Stimulation of CCK and GLP-1 secretion and expression in STC-1 cells by human jejunal contents and *in vitro* gastrointestinal digests from casein and whey proteins

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ABSTRACT

The present study evaluates casein and whey protein gastrointestinal digests as inducers of CCK and GLP-1 secretion and expression in STC-1 cells. *In vitro* digests were characterized regarding protein, peptide and free amino acid content. Digests from the intestinal phase containing small size peptides and free amino acids behaved as more potent CCK inducers than digests from the gastric phase. However, GLP-1 release was maximized with caseingastric digests and whey protein intestinal digests. Human jejunal digests from the samesubstrates showed a comparable response, except for casein jejunal digests which exerted a higher effect than *in vitro* casein gastrointestinal digests. The gene expression experiments also showed increased *CCK* and *GLP-1* mRNA levels but differences between gastric and gastrointestinal phases were not as pronounced as observed by quantifying the secreted hormone. Our results demonstrate that the degree of protein hydrolysis during digestion plays an important role on CCK and GLP-1 secretion.

KEYWORDS: anorexigenic hormones; STC-1 cells; casein; whey proteins; *in vitro* digestion; human jejunal digests; peptidomics; satiety

Highlights:

- CCK release was more pronounced with highly digested substrates (*in vitro* intestinal phase).
- GLP-1 secretion was induced by *in vitro* casein gastric- and whey protein intestinal-digests
- Human jejunal digests from casein and whey proteins induced CCK and GLP-1 secretion in STC-1 cells.
- In vitro and in vivo gastrointestinal digests from whey proteins showed a similar hormonal response in STC-1 cells

1. INTRODUCTION

It has been recognized the importance of nutrient sensing at the gut for glucose homeostasis and food intake. Food digestion products are detected by enteroendocrine cells located throughout the gastrointestinal epithelium and this triggers the secretion of gut hormones in response to these meal-related stimuli.[1, 2] Among the different macronutrients,

protein plays a key dietary role on the satiety effect of foods. While the mechanisms by which carbohydrates are detected in the gut are becoming better understood, the detection pathways followed by protein- and fat-digestion products are less described.[3]

A number of enteroendocrine cell types have been described, and are classified based on their morphology, distribution and the secretion of different gut peptides or hormones, such as I-cells producing cholecystokinin (CCK) or L-cells secreting glucagon-like peptide-1 (GLP-1). These two peripheral hormones, acting via the vagus nerve, are involved in the mechanism of food-induced satiety, being proteins strong inducers for their release.[4] Several studies have proven the release of gut-derived hormones from isolated enteroendocrine cells in response to protein ingestion. In these studies, murine secretin tumour cells, STC-1, are commonly used. This cell line possesses many features of native intestinal enteroendocrine cells, such as, the expression and secretion of a wide range of gut hormones in response to physiological stimuli although hormone levels may differ from native cells.[5]

Initially, it was shown that protein hydrolysates stimulated CCK secretion and gene transcription in STC-1 cells, while intact proteins or free amino acids induced only a modest effect in CCK secretion.[6] This activity was also described for the incretin, GLP-1, in isolated vascularly perfused rat intestine and STC-1 cells, confirming the direct effect of protein hydrolysates in enteroendocrine cells.[7] Later on, the G protein-coupled receptor, GPR93 (also known as GPR92 or LPAR5), sensitive to peptides and located on the surface of enteroendocrine cells, was involved in the protein-induced CCK release and expression in STC- 1 cells.8 It has been described a strong induction of CCK and GLP-1 release in STC-1 cells with casein, whey and pea intact proteins. However, in some cases, protein hydrolysis led to a reduction of the effect.[9, 10] Similarly, β-lactoglobulin (β-Lg) has proved to stimulate enteroendocrine cell proliferation and GLP-1 secretion, but the effect was lost after hydrolysis ofthe protein with trypsin or chymotrypsin.[11] Casein and casein fractions have also shown a

stimulatory effect on GLP-1 secretion in this cell line although it was significantly reduced upon hydrolysis with proteolytic enzymes.[12] However, other studies have revealed a higher secretion and expression of CCK and GLP-1 by haemoglobin digests compared to the non-digested protein[13], and the presence of aromatic residues within their sequences have been pointed as a structural characteristic for CCK-inducing peptides.[14] It has been suggested that the length of the peptide fragments derived from a protein during enzymatic digestion is relevant to this activity, and hence, to the satiating effect of proteins. It was concluded that intact proteins or partially hydrolysed proteins can be more potent than the products of their complete hydrolysis.[15]

The present study aims the examination of casein and whey protein gastrointestinal digests as inducers of CCK and GLP-1 secretion and expression in the enteroendocrine cell line, STC-1. For this purpose, *in vitro* gastrointestinal digests from casein and whey proteins were prepared by using an internationally harmonised digestion protocol that has demonstrated to reproduce physiological milk protein digestion.[16] In addition to the protein and free amino acids analysis, an exhaustive peptidomic characterization of the gastrointestinal digests was performed by HPLC-tandem mass spectrometry (HPLC-MS/MS), in order to establish a relationship between the observed effect and the digest peptide composition. CCK and GLP-1 secretion and expression in STC-1 cells were evaluated with samples taken along the digestion process. Moreover, with the purpose of assessing the physiological relevance of the assayed digests, human jejunal aspirates after oral administration of the same substrates were also tested in this cell line.

2. MATERIALS AND METHODS

2.1 *In vitro* simulated gastrointestinal digestion

In vitro simulated gastrointestinal digestion was carried out as described by Minekus, et al.[17] Briefly, casein or whey protein powders were dissolved in simulated salivary fluid at 60 mg of protein/mL, and the mix was 50% diluted with simulated gastric fluid pH 3.0, containing pepsin from porcine gastric mucosa (final pepsin concentration 2000 U/mL of gastric digest, EC 3.4.23.1, Sigma-Aldrich, St. Louis, MO, USA). Gastric samples were withdrawn at 30 and 120

min during gastric digestion and the reaction was stopped by adjusting the pH at 7.0 with NaOH 1 M followed by snap freezing in liquid nitrogen. Intestinal phase was carried out by mixing the end point from the gastric phase with the same volume of simulated intestinal fluid containing pancreatin from porcine pancreas (100 U trypsin activity/mL of final mixture, Sigma-Aldrich) and samples were taken at 30, 60 and 120 min. Bile salts at a final concentration of 10 mM in simulated intestinal fluid displayed cytotoxic effects and they were not included in the intestinal digestion phase. The intestinal digestion was stopped by heating 85ºC during 15 min, and snap freezing. Samples were freeze-dried and kept at -20ºC until analysis. Digestions of each protein powder were performed in duplicate. Enzyme activities were measured according to the assay described in the digestion protocol. Nitrogen content in freeze-dried samples was determined by elemental analysis and further analyses were performed on protein basis. The protein content of the freeze-dried casein digests was of 72% and 45% in the gastric and intestinal phase, respectively. Freeze-dried whey protein digests had a protein content of 67% and 41% in the gastric and intestinal phase, respectively.

2.2 Human jejunal effluents

Human jejunal effluents were obtained at the Human Nutrition Research Centre of Bobigny, as previously described. [18, 19] All procedures were approved by the Ethics Committee for Saint-Germain-en-Laye Hospital (Saint-Germain-en-Laye, France). Written informed consent was obtained from all participants. The study was registered under www.ClinicalTrials.gov (NCT00862329). Volunteers ingested a solution of 30 g of casein or whey dissolved in 500 mL of water (i.e. 6% w/v) in fasting conditions. Digest samples from six volunteers were obtained by aspiration with a nasointestinal tube that migrated to the proximal jejunum. Three of them had received the casein solution and three had ingested whey protein and samples were taken at 1 and 2 h after oral administration of the protein solution. Jejunal effluents were collected on ice, freeze-dried and kept at -80 °C until analysis. Nitrogen content in freeze-dried samples was determined by elemental analysis and further analyses were performed on protein basis. Protein content of jejunal effluents ranged from 27 to 44% in the group who had received casein and from 25 to 55% in the whey protein group. These jejunal digests were characterized by SDS-PAGE and HPLC-MS/MS, and compared to in vitro simulated digests of the same substrates. The results have been published by Sanchón et al. (2018)[16].

2.3 SDS-PAGE

SDS-PAGE was performed as previously described[16] but protein digests were dissolved at 0.7 mg of protein/mL in sample buffer. Gels were stained with Coomasie Blue (Instant Blue, Expedeon, Swavesey, UK) and images were taken with a Molecular Imager VersaDoc™ MP 5000 system (Bio-Rad, Hercules, CA, USA) and processed with Quantity One 1-D analysis software (Bio-Rad).

2.4 Analysis by HPLC-tandem mass spectrometry (HPLC-MS/MS)

Samples were analysed by HPLC-MS/MS in duplicate using an Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany), connected on-line to an Esquire 3000 linear ion trap mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with an electrospray ionization source. The column used was a reverse phase Mediterranea Sea_{18} column (150 × 2.1 mm, Teknokroma, Barcelona, Spain). The injection volume was 50 µL and the flow was set at 0.2 mL/min. Peptide elution was performed with a linear gradient from 0 to 45% of solvent B (acetonitrile: formic acid, 100:0.1, v:v) in 150 min. Nitrogen was used as the nebulising and drying gas and ion source parameters were: nebulizer pressure, 60 psi; drying gas, 8 L/min and dry temperature, 350ºC. The capillary was held at 4 kV and the helium pressure was set at 5 × 10-3 bar. The signal threshold to perform auto MS (n) analyses was 10,000 and the precursor ions were isolated within a range of 4.0 m/z and fragmented with a voltage ramp going from 0.3 to 1.5 V. The spectra were recorded over the mass/charge (m/z) ranges of 100-2100 and 100-1200, selecting 1200 and 750 as target mass, respectively.

Freeze-dried casein digests were reconstituted in solvent A (water: formic acid, 100:0.1, v:v) at 1 mg of protein/mL, and centrifuged at 11 000 × *g*, 10 min prior analysis. These digests were also subjected to a selective precipitation step of phosphorylated peptides as described by Sánchez-Rivera et al.[20] Freeze-dried whey digests were analyzed after a reducing step using dithiothreitol (DTT, Sigma-Aldrich), at a final concentration of 70 mM and pH 7, for 1 h at 37ºC, to aid the identification of disulfide-linked fragments.

Results were processed by using Data Analysis (version 4.0 Bruker Daltonics) and Biotools version 3. 2 and MASCOT v2.4 software (Matrix Science) were used for the interpretation of the matched MS/MS spectra. Homemade database of most abundant bovine casein and whey protein, including major genetic variants, was used for peptide sequencing in MASCOT. Peptide mass tolerance was set to 0.1% and 0.5 Da for MS and MS/MS analysis, respectively. Only individual scores indicating identity or extensive homology ($P < 0.05$) were used. In addition, peptide spectra of shorter peptides with low identification scores were revised manually.

Identified peptides were visualized by using the web application Peptigram.[21]

2.5. Determination of free amino-acids

Freeze-dried samples were dissolved at 4 mg of protein/mL in Milli-Q water. Then, proteins were precipitated with 5-sulfosalicylic acid for 1 h at 4ºC, and were centrifuged at 15000 × *g*, 15 min and 4ºC. The pH supernatant was adjusted to 2.2 with 0.3 M NaOH and filtered through 0.45 µm prior analysis. The analysis was carried out in a Biochrom 30 series Amino Acid Analyser (Biochrom Ltd., Cambridge, UK) equipped with a cation-exchange column. The post-column derivatization was achieved by mixing the eluent column with ninhydrin and by passing this mixture through a high temperature reaction coil. Finally, absorption was measured at 440 and 570 nm.

2.6. Secretion studies of CCK and GLP-1

STC-1 cells, provided by ATCC (ATCC® CRL3254), were cultured in Dulbecco's modified Eagle's Medium DMEM (containing 4.5 g/L with L-glutamine) (Life Technologies, Paisley, UK) and supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/L streptomycin and amphotericin, and incubated in a 5% CO_2 humidified atmosphere at 37ºC. When reaching 80% confluence, STC-1 cells were trypsinized and seeded according to each cell study requirement. Cells were used between passage numbers 15-40 upon reaching 80- 90% confluence.

Cells were seeded into 24-well plates (3×10^5 cell per well) and cultured for 48 h at 37ºC in a 5% CO_2 humidified atmosphere. Medium was removed and cells washed (×3) with HEPES buffer (20 mM HEPES 1M, 10 mM glucose, 140 mM NaCl, 4.5 mM KCl, 1.2 mM $CaCl_2$,

1.2 mM $MgCl_2$, pH 7.4) and then incubated for 1 h in HEPES buffer prior to adding buffer (control) or buffer supplemented with protein digests. All samples were dissolved in HEPES buffer followed by centrifugation at 11 000 × *g for* 10 min. Cell viability was evaluated with each sample at all tested concentrations, as described above. Samples of human jejunal effluents were heated at 85ºC during 15 min before centrifugation to stop the activity of digestive enzymes. A solution of free amino acids used for intravenous infusion was also used at 1% finalconcentration (Aminoplasmal 10% solution for infusion, B. Braun, Barcelona, Spain). Cells wereincubated for 2 h and the medium from each well was collected and stored at -80ºC with Halt protease and phosphatase inhibitor (ThermoFisher Scientific; Waltham, MA, USA). Measurement of the CCK and GLP-1 concentration was performed by using a commercial enzyme immunoassay CCK 26-33, non-sulfated EIA Kit (Phoenix Pharmaceuticals, Inc, Burlingame, CA, USA), and Glucagon Like Peptide-1 Active ELISA (EMD Millipore, Billerica,
MA, USA), respectively. . Because the CCK primary antiserum cross-reacts with human gastrin-1 and big gastrin-1, human jejunal effluents were also directly analysed with the CCK EIA kit at a concentration of 2 mg protein/ml. CCK concentration was below 6 pM for all assayed samples and therefore, background due to cross-reactivity was not considered. All experiments were performed at least two times by using three biological replicates and measurements were performed in duplicate. At the end of the incubation time, cells were collected to isolate RNA by adding on each well RA1 lysis buffer (Macherey-Nagel Gmbh & Co., Düren, Germany) and β- mercaptoethanol (1:10) and stored at -80ºC.

