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Release of bioactive peptides from whey proteins across digestion in adult humans and a static in vitro digestion model

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ABSTRACT

Proteolysis of whey proteins during gastrointestinal digestion releases bioactive peptides with potential health benefits. Our study examined whey-derived peptide release after digestion under static in vitro conditions and in three adults following whey protein isolate ingestion. Using LC-MS/MS-based peptidomics, we identified 1187 unique peptides in the in vitro gastric digesta, 449 in the in vitro intestinal digesta and 1041 in the human jejunum. Among these peptides, 61 were known to exert bioactivities including ACE inhibitory, antimicrobial, DPP-IV inhibitory, antioxidant, immunomodulatory, anticancer and opioid activities. The release of antimicrobial, antioxidant and opioid peptides suggests their potential role in promoting gut health. The peptide patterns produced across digestion in the in vitro model and in adult humans were strongly similar by amino acid frequency and moderately similar in terms of peptide abundances.

1. Introduction

From a nutritional perspective, whey protein is considered a high-quality protein source due to its high biological value—its ability to be used efficiently by the body—and richness in essential amino acids (Smithers, 2008). Beyond their function as a source of amino acids, whey proteins contain bioactive peptides that are released across digestion and that have potential health benefits distinct from those provided by intact whey proteins.

Upon consumption, as whey proteins pass through the gastrointestinal tract, digestive proteases, such as pepsin in the stomach and pancreatic enzymes in the small intestine, break down proteins and release the bioactive sequences encrypted within the parent proteins. These bioactive peptides can exert a wide range of biological functions, such as antimicrobial activities (Demers-Mathieu et al., 2013), antihypertensive effects (Lacroix & Li-Chan, 2014; Lacroix et al., 2016), antidiabetic activities (Lacroix et al., 2016; Jia et al., 2020), hypocholesterolemic effects (Liu et al., 2023), anti-cancer effects (Sah et al., 2016) and antioxidant properties (Báez et al., 2021). To exert these bioactivities, the corresponding bioactive peptide sequences must reach their target sites of action. For example, to exhibit antimicrobial activity and potentially prevent intestinal infection, an antimicrobial peptide must be present within the small intestine. However, the release and survival of whey protein–derived bioactive peptides across the gastrointestinal tract remains poorly described.

Although studying in vivo digestion in humans is the most physiologically relevant method of understanding the release of dietary protein-derived peptides across the gastrointestinal tract, in vitro digestion models offer a faster, cheaper and more reproducible alternative with fewer ethical concerns (Minekus et al., 2014). However, the accuracy of the in vitro digestion model in mimicking the complex physiological conditions of the human gastrointestinal tract needs to be further validated.

Comparisons of peptidomic profiles after milk protein digestion between in vivo and in vitro models have been made using Spearman's correlation, a non-parametric analysis, based on the frequency of appearance of each amino acid across protein sequences from peptidomic data. Egger et al. (2019) observed a high correlation in peptide patterns from β-casein (β-CN), β-lactoglobulin (BLG), α_{S1}-casein (α_{S1}-CN), α_{S2}-casein (α_{S2}-CN) and κ-casein (κ-CN) during the gastric phase and a moderate correlation during the intestinal phase across static in vitro, dynamic in vitro and in vivo adult pig models after skim milk powder digestion. Similarly, Sanchón et al. (2018) reported a strong correlation in peptide profiles in both healthy adult human jejunum and

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static in vitro intestinal conditions from BLG and α-lactalbumin (ALA) after digestion of whey protein isolate. However, the use of amino acid count data in these past studies decreased the potential variation that could be identified compared to if the researchers had used peptide abundance data, which could create the appearance of highly correlated data that may not be fully representative. Therefore, our study aimed to compare differences in peptidomic patterns between in vivo and in vitro data using both qualitative data of peptide patterns and quantitative peptide abundances to gain comprehensive understanding of overall peptide profiles after digestion.

The objective of this study was to identify whey-derived peptides released after in vivo digestion using jejunal fluids from three adults who ingested whey protein isolate and after static in vitro digestion following the harmonized COST INFOGEST protocols (Brodkorb et al., 2019; Minekus et al., 2014). Peptide identification was performed via LC-MS/MS-based peptidomics. Additionally, the identified peptides were searched against the Milk Bioactive Peptide Database (MBPDB; Nielsen et al., 2017; Nielsen et al., 2023) to determine their known bioactivities and elucidate their potential physiological effects and implications for consumer health.

2. Materials and methods

2.1. Feeding material

A spray-dried whey protein isolate (WPI; Provon 290) containing 92.84 % protein, 0.87 % fat, 4.17 % moisture and 2.35 % ash was provided by Glanbia Nutritionals (Twin Falls, ID, USA). The processing steps for WPI involved 1) pasteurizing raw milk, 2) pasteurizing sweet whey after casein separation and ultrafiltration and 3) spray-drying whey proteins.

2.2. Human feeding study

All study protocols were approved by the Good Samaritan Regional Medical Center (Corvallis, Oregon) and Oregon State University Institutional Review Boards (IRB number: IRB 18-088; Corvallis, OR, USA), as previously described (Qu et al., 2023). All volunteers were evaluated for eligibility by medical staff at the Good Samaritan Regional Medical Center, and individuals were excluded if they had lactose or dairy protein intolerance or allergies, gastrointestinal diseases or surgeries, nasal injuries or esophageal anomalies. Participants were thoroughly informed about the study's purpose and potential risks, and written informed consent was obtained. The study included three adult participants (one female and two males), aged 20–25 years, with BMIs ranging from 22.5 to 28.4 kg/m^2.

The volunteers were instructed to abstain from consuming dairy and dairy-based products for three days prior to the experiment to eliminate potential interference from other dairy proteins. After fasting overnight, they reported to the Good Samaritan Regional Medical Center on the sampling day. A 140-cm, 10-Fr nasojejunal tube (CORTRAK * 2 Nasogastric/Nasointestinal Feeding Tube with Electromagnetic Transmitting Stylet, 20-9551TRAK2; Avanos Medical, Alpharetta, GA, USA) was inserted through each participant's nose and monitored using the Cortrak magnetic system. Each participant consumed the WPI feed containing 40 g of WPI powder (based on one serving size of commercial whey protein supplements) dissolved in 500 mL of water, as well as 0.5 mg of polyethylene glycol-28 (PEG-28; Sigma-Aldrich, St. Louis, MO, USA), a non-absorbable marker that allowed the tracking of the dilution of digestive contents within the gut. Jejunal fluids were collected by gravity flow for up to 4 h post-consumption, after which the nasojejunal tube was removed. All biological samples were kept on dry ice and stored at −80 °C until analysis.

2.3. Static in vitro digestion of whey protein

In vitro gastric and intestinal digestions of WPI were conducted in duplicate based on the harmonized COST INFOGEST protocols (Brodkorb et al., 2019; Minekus et al., 2014). Enzyme activities and bile concentration were measured as described by Minekus et al. (2014). All simulated digestive fluids were pre-warmed at 37 °C before the digestion process. The WPI powder was dissolved in ultrapure water (18.2 MΩ. cm) at the same concentration as the feed used in the in vivo study (0.08 g/mL). The digestion began without the oral phase as WPI contained no starch and minimal lipids, the main macronutrients that would be impacted by salivary enzymes. Gastric digestion was started by mixing the WPI feed with simulated gastric fluid containing porcine pepsin (2000 U/mL of the final digesta mixture, Sigma-Aldrich) to achieve a final ratio of 1:1 by volume. The pH was adjusted to pH 3.0 with 1 M hydrochloric acid (Sigma-Aldrich) and the mixture was incubated at 37 °C with shaking at 300 rpm for 120 min. Gastric digesta were collected at 20, 60 and 120 min, adjusted to pH 7.0 with 1 M sodium hydroxide (Sigma-Aldrich) to stop pepsin digestion and immediately stored in a −80 °C freezer. To mimic different gastric emptying times, three separate gastric tubes were incubated at 20, 60 and 120 min before the intestinal phase was started. After each respective gastric endpoint, the chyme was neutralized to pH 7.0 with 1 M sodium hydroxide (Sigma-Aldrich), mixed with simulated intestinal fluid containing bovine bile mixture (10 mM in the final digesta, Sigma-Aldrich) and porcine pancreatin solution (trypsin activity of 100 U/mL in the final mixture, Sigma-Aldrich) and incubated at 37 °C with shaking at 300 rpm for 120 min to simulate the intestinal digestion. Intestinal phase samples were collected at 30, 60 and 120 min, mixed with Pefabloc® SC (Sigma-Aldrich) to achieve a final concentration of 5 mM to stop intestinal digestion and immediately frozen at −80 °C.

