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ARTICLE

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The objective of this study was to understand the possible interactions between whey protein and cranberry juice after processing that could impact either the protein digestibility or the bioaccessibility of cranberry antioxidants using an *in vitro* gastrointestinal digestion model. Whey protein isolate (27 or 54 mg of protein/mL) was dissolved in either cranberry juice or water and used as model beverage system. Beverages were either non-processed or underwent thermal (low: 85°C for 1 min, medium: 99°C for 10 s and long: 99°C for 5 min) or high-pressure processing (600 MPa for 4 min). After processing, beverages underwent oral (30s), gastric (2h) and small intestinal (2h) digestion. During in vitro digestion, protein hydrolysis was monitored by the O-phthalaldehyde (OPA) assay, SDS-PAGE, soluble amino acid content, and pepidomic profiling using Orbitrap mass-spectrometry. Antioxidant capacity was measured with Ferric Reducing Antioxidant Power (FRAP) and 2,2-azinobis (3-ethlybenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assays before and during in vitro digestion. Whey protein isolate dissolved in water had a significantly higher ($p < 0.05$) degree of hydrolysis and soluble amino acid content during small intestinal digestion compared to protein dissolved in cranberry juice, suggesting that cranberry juice had an effect on how protein was hydrolyzed during digestion. In all processing treatments except for long thermal processing, water and cranberry juice protein solutions had similar β-lactoglobulin digestibility ($p > 0.05$), suggesting that the cranberry juice interactions with the protein do not significantly decrease β-lactoglobulin resistance to hydrolysis by pepsin. Peptide formation also differed between whey protein dissolved in either water or juice. Cranberry juice protein solutions showed a slightly lower peptide count compared with whey protein isolate dissolved in water. Antioxidant bioaccessibility by FRAP during gastric digestion significantly increased in cranberry juice with addition of whey protein isolate. This trend might indicate a protective effect of whey protein isolate to cranberry antioxidant compounds.

Introduction

The increasing demand for functional foods with high nutritional value has led to the development of beverages containing fruit or vegetable juices with added protein [1-3].

Protein provides high nutritional value, due to its vital role in the human functioning such as development, repair, and energy, among others[4]. In addition, the consumption of fruit and vegetable juices has been related to the prevention of diseases related to oxidative stress as such as cancer and neurodegenerative diseases[5]. However, components of the juice matrix may interact with proteins or with digestive enzymes, impacting the nutritional benefits of both added proteins and fruit or vegetable antioxidants.

Previous studies on antioxidant-rich beverages during in vitro digestion have reported varying trends in protein digestibility[1, 6-8] and antioxidant activity or bioaccessibility[2, 3, 9]. Antioxidant activity measures the kinetics of the reaction between the antioxidant and the free radical that it scavenges whereas antioxidant bioaccessibility is the fraction of antioxidants available to react after digestion compared with the antioxidant available to react before digestion[10, 11].

Stojadinovic et al. (2013), studied the digestibility of β-lactoglobulin in the presence of coffee, cocoa, black and green tea polyphenol extracts during gastric and small intestinal in vitro digestion[8]. The presence of all the polyphenol extracts slowed down the hydrolysis of β-lactoglobulin by the end of the 6 h gastric phase. However, during the small intestinal phase, the green tea extract accelerated the rate of protein hydrolysis while coffee and cocoa extracts had the opposite effect. This might be influenced by the interaction of polyphenols with digestive enzymes, such as α-amylase, pepsin, and trypsin. Polyphenols may bind to these digestive enzymes, either

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binding directly to enzyme cavities, or causing enzyme precipitation, both of which inhibit enzyme activity[12, 13]. Proanthocyanidins with low degree of polymerization have been related to the inhibition of digestive enzymes such as pancreatic α-amylase, lipase, and trypsin due to their capacity to binding with the active sites of digestive enzymes [12]. As a result of the interactions of polyphenols with digestive enzymes, the protein digestibility may be decreased in the presence of polyphenols.

Cilla et al. (2012), studied the addition of skim milk (11% v/v) on the antioxidant activity of a fruit juice mix (apricot puree, grape and orange concentrate, sucrose, pectin and ascorbic acid) after in vitro gastrointestinal digestion[14]. The addition of skim milk to the fruit juice mix increased the antioxidant activity measured by oxygen radical absorbance capacity (ORAC) and 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay at the end of small intestinal digestion compared to juice without milk. Rodríguez-Roque et al. (2013), also studied the addition of milk on the antioxidant activity of a fruit juice mix of orange, kiwi, pineapple and mango juice after in vitro gastrointestinal digestion[15]. The addition of milk to the fruit juice significantly decreased the antioxidant activity measured by 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) for the model beverage after gastric and small intestinal digestion compared to the control juice without milk[15]. These conflicting results demonstrate that the impact of protein on natural antioxidant compounds needs additional study.

In addition to variations in formulation, antioxidant content, and protein composition, most beverages undergo thermal or non-thermal processing to assure food safety and extend shelf life [16, 17]. As a result of processing, protein and juice components may be modified further, impacting overall digestion. For example, previous studies have found that severe or prolonged thermal treatment increased whey protein isolate gastric digestibility [18-20]. Also, cranberry juice polyphenols are modified or degraded during thermal processing[21, 22]. The changes in polyphenols as a result of thermal processing might have affected their ability to interact with digestive enzymes[12]. Since large oligomers may only interact with the enzyme's surface, while smaller oligomers as able to interact with enzyme cavities, therefore affecting their activity[12]. In addition, the modifications to juice components that occur during processing in the presence of protein may influence their antioxidant properties.

In order to understand the possible interactions between proteins, juice polyphenols, and their behavior after processing, a model beverage of cranberry juice and whey protein isolate was selected for this study and was processed using either thermal or non-thermal treatments. Protein digestibility and antioxidant bioaccessibility were examined in the beverages before and during in vitro gastrointestinal digestion.

Materials and Methods

Raw Materials and Formulation

Beverages were formulated using cranberry juice (Just Cranberry, R.W. Knudsen, Chico, CA, USA) and whey protein isolate (WPI 8855; Fonterra, New Zealand). Whey protein isolate (0, 27, or 54 mg protein/mL) was mixed with either cranberry juice or deionized water (control beverage system) at 850 rpm until dissolved (approx. 25 min).

The two concentrations of whey protein isolate were selected based on FDA requirements for a label claim of "high", "rich in" or "excellent source of" (54 mg/mL) and "good source", "contains" or "provides" (27 mg/mL). To determine the recommended dietary allowance of protein, the average weight in North America used was 80.7 kg[23].

Processing

Samples of each beverage system were either thermally processed with three different time-temperature combinations (Low, Medium or Long thermal treatment), processed with high-pressure (HPP), or not processed (control). For the low thermal treatment (Low), glass tubes with 10 mL sample were heated in water at 100°C until the temperature reached 85°C (~1 min) and placed in a shaking water bath at 85°C for 1 min. For the medium thermal treatment (Medium), glass tubes with 5 mL sample were placed in a heating block at 180°C until the sample temperature reached 99°C (~70 sec) and held at 99°C for 10 seconds. For the long thermal treatment (Long), glass tubes with 5 mL sample were placed in a heating block at 180°C until it reached 99°C (~70 sec) followed by incubation in a shaking water bath for 5 min at 99°C. Immediately after processing, tubes were placed on ice.

For high-pressure processing, a 30 mL sample was sealed in a vacuum bag (Winpak Ltd., Winnipeg, MB, Canada) at 90% vacuum. Samples were processed in a high-pressure processing unit (2L-700 Lab System, Avure Technologies Inc, Kent, WA) with a pressure of 600 MPa for 4 min. The average come-up time to the target pressure was approximately 2 min. The average temperature of the water in the high-pressure chamber during processing was 32.5°C.

All samples were stored at 4°C and were analyzed within 1 day of processing.

Amino Acid Analysis

The amino acid composition of the whey protein isolate was analyzed using ion-exchange chromatography with a post column ninhydrin reaction detection system at 440 nm following[24] at the UC Davis Proteomics Core. Whey protein isolate was hydrolyzed with 6N hydrochloric acid containing 1% phenol at 110°C for 24 hours under vacuum. Samples were dissolved in sodium citrate buffer (Pickering Laboratories Inc, CA, USA) containing an internal standard (40 nmol/mL norleucine). 50 µL of the sample was injected for analysis by ion-exchange chromatography (L-8800 Hitachi Na-based analyzer, Tokyo, Japan). Amino acid analysis was performed in triplicate.

Protein Solubility

Samples were centrifuged for 30 min at 3000g to precipitate insoluble protein[25]. The supernatant was removed, and the insoluble protein was dried for 5 hours at 100°C[26]. The mass of insoluble protein was utilized to calculate the percent of soluble protein by difference.

***In vitro* Digestion**

Simulated saliva and simulated gastric fluids were prepared according to Bornhorst & Singh, (2013)[27]. Simulated intestinal fluid was prepared according to Roman, et al. (2012)[28]. Pepsin and trypsin activity were determined according to Minekus et al., (2014)[29]. Enzymes were added before the beginning of each digestion. Pepsin was added at a concentration of 2000 U/mL to the simulated gastric fluid and pancreatin was added at a concentration of 100 U/mL to the simulated intestinal fluid. The pH of both solutions was adjusted to 7 (saliva and intestinal fluid) or 1.8 (gastric fluid) with HCl or NaOH after addition of all components.

Simulated digestion was completed following the procedure of Bornhorst and Singh (2013) and Roman et al. (2012)[27, 28]. In addition to the protein solutions, a control sample consisting of water or juice only (no protein) was utilized to assess the influence of the simulated digestion fluids on the protein and antioxidant activity measurements. For all digestions, 5 mL sample was mixed with 3.3 mL simulated saliva for 30 sec. Following this, 6.66 mL of simulated gastric juice was added. The pH was adjusted to pH 3 with 1 M HCl. After 1 h of simulated gastric digestion, pH was adjusted to 2 using 1 M HCl. Following 2 h simulated gastric digestion, 10 mL simulated intestinal fluid was added and the pH was adjusted to 7 with 1 M NaOH. Simulated small intestinal digestion took place for 2 h. Samples were incubated at 37°C (100 rpm) in dark tubes. Simulated digestions were performed in triplicate for all treatments.

Samples were taken during gastric and small intestinal digestion to analyze the protein digestibility using the o-phthalaldehyde assay (OPA) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For OPA analysis, aliquots (100 μL) were taken after 0, 5, 15, 30, 45, 60, 90 and 120 min of gastric digestion and after 15, 30, 45, 60, 90 and 120 min of small intestinal digestion (135, 150, 165, 180, 210 and 240 min of total digestion time). Additional aliquots were taken after 0, 15, 30 and 120 min of gastric digestion (102 and 206 μL) and after 15, 30, 45 and 120 min of small intestinal digestion (167 and 343 μL) for SDS-PAGE analysis. Samples were taken after the gastric phase (120 min) and after the small intestinal phase (240 min total digestion time) for soluble amino acid content using ion-exchange chromatography and antioxidant activity analyses.

Protein Digestibility

All reagents were purchased from Biorad (Hercules, CA) unless otherwise specified

O-Phthalaldehyde (OPA) Assay

Free amino groups were measured using the o-phthalaldehyde assay (OPA)[30]. Prior to analysis, samples were diluted in deionized water to a protein concentration of 1.8 mg/mL for the gastric phase and 0.5-0.8 mg/mL for the small intestinal phase. 100 μL of each sample was used for analysis. Absorbance was measured at 340 nm. The free amino groups in each sample (mg NH_2/mL of protein solution) were calculated using glycine as a standard.