2.7. Cell viability

Cells were seeded into 96-well plates (5×10^4 cells per well) and cultured overnight at 37ºC in a 5% CO_2 humidified atmosphere. Cells were exposed to protein digests for 3 h after which medium was removed from the wells. Alamar Blue Cell Viability Reagent (ThermoFisher Scientific) was added (100 µL, diluted 1:10 v/v), and the plate was incubated (37ºC; 1 h). Finally, fluoresce was read using an excitation wavelength 570 nm and fluoresce emission at 600 nm in a microplate reader.

2.8. RNA isolation and gene expression

Total RNA was extracted from cultured cells using Nucleospin RNA kit (Macherey-Nagel Gmbh & Co.), according to the manufacturer's instructions. Concentration and purity of each sample were evaluated on a Nanodrop 1000 Spectrophotometer (ThermoFisher Scientific). cDNA was obtained by reverse transcription using a PrimeScript RT Reagent kit (RR037A, TaKaRa Bio Inc., Shiga, Japan). Quantitative RT-PCR amplification was carried out using a real-time thermocycler (Viia 7 Real-Time PCR system; Applied Biosystems, Foster, CA, USA) in384-well microplates (Axygen, Corning). The SYBR Green method was used and each assay was performed with cDNA samples in triplicate. Amplification was initiated at 50ºC for 2 min and at 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 60ºC 1 min. The following specific oligonucleotides were used: for *CCK* (accession no. NM_001284508.2) forward (F) 5′-CCAATTTTTCCTGCCCGCAT-3′ and reverse (R) 5′-AGAAGGAGCAGTCAAGCCAAA-3′; for *GLP-1* (accession no. NM_008100.4) (F) 5′-AGAGACATGCTGAAGGGACC-3′ and (R) 5′-CTTTCACCAGCCACGCAATG-3′[14]; and for reference gene *β-actin* (accession no. NM_007393.5): (F) 5'- AGCTGCGTTTTACACCCTTT-3' and (R) 5'-AAGCCATGCCAATGTTGTCT-3'. The relative expression levels of the target gene was calculated using the comparative critical threshold method (ΔΔCt) by normalizing data to the expression of β-actin. Experiments were performed at least three times in triplicate.

2.9. Statistical analysis

ELISA data were compared using one-way ANOVA with Tukey's post hoc test for pairwise comparisons and two-way ANOVA with Bonferroni post hoc tests to compare between samples at the same concentration. PCR results were compared using Mann-Whitney test. Groups were considered significant if $P < 0.05$. GraphPadPrism version 6.01 for Windows (La Jolla, CA, USA) was used for graphics and calculations.

3. RESULTS

3.1. Characterization of *in vitro* protein digests

Protein degradation during gastrointestinal digestion of casein and whey protein was followed by SDS-PAGE. As expected, the four main casein fractions (α_{s1}-, α_{s2}-, β- and κ-casein,

CN) were progressively degraded during the gastric phase and no bands consistent with intact proteins were detected at the end of the gastric phase (120 min) (Supplementary material, Figure S1). β-Lg band was visible during the gastric phase and was degraded within the first 30 min treatment with pancreatic enzymes.

Analysis of the peptide fraction from the different digests throughout the time was performed by HPLC-MS/MS. More than 500 and 700 different peptides were identified in whey protein and casein digests, respectively. Peptide sequences from the four major protein fractions β-CN, αs1-CN, β-Lg and α-lactalbumin (α-La) are given in Supplementary Tables S1- S4. These peptides were represented using the peptide profile tool of the Peptigram web application where each vertical bar corresponds to an amino acid identified as part of a peptide sequence; the height of this bar is proportional to the count of peptides overlapping this position and the colour intensity is proportional to the sum of the intensities of the peptides overlapping a given position. Figure 1 shows the peptide profiles obtained for the major casein fractions, αs1-, and β-CN, and the most abundant whey protein, β-Lg, at different time points during the gastric and the intestinal phase. During the gastric phase, degradation of αs1-CN started from N- and C-terminal domains of the protein since a higher number of peptides from these regions was found. Similarly, peptides belonging to the C-terminal region of β-CN were also more intense than those from other parts of the molecule, suggesting a higher susceptibility of this region the
action of pepsin. It has to be noted that by mass spectrometry, peptide intensity relies on peptide ionization capacity in addition to abundancy, and therefore, peptide intensity cannot directly be translated to peptide concentration. Due to the resistance of β-Lg to the action of pepsin, only few peptides were detected after 30 min gastric digestion and this time point is not shown in Figure 1. However, several β-Lg peptides were identified at the end of the gastric phase (120 min). Under our mass spectrometry conditions, peptides with sizes between 5 and 30 amino acids are preferably detected, although few peptides with longer sizes could be identified depending on their sequence. The blank regions observed in the peptide profile during the gastric phase probably correspond to peptide fragments too long to be solubilized or ionized under our analysis conditions while blank regions in the intestinal phase are more likely due to short peptides, free amino acids, or peptides with low ionization capacity. Due to further degradation of the proteins and long polypeptides during the intestinal phase, a higher number

of peptides along the entire sequences of milk proteins was achieved at the end of the intestinal

phase. Protein coverages at the end of the gastrointestinal digestion were 82, 97, 83 and 43% for β-CN, α_{s1}-CN, β-Lg and α-La, respectively. It has to be noted that these peptide profiles contain the identified casein phosphorylated sequences after selective precipitation which explain the coverage values reached. As expected, peptide size decreased with the digestion time. For instance, after 30 min of digestion with pepsin, only 13% of the αs1- and β-CN-derived peptides contained between 5-9 amino acids; while 72% of the casein-derived peptides belonged to this size range after 30 min of intestinal digestion, and it increased up to 75% at the end of the intestinal phase. Similarly, peptides longer than 20 amino acids derived from αs1- and β-CN represented 27% after 30 min digestion with pepsin but only few peptides between 15 and 30 amino acids were detected at the end of the intestinal phase (3%) (Supplementary material, Figure S2A). The digestion of whey proteins also resulted in the appearance of small size peptides during the intestinal phase, reaching the 80% of the total identified peptides at the end of the intestinal phase. At this time point, several medium-size peptides (15-19 amino acids) derived from β-Lg, were still identified in the soluble phase of the digests, which accounted for 7% of the peptides (Supplementary material Figure S2B).

The amino acid release during gastrointestinal digestion was followed by HPLC and post-column ninhydrin derivatization. Free amino acids were mainly released during the intestinal phase and only traces of Phe, Leu and Val could be quantified at the end of the gastricphase (Figure 2). An important amount of amino acids were released from the beginning of the incubation with pancreatic enzymes, and gradually increased over time. At the end of the intestinal digestion the total amount of free amino acids reached 2285 and 4802 nmol/mL for casein (Figure 2A) and whey proteins (Figure 2B), respectively, being Leu, Lys the most abundant, followed by Tyr, Phe and Arg.

3.2. Release of intestinal hormones induced by *in vitro* gastrointestinal digests from casein and whey proteins

Initially, cell viability under the assay conditions with all protein digests was evaluated. Gastric digests maintain cell viability at the three sample concentrations assayed (4, 1 and 0.25 mg protein digest/mL), but intestinal digests caused a notable decrease in cell viability except at

the lowest concentration (Figure 3 A). The cytotoxicity of the intestinal digests was attributed to the presence of the bile salts in these samples, since the digests prepared in absence of bile salts had no effect on cell viability (Figures 3 B and 3 C), and these were used in subsequent cell assays.

Casein and whey protein gastric digests stimulated a significant CCK secretion (from 2.8 to 7.6-fold) in comparison with the control cells (Figure 4 A, B). This increase was even higher (from 6.3 to 13.8-fold) with the casein and whey protein intestinal digests. A dose- response effect was observed for all samples. A mixture of free amino acids assayed at 1% did not show any effect on CCK secretion compared to the control cells. Comparing casein and whey protein digests, only the whey-protein intestinal digests at 30 min tested at 4 mg/mL showed statistically higher CCK secretion than the intestinal digests from casein at 30 or 120 min ($P < 0.001$).

Interestingly, GLP-1 secretion showed a markedly different behaviour (Figure 4 C, D). Its secretion was stimulated with the casein gastric digests at 30 and 120 min (6.3-10.5-fold) and with digests of whey proteins at 120 min (1.9-5.6-fold). For all these samples a dose-dependent effect was detected. On the contrary, casein intestinal digests at 30 or 120 min did not stimulate GLP-1 secretion at any concentration in this cell line. Similarly, whey proteins subjected to the incubation with pepsin (gastric phase) which contained intact β-Lg, as shown in figure S1, did not stimulate GLP-1 release. The solution of free amino acids caused a slight increase on GLP-1 release (vs. control cells) but it was not statistically different from those observed with casein intestinal digests. The GLP-1 response with intestinal whey protein digests at 120 min assayed at 4 and 1 mg/mL was statistically higher than that reached with gastric casein digests at 30 or 120 min ($P < 0.0001$).

3.3. Intestinal hormones release induced by human jejunal digests from casein and whey proteins

Cell viability with the human jejunal effluents was evaluated at the three concentrations assayed (4, 1 and 0.25 mg/mL) but the highest concentration caused a decrease in cell viability and was not used in these experiments (Figure 3D). Whey protein jejunal digests stimulated a significant and dose-dependent CCK secretion (from 2.2 to 12.6-fold) in comparison with the

control cells (Figure 5 B). This increase in CCK secretion was even higher with casein human digests (from 9.7 to 57.7-fold) (Figure 5 A). At 1 mg/mL, casein jejunal digests taken at 1 and 2 h were statistically higher than the corresponding whey jejunal digests with $P < 0.05$ and $P < 0.001$, respectively. Human jejunal digests also caused an increase in GLP-1 secretion compared with the control cells (from 1.0 to 5.6-fold). No significant differences on GLP-1 secretion were observed between human digests from casein and whey protein (Figures 5 C, D), except for the casein jejunal digests taken at 2 h and assayed at 0.25 mg/mL where a higher GLP-1 response was reached than with the corresponding jejunal effluents from the whey protein group ($P < 0.0001$).

The hormonal responses of the in vitro digests at the end of the in vitro gastrointestinal digestion were compared with those achieved with the human jejunal digests (Figure S3). The CCK levels reached with the *in vitro* and *in vivo* digests were comparable and only the jejunal sample from one volunteer who had received casein (subject B) induced significant higher CCK secretion than the *in vitro* intestinal digest and the other two casein human samples (Figures S3 A and B). The GLP-1 average value achieved with the human jejunal samples from the group receiving whey protein was also comparable to the secretion with the *in vitro* whey protein digest, although some differences could be evidenced depending on the volunteer and the dose (Figure S3 D). However, the GLP-1 secretagogue activity of the *in vitro* casein digest was significantly lower than the average value of the corresponding human samples or the individual responses (Figure S3 C).

3.4. Intestinal hormones gene expression induced by *in vitro* and jejunal digests from casein and whey proteins

Casein digests obtained at the end of the intestinal phase increased CCK mRNA levels with a maximum fold increase of 1.7. This increase was higher than that found for the casein jejunal samples on average; although a high interindividual variability was found and some individuals showed similar increase than the *in vitro* sample (Figure 6 A). In agreement with the hormone release results (Figure S3 C), *GLP-1* mRNA level induced by the *in vitro* casein digest at the end of the gastrointestinal digestion was significantly lower than the average value reached with the human whey digests ($P < 0.01$) (Figure 6 C). On the contrary, *CCK* and *GLP-1*

mRNA levels in cells incubated with the whey in vitro digest at the end of the digestion were comparable to those obtained with whey human jejunal effluents (Figures 6 B and D). GLP-1 mRNA levels with casein and whey human jejunal samples reached 2.6 and 3.0 maximum fold-change, respectively (Figures 6 C and D). In contrast to the effect on hormone secretion, not all samples showed a dose-response relationship in this assay.

4. DISCUSSION

This work aims to study the influence of characterized gastrointestinal digests from casein and whey protein on CCK and GLP-1 secretion and expression in enteroendocrine cells. *In vitro* gastrointestinal digests from casein and whey proteins were prepared by using an internationally harmonised digestion protocol, which had demonstrated good performance in samples of dairy origin by comparison with porcine and human samples.[16, 22] It has to be noted that SDS-PAGE and HPLC/MS-MS results are similar to those found by other authors[23] demonstrating the representability of these digests. In addition, human jejunal digests from the same substrates were also tested in the same cell model to validate the results obtained with the *in vitro* digests.

CCK release was increasedafter incubation of STC-1 cells with gastric digests from casein and whey proteins but its secretion was maximized with the intestinal digests of both substrates. The length of peptides was notably shortened during the intestinal phase for both substrates and, in addition, an important amount of free amino acids was released. However, a mixture of free amino acids assayed at 1% did not cause any increase on CCK release with respect the control cells. This suggests that CCK secretion is mainly induced by small size peptides, since most peptides at intestinal level were shorter than 10 amino acids, as determined by HPLC-MS/MS. These results confirm those previously reported where it was demonstrated that CCK secretion was caused by peptide molecules and not by free amino acids.[7, 24] Tulipano and co-workers[15] h a d suggested that the length of the peptides derived from a protein can be relevant for the CCK secretagogue activity. When working with synthetic peptides, this research group did not find any effect with di- or tri-peptides containing aliphatic side-chains on CCK secretion and only peptides with five or more amino acids were effective at stimulating CCK release from STC-1 cells. Our results confirm these previous findings with

casein and whey protein gastrointestinal digests in a dose-dependent manner. Regarding the amino acid composition, several authors had pointed out the importance of free aromatic amino acids (L-Phe and L-Trp) as CCK stimulators which has been postulated to be mediated by the calcium sensing receptor (CaSR).[25] This receptor was also involved in the CCK secretion induced by peptide fractions from various food protein hydrolysates.[26] Caron and co-workers[13] suggested that the CCK secretagogue activity of haemoglobin-derived peptides was linked to the high content of aromatic residues in their sequences. Although the total aromatic amino acid content (Phe+Tyr+Trp) is higher in casein than in whey proteins (111.8 *vs* 65.5 mg/g), the amount of aromatic free amino acids reached a higher value in whey protein than in casein digests (Figure 2). However, both intestinal digests (casein and whey protein) gave a similar response on CCK secretion in STC-1 and therefore, CCK secretion does not appear to be affected by the content of free aromatic amino acids. In addition to CaSR, GPR93 which is able to sense peptides, is expressed in this cell line, and had also been related to CCK secretion.[8] The involvement of GPR93 could support the poor CCK secretion level reached by the solution of free amino acids and the secretagogue role of the intestinal digests containing a high amount small size peptides.