2.4. Peptidomic sample preparation

2.4.1. Peptide enrichment via ethanol precipitation

All digestive samples (in vivo and in vitro digesta) were defrosted at 4 °C before sample preparation. Each sample was prepared in two 100 μL aliquots (technical duplicates) and subjected to the following extraction conditions. To remove initial large cellular interferences, 100 μL of the samples were centrifuged at 12,000 × *g* for 20 min at 4 °C before the liquid supernatant portion was collected. This process was repeated twice. To separate peptides from high molecular weight proteins in the digesta, ethanol precipitation was conducted by mixing 150 μL of chilled (−20 °C) pure ethanol (Sigma-Aldrich) with 30 μL of the pre-centrifuged samples. The resulting mixture was then stored at −20 °C for 1 h and centrifuged at 12,000 × *g* for 20 min at 4 °C. The liquid supernatant was collected and dried using a vacuum concentrator (Genevac, Gardiner, NY, USA) with low BP setting at 37 °C.

2.4.2. Reduction of disulfide bonds

To linearize potential circular peptides and enhance tandem MS fragmentation, the peptides were reduced to break the disulfide bonds and alkylated to prevent their reformation, thereby improving peptide identification and detection (Peng et al., 2020). The vacuum-dried samples were reconstituted in 100 μL of 50 mM ammonium bicarbonate (Thermo Scientific, Waltham, MA, USA). To reduce disulfide bonds, 2 μL of 550 mM dithiothreitol (Promega, Madison, WI, USA) was added in the sample and incubated for 50 min at 50 °C with shaking at 300 rpm. After reduction, 4 μL of 450 mM iodoacetamide (Sigma-Aldrich) was added, and the samples were incubated in the dark at room temperature for 1 h.

2.4.3. Peptide purification

C18 cartridge-based solid phase extraction was used to remove contaminants, desalt and concentrate the peptide samples. The elution buffer consisted of 80 % acetonitrile (Merck, Darmstadt, Germany), 0.1

% trifluoroacetic acid (Merck) and 19.9 % ultrapure water. The C18 cartridge (Sigma-Aldrich) was reconditioned by sequentially adding and washing with 5 mL of ultrapure water, 5 mL of elution buffer and 5 mL of ultrapure water. Before loading the samples, 1 mL of ultrapure water was added to adjust for low sample volume. The samples were rinsed with 5 mL ultrapure water and then eluted with 5 mL of the elution buffer. To remove the elution solvent, the samples were then dried using a vacuum concentrator (Genevac) with HPLC setting at 37 °C. Before LC-MS/MS analysis, the dried samples were fully dissolved in 100 μL of a reconstitution solvent containing 3 % acetonitrile, 0.1 % formic acid (Merck) and 96.9 % ultrapure water with a vortex mixer.

2.5. Peptide identification via LC-MS/MS

To remove any large particles, the reconstituted peptide samples were centrifuged at 12,000 × *g* at 4 °C for 5 min, and the supernatant liquid was collected. The supernatant was diluted 10-fold with the reconstitution solvent. The peptide samples were then analyzed once using Waters nanoACQUITY UPLC (Waters, Milford, MA, USA) with an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific, Waltham, MA, USA).

For the analysis of the in vivo digesta samples, a C18 180 μm × 20 mm, 5 μm bead nanoAcquity UPLC trap column (Waters) was used for enrichment and desalting, and separation was performed using a 100 μm × 100 mm, 1.7 μm bead Acquity UPLC Peptide BEH C18 column (Waters). The LC columns were changed for the analysis of the in vitro digesta samples. Enrichment and desalting were done using a nanoEase M/Z Symmetry C18 Trap Column, 100 Å, 5 μm, 180 μm × 20 mm (Waters), and separation was performed using a nanoEase M/Z Peptide BEH C18 Column, 130 Å, 1.7 μm, 75 μm × 100 mm (Waters). One microliter of each sample was injected at a separation flow rate of 0.5 μL/min over 60 min. The elution gradients using solvent A (0.1 % formic acid with 99.9 % ultrapure water) and solvent B (0.1 % formic acid with 99.9 % acetonitrile) were as follows: 3 % B (0–3 min), 3–15 % B (3–4.5 min for in vitro and 3–8 min for in vivo), 15–30 % B (4.5–45 min for in vitro and 8–40 min for in vivo and), 30–95 % B (40–50 min), 95 % B (50–55 min) and 95–3 % B (0.5 min). Additionally, the column was recalibrated with 97 % A for 5 min between samples.

The sample molecules were ionized using an electrospray voltage of 2350 V and a transfer tube temperature of 300 °C. Full-scan MS spectra were recorded in positive ionization mode, ranging from 300 to 2000 *m/z*, with a resolution of 60,000. The automatic gain control target was set at 4.0×10^5 with a maximum injection time of 50 ms and an MS cycle time of 3 s. The criteria for MS/MS precursor selection were an ion intensity of at least 5.0×10^4, a charge state between 2 and 8 and an exclusion time of 60 s. Higher-energy collision dissociation (HCD) was used to fragment precursor ions at 30 % normalized collision energy. All MS/MS spectra were acquired in positive ionization mode, at a resolution of 60,000 and with an automatic gain control target of 5.0×10^4 for all fragmentation methods.

To evaluate the impact of using different LC trap columns and analytical columns on peptide identifications for the in vivo and in vitro digesta, bottom-up proteomic analysis of WPI feeds was performed via LC-MS/MS using both sets of columns as controls. Four 100 μL aliquots of WPI feeds were subjected to protein extraction via ethanol precipitation (as described in section 2.4.1), with pellet portions collected and dried. The samples were treated with dithiothreitol and iodoacetamide (section 2.4.2) and digested with 1 μg/μL trypsin/Lys-C (Promega) at a 1:50 ratio of trypsin to protein and incubated at 37 °C for 16 h. The tryptic peptide samples were cleaned up (section 2.4.3) and reconstituted, then diluted 100-fold before LC-MS/MS analysis. Two WPI feed samples were analyzed using the same columns used for in vivo digesta, and two WPI feed samples were analyzed using the columns used for in vitro digesta, under the parameters and elution gradients described above.

2.6. Peptidomic data processing

Peptide identification was conducted using Proteome Discoverer (v3.1; Thermo Scientific) with a SequestHT search against an in-house cow milk protein database (n = 576). The precursor and fragment mass tolerances were 10 ppm and 0.1 Da, respectively. Fixed modifications included carbamidomethylation of cysteine. Oxidation of methionine and phosphorylation of serine and threonine were selected as dynamic modification. Peptides with high confidence (q-value < 0.01) were accepted. Peptide abundances were normalized by multiplying by dilution factors from PEG-28 concentration for the in vivo digesta (as described in 2.10) and by ratios of food to digestive fluids for the in vitro digesta (2 for gastric digesta and 4 for intestinal digesta). The normalized abundances were log10 transformed before statistical analysis.

2.7. Peptide sequence alignment and visualization

Heatmaps of identified peptide sequence alignment with protein origin, including BLG, ALA, β-CN and κ-CN, along with corresponding normalized peptide abundances represented as line plots, were generated using JupyterLab software (v.3.6.7), as previously described (Kuhfeld et al., 2024). Full protein sequences including signal sequences were used for peptide alignment and sequence numbering purposes.

2.8. Bioactive peptide search

Identified peptide sequences from both in vivo and in vitro digestion experiments were searched against the Milk Bioactive Peptide database (MBPDB; Nielsen et al., 2017; Nielsen et al., 2023) via https://mbpdb.nws.oregonstate.edu/peptide_search/ to determine their known biological functions. The search criteria included sequence as the search type, a similarity threshold of 100 % and cow (*Bos taurus*) as the selected species.

2.9. Quantification of PEG-28 via LC-MS/MS

PEG-28 concentrations in the feed and jejunal fluids were quantified via parallel reaction monitor LC-MS/MS and used to determine dilution factors to normalized peptide abundances in the in vivo digesta. WPI feed, in vivo digesta and a PEG-28 standard mixture (100 ng/μL, Sigma-Aldrich) were subjected to the following steps as described in Sections 2.4.1–2.4.3. The reconstituted samples were centrifuged at 12,000 × *g* and 4 °C for 5 min. Then the supernatant liquids from WPI feed and jejunal digesta samples were collected and used for LC-MS/MS analysis. The supernatant from PEG-28 standard was further diluted with the reconstitution solvent at the following factors to create standard dilution series: 20,000×, 10,000×, 4000×, 2000×, 1000×, 400×, 200×, 100×, 40× and 20×.