The degree of hydrolysis was calculated for each sample at each digestion time as follows[31]:

$$\mathrm{DH}(\%)=\frac{\left(\mathrm{NH_2}_{\text{digestion time point}}\text{-}\mathrm{NH_2}_{\text{Non-Dig}}\right)}{\text{Total } \mathrm{NH_2}}*100\% \quad (1)$$

where DH% is the degree of hydrolysis calculated as a percentage, $NH_{2digestion\ time\ point}$ is the free amino group concentration at a specific digestion time point (mg amino groups/mL), $NH_{2Non\text{-}Dig}$ is the amino group concentration at before digestion (mg amino groups/mL), and the $Total\ NH_2$ is the total number of amino groups based on the number of amino groups per amino acid (mg amino groups/mL), as measured by ion-exchange chromatography. Final degree of hydrolysis values were determined after subtraction of protein-free control solutions (water or cranberry juice) that also underwent in vitro digestion for each time point.

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Inglingstad et al., (2010) and Bornhorst et al., (2016)[32, 33]. Samples taken during digestion were diluted in water to a protein concentration of 2 mg/mL to allow for comparison between lanes during digestion of the same sample as well as between treatments with different protein concentration. SDS-PAGE analysis was performed on all samples from the triplicate digestions.

Optical densitometry analysis was completed using ImageJ[33] to give a measure of the protein digestibility of soluble β-lactoglobulin digestibility (selecting β-lactoglobulin band at 18.3 kDa). For imaging, gels were placed over a lightbox (AGPtek HL0163, color temperature 6000°K) with the same illumination settings for all images. Gel images were captured using a Canon EOS Rebel SL1 digital camera (18-megapixel, APS-C CMOS sensor, Canon USA, San Jose, CA). Camera setting were the same as previously described[34]. All images were taken using the same light intensity, camera distance from the gels, and camera settings[34].

For optical densitometry analysis, gel images were converted to 32-bit format. The background noise was subtracted using the "rolling ball" algorithm of 90 pixels. Within each gel, lanes were selected and converted into intensity plots. The area under the curve for the β-lactoglobulin band at 18.3 kDa in the intensity plot was calculated using the Gel Analyzer toolbox in ImageJ. Protein digestibility for each gel was calculated as follows:

$$Prot\ dig(\%) = \frac{\left(AUC_{Non-Dig} - AUC_{DTP}\right)}{AUC_{Non-Dig}} * 100\% \quad (2)$$

where $Prot\ dig(\%)$ represents the percent of the soluble β-lactoglobulin digestibility in percent, $AUC_{Non-Dig}$ represents the area under the curve in the intensity plot of β-lactoglobulin band for the non-digested sample (arbitrary units) and AUC_{DTP} represents the area under the curve in the intensity plot β-lactoglobulin band for each digestion time point (arbitrary units). Soluble β-lactoglobulin digestibility was calculated and compared to the non-digested sample within each gel to compensate for any difference in staining which might interfere with intensity values across different gels.

Soluble amino acid content using Ion-Exchange Chromatography

The soluble amino acid content using ion-exchange chromatography of the soluble fraction after sulfosalicylic acid (SSA) precipitation was analyzed for each treatment before and after gastric and small intestinal digestion according to Adibi and Mercer (1973)[35] with minor modifications. Aliquots of 1 mL were taken before or at the end of gastric and small intestinal digestion. Aliquots were heated at 95°C for 10 min. To precipitate the protein, SSA (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 2% (w/v). Samples were vortexed for 10 seconds and let to sit at room temperature for 15 min to increase precipitation. Samples were frozen overnight at -20°C and thawed the next day. After thawing, samples were centrifuged and the supernatant was subjected to hydrolysis with 6N hydrochloric acid with 1% phenol at 110°C for 24 hours under vacuum for measurement of total amino acid content as described in the Section Amino Acid Analysis. Total amino acid content was analyzed in duplicate for each treatment.

Peptide Analysis

Sample preparation for peptide analysis

Peptide analysis was performed on whey protein isolate solutions in water and cranberry juice (54 mg/mL) that were not processed or after long thermal processing (99°C for 5 min), as these samples showed the greatest differences in the soluble amino acid content. Aliquots of 1 mL were taken before digestion, at the end of gastric digestion (120 min) and at the end of small intestinal digestion (240 min) and heated at 95°C for 10 min. Samples were diluted in water to obtain a whey protein isolate concentration of 10 mg/mL. Peptides were extracted as described by Dallas et al. (2015)[36] with the following exceptions: at a volume ratio of 1:1, a solution of 200 g/L of trichloroacetic acid (TCA) was added to the samples and vortexed for 10 seconds. The samples were centrifuged at 2800 g for 30 min at 4°C and the supernatant was recovered. The supernatant containing the naturally occurring peptides was transferred to a new tube and was purified by microplate C18 (Glygen™ Corp., Columbia, MD, USA) solid phase extraction (SPE) as described previously[36]. Salts, sugars, and trichloroacetic acid were washed from the microplate with six column volumes of 1% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). Peptide solutions were dried, and the samples were re-dissolved in 25 µL 2% ACN/0.1% TFA for further analysis.

Peptide abundance determination

The appropriate amount of peptides were injected into the mass spectrometer, the peptide abundance was first estimated by using a fluorometric peptide assay (Pierce™ Quantitative Fluorometric Peptide Assay, Eugene, OR, USA). Samples taken before digestion and at the end of the small intestinal digestion were diluted in 2% ACN/0.1% TFA to obtain peptide abundance of 0.1 mg/mL and samples taken at the end of the gastric digestion were diluted in 2% ACN/0.1% TFA to a peptide abundance of 0.02 mg/mL. An aliquot of 10 µL was loaded to the Q-Exactive+, Easy 1200 UPLC liquid chromatographic (LC) column (Thermofisher scientific, Waltham, MA). Abundance of peptides were determined using mass-spectrometry [37].

Spectral analysis and peptide identification

Spectral analysis and identification of peptides were performed as described previously by Dallas et al. (2013)[38].

Functional peptide annotation

Peptide sequences identified in the samples were matched against an in-house milk bioactive peptide database search program, which compared the identified peptides with sequences that are known to be bioactive[39]. The peptides with a 100% match with the functional peptides were reported.

Antioxidant activity and Bioaccessibility

Antioxidant activity of cranberry juice (control, no protein added) and cranberry juice-protein solutions (27 or 54 mg/mL) was measured before and after simulated gastric and small intestinal digestion using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and Ferric Reducing Antioxidant Power (FRAP) methods. Trolox solutions from 0 to 1.6 mM were used as a standard curve to calculate the Trolox equivalent antioxidant activity of the samples (mM Trolox) (Gonzalez-Centeno et al., 2012). For each digestion, all samples were analysed in triplicate.

Antioxidant activity measured by FRAP and ABTS was used to calculate the gastric and small intestinal antioxidant bioaccessibility as follows[11]:

$$Bioaccessibility\ (\%) = \frac{AC_{\ digested}}{AC_{non-digested}} * 100\% \qquad (3)$$

where $Bioaccessibility$ is the gastric or small intestinal bioaccessibility calculated as a percentage, $AC_{\ digested}$ is the antioxidant activity measured by FRAP or ABTS after gastric or small intestinal digestion (mM Trolox), and $AC_{non-digested}$ is the antioxidant activity measured by FRAP or ABTS before digestion (mM Trolox).

Ferric Reducing Antioxidant Power (FRAP)

The antioxidant activity from the FRAP method was performed as described by Gonzalez-Centeno et al. (2012)[40] for 96-well microplates, with minor modifications. Three solutions were prepared: 0.01 M of 2,4,6-Tripyridyl-s-Triazine (TPTZ) in 0.04 M HCl, 0.03 M of $FeCl_3$ and acetate buffer (3.1 g/L of sodium acetate with 16 mL/L of glacial acetic acid mixed with water, pH 3.6). These solutions were mixed in a volume ratio of 1:1:10. 190 µL of the solution was transferred to each well in a 96-well microplate. The initial absorbance at 593 nm was read at 25°C for 5 min in a microplate reader (Synergy HTX, BioTek Instruments Inc., Winooski, VT, USA). 10 µL of sample or Trolox standard solution was added to each well. The mixture was incubated for 30 min at room temperature. The final absorbance was measured at 593 nm. The absorbance difference before and after incubation with the sample was used to determine the antioxidant activity

2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS)

The antioxidant activity by ABTS was performed as described by Re et al. (1999) with adaptations from Gonzalez-Centeno et al. (2012)[40] for 96-well microplates, with minor modifications. Briefly, a solution of the ABTS radical cation was prepared by reacting 7.5 mM ABTS and 2.5 mM potassium persulfate solutions (1:1, v:v). This solution reacted for 18 h at room

temperature (20 °C) in the dark and was used within 6 hours. Before analysis, 8 mL of the ABTS radical cation solution was diluted with EtOH/H2O (25:75, v:v). 190 μL of the diluted solution was transferred to each well in a 96-well microplate. The absorbance at 734 nm was read for 5 min at 25°C using a microplate reader (Synergy HTX, BioTek Instruments Inc., Winooski, VT, USA). A volume of 10 μL of sample or Trolox standard solution was then added to each well. The mixture was incubated for 30 min at room temperature and the final absorbance was measured at 734 nm.

Statistical Analysis

All statistical analysis was completed using SAS Enterprise 4.3 (Cary, NC, U.S.A.). Protein solubility was analyzed with a three-way analysis of variance (ANOVA) using a mixed model. The three fixed factors were: processing method (not processed, low thermal treatment, medium thermal treatment, long thermal treatment, or high-pressure processing), protein concentration (27 or 54 mg/mL) and solvent (cranberry juice or water). A three-way ANOVA using a mixed model with repeated measures was used to analyze differences in degree of protein hydrolysis and soluble β-lactoglobulin digestibility during digestion. The three fixed factors were: processing method, protein concentration and solvent. The repeated factor was digestion time, as samples from all digestion times were taken from the same test tube during digestion experiments. A two-way ANOVA using a mixed model with repeated measured was used to analyze differences in soluble amino acid content using ion-exchange chromatography. The factors were: processing method and solvent. The repeated factor was digestion phase, as samples from all digestion times were taken from the same test tube during digestion experiments.

To determine differences between the antioxidant bioaccessibility after gastric or small intestinal digestion of cranberry juice-protein solutions, a two-way ANOVA using a mixed model was utilized. The two fixed factors were processing method and protein concentration. Statistical significance was evaluated at $p < 0.05$. Where main effects were significant, the Tukey-Kramer test was utilized to evaluate differences between means. Results are shown as averages ± standard deviation.

Results and discussion

Amino Acid Analysis

The amino acid composition of the whey protein isolate was analyzed (Table 1) and was similar to previous studies on whey protein isolate[41 42]. The essential amino acid content of the whey protein isolate was 50 ± 0.32 g per 100 g of protein.

Protein Solubility

Protein solubility (Table 2) was significantly influenced by solvent ($p < 0.0001$), protein concentration ($p=0.0241$), processing ($p < 0.0001$), the interaction of solvent and protein concentration ($p=0.0099$), the interaction of protein concentration and processing ($p=0.0036$), and the solvent x protein concentration x processing interaction ($p=0.0221$).

Water-protein solutions had a significantly higher solubility (average 95.6 ± 2.1% dissolved, across all treatments) in comparison to cranberry juice-protein solutions (average 90.8 ± 4.0% dissolved, across all treatments). It should be noted that the true protein solubility in the cranberry juice-protein solution may be slightly higher than the reported values since the composition of the precipitate was not measured. It is possible that other insoluble aggregates such as polysaccharides also precipitated, resulting in slightly higher true protein solubility values[43].

The increased solubility of whey protein in water compared to cranberry juice might be related to the pH of the solutions. The pH of the water-protein solutions was 3.71 ± 0.19 (average across all solutions) and the pH of cranberry juice-protein solutions was 2.77 ± 0.09 (average across all solutions). pH can alter the protein net charge and promote conformational changes altering the accessibility of amino acid residues on exposed surfaces and therefore might have also affected the interaction between the whey protein and cranberry polyphenols[44].