GLP-1 response was markedly different from that observed for CCK. Its secretion was favoured with the casein gastric digests at 30 or 120 min and the whey protein digests at the end of the intestinal phase. Although this latter sample contains a high concentration of free amino acids, these were practically absent in the casein gastric digests. Our results are in agreement with a previous report, where GLP-1 secretion in intestinal enteroendocrine cellswas stimulated by different casein fractions and casein fractions digested with pepsin, but hydrolysis with trypsin and chymotrypsin reduced or abolished this stimulatory effect.[12] The behaviour observed for whey proteins was different, showing the highest stimulatory activity at the end of the simulated gastrointestinal digestion. Although it had been suggested that intact whey protein could induce GLP-1 release[9], gastric whey protein digests that contained intact β- Lg did not trigger GLP-1 secretion under our conditions. In addition, when comparing gastric casein and gastrointestinal whey digests, it was found that the latter behaved as a more potent stimulator of GLP-1 secretion. In agreement with our data, it had been reported that β-Lg

hydrolysed with chymotrypsin and trypsin retained GLP-1 secretagogue activity[11] and whey protein had been previously described as a greater stimulator or GLP-1 secretion than casein.[27]

Previous work on STC-1 cells had led different authors to suggest that STC-1 cells recognise some protein fragments and not others and therefore, that hormone stimulation could be peptide-specific.[12, 13, 28] Our results demonstrate not only that the nature of the protein may influence CCK and GLP-1 secretion but also that the degree of protein hydrolysis plays an important role. Since protein digestibility is affected by the technological process to which food is subjected, the release of digestion products with stimulatory effects on enteroendocrine cells could be influenced by food processing. To validate the results obtained with in vitro digests, previously characterized16 human jejunal digests were also assayed in this cell line. The jejunal digests of casein and whey proteins produced a significantly increase in CCK secretion compared to the control. The CCK secretatogue effect of the human digests from casein and whey proteins was comparable except subject B for which the stimulatory activity was higher. The amount of released CCK in these experiments with in vivo digests was also comparable to that obtained with the in vitro ones (ca. 300 pM CCK). However, the response of GLP-1 to incubation with the human jejunal casein digests was higher than that observed with the casein in vitro digests, although the effect of in vitro and in vivo digests from whey protein was similar. The different GLP-1 response with casein in vivo jejunal digests in comparison with the in vitro intestinal ones could be due to a slightly higher casein degradation observed in the in vitro digests in comparison with the human samples.[16] This finding could be in agreement with the peptide-specific effect claimed by different authors since several peptide sequences found in jejunal casein digests were not detected in the in vitro intestinal digests. Moreover, the effect of other endogenous molecules in the in vivo digests or the presence of bile salts cannot be ruled out. The secretagogue effect of human jejunal digests on STC-1 cells did not follow a dose-response pattern. In accordance with this, other authors had previously described an optimal concentration range of casein-derived products to act as GLP-1 inductors.[12] Similarly, some authors had reported whey protein to be a more efficient GLP-1 stimulator compared to casein[27], and our results with in vitro digests could support this. However, the GLP-1 secretion levels obtained with human jejunal digests from casein and whey proteins were similar for both

substrates, being the inter-individual variability between subjects more determinant in terms of GLP-1 secretion.

The gene expression experiments at the end of the incubation period with the *in vitro* digested samples (2 h) showed increased *CCK* and *GLP-1* mRNA levels but differences between casein and whey protein digests were not as pronounced as those observed by quantifying the secreted amount of hormone. Recently, it has been proposed that incretin hormones GLP and GIP are encoded on translationally regulated genes in STC-1 cells and pGIP/Neo sub-clone. Therefore, there is not a direct correlation between the number of proglucagon mRNA copies and the abundance of GLP-1 and GLP-2 peptides.29 These authors also found discrepancies between PYY and CCK expression and immunoreactivity of the protein in accordance with our results. In addition, comparison of the gene expression induced by in vitro gastrointestinal digests and the human jejunal counterparts was comparable and differences were attributable to the high inter-individual variability (Figure 6).

CCK is mainly produced by "I" cells which are concentrated in the proximal small intestine although ileal and colonic "L" cells are also known to express this hormone. GLP-1 is released by "L" cells which predominate in the ileum and colon and decrease in number towards the proximal end of the small intestine, although GLP-1 also occurs in subsets of duodenal "K" cells. However, it has been proposed that this division of enteroendocrine cells into subpopulations is not strict and several of these have a considerable overlap.[30] Our results suggest a higher stimulation of CCK release with small size peptides while GLP-1 secretion seems to be stimulated by longer peptides. Taking into account this physiological localization, it can be hypothesized that proteins easily digested during the gastric phase, such as caseins, may have a more pronounced stimulatory effect on CCK secretion while proteins rendering larger fragments during gastric digestion, such as whey proteins would preferably promote GLP-1 release. In a previous human study with milk protein fractions, a slightly lower GLP-1 secretion was observed in the group receiving casein compared to the group receiving whey protein or the mixture.[19] However, the translation of these results to satiety has to be carefully done sinceit has been stressed that anorexigenic hormones levels do not strictly correlate with satiety responses in humans.[31]

5. CONCLUSIONS

Casein and whey protein digests induced CCK and GLP-1 secretion in a dose-dependent manner and this effect was determined by the nature of the substrate and the protein digestion degree. Both, casein and whey protein digests behaved as CCK secretagogues although this effect is more pronounced with the intestinal digests, comprising mainly small size peptides (< 10 residues) and free amino acids, than with digests from the gastric phase. However, free amino acids are not strictly required for CCK secretion since gastric digests, i.e., digests where free amino acids were absent, are also able to induce the secretion of this hormone. In addition, a mixture of free amino acids did not trigger CCK release in this cell model. On the contrary, GLP-1 secretion was stimulated with casein gastric digests and whey protein digests. These results were confirmed with human jejunal digests from the same substrates which showed a comparable effect to the in vitro ones. Only the response of GLP-1 with the human jejunal casein digests was higher than that observed with the in vitro digests, probably due to the higher casein degradation observed in the in vitro digests versus the human samples. Studies are in progress to characterize the nature of peptides with a higher hormone secretagogue effect and the receptors involved in their sensing by enteroendocrine cells.

Conflict of interest statement

The authors declare no competing financial interest.

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FIGURE CAPTIONS

Figure 1. Peptides from α_{s1}-casein, β-casein identified in casein gastrointestinal digests and peptides from β-Lg identified in whey protein digests at different time points. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence. The height of the bar is proportional to the number of peptides overlapping this position and the colour intensity is proportional to the sum of the intensities of the peptides overlapping a given position. Each line corresponds to a different time point: 30G, 30 min gastric digestion; 120G, 120 min gastric digestion; 30I, 30 min gastrointestinal digestion; and 120I, 120 min gastrointestinal digestion.

Figure 2. Free amino acid contents (nmol/mL) in (A) casein and (B) whey protein digests at different time points: 30G, 30 min gastric digestion; 120G, 120 min gastric digestion; 30I, 30 min gastrointestinal digestion; and 120I, 120 min gastrointestinal digestion. Free amino acids were analyzed by cation exchange-HPLC and ninhydrin derivatization.

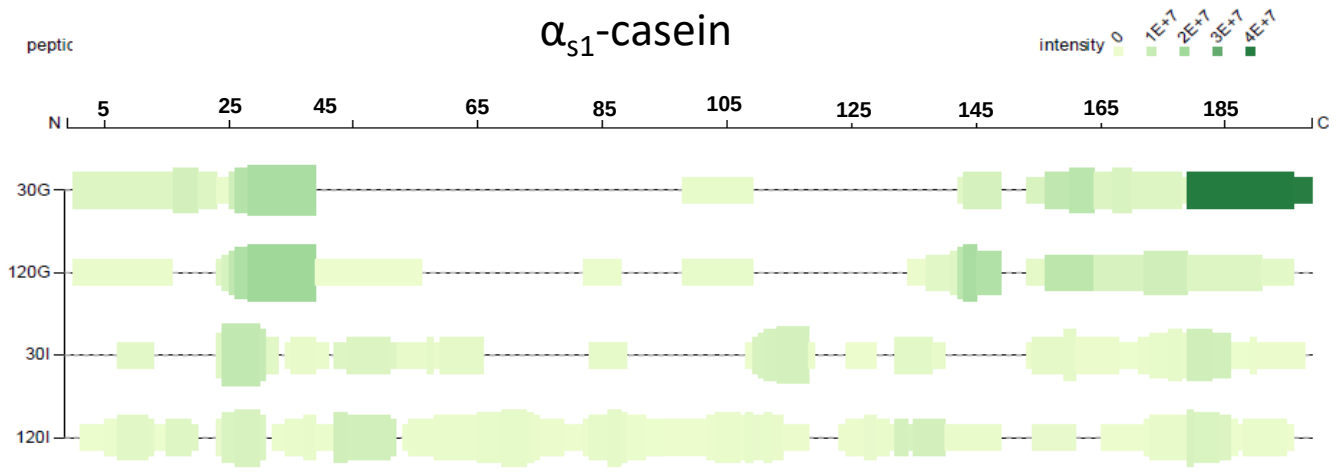
Figure 3. Cell viability in STC-1 cells (mean ± SEM, n=3) following 3 h incubation with (A) *in vitro* whey protein digests with bile salts, (B) whey protein *in vitro* digests without bile salts, (C) casein *in vitro* digests without bile salts, and (D) human jejunal effluents taken at 1 and 2 h afteringestion of casein (Subject B) or whey protein (Subject E). Digests were evaluated for cell viability at three different protein concentrations (4, 1 and 0.25 mg/mL). *In vitro* gastrointestinal digests were taken at different time points: at 30 and 120 min of gastric phase and 30 and 120 min of intestinal phase, except for whey protein where 30 min of gastric phase was omitted. Statistical significance compared with control (one way ANOVA with Tukey's post hoc test) is indicated by **P < 0.01, ****P < 0.0001.

Figure 4: CCK secretion after 2 h incubation of STC-1 cells with (A) casein and (B) whey protein *in vitro* gastrointestinal digests at 30 and 120 min of gastric phase (for whey protein only 120 min of gastric phase was tested) and 30 and 120 min of intestinal phase, at different protein concentrations (4, 1 and 0.25 mg/mL). GLP-1 secretion from STC-1 cells after incubation with (C) casein and (D) whey protein digests. CCK and GLP-1 secretion was determined by ELISA. FA 1%, free amino acid solution assayed at 1%. Error bars indicate SEM (n=3). Statistical

significance compared with control (one way ANOVA with Tukey's post hoc test) is indicated by *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. Different letters denote statistically significant differences (P < 0.05) between different time points and protein concentrations.

Figure 5: CCK secretion after 2 h incubation of STC-1 cells with human jejunal effluents taken at 1 and 2 h after oral administration of (A) casein and (B) whey protein, and assayed at different protein concentrations in this cell culture (1 and 0.25 mg/mL). GLP-1 secretion from STC-1 cells after incubation with human jejunal effluents after oral administration of (C) casein and (D) whey protein. CCK and GLP-1 secretion was determined by ELISA. Error bars indicate SEM (n=3). Statistical significance compared with control (one way ANOVA with Tukey's post hoc test) is indicated by *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. Different letters denote statistically significant differences (P < 0.05) between different time points and protein concentrations.