All samples were analyzed in triplicate using the same instrument, column and LC-MS/MS acquisition parameters used for the in vivo digesta samples, with some deviations in elution and recalibration conditions. The LC separation time was 20 min with the following elution gradient: 3–13 % B (0–5.5 min), 13–20 % B (5.5–11.5 min), 20–30 % B (11.5–13 min), 30–95 % B (13–14.5 min), 95 % B (14.5–16.5 min) and 95–3 % B (0.5 min). The column was recalibrated with 97 % A for 3 min. The selected PEG-28 precursor was fragmented at *m/z* 417.933 (z = 3) using collision-induced dissociation (CID) with 35 % normalized collision energy.

Peak areas from raw MS/MS files were processed using Skyline (MacCoss Lab, University of Washington, WA, USA) with a transition list as described by Kim et al. (2020). PEG-28 concentrations in the digesta were interpolated using a sigmoidal 4PL calibration curve of peak area versus five PEG-28 standards (0.25, 0.5, 1, 2 and 5 ng/μL). Dilution factors (Table S1) were calculated as follows: PEG-28 concentration in WPI feed ÷ PEG-28 concentration in the jejunal fluids. Peptide

abundance in the in vivo digesta was normalized by multiplying these dilution factors.

2.10. Statistics

All statical analyses were performed using JMP® Pro 17 (SAS Institute Inc., Cary, NC, USA). Pearson's correlation analyses were performed using log 10 normalized peptide abundances, and Spearman's correlation analyses were conducted based on the amino acid counts identified across selected parent protein sequences, including BLG, ALA, β-CN and κ-CN. Missing values were excluded from the correlation analysis. Relative percent difference analysis was used to evaluate percent variation in technical duplicates and differences in peptide abundances from two different sets of LC columns. Differences in total normalized peptide abundances between in vitro gastric digesta, in vitro intestinal digesta and in vivo jejunal digesta were evaluated via one-way ANOVA, followed by Tukey's post hoc test. Wilcoxon/Kruskal-Wallis tests were used to test differences in numbers of peptide counts. Significance threshold was set at p-value < 0.05.

3. Results and discussion

WPI was subjected to static in vitro digestion following the Harmonized COST INFOGEST protocols (Brodkorb et al., 2019; Minekus et al., 2014). Gastric digesta was collected at 20, 60 and 120 min after static in vitro gastric digestion. In vitro intestinal digestion was conducted after the following gastric endpoints: G20, G60 and G120, with intestinal samples collected at 30, 60 and 120 min of each gastric endpoint. Jejunal effluents were obtained from three adults over 4 h after WPI feed ingestion. Peptide identification in the digesta was conducted using LC-MS/MS, and the MS/MS spectra were searched against an in-house cow milk protein database. The MBDPB database (Nielsen et al., 2017; Nielsen et al., 2023) was used to identify known bioactivities of the identified peptides.

3.1. Reproducibility of peptide extraction

To assess reproducibility in peptide extraction via ethanol precipitation between technical duplicates, Pearson's correlation and relative percent difference (RPD) analyses were performed on log 10-normalized peptide abundances of all peptide sequences. The average correlation coefficients were 0.90 ± 0.16 for the in vivo samples and 0.90 ± 0.05 for the in vitro samples, with RPD values of 5.14 % ± 6.68 % and 5.70 % ± 1.68 %, respectively (Table S2). These results indicate strong reproducibility and consistency in peptidomic profiles between two technical duplicates. However, the two duplicates of one of the jejunal fluids samples showed a moderate correlation ($r = 0.43$) with slightly high RPD of 25.18 % (>20 % threshold), which might be due to inhomogeneous sample matrices during sample aliquoting prior to ethanol precipitation.

3.2. Impact of different LC columns on peptide identification

To evaluate variations in peptide identifications resulting from using slightly different LC columns to analyze the in vivo and in vitro digesta samples (as described in section 2.5), four duplicates of WPI feeds underwent identical protein extraction and bottom-up proteomics sample preparation steps and were analyzed via LC-MS/MS using both sets of LC columns ($n = 2$ for columns used for in vivo sample analysis and $n = 2$ for columns used for in vitro sample analysis). Pearson's correlation coefficients based on total abundance of all peptides from each protein ranged from 0.94 to 0.96 with an average RPD value of 3.64±3.54 %, when comparing WPI feeds analyzed with columns used to analyze the in vivo samples with WPI feeds analyzed with columns used to analyze the in vitro samples (Fig. S1). The low RPD and high correlation coefficients indicate similar proteomic profiles and no significant variations in peptide detection between the two sets of the LC columns.

3.3. Peptide profile after in vitro gastric digestion

After simulated gastric digestion of WPI, a total of 1187 unique peptides, including those with post-translational modifications, were identified (Fig. 1A) via LC-MS/MS. The peptide counts in the gastric samples (G20, G60 and G120), each with two technical duplicates ($n = 6$), predominantly consisted of β-CN from both A1 and A2 variants, with 306 unique sequences, averaging 260 ± 11 peptides across all samples and accounting for 26.9 % of the gastric peptide numbers (Fig. 2A and Table S3). Although caseins are not classified as whey proteins, small amounts of caseins found in WPI could result from 1) the presence of casein peptides released from intact caseins in milk by endogenous milk proteases; and 2) smaller fragments of caseins released by protease activity during cheesemaking migrating to the whey fraction. The high numbers of β-CN-derived peptides likely resulted from rapid gastric degradation of intact caseins, as reported by previous studies using both static and dynamic in vitro models to digest skim milk powder (Egger et al., 2019).

The second highest peptide counts in the gastric digesta were derived from BLG, with 229 unique sequences, averaging 186 ± 22 peptides (19.3 %). Though BLG was the main constituent in the WPI feed (68–70 % of protein content in the WPI feed), BLG-derived peptides were present in comparatively lower numbers, suggesting that BLG protein was partially resistant to gastric digestion and remained mostly in its intact form, as shown in previous studies (Egger et al., 2019; Sanchón et al., 2018). The third highest counts were from ALA, which accounted for 121 unique peptides, averaging 93 ± 19 peptides (9.7 %). Additional peptides included 65 unique sequences from glycosylation-dependent adhesion molecule 1 (GLYCAM1) and 63 unique sequences from bovine serum albumin (BSA), representing 5.9 % and 5.2 % of the total counts, respectively. Other casein-derived peptides included 59 unique sequences from κ-CN, which made up 5.0 % of the total count, 53 sequences from α_{S1}-CN, which made up 4.1 % of the total counts and 26 sequences from α_{S2}-CN, which made up 2.5 % of the total counts.

Several other minor whey proteins also contributed to the peptide counts in the gastric digesta, each accounting for less than 3 % of the totals. These peptide groups included those originating from IGHG1 immunoglobulin heavy constant gamma (IGHG1; accession: A0A3Q1N3I9), xanthine dehydrogenase/oxidase (XDH/XO), IGL@ protein (accession: Q3T101), lactoperoxidase (LPO), zinc-α2-glycoprotein (ZAG) and polymeric immunoglobulin receptor (PIGR). The remaining 11 % of peptide counts (129 unique sequences) were derived from 29 other proteins, including lactoferrin, osteopontin, butyrophilin subfamily 1 member, epididymal secretory protein and α1-acid glycoprotein (Table S5).

Despite β-CN being the primary source of peptide counts, GLYCAM1 (also known as lactophorin or proteose peptone component 3)-derived peptides showed the highest average peptide abundance in the gastric digesta (Fig. 2B and Table S4), representing 25.5 % of the total abundances. The high abundance from GLYCAM-1 (e.g., ^{19}ILNKPEDETHL^{29}; Fig. S2) suggests that it was preferentially hydrolyzed by pepsin during gastric digestion, likely due to its amino acid composition, which includes high levels of leucine and isoleucine (Kanno, 1989) which would provide cleavage sites for pepsin. κ-CN peptides were present at the next highest peptide abundance at 23.2 %, followed by ALA at 17.0 %, BLG at 15.2 % and β-CN at 9.2 %.