The effect of cranberry juice on whey protein isolate solubility is consistent with previous reports that showed a decrease of protein solubility in the presence of polyphenol extracts or polyphenols in comparison to protein solubility in water[7, 45, 46]. The decrease in solubility of cranberry juice-protein solutions compared to water-protein solutions might also be related to the interactions between protein and components in the cranberry juice such as polyphenols. In solution, proteins can interact with polyphenols; this type of interaction can modify properties such as solubility, foam formation, thermal stability and aggregation[47].

The interaction between whey protein and the polyphenols present in the juice matrix might also have been affected by the mineral content of both whey protein and cranberry juice. Calcium and magnesium can interact with polyphenols and formed metalo-polyphenol complexes or associate primarily with whey proteins.[44] However, mineral content was not measured within the scope of this study, but these specific interactions are an area for future investigation.

In addition, viscosity and flow behaviour may impact the behaviour of the protein solutions, as previous studies have indicated that increased viscosity leads to decreased mass transport during digestion[48], and may impact polyphenol bioaccessibility and bioavailability.[49] Viscosity and flow behaviour were not measured within the scope of this study. However, it is hypothesized that flow behaviour played a limited role in the trends observed in the current study. Based on visual observation of protein solutions, the viscosity was similar to water or juice, in contrast to some commercial products that have significantly higher viscosity than water or juice alone.

Protein Digestibility

O-Phthalaldehyde (OPA) Assay

Protein degree of hydrolysis (Equation 1, Table 3) was significantly ($p < 0.0001$) influenced by solvent, protein concentration, digestion time, and the interactions of: solvent and digestion time; protein concentration and digestion time; solvent and protein concentration, solvent, protein concentration and digestion time; protein concentration, processing, and digestion time, and solvent; protein

concentration, processing, and digestion time (p=0.0057). Processing did not significantly influence the degree of hydrolysis (p > 0.05) (Supplementary figure 1).

The degree of hydrolysis after 120 min of gastric digestion and after 240 min of gastrointestinal digestion for the non-processed water-protein solutions were similar to previously reported results of degree of hydrolysis of whey protein isolate during gastrointestinal digestion[50, 51]. For example, after 120 min of gastric digestion, the degree of hydrolysis reported by Mat et al., (2018) was 3.7 ± 0.9%[51]. In the current study, the degree of hydrolysis after 120 min of gastric digestion for non-processed water-protein solutions was 3.3 ± 0.4% (average across both protein concentrations). Also, the degree of hydrolysis after 240 min of gastrointestinal digestion in the current study was 47.2 ± 4.3% (average for non-processed water-protein solutions after 120 min of gastric digestion across both protein concentrations) which is similar to the values reported by Mat et al., (2016) (52 ± 2% after 2 hours of gastric digestion and 2 hour of small intestinal digestion)[50].

Degree of hydrolysis was significantly influenced (p=0.0065) by solvent during the small intestinal phase (135-210 min) except for the last time point (240 min). At these time points, the water-protein solutions had a significantly higher degree of hydrolysis in comparison to the cranberry juice-protein solution. For example, after 150 min, water-protein solutions had 32.6 ± 5.1% degree of hydrolysis compared to 28.2 ± 2.6% for cranberry juice-protein solutions (average across all processing methods and protein concentrations) (Supplementary figure 1). These results follow the same trend as described by Stojadinovic et al. (2013), who reported that during simulated small intestinal digestion of β-lactoglobulin in the presence of coffee and cocoa polyphenols, the time to digest 50% of the protein was almost 2 times longer in comparison to the absence of coffee and cocoa polyphenols[8]. However, in the same study by Stojadinovic et al. (2013) a different effect was observed by the addition of green tea extract[8]. The addition of green tea extract decreased the time to digest 50% of β-lactoglobulin almost 3 times compared to absence of green tea extract. The difference in trend can be related to the difference in structure and composition of the polyphenols in each extract. Stojadinovic et al. (2013) related the effect of polyphenols on the degree of hydrolysis to the loss of stability of the β-lactoglobulin secondary structure in the presence of polyphenols at pH 7.2[8].

Protein concentration significantly influenced (p < 0.0001) the degree of hydrolysis at 135 and 150 min small intestinal digestion. Protein solutions with 27 mg/mL whey protein isolate had a significantly higher degree of hydrolysis after 135 and 150 min small intestinal digestion (26.1 ± 3.6% and 32.9 ± 4.7%, respectively) in comparison to protein solutions with 54 mg/mL whey protein isolate (21.4 ± 3.2% and 27.9 ± 2.7%, respectively). The impact of protein concentration might be related to the higher enzyme/protein ratio in protein solution with 27 mg/mL in comparison to protein solution with 54 mg/mL[52].

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The proteins present in the whey protein isolate solutions before digestion (time 0) were identified according to their molecular weight compared to molecular weight standards used in each gel (Figure 1 & 2). The whey proteins identified were bovine serum albumin (66 kDa), dimer of bovine β-lactoglobulin (36.6 kDa), bovine β-lactoglobulin (18.3 kDa) and bovine α-lactalbumin (14.2 kDa)[53]. For all protein solutions, after 15 min of gastric digestion, serum albumin and α-lactalbumin were digested into small proteins or peptides which were no longer visible in the SDS-PAGE images (Figure 1 & 2). β-lactoglobulin was largely resistant to hydrolysis by pepsin during the gastric phase but was digested into small proteins or peptides that were no longer visible using SDS-PAGE after 15 min of small intestinal digestion (Figure 1 & 2). Rapid hydrolysis of α-lactalbumin and serum albumin after 15 min of gastric digestion has been reported during both *in vitro*[20, 54] and in vivo digestion[55], which is consistent with the observations in the current study. The resistance of β-lactoglobulin to pepsin hydrolysis has also been reported by several authors, and the trends are consistent with the current study[8, 56]. The β-lactoglobulin susceptibility to small intestinal digestion has also been observed during *in vitro* digestion[19].

Overall, water and cranberry-juice-protein solutions showed different protein breakdown profiles during digestion. The difference in protein breakdown profile between water and cranberry juice-protein solutions was more visible as the temperature of thermal processing increased (Figure 1 & 2). Differences can be observed in the molecular weight of the bands formed during *in vitro* digestion, which may indicate that β-lactoglobulin hydrolysis products are influenced by the solvent and type of processing before *in vitro* digestion.

Soluble β-lactoglobulin digestibility (Equation 2, Table 4), quantified during from SDS-PAGE image analysis was significantly (p < 0.05) influenced by the solvent, processing, digestion time, and the interactions between: solvent and processing; solvent and digestion time; protein concentration, solvent, and digestion time; solvent, processing, and digestion time; and solvent, protein concentration, processing, and digestion time. Soluble β-lactoglobulin had a significantly lower (p < 0.0001) resistance to pepsin hydrolysis during gastric digestion (15-120 min) for samples after long thermal processing compared to the other treatments. After 120 min of gastric digestion, protein solutions that were not processed and after high-pressure, low, and medium thermal processing had an average of 18 ± 7.2 % soluble β-lactoglobulin digestibility compared to 60 ± 25.0 % for protein solutions after long thermal processing (average across both protein concentrations and types of solvent).

An increase in susceptibility of β-lactoglobulin to hydrolysis during *in vitro* digestion after thermal treatment has been reported in the literature in milk[19], whey protein[20], and β-lactoglobulin[18]. In these studies, soluble β-lactoglobulin digestibility was dependent on the severity of the treatment and the source of protein. The increase in soluble β-

lactoglobulin digestibility due to severe thermal treatment (99 °C for 5 min) in the current study can be related to β-lactoglobulin denaturation during prolonged thermal treatment. Pepsin is a protease which preferentially cleaves amino acids with aromatic or aliphatic side chains[6]. β-lactoglobulin has a globular structure with hydrophobic amino acids buried inside the structure. Heating causes unfolding, making the hydrophobic amino acids more accessible for hydrolysis by pepsin[6, 18].

It should also be noted that the protein hydrolysis in the current study might also be impacted by the pH of the gastric juice used in the current study (pH =1.8 fasted state), since it is a lower pH than used in some in vitro models (pH=3.0 fed state)[57]. The lower pH used in this study after the first hour of gastric digestion (compared to a static pH of 3 as recommended by Brodkorb et al., 2019[57]) might have increased the protein hydrolysis as a result of the higher pepsin activity anticipated at pH 2 compared to pH 3.

The solvent (cranberry juice or water) did not significantly influence ($p > 0.05$) soluble β-lactoglobulin digestibility during the gastric phase for non-processed, high-pressure, low, and medium thermal processing (Table 4). After the long thermal treatment, water-protein solutions had a significantly higher ($p < 0.001$) soluble β-lactoglobulin digestibility during the gastric phase compared to cranberry juice-protein solutions. After 15 min of gastric digestion, water-protein solutions that underwent long thermal treatment had 47 ± 8.0 % soluble β-lactoglobulin digestibility compared to 24 ± 3.4 % for cranberry juice-protein solutions with the same treatment (Supplementary figure 1).

The effect of the solvent on protein digestibility during gastrointestinal digestion has been reported previously using fruit extracts[6-8] and single polyphenols that are abundant in fruit extracts and juices [58]. The effect of the solvent on protein digestibility depends on the type of protein, type of protein-polyphenol interaction (covalent or non-covalent), and the types of polyphenols present in the extract or juice. Previous studies have reported decreased in pepsin hydrolysis of milk and whey proteins due to the presence of tea or fruit extracts as well as single polyphenols[6, 8]. The decrease in protein hydrolysis may be related to the binding of polyphenols to amino acids which are at cleavage sites of the gastrointestinal enzymes. It has been previously demonstrated that the protein parameters that predict the non-covalent binding of β-lactoglobulin to polyphenols are the number of proline and aliphatic residues [59]. When denaturation of β-lactoglobulin occurs, hydrophobic amino acids are exposed and the number of possible hydrophobic interaction with polyphenols between β-lactoglobulin and polyphenols increase[60]. The preferential cleavage of amino acids by pepsin are amino acids with aromatic or aliphatic side chains[6]. Solutions processed through other thermal and high-pressure treatments did not show significant differences between water and cranberry juice. The difference in effect of solvent in the less severe thermal and high pressure cranberry juice-protein solutions may be related to their lack or partial denaturation of β-lactoglobulin, hampering the hydrophobic interaction of whey proteins with cranberry juice polyphenols[58].

Soluble amino acid content using Ion-Exchange Chromatography

Cation-exchange chromatography enabled separation and quantification of amino acids from acid-hydrolyzed peptides and free amino acids in individual samples. The soluble amino acid content using ion-exchange chromatography (Figure 3A) was significantly ($p < 0.05$) influenced by all the main effects and their interactions. The soluble amino acid content using ion-exchange chromatography significantly increased ($p < 0.0001$) during simulated digestion in both water and cranberry juice-protein solutions. This increase is likely related to the protein breakdown due to pepsin and pancreatin activity which resulted in the formation of soluble peptides[19]. The influence of processing method on the soluble amino acid content using ion-exchange chromatography was dependent on the digestion phase. During gastric digestion, processing method significantly ($p < 0.05$) influenced soluble amino acid content. However, at the end of the small intestinal phase, processing method did not have a significant influence ($p > 0.05$) on the soluble amino acid content. The influence of processing on whey protein isolate solutions during the gastric phase might be related to the increase of susceptibility of whey proteins to pepsin hydrolysis after thermal treatment, since whey protein such as β-lactoglobulin, in their natural structure are resistant to pepsin hydrolysis [18, 20]. These results align with the trend observed in soluble β-lactoglobulin digestibility for which samples that underwent long thermal processing were significantly ($p < 0.0001$) more susceptible to pepsin hydrolysis during gastric digestion (15-120 min).