Figure 6: *CCK* and *GLP-1* mRNA levels in STC-1 cells after 2 h incubation with *in vitro* gastrointestinal digests of casein and whey proteins at the end of the *in vitro* digestion protocol (120 min intestinal) and with human jejunal effluents from the same substrates taken at 1 h after oral administration. *CCK* mRNA levels after incubation with *in vitro* and *in vivo* digests from (A) casein, and (B) whey protein. *GLP-1* mRNA levels after incubation with *in vitro* and *in vivo* digests from (C) casein and (D) whey protein. Data are normalized using β-actin as reference gene and are expressed relative to the expression level of untreated cells (red line). Error bars correspond to SEM (n=9). Results were compared using Mann-Whitney test after Shapiro-Wilk normality test. Statistical significance compared with the normalised control is indicated by, $^{\#}$P <0.05, $^{\#\#}$P < 0.01, and $^{\#\#\#}$P < 0.001. Comparisons with the *in vitro* digest (two way ANOVA with Bonferroni post hoc test) are indicated by ***P < 0.001; ns, not significant.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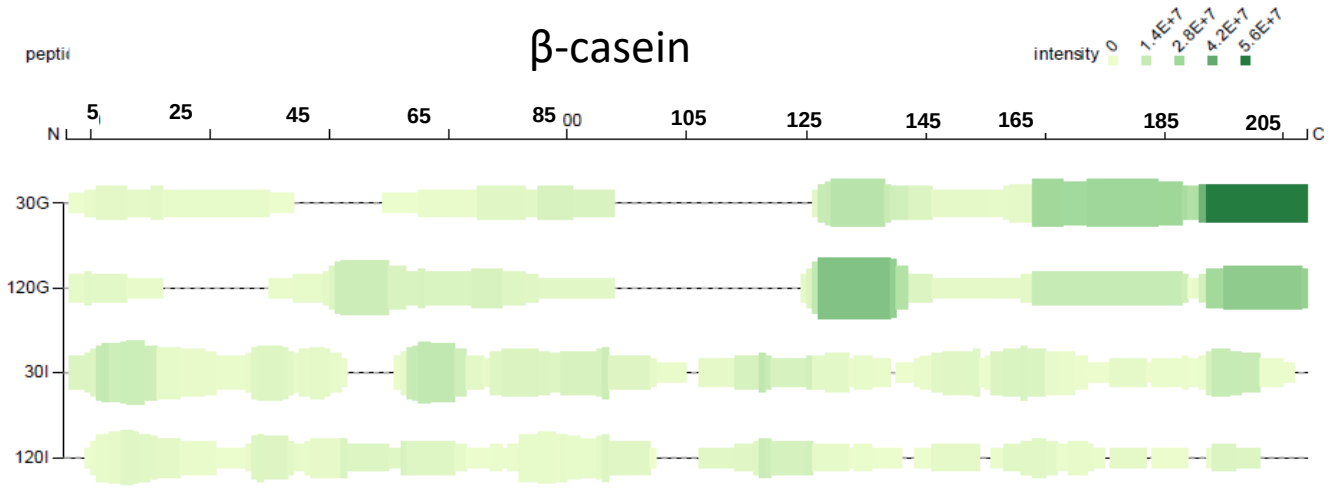
120G
30I
120I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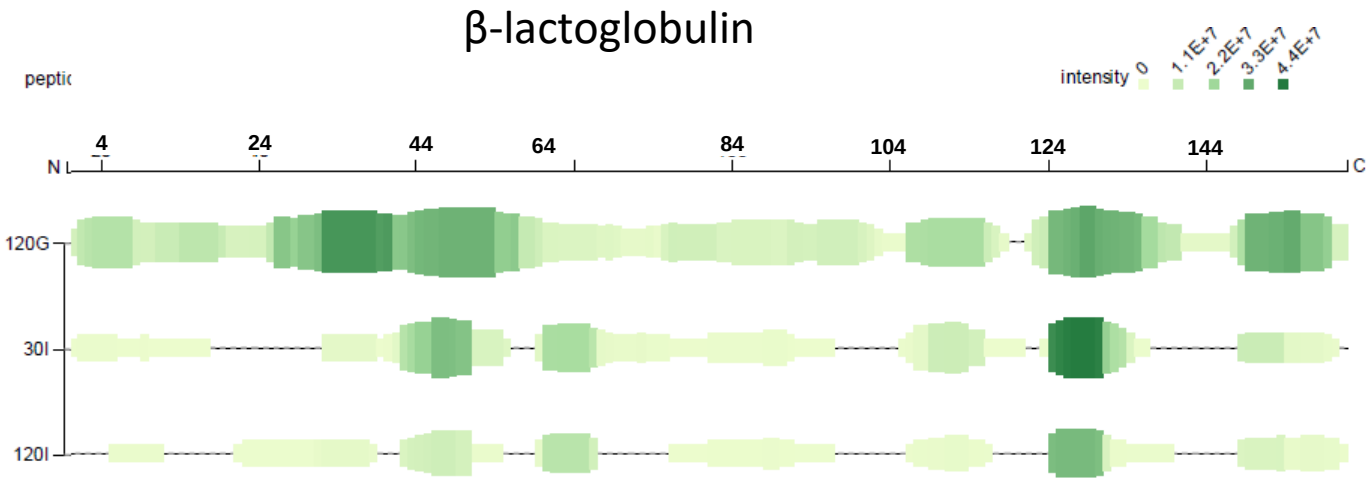
30I
120I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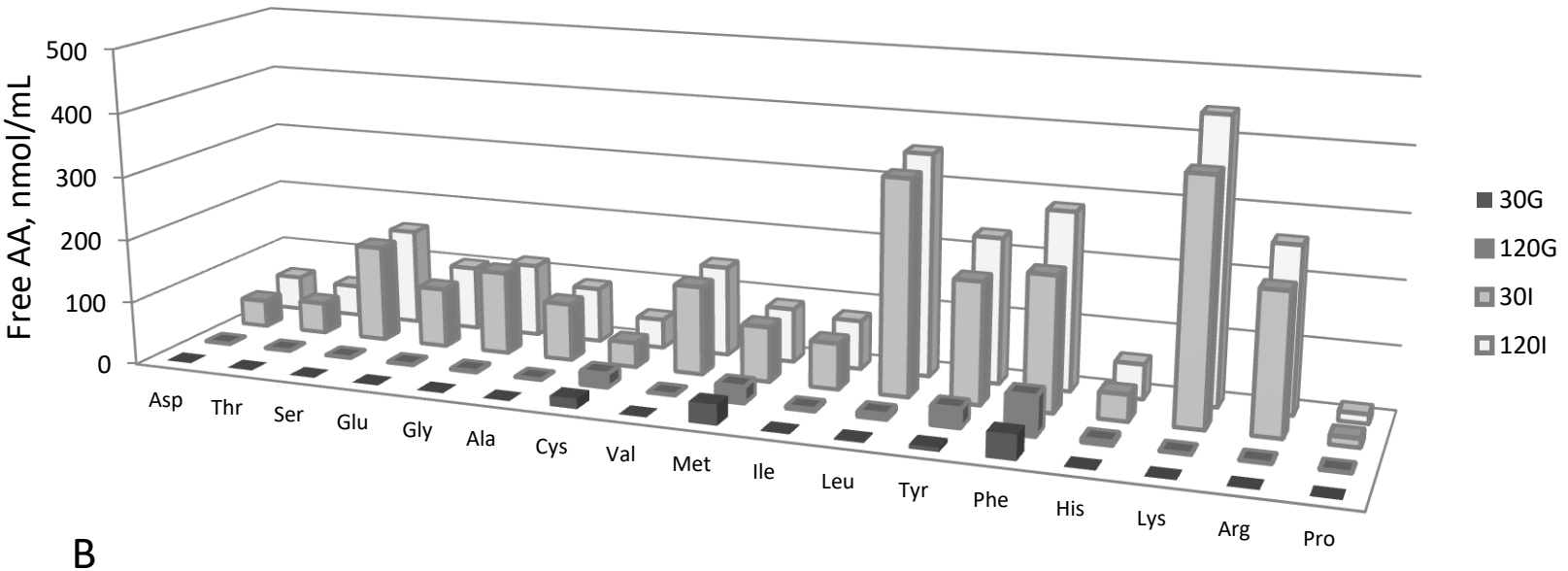
C
120G
30I
120I

A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500
400
300
200
100
0
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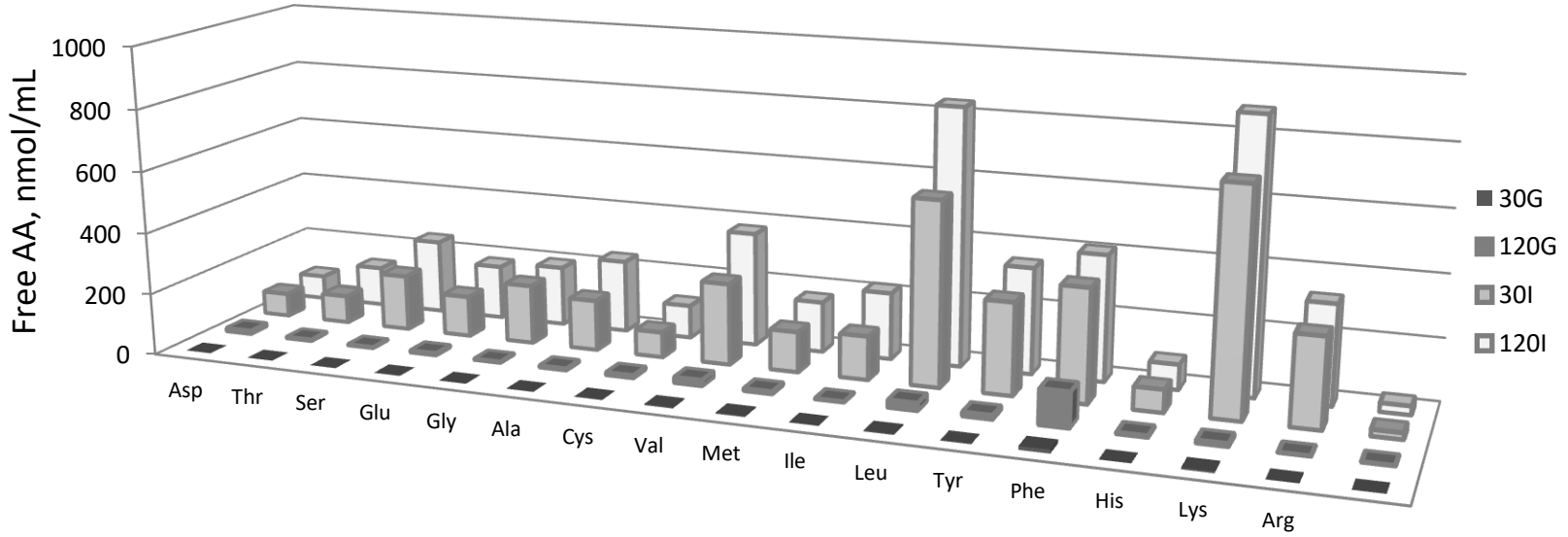
Thr
Ser
Glu
Gly
Ala
Cys
Val
Met
Ile
Leu
Tyr
Phe
His
Lys
Arg
Pro
30G
120G
30I
120I

B


Free AA, nmol/mL
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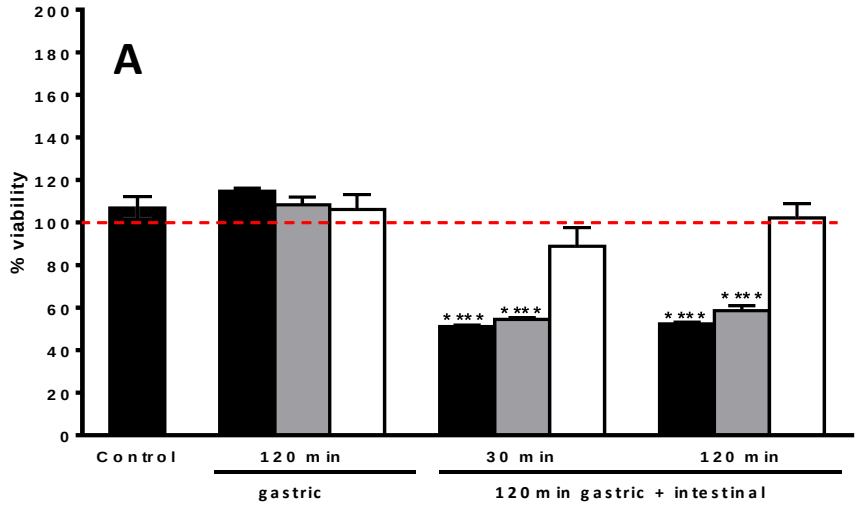
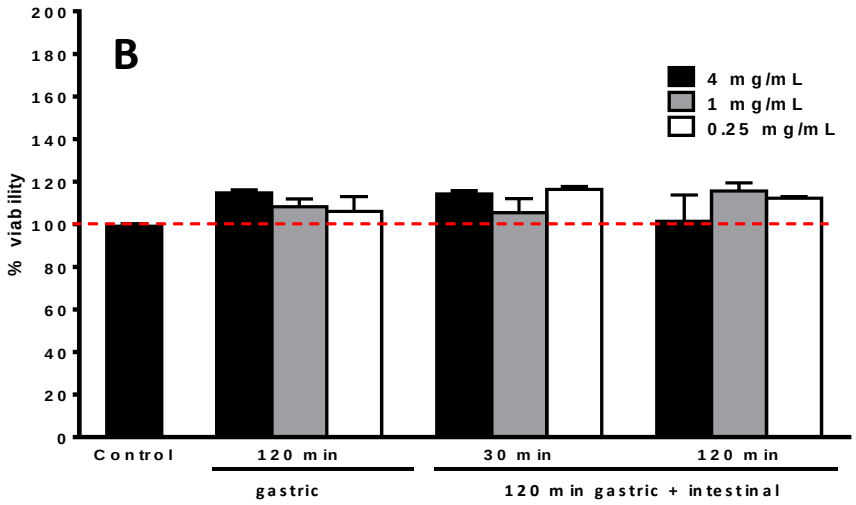
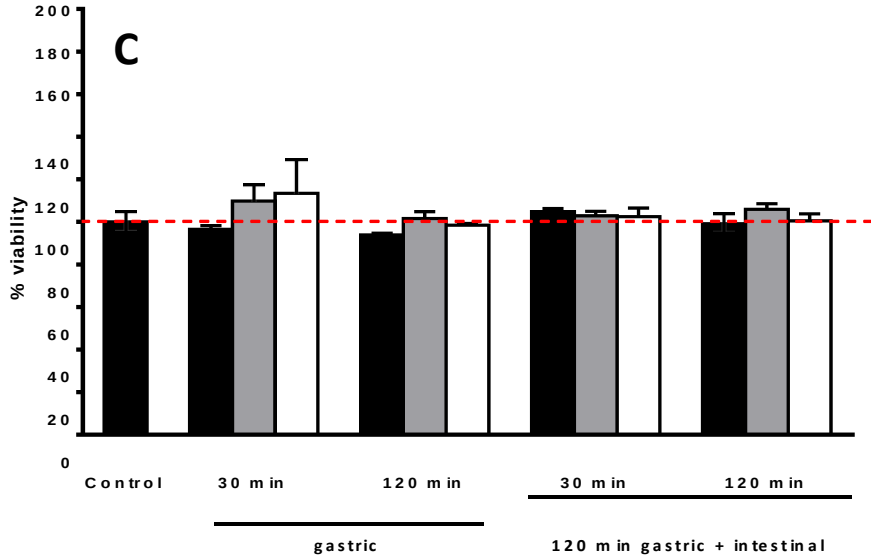
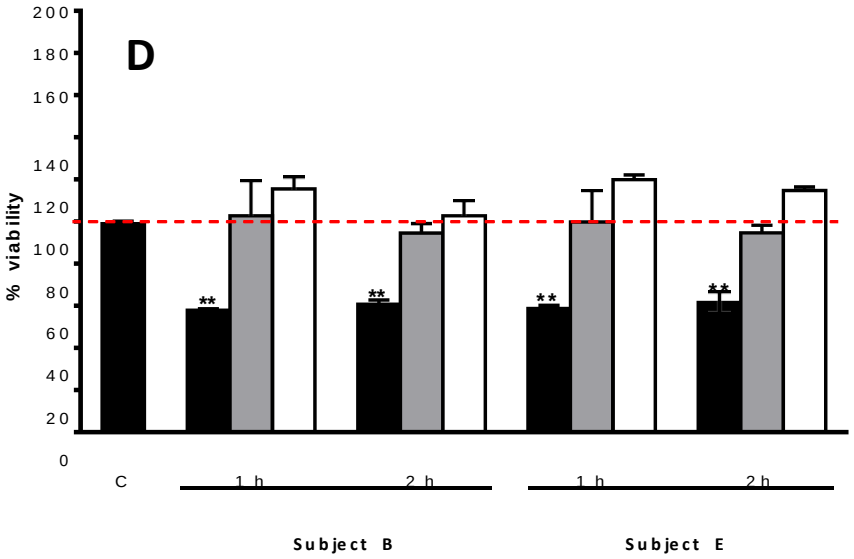
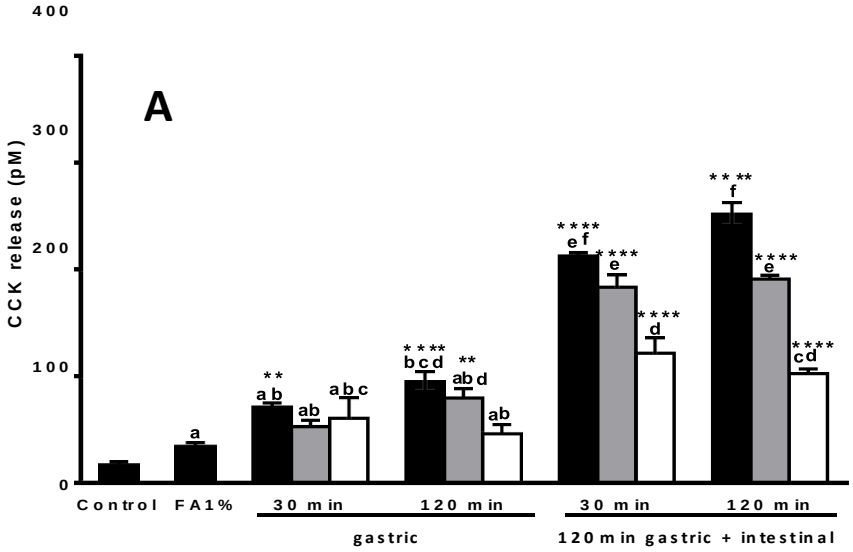
Phe
His
Lys
Arg
30G
120G
30I
120I