Certain regions of whey protein sequences demonstrated resistance to proteolysis by gastric digestion across 120 min. We identified resistant peptides to gastric digestion as those that were the shortest version of peptide arrays within a sequence range that persisted across 120 min. For β-CN, some of the resistant fragments included peptides from regions ^{96}PVVVPPF^{102}, ^{60}LQDKIHPF^{67} and ^{74}VYPFPGPI^{82} (Fig. 3A and Table S5). For BLG, resistant regions included ^{59}VEELKPTPE^{67}, ^{153}DKALKALPM^{161} and ^{62}LKPTPEGD^{69} (Fig. 3B), which aligns with the

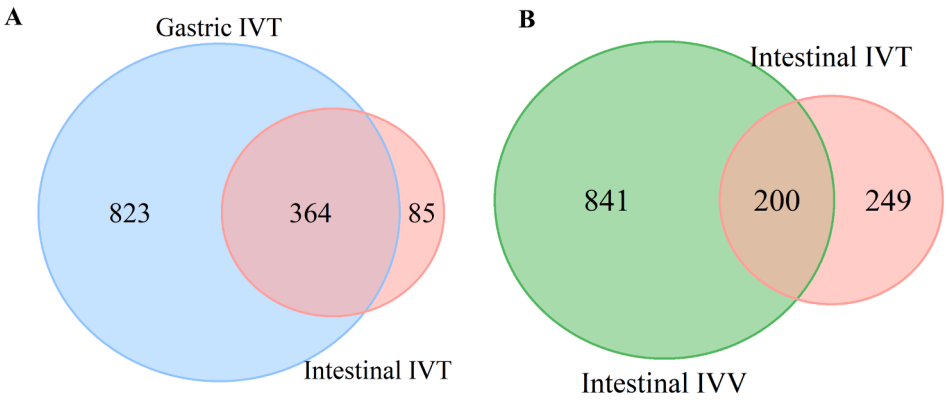

Fig. 1. Venn diagrams of the whey-derived peptide numbers identified after digestion of WPI under different digestion conditions. (A) Peptide identified in the in vitro gastric digesta (Gastric IVT, blue) vs. in vitro intestinal digesta (Intestinal IVT, red). (B) Peptides identified in the in vivo jejunal digesta (Intestinal IVV, green) vs. in vitro intestinal digesta (Intestinal IVT, red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

findings observed in a previous study using dynamic adult in vitro gastric digestion of whey protein concentrate (Miralles et al., 2017). The presence of proline in each of these resistant regions aligns with a previous study indicating that proline-containing peptides are resistant to gastric cleavage by pepsin (Vanhoof et al., 1995).

An MBPDB search of the gastric peptides revealed that 37 unique sequences were 100 % homologous to known bioactive peptides derived from β-CN, BLG, ALA, κ-CN and α_{S2}-CN, and that, in total, those sequences had 10 different identified biological functions (Fig. 4). The greatest number of these peptides (14 sequences) were ACE-inhibitory, followed by DPP-IV inhibitory (10 sequences), antioxidant (6 sequences) and antimicrobial (6 sequences; Table S6). Other peptides were associated with immunomodulatory, anticancer, antithrombotic, cytotoxic, cholesterol regulatory and prolyl endopeptidase-inhibitory activity. Among the casein-derived peptides, a total of 17 casein phosphopeptides (CPPs) were identified including 3 from β-CN, 7 from κ-CN, 6 from α_{S1}-CN and 1 from α_{S2}-CN (Table S5). Each peptide contained a single phosphorylation site at either serine or threonine.

In vitro gastric digestion produced various bioactive peptides, but the biological relevance of some functional categories in the stomach is limited, as many lack known sites of action in the stomach. For example, ACE-inhibitory or antihypertensive peptides (e.g. ALA-derived ^{34}LKGYGGVSLPEW^{45}; Otte et al., 2007) which inhibit the activity of angiotensin-converting enzyme, thereby regulating blood pressure and fluid balance, lack a known function within the stomach. However, peptides with antimicrobial and antioxidant properties might potentially exert their function within the stomach. Although the direct relevance of gastric-digested bioactive peptides may be limited, their stability through gastric digestion suggests they could reach and function in other parts of the digestive tract. For instance, antimicrobial peptides (i.e. BLG-derived ^{108}VLVLDTDYK^{116}) present in the stomach may be able to exert function in the upper small intestine and beyond if they remain resistant to intestinal proteases.

3.4. Peptide profiles after in vitro intestinal digestion

In vitro intestinal digestion of WPI from three different gastric endpoints (G20, G60 and G120) with intestinal samples collected at 30, 60 and 120 min (n = 18) generated a total of 499 unique peptides (Fig. 1A). Among the 499 peptides present in the intestinal samples, 127 unique sequences were generated from β-CN, with 50 ± 15 peptides identified on average across all samples, representing 27.6 % of total peptide numbers (Fig. 2A). A hundred and three unique peptides were derived from BLG, with an average of 37 ± 16 peptides, representing 20.3 % of the total peptide numbers. Minor contributing peptides included 42 unique sequences from ALA, 31 from κ-CN, 21 from BSA and 21 from α_{S1}-CN, representing 9.6 %, 8.5 %, 3.8 % and 3.8 % of the total peptide numbers, respectively. Additionally, proteins contributing less than 3 % each included GLYCAM1, PIGR, IGHG1, ZAG, LPO, IGL@ protein, XDH/XO and α_{S2}-CN. The remaining 29 proteins collectively made up 14.2 % of the total peptide counts (Table S3). We identified a larger number of peptides than did a previous study by Corrochano et al. (2018), which found, from static in vitro intestinal digestion of whey proteins, 47 peptides from BLG, 3 from ALA, 2 from BSA and 2 from lactoferrin. The present study's identification of a higher number of peptides could be attributed to its employment of peptidomic sample preparation steps that Corrochano et al. did not use; the present study used selective enrichment via ethanol precipitation, which separated peptides from larger proteins, and peptide purification, which concentrated the peptides and removed interfering molecules that could compete for ionization during mass spectrometry analysis. Peptides derived from κ-CN had the highest abundance, at 45.9 % of the total abundance in the intestinal digesta, followed by BLG at 33.8 %, PIGR at 8.0 %, β-CN at 7.9 % and other proteins at 4.4 % (Fig. 2B and Table S4). The specific region of κ-CN with the highest peptide abundances after in vitro intestinal digestion was ^{175}ESPPEINT^{182} (Fig. 3D and Table S5), which is part of the glycomacropeptide (GMP), a 64 amino acid fragment of κ-CN (region 127–190) present in whey portion after rennet hydrolysis of κ-CN during cheese production. Glycosylation present in GMP fragments may enhance its resistance to proteolytic enzymes due to steric hindrance, which can protect the peptide from enzymatic cleavage (Boutrou et al., 2008).

Several peptide fragments resisted degradation not only during the gastric phase but also through the intestinal phase. Examples include β-CN-derived peptides such as ^{96}PVVVPPF^{102} and ^{74}VYPFPGP^{80}, as well as the BLG-derived peptide ^{61}ELKPTPE^{67} (Table S5). These fragments are rich in proline, which likely contributes to their resistance to gastrointestinal digestion (Sanchón et al., 2018).

After intestinal digestion, a total of 34 unique peptides were identified as bioactive, each associated with one or more of 16 distinct biological functions (Fig. 4 and Table S6). Among these bioactive peptides, 15 that derived from β-CN, BLG, ALA and α_{S1}-CN exhibited ACE-inhibitory properties. Eight peptides derived from β-CN, BLG and κ-CN were identified as antimicrobial. Another eight peptides showed DPP-IV inhibitory activity. Six peptides exhibited antioxidant properties, with four derived from β-CN, one from ALA and one from κ-CN. Additionally, two β-CN-derived peptides were identified as immunomodulatory, and two were associated with increased mucin secretion. Other bioactivities identified included antithrombotic, bradykinin-potentiating, cathepsin B inhibitory, cholesterol regulation, anticancer, anti-anxiety, increased calcium uptake, opioid, prolyl endopeptidase-inhibitory and satiety. A total of 5 CPPs were detected with 2 from β-CN, 2 from α_{S1}-CN and 1