However, during the small intestinal phase, enzymes present in pancreatin such trypsin and chymotrypsin, are capable to hydrolyze whey proteins regardless of whether they are processed or not[61].

The soluble amino acid concentration in each of the samples after gastric and small intestinal digestion is shown in Table 1. The amount of each amino acid in the soluble phase after digestion was similar across processing treatments and in either juice or water-protein solutions. This indicates that the soluble amino acids released as a result of the protein hydrolysis were similar across all treatments. This lack of differences would suggest minimal impact on protein digestibility scores, such as the protein digestibility corrected amino acid score (PDCAAS). Determination of the impact of thermal treatment and beverage system on PDCAAS and protein digestibility in vivo is an area recommended for future study.

In order to quantify the relative magnitude of the changes during gastrointestinal digestion, (as the initial values for each solution were significantly different), soluble amino acid content after digestion was normalized with the initial value for each sample and compared in Figure 3B. The normalized soluble amino acid content using ion-exchange chromatography was significantly influenced by solvent, processing method, digestion phase, and their interactions ($p < 0.001$).

Water-protein solutions had a significantly higher ($p < 0.001$) normalized soluble amino acid content compared to the

cranberry juice-protein solutions (Figure 3B). For example, the normalized soluble amino acid content of water-protein solutions after the small intestinal phase was 2.4 ± 1.0 times higher than the non-digested sample, while cranberry juice-protein solutions increased only 1.8 ± 0.5 times compared to the non-digested sample. The lower rate of normalized soluble amino acid content of water-protein solutions compared to cranberry juice-protein solutions might be related to the interaction of whey proteins with the polyphenols present in the cranberry juice. Polyphenols can bind and precipitate digestive enzymes such as α-amylase, pepsin, trypsin and consequently inhibit their activity[13]. Proanthocyanidins, the most abundant type of polyphenol in cranberry juice[62], has been related to decreased in activity of pancreatic α-amylase, lipase, and trypsin[12]. However, proanthocyanidins effect depends on their level of polymerization and the concentration of the polyphenols present. Another possible explanation of the higher rate of normalized soluble amino acid content of water-protein solutions compared to cranberry juice-protein solutions might be the interaction of peptides formed during the small intestinal digestion with the polyphenols present in the cranberry juice, which may cause their precipitation during the sample processing[56].

The long thermal treatment had the greatest increase in normalized soluble amino acid content during digestion, with an average increase of 2.4 ± 1.2 (average across both solvents and digestion phases).

The trends observed here are similar to those described above and quantified by the SDS-PAGE method, which were similar to previously reported results[6, 8, 18, 20]. It is difficult to compare these results with previous studies, as this is the first report of soluble amino acid content using ion-exchange chromatography of whey protein isolate solutions that underwent in vitro gastrointestinal digestion.

Peptide analysis

Peptide sequences in water and cranberry juice-protein solutions were identified at different stages of digestion by Orbitrap mass spectrometry. The peptide sequences are shown in Supplementary Table 1.

Peptide identification by LC–Orbitrap MS/MS

In the present study, high-resolution Orbitrap mass spectrometry and related peptidomic techniques were utilized to identify peptides in selected samples before and after in vitro digestion.

The statistically significant differences ($p < 0.001$) between the long thermal processing and the rest of other protein solutions evidenced by the SDS-PAGE analysis suggested that more severe thermal processing affected protein breakdown after in-vitro digestion of whey protein isolate in water and cranberry juice solutions. As a result, the treatments that were not processed or after long thermal processing (99°C for 5 min) were selected for further analysis of peptides.

The number of peptides identified varied between 80 peptides in the water-protein solution that underwent long thermal processing after small intestinal digestion and 2055 peptides in the water-protein solution that was not processed and did not undergo in vitro digestion (Figure 4). Studies employing similar analytical techniques reported an average peptide count of 1421 ± 42 peptides for kefir samples, which included the added proteolytic activity of kefir microorganisms[37] and an average of 1732 peptides in a set of 10 commercial dairy products, including 8 milk-based and 2 yoghurt samples[63]. Another study on validating the static in-vitro digestion protocol published by Minekus et al., 2014[29] towards in vivo data confirmed that the harmonized in vitro protein digestion of skim milk powder, correlated with in vivo gastric samples ($r = 0.8$) and intestinal in vitro digestion correlated best with in vivo samples collected from the median jejunum ($r = 0.57$) in pigs. Protein hydrolysis at different levels was analysed by various analytical methods, including mass spectrometry[64, 65]. The apparently lower peptide numbers identified in the present study, as compared to the above referenced studies, could be attributed to the samples undergoing in vitro digestion resulting in wide difference between the lowest and highest number of peptides. These findings illustrate the capability of high-throughput mass spectrometry platforms in identifying a multitude of peptides for peptidomic profiling.

Even though the product used in this study was whey protein isolate, the majority of peptides identified in the starting material originated from caseins (Table 5). This finding is not surprising when considering that caseins' rheomorphic structure renders them more prone to hydrolysis by endogenous milk enzymes as well as by the enzymes in starters cultures used in cheese making[38]. It has been previously demonstrated that a high number of naturally occurring peptides from caseins were found in whey permeate, a co-product of whey protein isolate production[66]. It is well-known that globular proteins found in whey, such as β-lactoglobulin, are intrinsically more resistant to enzymatic digestion. Nonetheless, it is demonstrated here that peptides derived from β-lactoglobulin dominated over casein-derived peptides during the more advanced stages of in vitro digestion (Table 5). Figure 3A shows that in the gastric phase, the abundance of peptides in the long thermal processing sample led to a higher degree of hydrolysis of whey proteins. This hypothesis is supported by the results from Table 5, which showed 608 peptides from whey proteins vs 252 peptides from caseins (average across both solvents and processing methods) during gastric phase. Also, a current study supports the hypothesis that heat treatment strongly influences β-lactoglobulin degradation during the gastric phase[19].

The number of peptides of whey protein isolate solutions that were either non-processed or underwent long thermal processing decreased as digestion progressed (Figure 4). A lower number of peptides identified in juice protein solutions (1209 versus 1169 peptides) compared to water protein solution (2055 versus 1899 peptides) before digestion could be attributed to the interaction of polyphenols with peptides[60, 67], as the polyphenols might interact with peptides from whey proteins resulting into their precipitation during the sample processing[67] (Figure 4). Thus, binding of peptides and polyphenols in juice-protein solutions rendered them unextractable for peptidomic analysis affecting the total number of identified peptides enlisted in Supplementary Table

2. For example, ACE-inhibitory peptides from αs1 casein (amino acid sequence 201-212)[68], ß casein (amino acid sequence 208-217)[69], αs1 casein (amino acid sequence 16-24)[70] and an antimicrobial and immunomodulatory peptide from β-lactoglobulin (amino acid sequence 100–107)[71] were missing in juice protein solutions, but were found exclusively in water-protein solutions before digestion, possibly affected by polyphenol-peptide interaction.

Complementing a higher number of peptides in water and juice protein solutions before digestion (>1000 peptides; Figure 4), the soluble amino acid content (representing peptides and free amino acids) demonstrated (Figure 3A) that such peptides are found in low amounts and proteins are then further degraded during in vitro digestion. However, the average number of peptides across both solvents and processing methods decreased from gastric (856 peptides) to intestinal digestion (88 peptides). The decrease in the number of peptides is the result of increased hydrolysis into di- and tripeptides and amino acids during gastric and small intestinal digestion of proteins. As evidenced by the literature on peptidomics[72, 73], the current inability of measuring short peptides is a limitation of the technique. Further method development by the scientific community will be necessary in order to enable quantification of the lower molecular weight peptides.

During in vitro digestion, the difference between the number of peptides of water and cranberry juice-protein solutions was small, suggesting that the number of peptides were not affected by digestion. Although, during gastric phase, a higher number of peptides observed in juice protein solution which did not undergo processing compared to the juice protein that underwent long thermal treatment (1077 peptides vs 643 peptides) could be attributed to the binding of small oligomers of polyphenols such as proanthocyanidins[12, 62] to digestive enzymes such as α-amylase, trypsin[12] and pepsin[13], leading to precipitation and inhibition of their activity. This possibly explains the lower number of peptides in thermally treated juice-protein solution. Thermal treatment such as long thermal processing might lead to break-down of polymers and oligomers of medium and high molecular weight to small oligomers, which could bind and inhibits the activity of digestive enzymes in the juice-protein solutions that underwent long thermal processing.

Through utilization of various techniques for quantifying protein digestion in the current study, it is possible to gain a more complete understanding of the digestion process, within the limitations of each analytical method. For example, the SDS-PAGE images of water and cranberry juice protein solutions that were not processed showed similar protein profiles during the gastric phase (Figure 1 & 2) which aligns with the results that the soluble β-lactoglobulin digestibility (%) was not significantly different ($p > 0.05$) between water and cranberry juice (Table 4). However, water-protein solutions that underwent thermal processing showed a different protein profile and significantly lower resistance to pepsin hydrolysis ($p < 0.001$) during gastric digestion compared to cranberry juice-protein solutions. Similar results were observed during the quantification of soluble amino acid using ion-exchange chromatography (Figure 3A), in which water-protein solutions that underwent long thermal processing had relatively higher soluble amino acid content (48.3 ± 0.034) compared to cranberry juice-protein solutions with the same type of processing (40.9 ± 2.2), although these values were not significantly different. Similarly, the peptide count was 699 and 643 peptides for the water and cranberry juice-protein solutions after long thermal processing, respectively.

The water and cranberry-juice-protein solutions showed similar SDS-PAGE protein breakdown profiles during small intestinal digestion. After 240 min, whey protein isolate was digested into small proteins or peptides that were no longer visible using SDS-PAGE, therefore we cannot resolve differences in peptides based on SDS-PAGE. Peptides that are present in the sample vary in molecular weight range, so they do not resolve as a band even if they are present (Figure 1 & 2). Complementary information was obtained from the peptidomic profiling which presented 81 peptides. Considering that peptides below 5 amino acid length are not measured[74], due to limitation of mass spectrometry as explained above, it is reasonable to assume an intrinsic underestimate in the peptide numbers for the samples obtained at the end of small intestinal digestion.

The peptide profiles reported in the current study are similar to previous studies that have identified bioactive peptides in whey protein concentrates, isolates, and related dairy fractions (Supplementary Table 2). A dipeptidyl peptidase- IV inhibitory peptide from α-lactalbumin (amino acid sequence 123-136) identified in a study on pepsin-treated whey protein isolate[75] was also identified in all 4 samples subjected to gastric digestion. The presence of this peptide in the gastric digests in present study could be a result of hydrolysis by pepsin, an enzyme present during gastric digestion. Another dipeptidyl peptidase- IV inhibitory peptide from α-lactalbumin with amino acid sequence 123-129[76] was unique to water and cranberry juice-protein solutions that underwent long thermal processing and gastric digestion, suggesting its formation could be due to changes in protein structure during the long-thermal treatment followed by pepsin hydrolysis during digestion. A recent publication on α-lactalbumin-based Maillard reaction products (MRPs) have shown to exhibit increased surface hydrophobicity and antioxidant capacity, compared to the unprocessed α-lactalbumin[77]. In contrast, an immunomodulatory peptide from β-lactoglobulin (amino acid sequence 158–164)[78, 79] was unique to water-protein solution that had not undergone long-thermal treatment and digestion, suggesting that this could be a naturally occurring peptide.