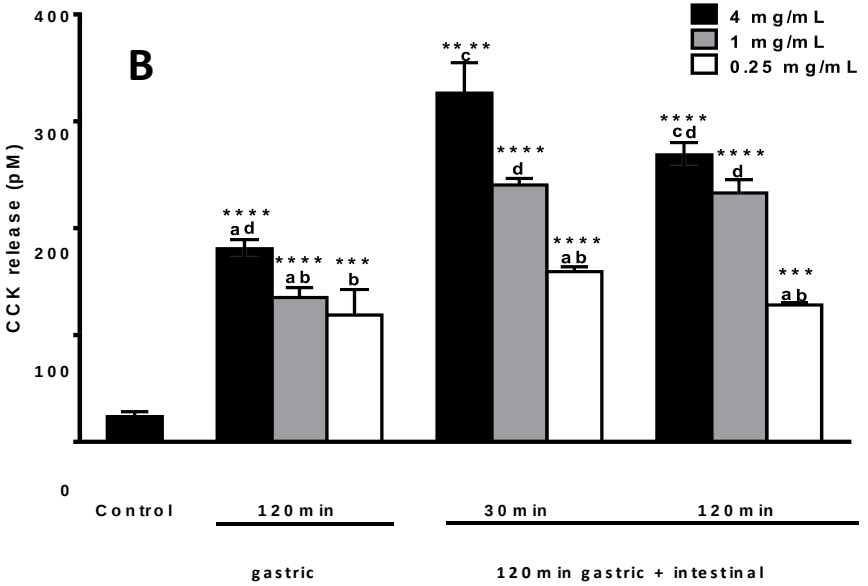

A
% viability
200
180
160
140
120
100
80
60
40
20
0
***
***
***
***
Control
120 min
30 min
120 min
gastric
120 min gastric + intestinal

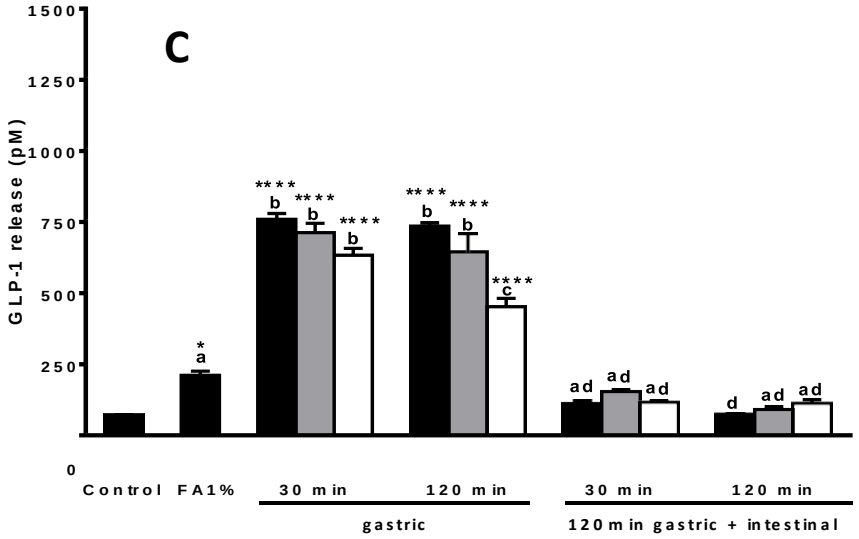

B
% viability
4 mg/mL
1 mg/mL
0.25 mg/mL
Control
120 min
30 min
120 min
gastric
120 min gastric + intestinal

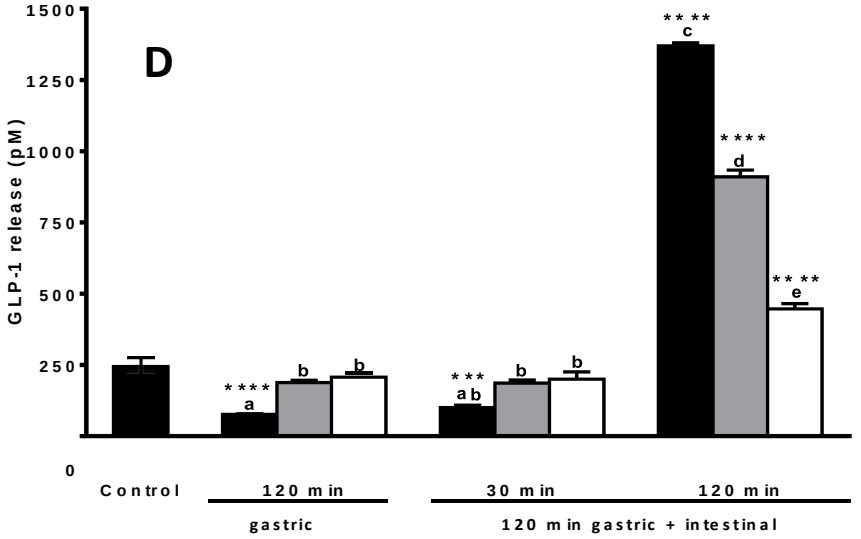

C
% viability
Control
30 min
120 min
30 min
120 min
gastric
120 min gastric + intestinal


D
% viability
**
**
**
**
C
1 h
2 h
1 h
2 h
Subject B
Subject E


A
CCK release (pM)
400
300
200
100
0
Control
FA1%
30 min
120 min
30 min
120 min
gastric
120 min gastric + intestinal


B
4 mg/mL
1 mg/mL
0.25 mg/mL
CCK release (pM)
400
300
200
100
0
Control
120 min
30 min
120 min
gastric
120 min gastric + intestinal


C
GLP-1 release (pM)
1500
1250
1000
750
500
250
0
Control
FA1%
30 min
120 min
30 min
120 min
gastric
120 min gastric + intestinal


D
GLP-1 release (pM)
1500
1250
1000
750
500
250
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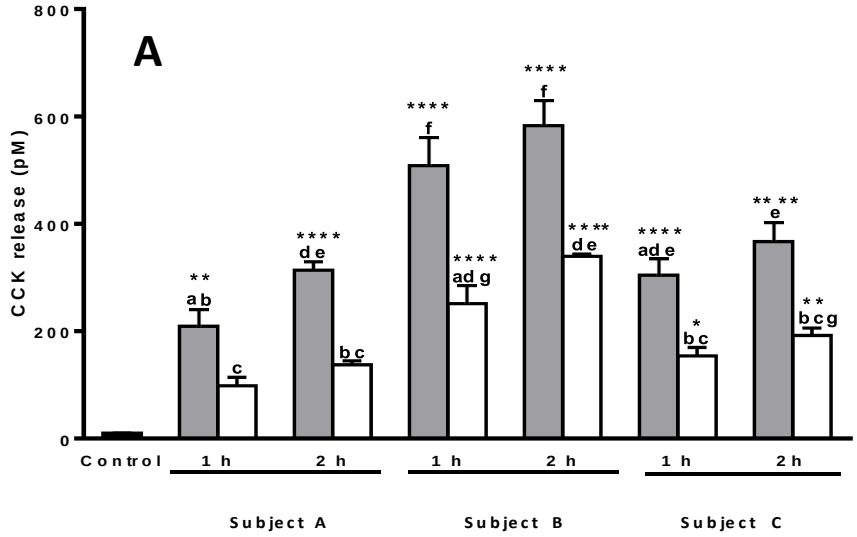
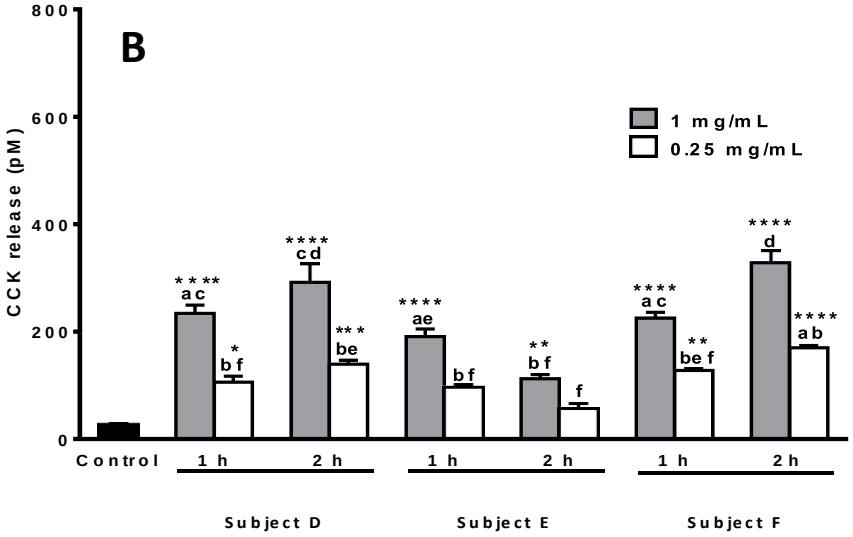
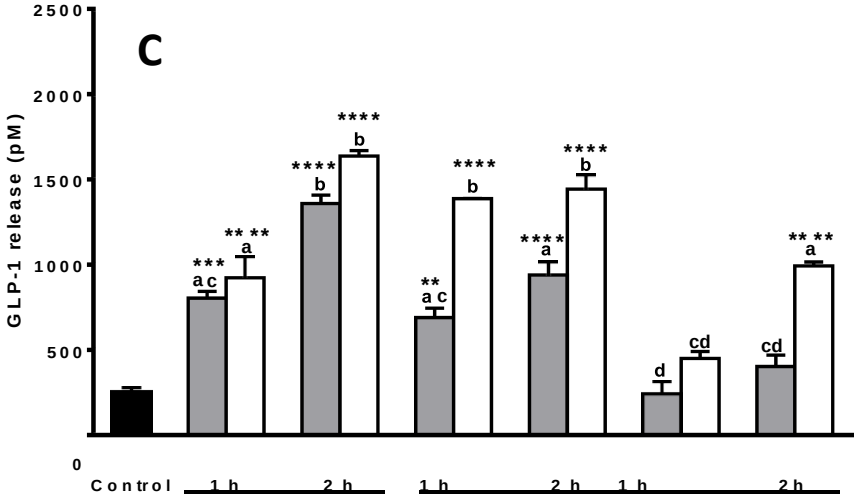
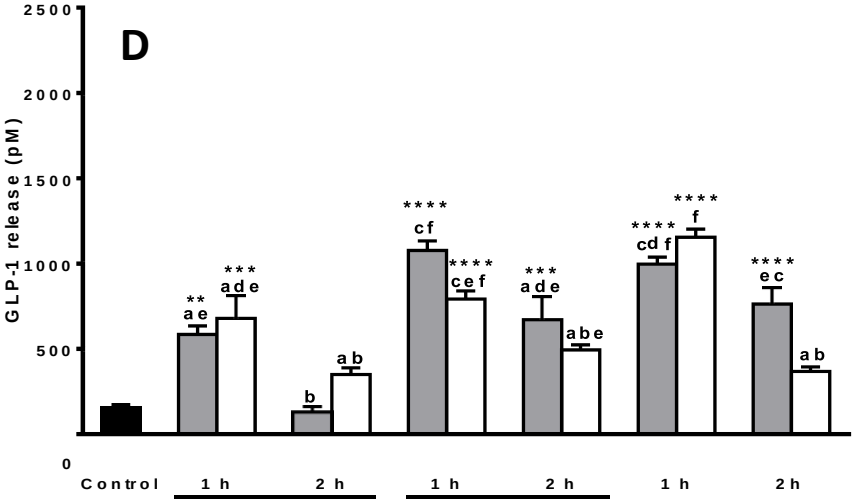
Control
120 min
30 min
120 min
gastric
120 min gastric + intestinal