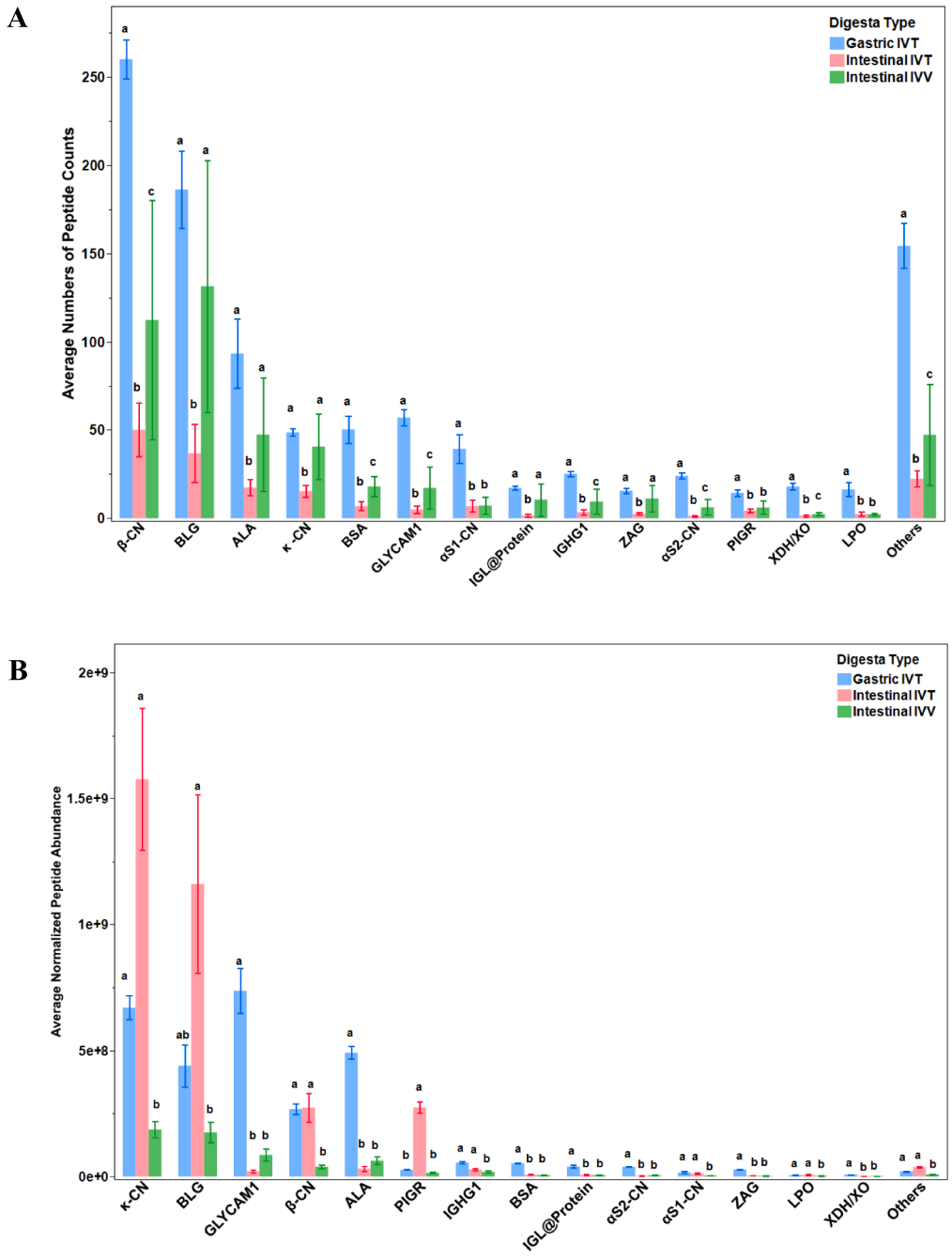

Fig. 2. Comparisons of (A) average number of peptides and (B) average normalized peptide abundances derived from each parent protein across different digestion conditions, including in vitro gastric digestion ('Gastric IVT', n = 6, blue), in vitro intestinal digestion ('Intestinal IVT', n = 18, red) and in vivo intestinal digestion ('Intestinal IVV', n = 22, green). For each protein, digesta types with different letter (a, b or c) were significantly different (p-value < 0.05). Data are represented as mean ± standard deviation for (A) and standard error for (B). β-CN, β-casein; BLG, β-lactoglobulin; ALA, α-lactalbumin; κ-CN, κ-casein; BSA, bovine serum albumin; GLYCAM1, glycosylation-dependent cell adhesion molecule 1, αS1-CN, α_{S1}-casein, IGHG1, immunoglobulin heavy constant gamma1; ZAG, zinc-α2-glycoprotein; αS2-CN, α_{S2}-casein; PIGR, polymeric immunoglobulin receptor; LPO, lactoperoxidase; Others, other 41 proteins. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

from κ-CN.

Several bioactive peptides released after intestinal digestion are linked to multifunctional properties. For example, the β-CN-derived peptide ^{75}YPFPGPI^{81}, also known as β-casomorphin-7, exhibits a range of bioactivities. It has been shown to have ACE-inhibitory effects (Amigo, Martínez-Maqueda, & Hernández-Ledesma, 2020; Hernández-Ledesma et al., 2004), anti-anxiety properties (Dubynin et al., 1998), anticancer activity (Hatzoglou et al., 1996), antioxidant capabilities (Amigo et al., 2020; Yin et al., 2012) and immunomodulatory effects (Haq et al., 2014). Additionally, β-casomorphin-7 can increase mucin secretion (Claustre et al., 2002; Trompette et al., 2003; Zoghbi et al., 2006), act as an opioid agonist (Martinez-Maqueda et al., 2012) and promote satiety (Osborne et al., 2014).

Within the small intestine, several peptide biological functions are particularly relevant due to their potential roles in maintaining overall intestinal health. These bioactivities include antimicrobial effects,

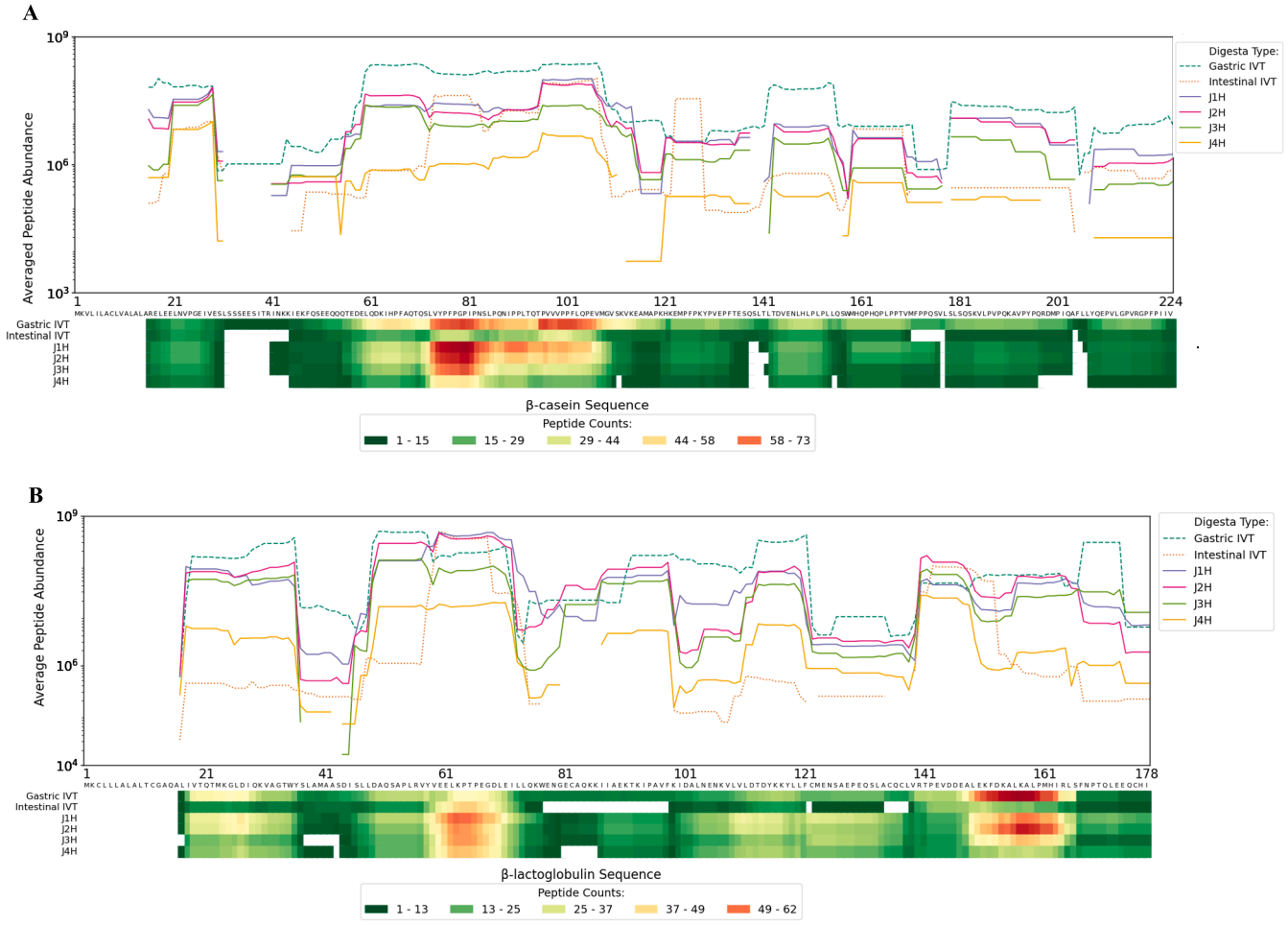

Fig. 3. Whey-derived peptide profiles from different parent proteins: (A) β-casein, (B) β-lactoglobulin, (C) α-lactalbumin and (D) κ-casein, after in vitro (gastric and intestinal) and in vivo digestion. The conditions include in vitro gastric digestion ('Gastric IVT', n = 6, green dot line); in vitro intestinal digestion ('Intestinal IVT', n = 18, yellow dot line) and in vivo digestion at 1 h ('J1H', n = 6, purple line), 2 h (J2H, n = 6, pink line), 3 h (J3H, n = 4, green line) and 4 h (J4H, n = 6, yellow line) post-WPI ingestion from the jejunal fluids of three participants. The heatmaps on the x-axis represent the frequency of peptide counts identified as part of the parent proteins, whereas the line plots show the average normalized abundances of the peptide sequences on a logarithmic scale for each digestion condition. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

antioxidant activity, mucin-stimulatory, immunomodulatory properties and opioid activities. For example, the β-CN-derived peptide ^{113}VKEAMAPK^{120} has been shown to exert direct bactericidal effects against gram-negative bacteria (Sedaghati et al., 2016), which could help prevent bacterial infection within the gut.