A previous study on plant protease-hydrolyzed whey protein reported presence of an ACE-inhibitory peptide from β-lactoglobulin (amino acid sequence 49-58) in the 3 kDa fraction of whey protein concentrate[80]. This same peptide was also identified in all 4 samples subjected to gastric digestion in the current study. Similarly, a previous study analysed nanofiltration retentate and permeate of whey protein tryptic hydrolysate. A peptide from β-lactoglobulin (amino acid sequence 141–151) identified in the nanofiltration retentate, as reported by Demers-Mathieu et al. (2013)[71] was also found in all 4 samples subjected to small intestinal digestion, suggesting

that trypsin, a predominant intestinal enzyme, could be responsible for generating this peptide. In the cited study, the authors also noted that a peptide from β-lactoglobulin (amino acid sequence 141–151) was an effective antimicrobial against *Listeria, Staphylococcus aureus* and *Escherichia coli*. In the current study, this antimicrobial peptide was also found in water and cranberry juice-protein solutions before digestion. A peptide from β-casein (123-128) identified in bovine milk-based formulas after in vitro digestion with pepsin and pancreatin (which contains trypsin)[81] was also present in both the samples that were not processed and that underwent long thermal processing during intestinal digestion, suggesting that trypsin could be responsible for generating this peptide. An antimicrobial peptide from αs1 casein (amino acid sequence 95-117) identified in a previous study[82] was also identified in water and cranberry juice-protein solutions that underwent long thermal processing before digestion. These peptides could have been potentially generated during industrial processing of whey protein isolate and are likely increased during small intestinal digestion due to the presence of trypsin.

Antioxidant Bioaccessibility

Antioxidant Bioaccessibility by FRAP

Gastric antioxidant bioaccessibility from the FRAP method (Equation 3; Figure 5 A1) was significantly ($p < 0.001$) influenced by the protein concentration, processing method, and their interaction. Overall, the addition of 27 and 54 mg/mL of whey protein isolate significantly ($p < 0.0001$) increased the gastric antioxidant bioaccessibility in cranberry juice determined by the FRAP method (101.8 ± 11.2 %) compared to cranberry juice without protein (86.9 ± 9.7 %). However, the specific amount of whey protein isolate (27 or 54 mg/mL) added to the cranberry juice-protein solutions did not significantly affect ($p > 0.05$) the gastric antioxidant bioaccessibility. To determine the impact of whey protein on the bioaccessibility from the FRAP method, antioxidant activity by FRAP of water-protein solutions with 0, 27 and 54 mg/mL were measured before and during in vitro gastrointestinal digestion (data not shown). All water-protein solutions had an antioxidant capacity by FRAP that was not significantly different from 0 ($p>0.05$). Since the FRAP method does not directly measure the protein capacity to donate electrons[83], the increase in antioxidant activity in cranberry juice protein solutions by FRAP from non-digested to after gastric digestion might indicate a protective effect of whey protein isolate to cranberry antioxidant compounds during gastric digestion. A protective effect of proteins on antioxidant compounds may have facilitated the slight increase in antioxidant activity during the gastric phase compared to the antioxidant activity of the cranberry juice without protein (0 mg/mL).

Small intestinal antioxidant bioaccessibility determined by the FRAP method (Figure 5 B1) was not significantly ($p > 0.05$) influenced by protein concentration, processing or their interactions.

The lack of significance of processing method on small intestinal antioxidant bioaccessibility is similar to results observed in a previous study. Mennah-Govela & Bornhorst (2017) measured the small intestinal antioxidant bioaccessibility of orange juice that was not processed and after thermal (99°C for 10s, 85°C for 1, 5 and 15 min) and high-pressure processing (600 MPa for 4 min). In this study, the small intestinal antioxidant bioaccessibility by FRAP was not significantly influenced ($p < 0.05$) by orange variety, processing method or their interaction[11]. Although the type of juice was different (orange juice) compared to one used in the current study (cranberry juice) a similar trend with the relationship to processing method was observed. This might indicate that even though the antioxidants and polyphenols present in cranberry juice changed during processing, their capacity to donate electrons at the end of the small intestinal digestion was only slightly affected.

Antioxidant Bioaccessibility by ABTS

Gastric antioxidant bioaccessibility from the ABTS method (Figure 5 A2) was significantly ($p < 0.0001$) influenced by protein concentration, processing method, and their interaction. Cranberry juice-protein solutions that underwent long thermal processing had a significantly higher ($p=0.03$) gastric antioxidant bioaccessibility from ABTS (116.1 ± 11.0 %) compared to cranberry juice-protein solutions that were not processed or underwent other processing methods (81.5 ± 11.8 %) (Figure 5 A2). However, cranberry juice samples without protein (0 mg/mL) that underwent long thermal processing had a significantly lower ($p=0.03$) gastric antioxidant bioaccessibility by the ABTS method (63.2 ± 14.0 %) compared to cranberry juice that was not processed or that underwent high-pressure processing or low thermal processing (88.7 ± 7.5 %).

The effect of processing on the gastric and small intestinal antioxidant bioaccessibility by ABTS was dependent on protein concentration. The significantly lower ($p=0.03$) gastric antioxidant bioaccessibility measured by ABTS of cranberry juice without protein after long thermal processing (63.2 ± 14.0 %) might be related to the severe thermal treatment, which may have changed the thermally-sensitive polyphenols and antioxidant structures present in the solution during gastric digestion and decreased their antioxidant activity and bioaccessibility[84]. Conversely, cranberry juice-protein solutions (27 and 54 mg/mL) that underwent long thermal processing (99°C for 5 min) had a significantly higher ($p < 0.05$) gastric antioxidant bioaccessibility by ABTS (116.1 ± 11.0 %) compared to the non-processed cranberry juice-protein solutions or those that underwent other types of processing (81.5 ± 11.8 %). The increase of gastric antioxidant bioaccessibility of cranberry juice-protein solutions that underwent long thermal processing might be attributed to increased products of protein hydrolysis, as well as exposure of amino acids with antioxidant activity measurable by ABTS during digestion, such as tryptophan, tyrosine, cysteine, phenilalanine and histidine[85]. An increase in whey protein isolate antioxidant activity after in vitro digestion in samples with increases in thermal treatment has been reported previously [86]. This may be due to protein hydrolysis products that react with ABTS cations, increasing antioxidant activity. In the current study, cranberry juice-protein solutions that underwent the long thermal treatment had a significantly

higher soluble protein digestibility compared to the other cranberry juice-protein solution treatments at the end of the gastric phase. The increased number of protein digestion products in these samples may have reacted with ABTS cations, influencing the gastric antioxidant bioaccessibility. Antioxidant activity of whey protein (27 and 54 mg/mL) dissolved in water were measured to demostrate the interaction of ABTS cations with protein in the absence of cranberry polyphenols.

Small intestinal antioxidant bioaccessibility by the ABTS method was significantly influenced by the protein concentration ($p < 0.0001$) and the interaction of protein concentration and processing method ($p=0.0164$). The small intestinal antioxidant bioaccessibility by the ABTS method ($295.2 \pm 71.1\%$, average of cranberry juice-protein solutions with 27 and 54 mg/mL of whey protein isolate across all processing methods) was significantly greater than cranberry juice without protein (95.2 ± 43.4 %, average across all processing methods). The relationship between the increase of antioxidant activity by ABTS with the hydrolysis of whey proteins might also explain the significant increase ($p < 0.0001$) of small intestinal antioxidant bioaccessibility of cranberry juice-protein solutions (27 and 54 mg/mL) compared to cranberry juice without protein (0 mg/mL). Therefore in cranberry juice protein solutions, ABTS method likely captures both the antioxidant activity of both the cranberry juice and the protein. Interaction of whey protein and milk products after digestion with ABTS cations has been previosuly reported[86]. Another possible explanation of the increase of antioxidant activity by ABTS during the small intestinal phase might be related to the dependance of the ABTS reagent to the pH of the sample, increasing its capacity with higher pH value[87]. The initial pH of the cranberry juices tested ranged between 2.77 ± 0.09. At the end of the gastric phase, the pH of digested cranberry juice-protein decreased to pH 2.06 ± 0.01, however at the end of the small intestinal phase the digested juice increased to up to pH 7.11 ± 0.09. Some studies have suggested that the changes of pH during digestion might affect the ABTS reaction due to its lack of buffering capacity[87]. However, a previous study by Mennah-Govela & Bornhorst (2017) that used the same methodology as in the current study measured the antioxidant capacity of L-ascorbic solutions before and after gastrointestinal digestion, both at the pH of the intestinal fluids (~7.2) as well as at the initial pH of the sample (~3.0). In this study, they observed that there were no significant differences ($p > 0.05$) between the pH of the sample on the antioxidant activity from the ABTS assay[11]. Due the similarity of the methods used, it is hypothesized that the increases in antioxidant capacity and the resulting antioxidant bioaccessibility after digestion were due to the digestion of the cranberry juice and proteins, and not only due to sample pH.

Conclusions

The influence of processing on protein solubility of cranberry juice and water-whey protein isolate solutions was dependent on the severity of the thermal treatment. Higher temperatures and longer thermal treatments resulted in lower whey protein isolate solubility. The solvent in which whey protein isolate was dissolved also influenced protein solubility, where the solubility of whey protein isolate in water was greater compared to cranberry juice. Processing method and solvent significantly influenced whey protein isolate digestibility. Whey protein isolate dissolved in water had a significantly higher degree of hydrolysis and soluble amino acid content using ion-exchange chromatography during small intestinal digestion. However, the specific influence of processing on protein digestibility depended on the severity of the thermal treatment. Water-protein solutions only had higher protein digestibility during the gastric phase after long thermal processing (99°C for 5 min) compared to cranberry juice-protein solutions. The slightly lower peptide count measured for cranberry protein solutions compared with whey protein isolate in water could be due to interaction of polyphenols with peptides. The influence of processing on antioxidant bioaccessibility of cranberry juice with whey protein isolate depended on the measurement method. Antioxidant bioaccessibility by ABTS was significantly influenced by the presence of protein due to the capacity of ABTS cations to react with protein hydrolysis products. In contrast, antioxidant bioaccessibility by FRAP significantly increased in cranberry juice with the addition of 27 and 54 mg/mL of whey protein isolate, although the FRAP method does not take into account changes due to protein digestion. This trend might indicate a protective effect of whey protein isolate on cranberry antioxidant compounds during gastric digestion. The information from this study can be utilized to optimize the processing and formulation of high-protein juice products to increase both the protein digestibility and antioxidant bioaccessibility after digestion.