A
CCK release (pM)
800
600
400
200
0
Control
1 h
2 h
Subject A
Subject B
Subject C


B
CCK release (pM)
1 mg/mL
0.25 mg/mL
Control
1 h
2 h
Subject D
Subject E
Subject F


C
GLP-1 release (pM)
2500
2000
1500
1000
500
0
Control
1 h
2 h


D
GLP-1 release (pM)
2500
2000
1500
1000
500
0
Control
1 h
2 h

Subject A

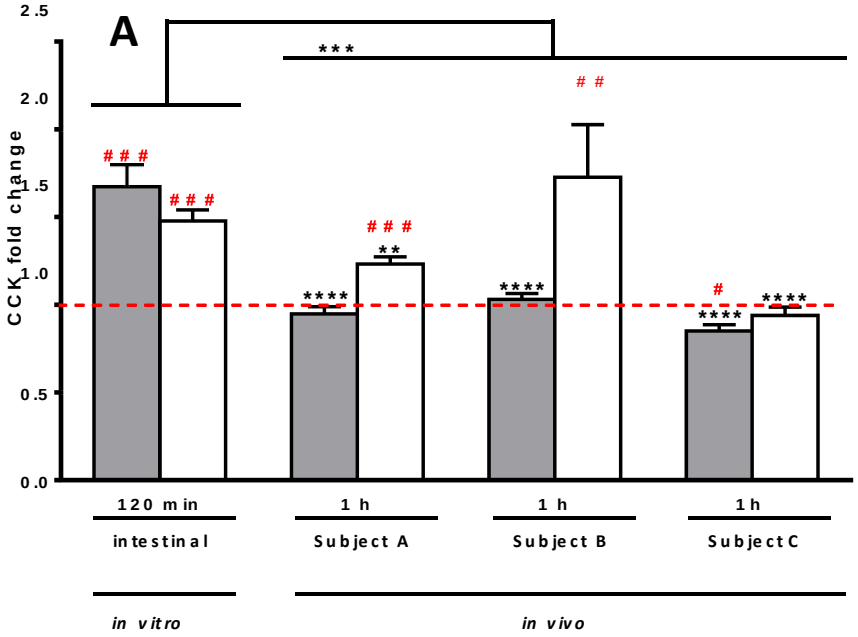
Subject B

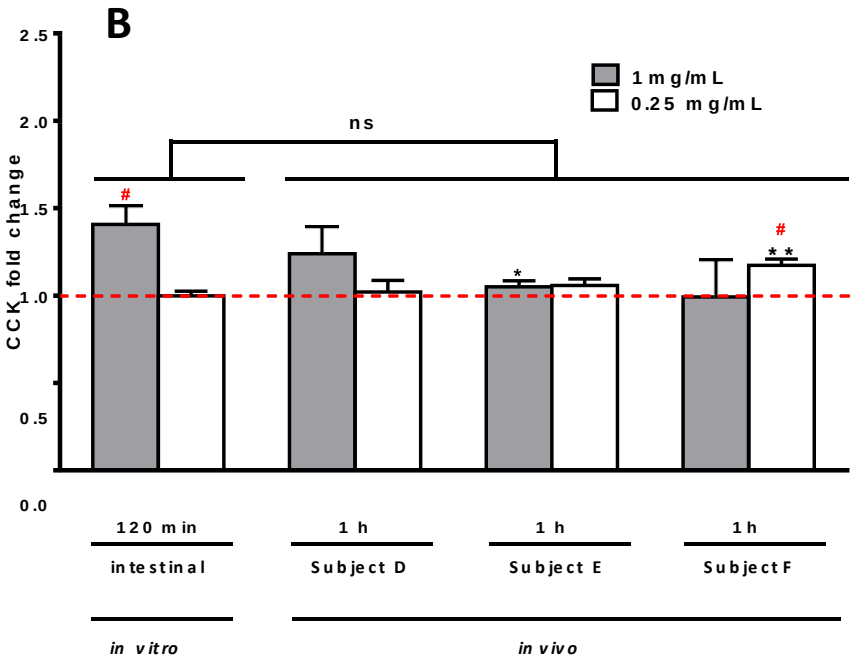
Subject C

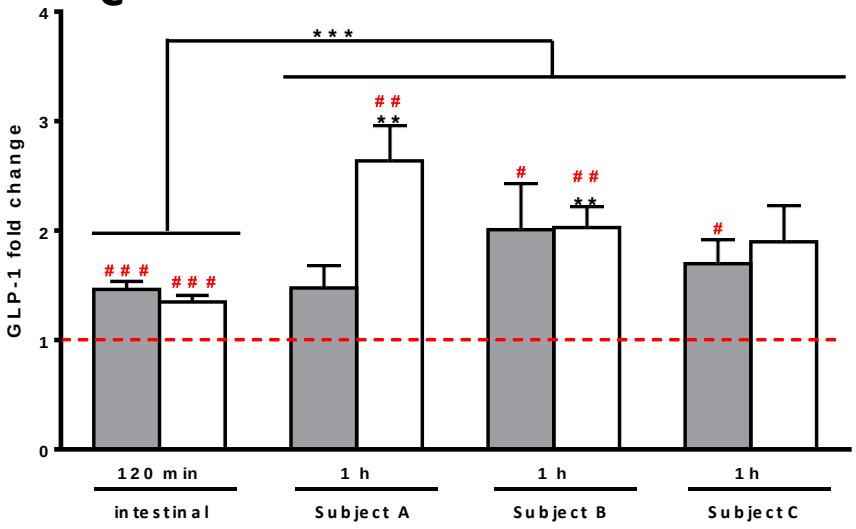
Subject D

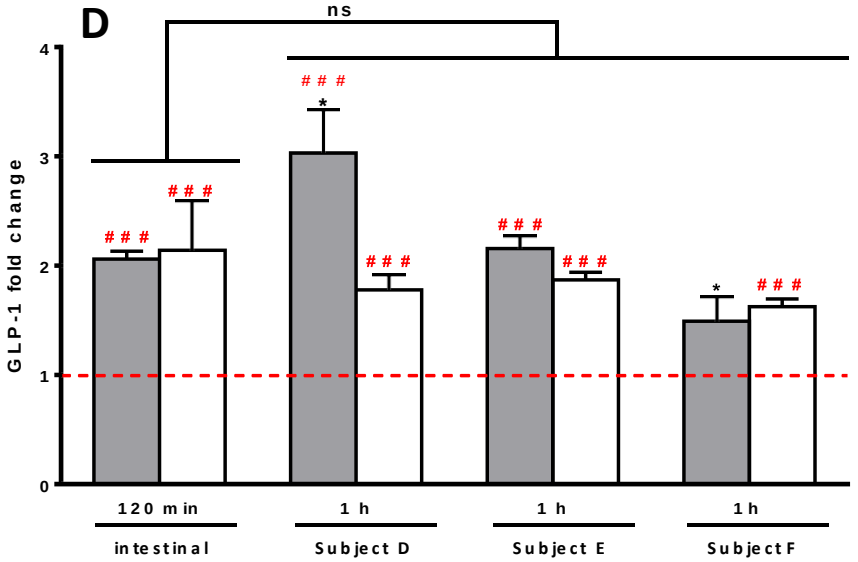
Subject E

Subject F

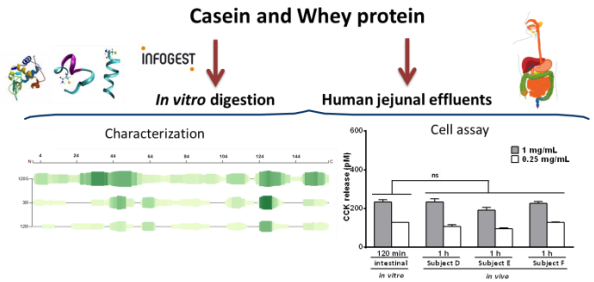

A
CCK fold change
2.5
2.0
1.5
1.0
0.5
0.0
***
# #
# # #
# # #
**
****
****
****
#
****
120 min
1 h
1 h
1 h
intestinal
Subject A
Subject B
Subject C
in vitro
in vivo


B
1 mg/mL
0.25 mg/mL
CCK fold change
2.5
2.0
1.5
1.0
0.5
0.0
ns
#
*
#
**
120 min
1 h
1 h
1 h
intestinal
Subject D
Subject E
Subject F
in vitro
in vivo


C
GLP-1 fold change
4
3
2
1
0
***
# #
**
# # #
# # #
#
# #
**
#
120 min
1 h
1 h
1 h
intestinal
Subject A
Subject B
Subject C


D
GLP-1 fold change
4
3
2
1
0
ns
# # #
*
# # #
# # #
# # #
# # #
# # #
*
# # #
120 min
1 h
1 h
1 h
intestinal
Subject D
Subject E
Subject F

in vitro	*in vivo*	*in vitro*	*in vivo*


Casein and Whey protein
INFOGEST
In vitro digestion
Human jejunal effluents
Characterization
Cell assay
1 mg/mL
0.25 mg/mL
ns
CCK release (pM)
600
400
200
0
120 min intestinal
1 h Subject D
1 h Subject E
1 h Subject F
in vitro
in vivo

Stimulation of CCK and GLP-1 secretion and expression in STC-1 cells by human jejunal contents and *in vitro* gastrointestinal digests from casein and whey proteins

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1. SDS-PAGE ANALYSIS

The methodology is described in the manuscript

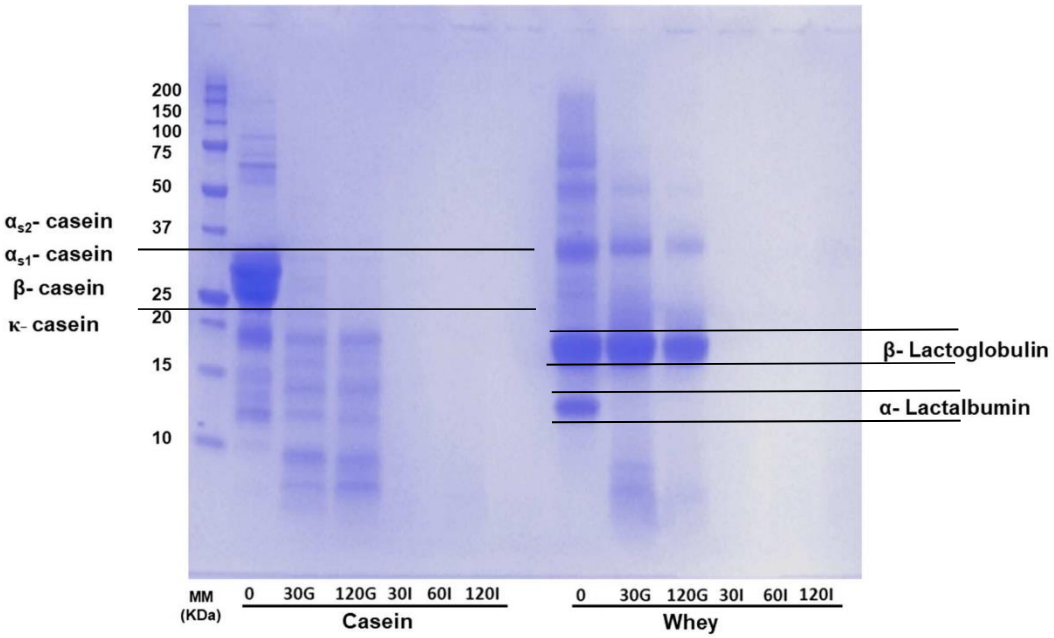

Figure S1: SDS-PAGE analysis of casein and whey *in vitro* gastrointestinal digests. Each lane corresponds to a different time points of gastric (G) and gastrointestinal (I) digestion. MM, molecular weight marker; 30G, 30 min gastric digestion; 120G, 120 min gastric digestion; 30I, 30 min gastrointestinal digestion; and 120I, 120 min gastrointestinal digestion.