3.5. Comparison between in vitro gastric and intestinal digestion

Proteolysis of WPI under in vitro intestinal digestion resulted in a loss of 823 peptides from the gastric digesta, leaving 364 peptides that persisted into the intestinal phase (Fig. 1A). Additionally, 85 new peptides were released during intestinal digestion. The average peptide counts from a broad array of proteins, including β-CN, BLG, ALA, κ-CN, BSA and GLYCAM1 significantly decreased after intestinal digestion (Fig. 2A). Though large changes in peptide profile based on unique peptides were apparent, Spearman's correlation coefficient based on the frequency of amino acid appearances across protein sequences for β-CN, BLG, ALA and κ-CN in gastric and intestinal conditions indicated strong similarity (r = 0.82 ± 0.07) (Table S7). However, missing fragments were observed after intestinal digestion, as represented in the heat maps in Fig. 3A–D.

The abundances of GLYCAM1, ALA, BSA, IGL@ protein, α_{S2}-CN, ZAG and XDH/XO-derived peptides significantly decreased from gastric digestion to intestinal digestion, indicating degradation and reduced stability in the intestinal condition. However, the abundances of PIGR-derived peptides significantly increased (Fig. 2B), particularly for the fragment ^{610}AGGPGAPADPGRPT^{623} (Table S4), suggesting that this fragment was resistant to further proteolysis by pancreatin during intestinal digestion. No significant differences in abundances were observed for peptides derived from κ-CN, BLG, β-CN, IGHG1, α_{S1}-CN, LPO, and 29 other proteins. The abundances of each peptide between in vitro gastric and intestinal samples were not correlated (r = 0.06), which indicates that gastric and intestinal phase peptides were highly different in terms of abundances. This lack of correlation is expected due to the presence of differing enzymes in each phase, with further degradation occurring in the intestinal phase by pancreatic enzymes.

Though some peptides initially found in the gastric digesta were degraded during in vitro intestinal digestion, new bioactive peptides were liberated during the intestinal phase. A total of seven new bioactive peptides were generated after intestinal digestion, including β-CN-derived peptides such as ^{75}YPFPGPI^{81}, ^{123}EMPFPK^{128} (which has ACE-inhibitory, antimicrobial, bradykinin-potentiating and increased mucin secretion properties) and ^{208}YQEPVLGPVRGPFPIIV^{224} (which has ACE-inhibitory, antimicrobial, antithrombotic, anticancer and

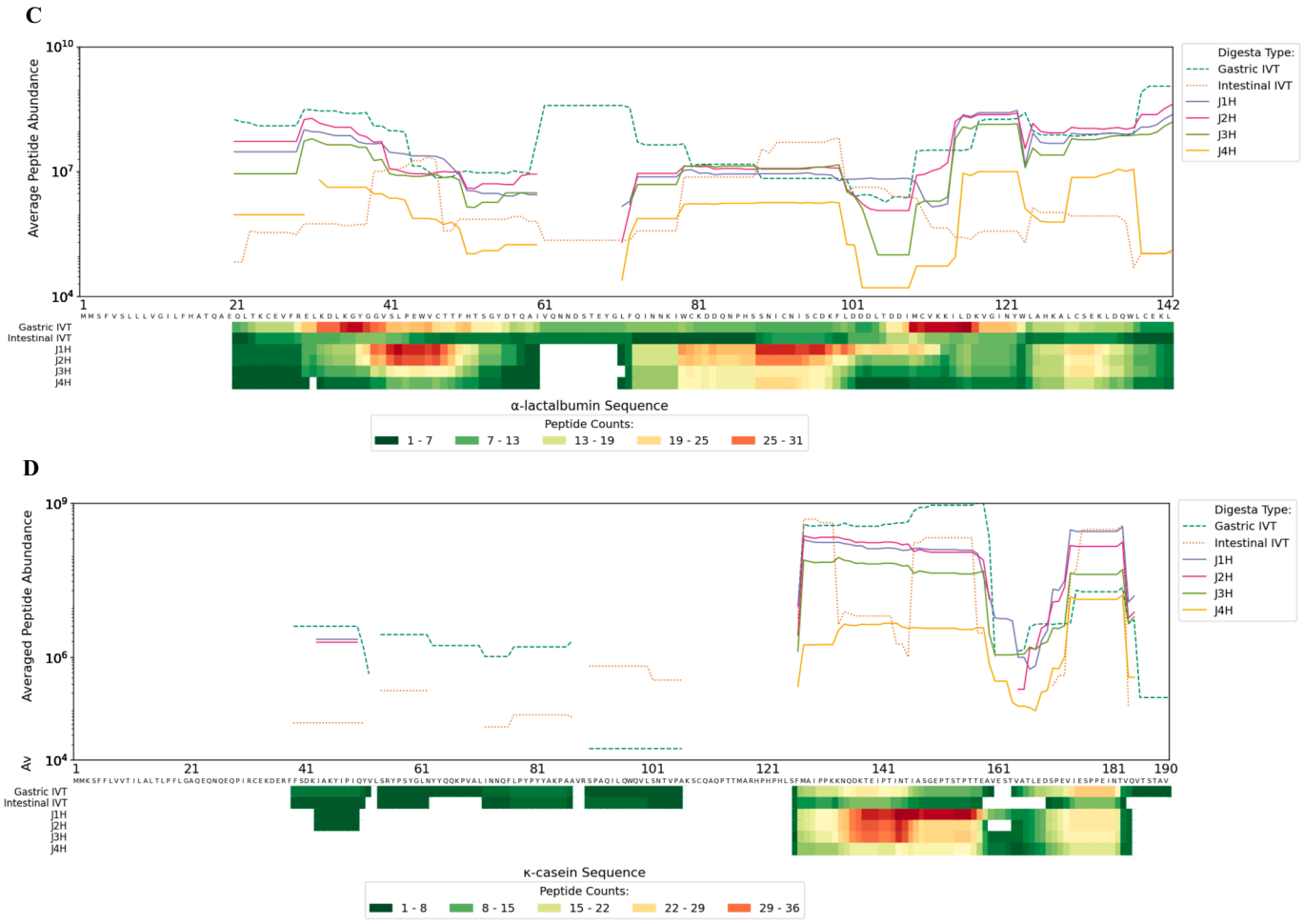

Fig. 3. (*continued*).

immunomodulatory properties; Table S3).

In contrast, 11 bioactive peptides were no longer detected after intestinal digestion, indicating their susceptibility to pancreatic enzymes. These bioactive peptides included some derived from ALA (e.g., ^{34}LKGYGGVSLPEW^{45}), β-CN (e.g., ^{181}SQSKVLPVPQKAVPYPQ^{197}), α_{S2}-CN (i.e., ^{163}TKKTKLTEEEKNRL^{176}) and BLG (e.g., ^{17}LIVTQTMK^{24}; Table S3).

Twenty-five bioactive peptides remained stable across gastric and intestinal digestions. These peptides included those-derived from β-CN (e.g., ^{21}LNVPGEIVE^{29} [ACE-inhibitory] and ^{113}VKEAMAPK^{120} [antimicrobial]), BLG (e.g., ^{28}IQKVAGTW^{35} [ACE-inhibitory and DPP-IV inhibitory]), ALA (e.g., ^{34}LKGYGGVSLPE^{44} [DPP-IV inhibitory]) and κ–CN (e.g., ^{127}MAIPPKKNQDKTEIPTINT^{145} [antimicrobial]).