Conflicts of interest

There are no conflicts to declare

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1. Z. He, B. Yuan, M. Zeng, G. Tao and J. Chen, Effect of simulated processing on the antioxidant capacity and in vitro protein digestion of fruit juice-milk beverage model systems, *Food Chem*, 2015, **175**, 457-464.
2. F. J. Barba, C. Cortés, M. J. Esteve and A. Frígola, Study of Antioxidant Capacity and Quality Parameters in An Orange Juice–Milk Beverage After High-Pressure Processing Treatment, *Food and Bioprocess Technology*, 2011, **5**, 2222-2232.
3. T. F. Wegrzyn, J. M. Farr, D. C. Hunter, J. Au, M. W. Wohlers, M. A. Skinner, R. A. Stanley and D. Sun-Waterhouse, Stability of antioxidants in an apple polyphenol-milk model system, *Food Chem*, 2008, **109**, 310-318.
4. D. C. Whitcomb and M. E. Lowe, Human pancreatic digestive enzymes, *Dig Dis Sci*, 2007, **52**, 1-17.
5. J. L. Slavin and B. Lloyd, Health benefits of fruits and vegetables, *Adv Nutr*, 2012, **3**, 506-516.
6. S. Lamothe, N. Azimy, L. Bazinet, C. Couillard and M. Britten, Interaction of green tea polyphenols with dairy matrices in a simulated gastrointestinal environment, *Food Funct*, 2014, **5**, 2621-2631.
7. N. J. Plundrich, B. L. White, L. L. Dean, J. P. Davis, E. A. Foegeding and M. A. Lila, Stability and immunogenicity of hypoallergenic peanut protein-polyphenol complexes during in vitro pepsin digestion, *Food Funct*, 2015, **6**, 2145-2154.
8. M. Stojadinovic, J. Radosavljevic, J. Ognjenovic, J. Vesic, I. Prodic, D. Stanic-Vucinic and T. Cirkovic Velickovic, Binding affinity between dietary polyphenols and beta-lactoglobulin negatively correlates with the protein susceptibility to digestion and total antioxidant activity of complexes formed, *Food Chem*, 2013, **136**, 1263-1271.
9. Z. He, Y. Tao, M. Zeng, S. Zhang, G. Tao, F. Qin and J. Chen, High pressure homogenization processing, thermal treatment and milk matrix affect in vitro bioaccessibility of phenolics in apple, grape and orange juice to different extents, *Food Chem*, 2016, **200**, 107-116.
10. R. Apak, S. Gorinstein, V. Böhm, K. M. Schaich, M. Özyürek and K. Güçlü, Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report), *Pure and Applied Chemistry*, 2013, **85**, 957-998.
11. Y. A. Mennah-Govela and G. M. Bornhorst, Fresh-Squeezed Orange Juice Properties Before and During In Vitro Digestion as Influenced by Orange Variety and Processing Method, *J Food Sci*, 2017, **82**, 2438-2447.
12. A. A.-O. Vazquez-Flores, A. A.-O. Martinez-Gonzalez, E. A.-O. Alvarez-Parrilla, G. A.-O. Díaz-Sánchez Á, L. A.-O. X. de la Rosa, G. A. González-Aguilar and C. A.-O. Aguilar, Proanthocyanidins with a Low Degree of Polymerization are Good Inhibitors of Digestive Enzymes Because of their Ability to form Specific Interactions: A Hypothesis, 2018.
13. Q. He, Y. Lv and K. Yao, Effects of tea polyphenols on the activities of α-amylase, pepsin, trypsin and lipase, *Food Chemistry*, 2007, **101**, 1178-1182.
14. A. Cilla, A. Alegria, B. de Ancos, C. Sanchez-Moreno, M. P. Cano, L. Plaza, G. Clemente, M. J. Lagarda and R. Barbera, Bioaccessibility of tocopherols, carotenoids, and ascorbic acid from milk- and soy-based fruit beverages: influence of food matrix and processing, *J Agric Food Chem*, 2012, **60**, 7282-7290.
15. M. J. Rodriguez-Roque, M. A. Rojas-Grau, P. Elez-Martinez and O. Martin-Belloso, Changes in vitamin C, phenolic, and carotenoid profiles throughout in vitro gastrointestinal digestion of a blended fruit juice, *J Agric Food Chem*, 2013, **61**, 1859-1867.
16. A. Patras, N. P. Brunton, C. O'Donnell and B. K. Tiwari, Effect of thermal processing on anthocyanin stability in foods; mechanisms and kinetics of degradation, *Trends in Food Science & Technology*, 2010, **21**, 3-11.
17. B. K. Tiwari, C. P. O'Donnell and P. J. Cullen, Effect of non thermal processing technologies on the anthocyanin content of fruit juices, *Trends in Food Science & Technology*, 2009, **20**, 137-145.
18. M. R. Peram, S. M. Loveday, A. Ye and H. Singh, In vitro gastric digestion of heat-induced aggregates of beta-lactoglobulin, *J Dairy Sci*, 2013, **96**, 63-74.
19. K. A. Kopf-Bolanz, F. Schwander, M. Gijs, G. Vergères, R. Portmann and L. Egger, Impact of milk processing on the generation of peptides during digestion, *International Dairy Journal*, 2014, **35**, 130-138.
20. S. B. Kim, K. S. Ki, M. A. Khan, W. S. Lee, H. J. Lee, B. S. Ahn and H. S. Kim, Peptic and tryptic hydrolysis of native and heated whey protein to reduce its antigenicity, *J Dairy Sci*, 2007, **90**, 4043-4050.
21. E. Pappas and K. M. Schaich, Phytochemicals of cranberries and cranberry products: characterization, potential health effects, and processing stability, *Crit Rev Food Sci Nutr*, 2009, **49**, 741-781.
22. B. L. White, L. R. Howard and R. L. Prior, Impact of different stages of juice processing on the anthocyanin, flavonol, and procyanidin contents of cranberries, *J Agric Food Chem*, 2011, **59**, 4692-4698.
23. S. C. Walpole, D. Prieto-Merino, P. Edwards, J. Cleland, G. Stevens and I. Roberts, The weight of nations: an estimation of adult human biomass, *BMC Public Health*, 2012, **12**, 439.
24. F. D. Macchi, F. J. Shen, R. G. Keck and R. J. Harris, *Amino Acid Analysis Protocol*, Humana Press, 2000.
25. C. E. LaClair and M. R. Etzel, Ingredients and pH are key to clear beverages that contain whey protein, *J Food Sci*, 2010, **75**, C21-27.
26. A. International, *Official methods of analysis of AOAC International.*, AOAC International, 1995.
27. G. M. Bornhorst and R. P. Singh, Kinetics of in Vitro Bread Bolus Digestion with Varying Oral and Gastric Digestion Parameters, *Food Biophysics*, 2013, **8**, 50-59.
28. M. J. Roman, B. J. Burri and R. P. Singh, Release and bioaccessibility of beta-carotene from fortified almond butter during in vitro digestion, *J Agric Food Chem*, 2012, **60**, 9659-9666.
29. M. Minekus, M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carriere, R. Boutrou, M. Corredig, D. Dupont, C. Dufour, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, S. Marze, D. J. McClements, O. Menard, I. Recio, C. N. Santos, R. P. Singh, G. E. Vegarud, M. S. Wickham, W. Weitschies and A. Brodkorb, A standardised static in vitro digestion method suitable for food - an international consensus, *Food Funct*, 2014, **5**, 1113-1124.
30. F. C. Church, D. H. Porter, G. L. Catignani and H. E. Swaisgood, An o-phthalaldehyde spectrophotometric assay for proteinases, *Anal Biochem*, 1985, **146**, 343-348.
31. P. M. Nielsen, D. Petersen and C. Dambmann, Improved Method for Determining Food Protein Degree of

Hydrolysis., *Food Chemistry and Toxicology*, 2001, **66**, 642–646.
32. R. A. Inglingstad, T. G. Devold, E. K. Eriksen, H. Holm, M. Jacobsen, K. H. Liland, E. O. Rukke and G. E. Vegarud, Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes, *Dairy Science & Technology*, 2010, **90**, 549-563.
33. G. M. Bornhorst, K. C. Drechsler, C. A. Montoya, S. M. Rutherfurd, P. J. Moughan and R. P. Singh, Gastric protein hydrolysis of raw and roasted almonds in the growing pig, *Food Chem*, 2016, **211**, 502-508.
34. C. Swackhamer, Z. Zhang, A. Y. Taha and G. M. Bornhorst, Fatty acid bioaccessibility and structural breakdown from in vitro digestion of almond particles, *Food Funct*, 2019, **10**, 5174-5187.
35. S. A. Adibi and D. W. Mercer, Protein digestion in human intestine as reflected in luminal, mucosal, and plasma amino acid concentrations after meals, *J Clin Invest*, 1973, **52**, 1586-1594.
36. D. C. Dallas, C. J. Smink, R. C. Robinson, T. Tian, A. Guerrero, E. A. Parker, J. T. Smilowitz, K. A. Hettinga, M. A. Underwood, C. B. Lebrilla, J. B. German and D. Barile, Endogenous human milk peptide release is greater after preterm birth than term birth, *J Nutr*, 2015, **145**, 425-433.
37. D. C. Dallas, F. Citerne, T. Tian, V. L. Silva, K. M. Kalanetra, S. A. Frese, R. C. Robinson, D. A. Mills and D. Barile, Peptidomic analysis reveals proteolytic activity of kefir microorganisms on bovine milk proteins, *Food Chem*, 2016, **197**, 273-284.
38. D. C. Dallas, A. Guerrero, N. Khaldi, P. A. Castillo, W. F. Martin, J. T. Smilowitz, C. L. Bevins, D. Barile, J. B. German and C. B. Lebrilla, Extensive in vivo human milk peptidomics reveals specific proteolysis yielding protective antimicrobial peptides, *J Proteome Res*, 2013, **12**, 2295-2304.
39. S. D. Nielsen, R. L. Beverly, Y. Qu and D. C. Dallas, Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization, *Food Chem*, 2017, **232**, 673-682.
40. M. R. Gonzalez-Centeno, M. Jourdes, A. Femenia, S. Simal, C. Rossello and P. L. Teissedre, Proanthocyanidin composition and antioxidant potential of the stem winemaking byproducts from 10 different grape varieties (Vitis vinifera L.), *J Agric Food Chem*, 2012, **60**, 11850-11858.
41. R. Sinha, C. Radha, J. Prakash and P. Kaul, Whey protein hydrolysate: Functional properties, nutritional quality and utilization in beverage formulation, *Food Chemistry*, 2007, **101**, 1484-1491.
42. M. Greenwood, D. S. Kalman and J. Antonio, *Nutritional Supplements in Sports and Exercise*, Springer Berlin Heidelberg, 2008.
43. A.-S. Stübler, U. Lesmes, V. Heinz, C. Rauh, A. Shpigelman and K. Aganovic, Digestibility, antioxidative activity and stability of plant protein-rich products after processing and formulation with polyphenol-rich juices: kale and kale–strawberry as a model, *European Food Research and Technology*, 2019, **245**, 2499-2514.
44. S. Moser, M. Chegeni, O. G. Jones, A. Liceaga and M. G. Ferruzzi, The effect of milk proteins on the bioaccessibility of green tea flavan-3-ols, *Food Research International*, 2014, **66**, 297-305.
45. M. von Staszewski, F. L. Jara, A. L. T. G. Ruiz, R. J. Jagus, J. E. Carvalho and A. M. R. Pilosof, Nanocomplex formation between β-lactoglobulin or caseinomacropeptide and green tea polyphenols: Impact on protein gelation and polyphenols antiproliferative activity, *Journal of Functional Foods*, 2012, **4**, 800-809.
46. M. Schneider, D. Esposito, M. A. Lila and E. A. Foegeding, Formation of whey protein-polyphenol meso-structures as a natural means of creating functional particles, *Food Funct*, 2016, **7**, 1306-1318.
47. K. Brudzynski and L. Maldonado-Alvarez, Polyphenol-Protein Complexes and Their Consequences for the Redox Activity, Structure and Function of Honey. A Current View and New Hypothesis – a Review, *Polish Journal of Food and Nutrition Sciences*, 2015, **65**, 71-80.
48. A. Tharakan, I. T. Norton, P. J. Fryer and S. Bakalis, Mass transfer and nutrient absorption in a simulated model of small intestine, *J Food Sci*, 2010, **75**, E339-346.
49. T. Bohn, Dietary factors affecting polyphenol bioavailability, *Nutr Rev*, 2014, **72**, 429-452.
50. D. J. L. Mat, S. Le Feunteun, C. Michon and I. Souchon, In vitro digestion of foods using pH-stat and the INFOGEST protocol: Impact of matrix structure on digestion kinetics of macronutrients, proteins and lipids, *Food Research International*, 2016, **88**, 226-233.
51. D. J. L. Mat, T. Cattenoz, I. Souchon, C. Michon and S. Le Feunteun, Monitoring protein hydrolysis by pepsin using pH-stat: In vitro gastric digestions in static and dynamic pH conditions, *Food Chem*, 2018, **239**, 268-275.
52. G. Shu, B. Zhang, Q. Zhang, H. Wan and H. Li, Effect of Temperature, pH, Enzyme to Substrate Ratio, Substrate Concentration and Time on the Antioxidative Activity of Hydrolysates from Goat Milk Casein by Alcalase *Acta Universitatis Cibiniensis. Series E: Food Technology*, 2016, **20**.
53. A. Grácia-Juliá, M. René, M. Cortés-Muñoz, L. Picart, T. López-Pedemonte, D. Chevalier and E. Dumay, Effect of dynamic high pressure on whey protein aggregation: A comparison with the effect of continuous short-time thermal treatments, *Food Hydrocolloids*, 2008, **22**, 1014-1032.
54. S. Zhang, Z. Zhang and B. Vardhanabhuti, Effect of charge density of polysaccharides on self-assembled intragastric gelation of whey protein/polysaccharide under simulated gastric conditions, *Food Funct*, 2014, **5**, 1829-1838.
55. L. M. Sullivan, J. J. Kehoe, L. Barry, M. J. Buckley, F. Shanahan, K. H. Mok and A. Brodkorb, Gastric digestion of alpha-lactalbumin in adult human subjects using capsule endoscopy and nasogastric tube sampling, *Br J Nutr*, 2014, **112**, 638-646.
56. G. Picariello, P. Ferranti, O. Fierro, G. Mamone, S. Caira, A. Di Luccia, S. Monica and F. Addeo, Peptides surviving the simulated gastrointestinal digestion of milk proteins: biological and toxicological implications, *J Chromatogr B Analyt Technol Biomed Life Sci*, 2010, **878**, 295-308.
57. A. Brodkorb, L. Egger, M. Alminger, P. Alvito, R. Assuncao, S. Ballance, T. Bohn, C. Bourlieu-Lacanal, R. Boutrou, F. Carriere, A. Clemente, M. Corredig, D. Dupont, C. Dufour, C. Edwards, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. R. Mackie, C.

