected to generate equivalent glucose content. However only about half of sucrose's weight will be converted into glucose as it is a disaccharide composed of glucose and fructose. Based on the 60% carbohydrate content (of which 23% sugars), after deducting the theoretical fructose content of sucrose ingredient, maximum glucose (*i.e.* 100% digestion) that could be expected to be released from digestion would be about 70% of the total carbohydrate fraction. Our results showed increased glucose release with time, maximized at around 64% glucose after 3 h of intestinal digestion, *i.e.* about 90% digestion, this demonstrating nearly complete *in vitro* digestibility of carbohydrate fraction. Also, *in vitro* digestion of corn oil, lipid component of Ensure, resulted in 11% FFA (w/w) released during the gastric phase and 66% FFAs (w/w) after 4 h small intestinal digestion, the later value indicating high digestibility. Corn oil is a well-absorbed fat for which *in vivo* digestibility coefficient in human subjects has been reported to range between 95.4% and 97.4% [48] [49]. Finally, *in vitro* digestion of the mixed protein fraction in Ensure (including sodium and calcium caseinates and soy protein isolate), while at an apparently slower rate, showed an extent at the end of the small intestinal digestion comparable to that obtained with reference single ingredient whey protein isolate. *In vivo* data has shown slower casein and soy protein digestion than whey protein, mostly evidenced by more rapid increase in plasma amino acid levels after ingesting whey protein [50] [51] [52]. However, efficiency of casein and soy breakdown *in vivo* is comparable to that of whey protein as indicated by high true digestibility levels [53] [54].

Despite the added complexity in composition of final digestive solution in this model due to the integration of small intestinal enzyme extract, our results were consistent and reproducible, as shown by lower than 8% CV at different times of small intestinal digestion for both corn starch and whey protein isolate, obtained from 3 independent experiment including 3 different batches of small intestinal enzyme extract tested, for each substrate, in triplicate.

Future perspectives of the model include its application to the understanding of how food composition, food matrix, particle size, processing and other conditions, impact the digestibility of either one or all three macronutrients in a food, this knowledge being relevant for the development of innovative food ingredients and foods that target delivery of nutrients to specific points of the gastrointestinal tract as well as foods with optimal nutritional properties.

5. Conclusion

In conclusion, the present study reports the development of an *in vitro* digestion

model that includes intestinal mucosal enzymes as an integral parameter in the small intestinal phase of digestion and demonstrates its suitability to evaluate the digestive profiles of all three macronutrients. The good reproducibility, together with the digestion profiles observed for reference materials being consistent with *in vitro* and vivo data, supports the validity of this method as an advanced and robust *in vitro* tool for screening digestion of all three macronutrients, carbohydrates, proteins and lipids, whether separately or in a food, in a single digestion reaction.

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Author Contributions

Vicenta Garcia-Campayo (corresponding author; data acquisition, analysis and interpretation; writing of the manuscript), Sonia Han (data acquisition and analysis), Ronny Vercauteren (expertise and experimental protocol for intestinal mucosal enzymes), Anne Franck (conception of the work, discussion of data and review of the manuscript). All authors have read and approved the manuscript.

Conflict of Interest Declaration

Authors are employed by Cargill.

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