3.6. Peptidomic profiles of WPI within the human jejunum

After ingestion of WPI, a total of 1041 peptide sequences were released within the jejunum of the three participants over 4 h (n = 22; Fig. 1B). This number is significantly higher compared to that found by a previous study, which analyzed jejunal effluents of eight adults over 6 h after ingestion of whey proteins and identified 146 peptides (Boutrou et al., 2013). Again, the increase in peptide numbers could be attributed to the peptide extraction method used in our study, as Boutrou et al. did not extract the peptides prior to injection in the LC-MS/MS (Boutrou et al., 2013). Additionally, the use of more advanced LC-MS/MS instrumentation in our study likely also enhanced resolution, accuracy and sensitivity in peptide detection.

Among the 1041 peptides identified in the human jejunum, those originating from BLG were the most numerous, with 287 unique sequences (averaging 131 ± 71 peptides) accounting for 28.3 % of total peptide counts in the jejunal digesta identified (Fig. 2A and Table S1). Additionally, 246 unique peptides were derived from β-CN (averaging 112 ± 68 peptides; 24.1 % of the total), followed by 124 sequences from ALA (averaging 47 ± 32 peptides; 10.2 % of the total) and 87 unique sequences of κ-CN (averaging 41 ± 19 peptides; 8.7 % of the total). Fourteen also derived from PIGR (averaging 6 ± 4 peptides; 1.3 %), 3 from XDH/XO (averaging 2 ± 1 peptides; 0.5 %), 3 from LPO (averaging 2 ± 1 peptides; 0.5 %) and 85 from other proteins (averaging 42 ± 18 peptides; 11.5 %). Additional contributing parent proteins in the jejunal digesta included BSA, GLYCAM1, ZAG, IGL@ protein, IGHG1, α_{S1}-CN and α_{S2}-CN at 3.9 %, 3.7 %, 2.4 %, 2.3 %, 2.0 %, 1.6 % and 1.3 % of the total, respectively. Similar to the in vitro intestinal digestion, in the in vivo digestion, the average abundances of peptides derived from κ-CN were the highest, at 30.4 % in the human jejunum (Table S4). Some of the highest abundance κ-CN-derived peptides, specifically from the GMP region, were ^{146}IASGEPTSTPTTE^{158} and ^{173}VIESPPEINT^{182} which were identified in all three participant jejunums over at least 2 h (Fig. 3D and Table S5). These highly abundant κ-CN sequences are consistent with those reported by Koh et al. (2022), who studied three adults fed purified GMP.

The next highest abundance peptides were from BLG at 28.6 %, followed by GLYCAM1 at 13.9 %, ALA at 10.2 %, β-CN at 6.3 %, PIGR at 2.4 % and the remaining proteins at 8.2 % (Fig. 2B). Specific regions of proteins β-CN (e.g., ^{95}TPVVVPPFLQPEVM^{108}), BLG (e.g., ^{57}VYVEELKPTPEGDL^{70}), ALA (e.g., ^{38}GGVSLPEWVC^{47}) and κ-CN (e.g., ^{173}VIESPPEINT^{182}) decreased in abundances over 4 h of digestion in the

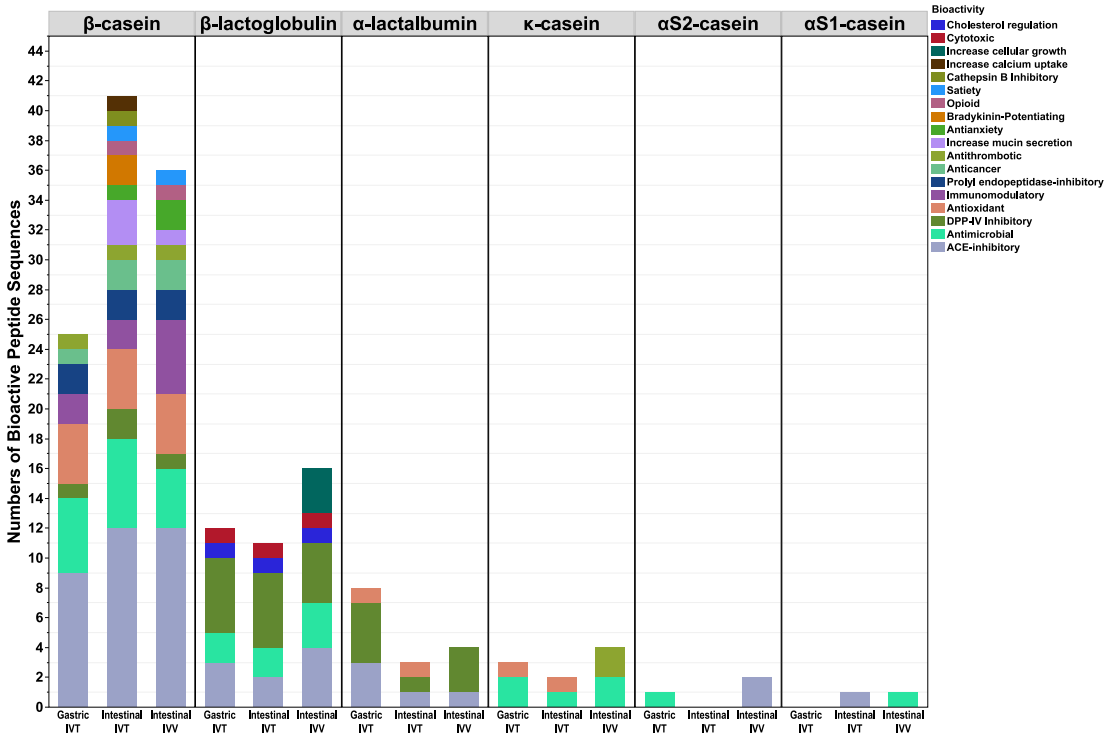

Fig. 4. Number of bioactive peptide sequences derived from parent proteins (β-casein, β-lactoglobulin, α-lactalbumin, κ-casein, α_{S2}-casein and α_{S1}-casein) with 100 % homology to known biological functions from in vitro gastric (denoted as 'Gastric IVT'), in vitro intestinal (denoted as 'Intestinal IVT') and in vivo intestinal (denoted as 'Intestinal IVV') digestive samples. Each color represents a different bioactivity.

jejunum (Fig. 3A–D) with average peptide abundance across all proteins decreased over 4 h post ingestion (Fig. S3).

The in vivo intestinal digestion of WPI resulted in the release of 41 unique bioactive peptides derived from β-CN, BLG, ALA, κ-CN, α_{S1}-CN and α_{S2}-CN within the human jejunum with 18 biological functions that are associated with ACE-inhibitory, DPP-IV inhibitory, antimicrobial, antioxidant, immunomodulatory, anticancer, antianxiety, prolyl endopeptidase-inhibitory, mucin-stimulatory, cathepsin B inhibitory, antithrombotic, cholesterol regulation and opioid activities (Fig. 4 and Table S6). Among the casein-derived peptides, 17 were phosphorylated, including 2 β-CN, from 3 from α_{S1}-CN, 2 from α_{S2}-CN and 10 from κ-CN. Twenty-five bioactive peptides, including those derived from β-CN (e.g., ^{74}VYPFPGPIPN^{83} and ^{74}VYPFPGPI^{81}) and BLG (e.g., ^{59}VYPFPGPI^{66} and ^{141}TPEVDDEALEK^{151}) were identified across the jejunum of the three participants (Table S5), indicating a consistent and reproducible pattern of bioactive peptide release among different individuals. The number of bioactive peptides identified was higher than in previous work, which identified no whey protein-derived bioactive peptides in the jejunal effluents of eight adults fed whey protein (Boutrou et al. 2013). The use of an extensive, contemporary database in our study enabled the identification of a substantially higher number of bioactive peptides.

The impact of ACE-inhibitory and DPP-IV inhibitory peptides identified in the jejunum may be of limited physiological relevance because, to regulate blood pressure and blood glucose, respectively, these peptides must enter the bloodstream intact. These peptides would have to survive exposure to intestinal brush border peptidases and be absorbed to exert these activities.

In the human jejunum, bioactive peptides such as antimicrobial, mucin-secretion-enhancing and opioid peptides may play significant biological roles in promoting gut health. For example, BLG-derived ^{141}TPEVDDEALEK^{151} can inhibit growth of pathogenic gram-positive bacteria, including *Listeria monocytogenes* and *Staphylococcus aureus*, in a concentration-dependent manner, with a minimum concentration of 10 mg/mL (Demers-Mathieu et al., 2013). Moreover, β-casomorphin-7 has been shown to increase mucin secretion in rat jejunum (Claustre et al., 2002), which means that its presence in the small intestine could modulate intestinal physiology and health. Intestinal mucin plays an important role in lubricating and protecting the intestinal mucosal surfaces against microbial invasion, acids and proteolytic enzymes (Forstner, 1978), and abnormalities in mucus secretion and/or mucin expression may be associated with several diseases, including inflammatory bowel diseases and cancer (Claustre et al., 2002; Ho et al., 1993). Thus, β-casomorphin-7 could contribute to intestinal health in important ways.