Martins, S. Marze, D. J. McClements, O. Menard, M. Minekus, R. Portmann, C. N. Santos, I. Souchon, R. P. Singh, G. E. Vegarud, M. S. J. Wickham, W. Weitschies and I. Recio, INFOGEST static in vitro simulation of gastrointestinal food digestion, *Nat Protoc*, 2019, **14**, 991-1014.

58. H. M. Rawel, J. Kroll and U. C. Hohl, Model studies on reactions of plant phenols with whey proteins, *Nahrung*, 2001, **45**, 72-81.
59. K. Nagy, M. C. Courtet-Compondu, G. Williamson, S. Rezzi, M. Kussmann and A. Rytz, Non-covalent binding of proteins to polyphenols correlates with their amino acid sequence, *Food Chem*, 2012, **132**, 1333-1339.
60. A. Shpigelman, G. Israeli and Y. D. Livney, Thermally-induced protein–polyphenol co-assemblies: beta lactoglobulin-based nanocomplexes as protective nanovehicles for EGCG, *Food Hydrocolloids*, 2010, **24**, 735-743.
61. T. T. P. Nguyen, B. Bhandari, J. Cichero and S. Prakash, Gastrointestinal digestion of dairy and soy proteins in infant formulas: An in vitro study, *Food Res Int*, 2015, **76**, 348-358.
62. J. B. Blumberg, T. A. Camesano, A. Cassidy, P. Kris-Etherton, A. Howell, C. Manach, L. M. Ostertag, H. Sies, A. Skulas-Ray and J. A. Vita, Cranberries and their bioactive constituents in human health, *Adv Nutr*, 2013, **4**, 618-632.
63. M. Bhattacharya, J. Salcedo, R. C. Robinson, B. M. Henrick and D. Barile, Peptidomic and glycomic profiling of commercial dairy products: identification, quantification and potential bioactivities, *NPJ Sci Food*, 2019, **3**, 4.
64. L. Egger, P. Schlegel, C. Baumann, H. Stoffers, D. Guggisberg, C. Brugger, D. Durr, P. Stoll, G. Vergeres and R. Portmann, Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion, *Food Res Int*, 2017, **102**, 567-574.
65. L. Egger, P. Schlegel, C. Baumann, H. Stoffers, D. Guggisberg, C. Brugger, D. Durr, P. Stoll, G. Vergeres and R. Portmann, Mass spectrometry data of in vitro and in vivo pig digestion of skim milk powder, *Data Brief*, 2018, **21**, 911-917.
66. D. C. Dallas, A. Guerrero, E. A. Parker, L. A. Garay, A. Bhandari, C. B. Lebrilla, D. Barile and J. B. German, Peptidomic profile of milk of Holstein cows at peak lactation, *J Agric Food Chem*, 2014, **62**, 58-65.
67. A. J. Charlton, N. J. Baxter, M. L. Khan, A. J. Moir, E. Haslam, A. P. Davies and M. P. Williamson, Polyphenol/peptide binding and precipitation, *J Agric Food Chem*, 2002, **50**, 1593-1601.
68. M. Hayes, C. Stanton, H. Slattery, O. O'Sullivan, C. Hill, G. F. Fitzgerald and R. P. Ross, Casein fermentate of Lactobacillus animalis DPC6134 contains a range of novel propeptide angiotensin-converting enzyme inhibitors, *Appl Environ Microbiol*, 2007, **73**, 4658-4667.
69. Y. Lu, S. Govindasamy-Lucey and J. A. Lucey, Angiotensin-I-converting enzyme-inhibitory peptides in commercial Wisconsin Cheddar cheeses of different ages, *J Dairy Sci*, 2016, **99**, 41-52.
70. T. Saito, T. Nakamura, H. Kitazawa, Y. Kawai and T. Itoh, Isolation and Structural Analysis of Antihypertensive Peptides That Exist Naturally in Gouda Cheese, *Journal of Dairy Science*, 2000, **83**, 1434-1440.
71. V. Demers-Mathieu, S. F. Gauthier, M. Britten, I. Fliss, G. Robitaille and J. Jean, Antibacterial activity of peptides extracted from tryptic hydrolyzate of whey protein by nanofiltration, *International Dairy Journal*, 2013, **28**, 94-101.
72. J. A. Gomez-Ruiz, M. Ramos and I. Recio, Identification and formation of angiotensin-converting enzyme-inhibitory peptides in Manchego cheese by high-performance liquid chromatography-tandem mass spectrometry, *J Chromatogr A*, 2004, **1054**, 269-277.
73. M. Lund and Y. Ardo, Purification and identification of water-soluble phosphopeptides from cheese using Fe(III) affinity chromatography and mass spectrometry, *J Agric Food Chem*, 2004, **52**, 6616-6622.
74. V. R. Koskinen, P. A. Emery, D. M. Creasy and J. S. Cottrell, Hierarchical clustering of shotgun proteomics data, *Mol Cell Proteomics*, 2011, **10**, M110 003822.
75. I. M. Lacroix and E. C. Li-Chan, Isolation and characterization of peptides with dipeptidyl peptidase-IV inhibitory activity from pepsin-treated bovine whey proteins, *Peptides*, 2014, **54**, 39-48.
76. I. M. E. Lacroix, G. Meng, I. W. Y. Cheung and E. C. Y. Li-Chan, Do whey protein-derived peptides have dual dipeptidyl-peptidase IV and angiotensin I-converting enzyme inhibitory activities?, *Journal of Functional Foods*, 2016, **21**, 87-96.
77. Y. Joubran, A. Moscovici, R. Portmann and U. Lesmes, Implications of the Maillard reaction on bovine alpha-lactalbumin and its proteolysis during in vitro infant digestion, *Food Funct*, 2017, **8**, 2295-2308.
78. A. Yamada, T. Sakurai, D. Ochi, E. Mitsuyama, K. Yamauchi and F. Abe, Antihypertensive effect of the bovine casein-derived peptide Met-Lys-Pro, *Food Chem*, 2015, **172**, 441-446.
79. M. M. Mullally, H. Meisel and R. J. FitzGerald, Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine beta-lactoglobulin, *FEBS Lett*, 1997, **402**, 99-101.
80. T. Tavares, M. Contreras Mdel, M. Amorim, M. Pintado, I. Recio and F. X. Malcata, Novel whey-derived peptides with inhibitory effect against angiotensin-converting enzyme: in vitro effect and stability to gastrointestinal enzymes, *Peptides*, 2011, **32**, 1013-1019.
81. B. Hernández-Ledesma, A. Quirós, L. Amigo and I. Recio, Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin, *International Dairy Journal*, 2007, **17**, 42-49.
82. Y. Liu, J. Eichler and M. Pischetsrieder, Virtual screening of a milk peptide database for the identification of food-derived antimicrobial peptides, *Mol Nutr Food Res*, 2015, **59**, 2243-2254.
83. L. Cerretani and A. Bendini, *Rapid Assays to Evaluate the Antioxidant Capacity of Phenols in Virgin Olive Oil*, Academic Press, Inc., San Diego, USA, 2010.
84. M. J. Rodríguez-Roque, B. de Ancos, C. Sánchez-Moreno, M. P. Cano, P. Elez-Martínez and O. Martín-Belloso, Impact of food matrix and processing on the in vitro bioaccessibility of vitamin C, phenolic compounds, and hydrophilic antioxidant activity from fruit juice-based beverages, *Journal of Functional Foods*, 2015, **14**, 33-43.
85. J. Chen, H. Lindmark-Månsson, L. Gorton and B. Åkesson, Antioxidant capacity of bovine milk as assayed by spectrophotometric and amperometric methods, *International Dairy Journal*, 2003, **13**, 927-935.

86. M. Goudarzi, A. Madadlou, M. E. Mousavi and Z. Emam-Djomeh, Optimized preparation of ACE-inhibitory and antioxidative whey protein hydrolysate using response surface method, *Dairy Science & Technology*, 2012, **92**, 641-653.
87. D. Tagliazucchi, E. Verzelloni, D. Bertolini and A. Conte, In vitro bio-accessibility and antioxidant activity of grape polyphenols, *Food Chemistry*, 2010, **120**, 599-606.

ARTICLE

Interactions between whey proteins and cranberry juice after thermal or non-thermal processing during in vitro gastrointestinal digestion

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The objective of this study was to understand the possible interactions between whey protein and cranberry juice after processing that could impact either the protein digestibility or the bioaccessibility of cranberry antioxidants using an *in vitro* gastrointestinal digestion model. Whey protein isolate (27 or 54 mg of protein/mL) was dissolved in either cranberry juice or water and used as model beverage system. Beverages were either non-processed or underwent thermal (low: 85°C for 1 min, medium: 99°C for 10 s and long: 99°C for 5 min) or high-pressure processing (600 MPa for 4 min). After processing, beverages underwent oral (30s), gastric (2h) and small intestinal (2h) digestion. During in vitro digestion, protein hydrolysis was monitored by the O-phthalaldehyde (OPA) assay, SDS-PAGE, soluble amino acid content, and pepidomic profiling using Orbitrap mass-spectrometry. Antioxidant capacity was measured with Ferric Reducing Antioxidant Power (FRAP) and 2,2-azinobis (3-ethlybenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assays before and during in vitro digestion. Whey protein isolate dissolved in water had a significantly higher ($p < 0.05$) degree of hydrolysis and soluble amino acid content during small intestinal digestion compared to protein dissolved in cranberry juice, suggesting that cranberry juice had an effect on how protein was hydrolyzed during digestion. In all processing treatments except for long thermal processing, water and cranberry juice protein solutions had similar β-lactoglobulin digestibility ($p > 0.05$), suggesting that the cranberry juice interactions with the protein do not significantly decrease β-lactoglobulin resistance to hydrolysis by pepsin. Peptide formation also differed between whey protein dissolved in either water or juice. Cranberry juice protein solutions showed a slightly lower peptide count compared with whey protein isolate dissolved in water. Antioxidant bioaccessibility by FRAP during gastric digestion significantly increased in cranberry juice with addition of whey protein isolate. This trend might indicate a protective effect of whey protein isolate to cranberry antioxidant compounds.