2. Analysis by HPLC-tandem mass spectrometry

Table S1. β-casein-derived peptides identified in casein *in vitro* digests taken at 30 and 120 min of the gastric phase, and 30 and 120 min of the intestinal phase

Range		Sequence	Range		Sequence
1	5	RELEE	48	58	KIHPFAQTQSL
1	6	RELEEL	55	86	TQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPP
1	11	RELEELNVPGE	57	62	SLVYPF
2	8	ELEELNV	57	66	SLVYPFPGPI
5	17	ELNVPGEIVESLS	57	68	SLVYPFPGPIPN
5	34	ELNVPGEIVESLSSSEESITRINKKIEKFQ	58	66	LVYPFPGPI
6	12	LNVPGEI	58	68	LVYPFPGPIPN
6	14	LNVPGEIVE	59	66	VYPFPGPI
6	16	LNVPGEIVESL	59	68	VYPFPGPIPN
6	26	LNVPGEIVESLSSSEESITRI	59	80	VYPFPGPIPNSLPQNIPPLTQT
7	13	NVPGEIV	60	66	YPFPGPI
7	14	NVPGEIVE	60	68	YPFPGPIPN
7	15	NVPGEIVES	61	68	PFPGPIPN
7	16	NVPGEIVESL	61	71	PFPGPIPNSLP
7	35	NVPGEIVESLSSSEESITRINKKIEKFQS	61	78	PFPGPIPNSLPQNIPPLT
7	41	NVPGEIVESLSSSEESITRINKKIEKFQSEEQQQT	67	74	PNSLPQNI
8	37	VPGEIVESLSSSEESITRINKKIEKFQSEE	69	80	SLPQNIPPLTQT
9	14	PGEIVE	69	82	SLPQNIPPLTQTPV
9	26	PGEIVESLSSSEESITRI	70	80	LPQNIPPLTQT
11	20	EIVESLSSSE	71	93	PQNIPPLTQTPVVVPPFLQPEVM
12	24	IVESLSSSEESIT	73	80	NIPPLTQT
16	39	LSSSEESITRINKKIEKFQSEEQQ	73	82	NIPPLTQTPV
17	26	SSSEESITRI	73	92	NIPPLTQTPVVVPPFLQPEV
32	38	KFQSEEQ	75	82	PPLTQTPV
33	38	FQSEEQ	76	83	PLTQTPVV
33	39	FQSEEQQ	78	82	TQTPV
33	40	FQSEEQQQ	78	85	TQTPVVVP
34	39	QSEEQQ	78	87	TQTPVVVPPF
35	44	SEEQQQTEDE	81	92	PVVVPPFLQPEV
36	61	KVNELSKDIGSESTEDQAMEDIKQME	81	93	PVVVPPFLQPEVM
40	45	QTEDEL	83	91	VVPPFLQPE
40	55	LSKDIGSESTEDQAME	83	92	VVPPFLQPEV
41	45	TEDEL	85	92	PPFLQPEV
41	47	TEDELQD	91	99	EVMGVSKVK
41	48	TEDELQDK	92	99	VMGVSKVK
42	47	EDELQD	92	100	VMGVSKVKE
43	48	DELQDK	100	105	EAMAPK
45	58	LQDKIHPFAQTQSL	108	113	EMPFPK
46	55	QDKIHPFAQT	108	116	EMPFPKYPV
46	58	QDKIHPFAQTQSL	114	118	YPVEP
47	55	DKIHPFAQT	114	119	YPVEPF
47	74	ESTEDQAMEDIKQMEAESISSSEEIVPN	117	136	EPFTESQSLTLTDVENLHLP

Range		Sequence
118	126	PFTESQSLT
119	125	FTESQSL
125	140	LTLTDVENLHLPLPLL
125	142	LTLTDVENLHLPLPLLQS
126	132	TLTDVEN
126	140	TLTDVENLHLPLPLL
126	142	TLTDVENLHLPLPLLQS
127	132	LTDVEN
127	139	LTDVENLHLPLPL
127	140	LTDVENLHLPLPLL
127	142	LTDVENLHLPLPLLQS
128	140	TDVENLHLPLPLL
128	142	TDVENLHLPLPLLQS
128	146	TDVENLHLPLPLLQSWMHQ
129	138	DVENLHLPLP
129	139	DVENLHLPLPL
130	138	VENLHLPLP
134	139	HLPLPL
134	141	HLPLPLLQ
141	163	QSWMHQPHQPLPPTVMFPPQSVL
141	170	QSWMHQPHQPLPPTVMFPPQSVLSLSQSKV
143	156	WMHQPHQPLPPTVM
144	154	MHQPHQPLPPT
145	154	HQPHQPLPPT
147	154	PHQPLPPT
149	154	QPLPPT
154	164	TVMFPPQSVLS
156	162	MFPPQSV
157	162	FPPQSV
157	163	FPPQSVL
157	165	FPPQSVLSL

Range		Sequence
159	167	PQSVLSLSQ
159	184	PQSVLSLSQSKVLPVPQKAVPYPQRD
160	168	QSVLSLSQS
161	171	SVLSLSQSKVL
162	170	VLSLSQSKV
162	172	VLSLSQSKVLP
162	190	GAWYYVPLGTQYTDAPSFSDIPNPIGSEN
164	173	SLSQSKVLPV
164	188	SLSQSKVLPVPQKAVPYPQRDMPIQ
164	189	SLSQSKVLPVPQKAVPYPQRDMPIQA
164	190	SLSQSKVLPVPQKAVPYPQRDMPIQAF
164	191	SLSQSKVLPVPQKAVPYPQRDMPIQAFL
164	192	SLSQSKVLPVPQKAVPYPQRDMPIQAFLL
165	172	LSQSKVLP
168	196	SKVLPVPQKAVPYPQRDMPIQAFLLYQEP
170	175	VLPVPQ
173	188	VPQKAVPYPQRDMPIQ
177	182	AVPYPQ
184	189	DMPIQA
189	205	AFLLYQEPVLGPVRGPF
190	209	FLLYQEPVLGPVRGPFPIIV
191	209	LLYQEPVLGPVRGPFPIIV
192	201	LYQEPVLGPV
192	209	LYQEPVLGPVRGPFPIIV
193	197	YQEPV
193	198	YQEPVL
193	201	YQEPVLGPV
193	208	YQEPVLGPVRGPFPII
193	209	YQEPVLGPVRGPFPIIV
194	201	QEPVLGPV
196	209	PVLGPVRGPFPIIV
202	207	RGPFPI

Table S2. α_{s1}- casein-derived peptides identified in casein *in vitro* digests taken at 30 and 120 min of the gastric phase, and 30 and 120 min of the intestinal phase

Range		Sequence	Range		Sequence
1	16	RPKHPIKHQGLPQEVL	70	74	EIVPN
1	20	RPKHPIKHQGLPQEVLNENL	83	88	KEDVPS
1	23	RPKHPIKHQGLPQEVLNENLLRF	84	89	EDVPSE
2	11	PKHPIKHQGL	87	95	PSERYLGYL
6	12	IKHQGLP	87	104	PSERYLGYLEQLLRLKKY
8	13	HQGLPQ	96	114	EQLLRLKKYKVPQLEIVPN
12	19	PQEVLNEN	97	103	QLLRLKK
16	20	LNENL	98	129	LLRLKKYKVPQLEIVPNSAEERLHSMKEGIHA
17	23	NENLLRF	99	109	LRLKKYKVPQL
24	30	FVAPFPE	100	129	RLKKYKVPQLEIVPNSAEERLHSMKEGIHA
24	31	FVAPFPEV	102	107	KKYKVP
24	33	FVAPFPEVFG	103	107	KYKVP
24	39	FVAPFPEVFGKEKVNE	104	109	YKVPQL
25	30	VAPFPE	105	112	KVPQLEIV
25	31	VAPFPEV	109	114	LEIVPN
25	33	VAPFPEVFG	109	118	LEIVPNSAEE
25	39	VAPFPEVFGKEKVNE	110	118	EIVPNSAEE
26	39	APFPEVFGKEKVNE	111	118	IVPNSAEE
27	31	PFPEV	112	118	VPNSAEE
27	39	PFPEVFGKEKVNE	114	119	NSAEER
29	39	PEVFGKEKVNE	120	125	LHSMKE
33	39	GKEKVNE	124	131	KEGIHAQQ
35	39	EKVNE	125	129	EGIHA
35	45	EKVNELSKDIG	128	134	HAQQKEP
36	41	KVNELS	133	138	EPMIGV
38	44	NELSKDI	133	140	EPMIGVNQ
40	47	LSKDIGSE	135	145	MIGVNQELAYF
40	56	LSKDIGSESTEDQAMED	136	141	IGVNQE
41	77	SKDIGSESTEDQAMEDIKQMEAESISSSEEIVPNSVE	136	149	IGVNQELAYFYPEL
43	51	DIGSESTED	138	145	VNQELAYF
43	52	DIGSESTEDQ	142	149	LAYFYPEL
43	53	DIGSESTEDQA	143	149	AYFYPEL
45	51	GSESTED	144	149	YFYPEL
45	69	GSESTEDQAMEDIKQMEAESISSSE	154	161	YQLDAYPS
53	58	AMEDIK	154	164	YQLDAYPSGAW
54	79	MEDIKQMEAESISSSEEIVPNSVEQK	155	161	QLDAYPS
55	63	EDIKQMEAE	157	164	DAYPSGAW
58	66	KQMEAESIS	160	168	PSGAWYYVP
59	64	QMEAES	160	181	PSGAWYYVPLGTQYTDAPSFSD
60	66	MEAESIS	160	190	PSGAWYYVPLGTQYTDAPSFSDIPNPIGSEN
64	75	SISSSEEIVPNS	161	170	SGAWYYVPLG
65	92	ISSSEEIVPNSVEQKHIQKEDVPSERYL	165	172	YYVPLGTQ
66	73	SSSEEIVP	165	179	YYVPLGTQYTDAPSF
69	77	EEIVPNSVE	166	196	YVPLGTQYTDAPSFSDIPNPIGSENSEKTTM

Range		Sequence
168	178	PLGTQYTDAPS
171	191	TQYTDAPSFSDIPNPIGSENS
172	178	QYTDAPS
173	178	YTDAPS
173	179	YTDAPSF
173	181	YTDAPSFSD
173	191	YTDAPSFSDIPNPIGSENS
174	180	TDAPSFS
174	186	TDAPSFSDIPNPI
177	183	PSFSDIP
178	186	SFSDIPNPI
179	187	FSDIPNPIG
180	184	SDIPN
180	186	SDIPNPI
180	196	SDIPNPIGSENSEKTTM
180	199	SDIPNPIGSENSEKTTMPLW
189	195	ENSEKTT
190	198	NSEKTTMPL

Table S3. β-lactoglobulin-derived peptides identified in whey protein *in vitro* digests taken at 120 min of the gastric phase, and 30 and 120 min of the intestinal phase.

Range		Sequence	Range		Sequence
1	8	LIVTQTMK	42	49	YVEELKPT
1	10	LIVTQTMKGL	42	51	YVEELKPTPE
1	14	LIVTQTMKGLDIQK	42	54	YVEELKPTPEGDL
2	6	IVTQT	42	55	YVEELKPTPEGDLE
2	8	IVTQTMK	42	57	YVEELKPTPEGDLEIL
2	9	IVTQTMKG	42	59	YVEELKPTPEGDLEILLQ
2	10	IVTQTMKGL	42	60	YVEELKPTPEGDLEILLQK
2	11	IVTQTMKGLD	43	49	VEELKPT
3	8	VTQTMK	43	51	VEELKPTPE
4	11	TQTMKGLD	43	52	VEELKPTPEG
6	12	TMKGLDI	43	54	VEELKPTPEGDL
9	20	GLDIQKVAGTWY	43	55	VEELKPTPEGDLE
9	23	GLDIQKVAGTWYSLA	43	57	VEELKPTPEGDLEIL
10	18	LDIQKVAGT	44	51	EELKPTPE
10	19	LDIQKVAGTW	44	54	EELKPTPEGDL
11	19	DIQKVAGTW	44	60	EELKPTPEGDLEILLQK
12	19	IQKVAGTW	45	51	ELKPTPE
12	28	IQKVAGTWYSLAMAASD	45	54	ELKPTPEGDL
15	20	VAGTWY	45	55	ELKPTPEGDLE
15	26	VAGTWYSLAMAA	46	51	LKPTPE
21	26	SLAMAA	46	54	LKPTPEGDL
22	31	LAMAASDISL	47	51	KPTPE
23	32	AMAASDISLL	47	55	KPTPEGDLE
24	41	MAASDISLLDAQSAPLRV	47	56	KPTPEGDLEI
26	32	ASDISLL	48	56	PTPEGDLEI
26	41	ASDISLLDAQSAPLRV	50	55	PEGDLE
27	32	SDISLL	55	62	EILLQKWE
27	39	SDISLLDAQSAPL	56	67	ILLQKWENDECA
27	41	SDISLLDAQSAPLRV	57	70	LLQKWENDECAQKK
27	42	SDISLLDAQSAPLRVY	58	69	LQKWENDECAQK
28	54	DISLLDAQSAPLRVYVEELKPTPEGDL	58	74	LQKWENDECAQKKIIAE
30	39	SLLDAQSAPL	60	66	KWENDEC
30	41	SLLDAQSAPLRV	60	67	KWENDECA
32	38	LDAQSAP	61	66	WENDEC
32	41	LDAQSAPLRV	61	67	WENDECA
33	39	DAQSAPL	61	68	WENDECAQ
33	40	DAQSAPLR	61	73	WENDECAQKKIIA
33	41	DAQSAPLRV	62	67	ENDECA
33	42	DAQSAPLRVY	63	69	NDECAQK
34	38	AQSAP	68	76	QKKIIAEKT
40	57	RVYVEELKPTPEGDLEIL	69	77	KKIIAEKTK
41	48	VYVEELKP	73	91	AEKTKIPAVFKIDALNENK
41	57	VYVEELKPTPEGDLEIL	74	80	EKTKIPA

Range		Sequence
74	82	EKTKIPAVF
75	82	KTKIPAVF
76	82	TKIPAVF
77	82	KIPAVF
77	90	KIPAVFKIDALNEN
82	91	FKIDALNENK
82	92	FKIDALNENKV
83	89	KIDALNE
83	91	KIDALNENK
83	92	KIDALNENKV
83	93	KIDALNENKVL
83	102	KIDALNENKVLVLDTDYKKY
85	91	DALNENK
86	95	ALNENKVLVL
89	97	ENKVLVLDT
90	95	NKVLVL
92	100	VLVLDTDYK
92	101	VLVLDTDYKK
96	102	DTDYKKY
96	103	DTDYKKYL
96	104	DTDYKKYLL
105	116	FCMENSAEPEQS
106	114	CMENSAEPE
107	112	MENSAE
107	114	MENSAEPE
107	116	MENSAEPEQS
107	117	MENSAEPEQSL
107	118	MENSAEPEQSLV
107	119	MENSAEPEQSLVC
108	113	ENSAEP
108	114	ENSAEPE
108	116	ENSAEPEQS
109	117	NSAEPEQSL
110	116	SAEPEQS
110	118	SAEPEQSLV
112	117	EPEQSL
112	121	EPEQSLVCQC
122	141	LVRTPEVDDEALEKFDKALK
123	130	VRTPEVDD
123	131	VRTPEVDDE
123	132	VRTPEVDDEA
123	133	VRTPEVDDEAL
123	141	VRTPEVDDEALEKFDKALK
124	130	RTPEVDD