3.7. Comparison between in vivo and in vitro intestinal digestion

In vivo and static in vitro intestinal digestion of WPI liberated 200 identical peptide sequences from 23 common protein precursors (Fig. 1B), accounting for 40 % of unique peptide counts identified in vitro and 19 % of peptides identified in vivo. These findings indicate that the peptide profile generated in vivo is more diverse than that generated in vitro, possibly because of the presence of a broader array of proteases in the gut. The human jejunum samples contained significantly higher numbers of peptides derived from the majority of proteins, including β-CN, BLG, ALA, κ-CN, BSA, GLYCAM1, IGL@ Protein, IGHG1, ZAG, α_{S2}-CN, XDH/XO and a group of 29 other proteins, compared with the in vitro intestinal digestion samples (Fig. 2A). No significant differences in peptide counts were observed between these two conditions for peptides derived from α_{S1}-CN, PIGR and LPO.

The abundances of the 200 shared peptides, on average, represented 45 % of the total peptide abundance in vitro and 20 % of total peptide abundance in vivo. There were no significant differences in the abundances of peptides derived from several proteins, including GLYCAM1, ALA, BSA, α_{S2}-CN, IGL@ Protein, ZAG and XDH/XO. Peptide abundances from κ-CN, BLG, β-CN, PIGR, IGHG1, α_{S1}-CN and LPO were significantly higher in the in vitro intestinal digesta than in the in vivo (Fig. 2B). These differences between in vitro and in vivo samples in terms of abundances could be due to differences in enzymatic activities, concentrations and various types of endogenous enzymes present in the

human gastrointestinal tract.

To compare the similarity between peptide sequences released after intestinal digestions of WPI under in vivo and in vitro conditions, a rank-based Spearman's correlation analysis was conducted, using the frequency of amino acid appearance across major proteins, including β-CN, BLG, ALA, κ-CN, α_{S1}-CN and α_{S2}-CN. Spearman's correlation coefficients revealed moderate correlations between static in vitro and in vivo intestinal digestion for β-CN ($r = 0.61$), BLG ($r = 0.74$), κ-CN ($r = 0.65$), α_{S1}-CN ($r = 0.61$), α_{S2}-CN ($r = 0.57$) and all proteins combined ($r = 0.68$), except for ALA, which showed a poor correlation of 0.44 (Table S7). These findings are relatively consistent with previous research by Sanchón et al. (2018), who reported similar correlation coefficients ($r = 0.74 \pm 0.16$) for the combination of β-CN and α_{S1}-CN when comparing in vivo (in four adults) with static in vitro intestinal digesta of WPI. Furthermore, the moderate correlation coefficients of peptides derived from β-CN (Fig. 3A), BLG (Fig. 3B) and κ-CN (Fig. 3D) in our study are reflected in the relatively similar peptide patterns shown in the heatmaps when comparing intestinal in vitro digestion to human jejunum across 4 h. However, ALA's peptide pattern showed weak similarity between the in vivo and in vitro conditions as indicated by the low correlation coefficient (Fig. 3C). The Spearman correlation values between in vitro and in vivo intestinal herein are similar to those between in vitro gastric and in vitro intestinal ($r = 0.73 \pm 0.11$).

To provide a more comprehensive analysis of peptide quantities, we also compared peptidomic profiles using peptide abundance data. The results show mixed correlation coefficients. Specifically, Pearson's correlation coefficients based on log10 normalized peptide abundances indicated high correlation for κ-CN ($r = 0.84$), moderate correlation for BLG ($r = 0.59$) and β-CN ($r = 0.59$), poor correlation for α_{S1}-CN ($r = 0.33$) and α_{S2}-CN ($r = 0.45$) and a negative correlation for ALA ($r = -0.43$). These findings indicate the complex nature of peptide quantity release from different parent proteins. Careful consideration is needed when interpreting in vitro digestion results as the peptide patterns seem to be protein specific. When considering peptide abundances of all proteins combined, the in vivo and in vitro conditions were moderately correlated ($r = 0.54$), indicating moderate similarity in overall peptide quantities produced between in vivo and in vitro models. Our study is the first to assess comprehensive peptide profiles between in vivo and in vitro intestinal digestion using peptide abundances. This finding indicates that the capacity of the currently used static in vitro digestion model to replicate adult human intestinal digestion in terms of specific peptides released and their abundances is moderate. Further optimization of current in vitro models to enhance their ability to represent in vivo human digestion in terms of peptide release is needed.

Similarly, the ability of in vitro intestinal digestion to reproduce the same bioactive peptides as in vivo digestion was limited. Twenty-four bioactive peptides were common to both conditions, representing 58 % of bioactive peptide counts and 49 % of bioactive peptide abundance in vivo, and 70 % of counts and 56 % of abundance in vitro. Twelve bioactive peptides were uniquely found in vitro, whereas 19 were exclusive to in vivo digestion. The relatively high percentage of common bioactive peptide counts indicates some degree of consistency in peptide generation. However, the correlation of peptide abundances of these 24 sequences was quite poor ($r = 0.23$), indicating significant differences in the relative quantities of these peptides between the two conditions. These discrepancies likely occurred because of differences in enzymes present, enzyme activities, enzyme concentrations and other environmental factors between in vivo digestion and the in vitro model applied.

4. Conclusion

A wide array of whey-derived peptides, including those with bioactive properties, were released across the gastrointestinal tract after both in vivo and in vitro digestion, which may have the potential to cross the intestinal barrier. These digestion-generated bioactive peptides, such as those with antimicrobial effects, antioxidant properties, the ability to increase mucin secretion and opioid activity, could potentially exert their biological functions within the gastrointestinal tract, thus supporting gut health. Identifying these whey-derived bioactive peptides and their relative abundances is a crucial step in evaluating their potential to exert biological functions at their target sites. However, to determine if these bioactive peptides are present at physiologically relevant concentrations to exert their bioactivities, absolute quantification of peptide concentrations is necessary.

Peptide profiles based on amino acid counts identified across major proteins, including β-CN, BLG, κ-CN, α_{S1}-CN and α_{S2}-CN, were highly similar between in vivo and in vitro intestinal digestion, whereas those identified in ALA showed distinct differences. However, when considering peptidomic profiles based on peptide abundances of all proteins combined, moderate correlation between intestinal in vivo and in vitro digestion was observed. Moreover, in vitro intestinal digestion has a limited capacity to produce the same bioactive peptides in similar abundances as seen in vivo. These variations in peptide abundance reflect the differences in enzyme types, activities, concentrations and environmental conditions between in vivo and in vitro digestion models. Current static in vitro digestion models need to be improved to accurately replicate intestinal digestion in adult humans specifically for peptide release.

A limitation of our research was the small number of participants in the in vivo feeding study. The invasive nature of collecting jejunal fluid via nasojejunal tubes significantly constrained participant recruitment, which may limit the generalizability of our findings to the larger population, particularly in terms of individual differences in digestive physiology. To validate our results and enhance their applicability to the broader population, clinical trials with larger and more diverse participant pools should be conducted.

This study's identification of more bioactive peptides derived from β-CN than other proteins reflects a bias in the existing literature, rather than a biological reality. The extensive focus on β-CN in the past research has led to the identification of its bioactive peptides more frequently, which may create the impression that β-CN is more bioactive than other milk proteins. This focus, however, overlooks the potential bioactivity of other milk proteins. Thus, it is possible that many released peptides identified in our study may have undiscovered bioactive functions. Future studies should investigate bioactivities of other milk proteins, such as lactoferrin, GLYCAM1, BSA and LPO, for digestion-released functional peptides.

Ethical statement

Ethical approval for the involvement of human subjects in this study was granted by Oregon State University Research Ethics Committee, IRB number: IRB 18-088, Corvallis, OR, USA.

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CRediT authorship contribution statement

Suwimon Sutantawong: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bum Jin Kim:** Writing – review & editing, Validation, Methodology, Conceptualization. **Russell F. Kuhfeld:** Writing – review & editing, Visualization, Software, Data curation. **Yunyao Qu:** Writing – review & editing, Methodology. **David C. Dallas:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: David C. Dallas reports financial support was provided by BUILD Dairy. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2024.106540.

Data availability

Data will be made available on request.

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