Range		Sequence
124	131	RTPEVDDE
124	138	RTPEVDDEALEKFDK
125	130	TPEVDD
125	131	TPEVDDE
125	132	TPEVDDEA
125	134	TPEVDDEALE
125	135	TPEVDDEALEK
125	136	TPEVDDEALEKF
125	137	TPEVDDEALEKFD
125	138	TPEVDDEALEKFDK
125	139	TPEVDDEALEKFDKA
125	140	TPEVDDEALEKFDKAL
125	141	TPEVDDEALEKFDKALK
126	131	PEVDDE
126	132	PEVDDEA
126	133	PEVDDEAL
127	134	EVDDEALE
127	135	EVDDEALEK
127	136	EVDDEALEKF
128	133	VDDEAL
128	138	VDDEALEKFDK
129	141	DDEALEKFDKALK
137	143	DKALKAL
142	148	ALPMHIR
148	156	RLSFNPTQL
148	160	RLSFNPTQLEEQC
148	162	RLSFNPTQLEEQCHI
149	154	LSFNPT
149	156	LSFNPTQL
149	159	LSFNPTQLEEQ
149	160	LSFNPTQLEEQC
149	162	LSFNPTQLEEQCHI
150	154	SFNPT
150	156	SFNPTQL
150	159	SFNPTQLEEQ
150	160	SFNPTQLEEQC
150	162	SFNPTQLEEQCHI
152	162	NPTQLEEQCHI
153	162	PTQLEEQCHI
154	161	TQLEEQCH
155	159	QLEEQ
155	160	QLEEQC
156	161	LEEQCH
157	161	EEQCH
157	162	EEQCHI

Table S4. α-lactalbumin-derived peptides identified in whey proteins *in vitro* digests taken at 120 min of the gastric phase, and 30 and 120 min of the intestinal phase.

Range		Sequence	Range		Sequence
1	9	EQLTKCEVF	59	64	IWCKDD
1	11	EQLTKCEVFRE	59	73	IWCKDDQNPHSSNIC
4	9	TKCEVF	61	68	CKDDQNPH
8	14	VFRELKD	62	77	KDDQNPHSSNICNISC
10	17	RELKDLKG	62	79	KDDQNPHSSNICNISCDK
10	18	RELKDLKGY	63	68	DDQNPH
12	16	LKDLK	69	76	SSNICNIS
13	20	KDLKGYGG	69	78	SSNICNISCD
15	35	LKGYGGVSLPEWVCTTFHTSG	74	80	NISCDKF
19	25	GGVSLPE	74	88	NISCDKFLDDDLTDD
19	27	GGVSLPEWV	75	88	ISCDKFLDDDLTDD
19	28	GGVSLPEWVC	76	83	SCDKFLDD
19	29	GGVSLPEWVCT	76	85	SCDKFLDDDL
19	30	GGVSLPEWVCTT	80	97	FLDDDLTDDIMCVKKILD
19	31	GGVSLPEWVCTTF	81	87	LDDDLTD
19	35	GGVSLPEWVCTTFHTSG	81	88	LDDDLTDD
19	36	GGVSLPEWVCTTFHTSGY	82	88	DDDLTDD
19	40	GGVSLPEWVCTTFHTSGYDTQA	82	89	DDDLTDDI
21	30	VSLPEWVCTT	83	89	DDLTDDI
22	29	SLPEWVCT	89	97	IMCVKKILD
24	28	PEWVC	89	103	IMCVKKILDKVGINY
24	29	PEWVCT	90	103	MCVKKILDKVGINY
24	30	PEWVCTT	91	103	CVKKILDKVGINY
24	31	PEWVCTTF	92	99	VKKILDKV
24	35	PEWVCTTFHTSG	92	121	VKKILDKVGINYWLAHKALCSEKLDQWLCE
24	39	PEWVCTTFHTSGYDTQ	94	99	KILDKV
24	40	PEWVCTTFHTSGYDTQA	94	103	KILDKVGINY
26	31	WVCTTF	95	103	ILDKVGINY
26	35	WVCTTFHTSG	97	102	DKVGIN
30	40	TFHTSGYDTQA	97	103	DKVGINY
32	39	HTSGYDTQ	97	104	DKVGINYW
32	40	HTSGYDTQA	98	103	KVGINY
40	49	AIVQNNDSTE	104	117	WLAHKALCSEKLDQ
40	52	AIVQNNDSTEYGL	105	117	LAHKALCSEKLDQ
41	48	IVQNNDST	106	117	AHKALCSEKLDQ
41	49	IVQNNDSTE	106	119	AHKALCSEKLDQWL
41	50	IVQNNDSTEY	109	117	ALCSEKLDQ
41	51	IVQNNDSTEYG	110	117	LCSEKLDQ
41	52	IVQNNDSTEYGL	111	117	CSEKLDQ
45	51	NDSTEYG	111	118	CSEKLDQW
53	57	FQINN	117	123	QWLCEKL
53	58	FQINNK	118	123	WLCEKL

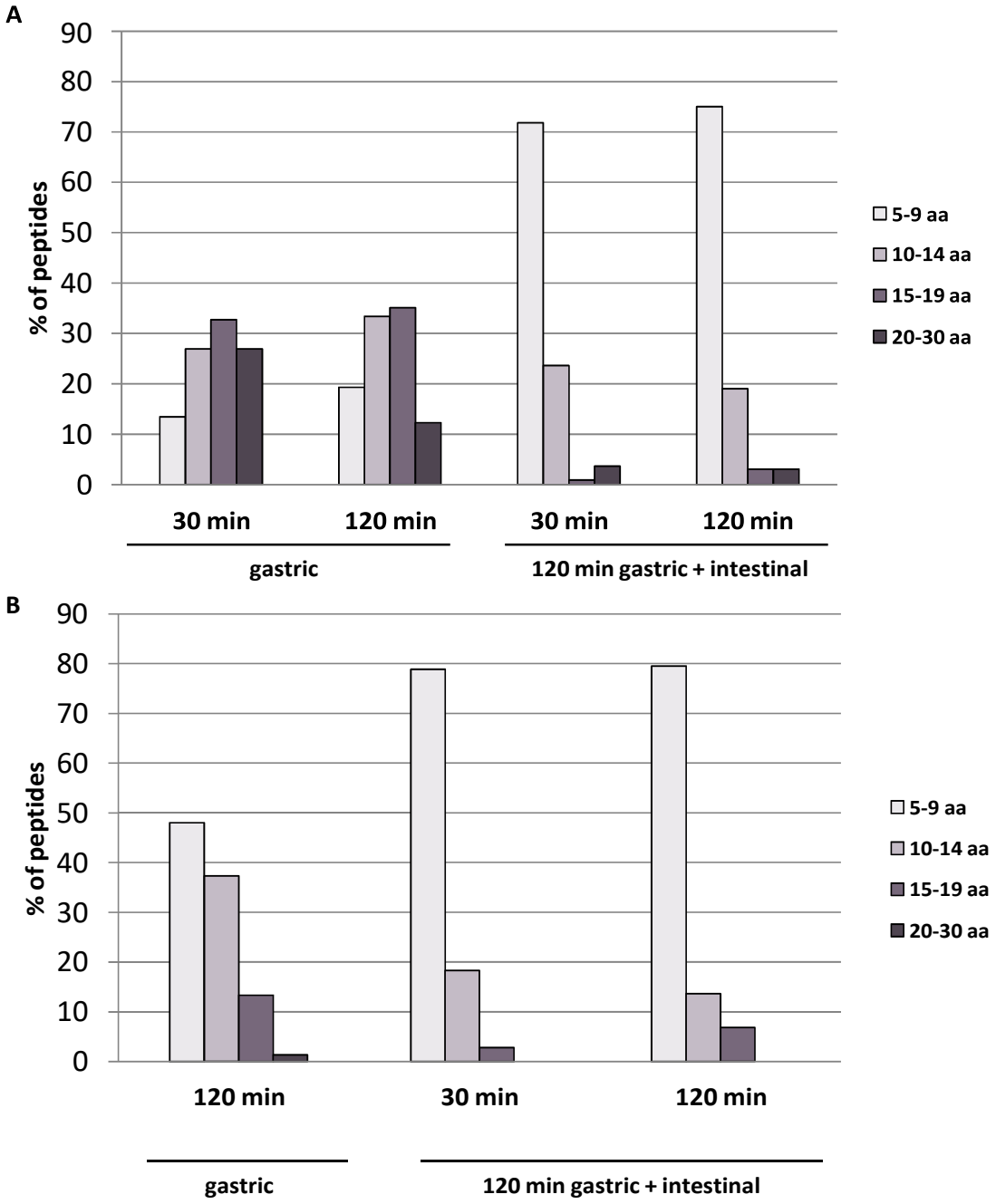

Figure S2: Size of peptides identified by HPLC-tandem mass spectrometry in (A) casein and (B) whey protein *in vitro* digests. The peptide size is expressed as number of amino acids (aa) and represented as percentage of the total identified peptides (y-axis) at each digestion time. Gastric digestion at 30 min is not shown due to the low hydrolysis degree in this sample. Each colour corresponds to a different peptide size range.

3. Comparison of CCK and GLP-1 secretion between *in vitro* and *in vivo* digests

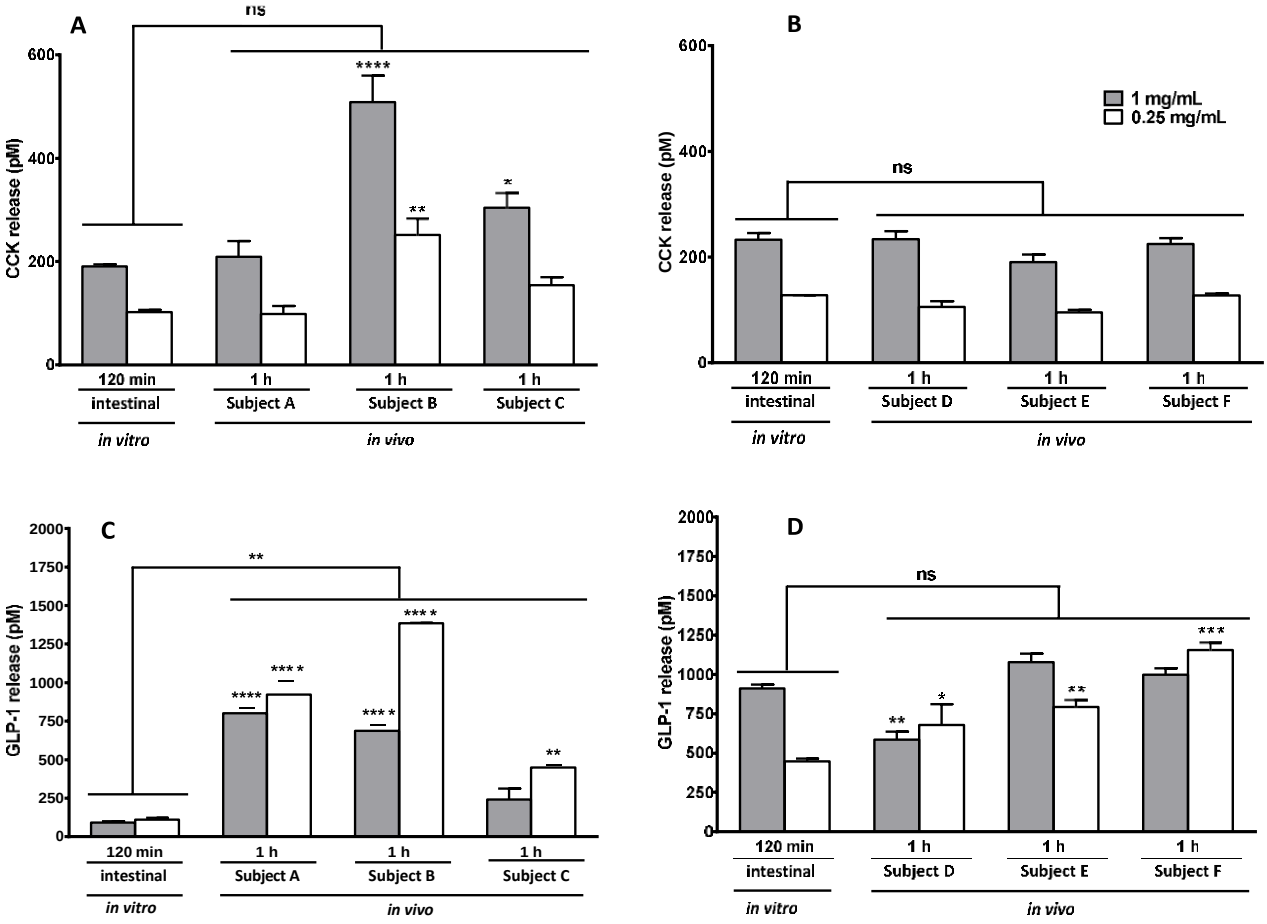

Figure S3: CCK secretion after 2 h incubation of STC-1 cells with (A) casein and (B) whey protein *in vitro* digests at the end of the digestion protocol (intestinal phase 120 min) and humanjejunal effluents (*in vivo* digests*)* taken at 1 h after oral administration of the same substrates. Samples were incubated at two different protein concentrations, 1 and 0.25 mg/mL. GLP-1 secretion after 2 h incubation of STC-1 cells with (C) casein and (D) whey protein *in vitro* and *invivo* digests. CCK and GLP-1 secretion was determined by ELISA. Error bars indicate SEM (n=3). Comparisons between samples tested at the same protein concentration and group comparisons were performed by two way ANOVA with Bonferroni post hoc test and is indicated by *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.