Introduction

The increasing demand for functional foods with high nutritional value has led to the development of beverages containing fruit or vegetable juices with added protein [1-3].

Protein provides high nutritional value, due to its vital role in the human functioning such as development, repair, and energy, among others[4]. In addition, the consumption of fruit and vegetable juices has been related to the prevention of diseases related to oxidative stress as such as cancer and neurodegenerative diseases[5]. However, components of the juice matrix may interact with proteins or with digestive enzymes, impacting the nutritional benefits of both added proteins and fruit or vegetable antioxidants.

Previous studies on antioxidant-rich beverages during in vitro digestion have reported varying trends in protein digestibility[1, 6-8] and antioxidant activity or bioaccessibility[2, 3, 9]. Antioxidant activity measures the kinetics of the reaction between the antioxidant and the free radical that it scavenges whereas antioxidant bioaccessibility is the fraction of antioxidants available to react after digestion compared with the antioxidant available to react before digestion[10, 11].

Stojadinovic et al. (2013), studied the digestibility of β-lactoglobulin in the presence of coffee, cocoa, black and green tea polyphenol extracts during gastric and small intestinal in vitro digestion[8]. The presence of all the polyphenol extracts slowed down the hydrolysis of β-lactoglobulin by the end of the 6 h gastric phase. However, during the small intestinal phase, the green tea extract accelerated the rate of protein hydrolysis while coffee and cocoa extracts had the opposite effect. This might be influenced by the interaction of polyphenols with digestive enzymes, such as α-amylase, pepsin, and trypsin. Polyphenols may bind to these digestive enzymes, either

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binding directly to enzyme cavities, or causing enzyme precipitation, both of which inhibit enzyme activity[12, 13]. Proanthocyanidins with low degree of polymerization have been related to the inhibition of digestive enzymes such as pancreatic α-amylase, lipase, and trypsin due to their capacity to binding with the active sites of digestive enzymes [12]. As a result of the interactions of polyphenols with digestive enzymes, the protein digestibility may be decreased in the presence of polyphenols.

Cilla et al. (2012), studied the addition of skim milk (11% v/v) on the antioxidant activity of a fruit juice mix (apricot puree, grape and orange concentrate, sucrose, pectin and ascorbic acid) after in vitro gastrointestinal digestion[14]. The addition of skim milk to the fruit juice mix increased the antioxidant activity measured by oxygen radical absorbance capacity (ORAC) and 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay at the end of small intestinal digestion compared to juice without milk. Rodríguez-Roque et al. (2013), also studied the addition of milk on the antioxidant activity of a fruit juice mix of orange, kiwi, pineapple and mango juice after in vitro gastrointestinal digestion[15]. The addition of milk to the fruit juice significantly decreased the antioxidant activity measured by 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) for the model beverage after gastric and small intestinal digestion compared to the control juice without milk[15]. These conflicting results demonstrate that the impact of protein on natural antioxidant compounds needs additional study.

In addition to variations in formulation, antioxidant content, and protein composition, most beverages undergo thermal or non-thermal processing to assure food safety and extend shelf life [16, 17]. As a result of processing, protein and juice components may be modified further, impacting overall digestion. For example, previous studies have found that severe or prolonged thermal treatment increased whey protein isolate gastric digestibility [18-20]. Also, cranberry juice polyphenols are modified or degraded during thermal processing[21, 22]. The changes in polyphenols as a result of thermal processing might have affected their ability to interact with digestive enzymes[12]. Since large oligomers may only interact with the enzyme's surface, while smaller oligomers as able to interact with enzyme cavities, therefore affecting their activity[12]. In addition, the modifications to juice components that occur during processing in the presence of protein may influence their antioxidant properties.

In order to understand the possible interactions between proteins, juice polyphenols, and their behavior after processing, a model beverage of cranberry juice and whey protein isolate was selected for this study and was processed using either thermal or non-thermal treatments. Protein digestibility and antioxidant bioaccessibility were examined in the beverages before and during in vitro gastrointestinal digestion.

Materials and Methods

Raw Materials and Formulation

Beverages were formulated using cranberry juice (Just Cranberry, R.W. Knudsen, Chico, CA, USA) and whey protein isolate (WPI 8855; Fonterra, New Zealand). Whey protein isolate (0, 27, or 54 mg protein/mL) was mixed with either cranberry juice or deionized water (control beverage system) at 850 rpm until dissolved (approx. 25 min).

The two concentrations of whey protein isolate were selected based on FDA requirements for a label claim of "high", "rich in" or "excellent source of" (54 mg/mL) and "good source", "contains" or "provides" (27 mg/mL). To determine the recommended dietary allowance of protein, the average weight in North America used was 80.7 kg[23].

Processing

Samples of each beverage system were either thermally processed with three different time-temperature combinations (Low, Medium or Long thermal treatment), processed with high-pressure (HPP), or not processed (control). For the low thermal treatment (Low), glass tubes with 10 mL sample were heated in water at 100°C until the temperature reached 85°C (~1 min) and placed in a shaking water bath at 85°C for 1 min. For the medium thermal treatment (Medium), glass tubes with 5 mL sample were placed in a heating block at 180°C until the sample temperature reached 99°C (~70 sec) and held at 99°C for 10 seconds. For the long thermal treatment (Long), glass tubes with 5 mL sample were placed in a heating block at 180°C until it reached 99°C (~70 sec) followed by incubation in a shaking water bath for 5 min at 99°C. Immediately after processing, tubes were placed on ice.

For high-pressure processing, a 30 mL sample was sealed in a vacuum bag (Winpak Ltd., Winnipeg, MB, Canada) at 90% vacuum. Samples were processed in a high-pressure processing unit (2L-700 Lab System, Avure Technologies Inc, Kent, WA) with a pressure of 600 MPa for 4 min. The average come-up time to the target pressure was approximately 2 min. The average temperature of the water in the high-pressure chamber during processing was 32.5°C.

All samples were stored at 4°C and were analyzed within 1 day of processing.

Amino Acid Analysis

The amino acid composition of the whey protein isolate was analyzed using ion-exchange chromatography with a post column ninhydrin reaction detection system at 440 nm following[24] at the UC Davis Proteomics Core. Whey protein isolate was hydrolyzed with 6N hydrochloric acid containing 1% phenol at 110°C for 24 hours under vacuum. Samples were dissolved in sodium citrate buffer (Pickering Laboratories Inc, CA, USA) containing an internal standard (40 nmol/mL norleucine). 50 µL of the sample was injected for analysis by ion-exchange chromatography (L-8800 Hitachi Na-based analyzer, Tokyo, Japan). Amino acid analysis was performed in triplicate.

Protein Solubility

Samples were centrifuged for 30 min at 3000g to precipitate insoluble protein[25]. The supernatant was removed, and the insoluble protein was dried for 5 hours at 100°C[26]. The mass of insoluble protein was utilized to calculate the percent of soluble protein by difference.

***In vitro* Digestion**

Simulated saliva and simulated gastric fluids were prepared according to Bornhorst & Singh, (2013)[27]. Simulated intestinal fluid was prepared according to Roman, et al. (2012)[28]. Pepsin and trypsin activity were determined according to Minekus et al., (2014)[29]. Enzymes were added before the beginning of each digestion. Pepsin was added at a concentration of 2000 U/mL to the simulated gastric fluid and pancreatin was added at a concentration of 100 U/mL to the simulated intestinal fluid. The pH of both solutions was adjusted to 7 (saliva and intestinal fluid) or 1.8 (gastric fluid) with HCl or NaOH after addition of all components.

Simulated digestion was completed following the procedure of Bornhorst and Singh (2013) and Roman et al. (2012)[27, 28]. In addition to the protein solutions, a control sample consisting of water or juice only (no protein) was utilized to assess the influence of the simulated digestion fluids on the protein and antioxidant activity measurements. For all digestions, 5 mL sample was mixed with 3.3 mL simulated saliva for 30 sec. Following this, 6.66 mL of simulated gastric juice was added. The pH was adjusted to pH 3 with 1 M HCl. After 1 h of simulated gastric digestion, pH was adjusted to 2 using 1 M HCl. Following 2 h simulated gastric digestion, 10 mL simulated intestinal fluid was added and the pH was adjusted to 7 with 1 M NaOH. Simulated small intestinal digestion took place for 2 h. Samples were incubated at 37°C (100 rpm) in dark tubes. Simulated digestions were performed in triplicate for all treatments.

Samples were taken during gastric and small intestinal digestion to analyze the protein digestibility using the o-phthalaldehyde assay (OPA) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For OPA analysis, aliquots (100 μL) were taken after 0, 5, 15, 30, 45, 60, 90 and 120 min of gastric digestion and after 15, 30, 45, 60, 90 and 120 min of small intestinal digestion (135, 150, 165, 180, 210 and 240 min of total digestion time). Additional aliquots were taken after 0, 15, 30 and 120 min of gastric digestion (102 and 206 μL) and after 15, 30, 45 and 120 min of small intestinal digestion (167 and 343 μL) for SDS-PAGE analysis. Samples were taken after the gastric phase (120 min) and after the small intestinal phase (240 min total digestion time) for soluble amino acid content using ion-exchange chromatography and antioxidant activity analyses.

Protein Digestibility

All reagents were purchased from Biorad (Hercules, CA) unless otherwise specified

O-Phthalaldehyde (OPA) Assay

Free amino groups were measured using the o-phthalaldehyde assay (OPA)[30]. Prior to analysis, samples were diluted in deionized water to a protein concentration of 1.8 mg/mL for the gastric phase and 0.5-0.8 mg/mL for the small intestinal phase. 100 μL of each sample was used for analysis. Absorbance was measured at 340 nm. The free amino groups in each sample (mg NH_2/mL of protein solution) were calculated using glycine as a standard.

The degree of hydrolysis was calculated for each sample at each digestion time as follows[31]:

$$\mathrm{DH}(\%)=\frac{\left(\mathrm{NH_2}_{\text{digestion time point}}\text{-}\mathrm{NH_2}_{\text{Non-Dig}}\right)}{\text{Total } \mathrm{NH_2}}*100\% \quad (1)$$

where DH% is the degree of hydrolysis calculated as a percentage, $NH_{2digestion\ time\ point}$ is the free amino group concentration at a specific digestion time point (mg amino groups/mL), $NH_{2Non\text{-}Dig}$ is the amino group concentration at before digestion (mg amino groups/mL), and the $Total\ NH_2$ is the total number of amino groups based on the number of amino groups per amino acid (mg amino groups/mL), as measured by ion-exchange chromatography. Final degree of hydrolysis values were determined after subtraction of protein-free control solutions (water or cranberry juice) that also underwent in vitro digestion for each time point.

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Inglingstad et al., (2010) and Bornhorst et al., (2016)[32, 33]. Samples taken during digestion were diluted in water to a protein concentration of 2 mg/mL to allow for comparison between lanes during digestion of the same sample as well as between treatments with different protein concentration. SDS-PAGE analysis was performed on all samples from the triplicate digestions.

Optical densitometry analysis was completed using ImageJ[33] to give a measure of the protein digestibility of soluble β-lactoglobulin digestibility (selecting β-lactoglobulin band at 18.3 kDa). For imaging, gels were placed over a lightbox (AGPtek HL0163, color temperature 6000°K) with the same illumination settings for all images. Gel images were captured using a Canon EOS Rebel SL1 digital camera (18-megapixel, APS-C CMOS sensor, Canon USA, San Jose, CA). Camera setting were the same as previously described[34]. All images were taken using the same light intensity, camera distance from the gels, and camera settings[34].

For optical densitometry analysis, gel images were converted to 32-bit format. The background noise was subtracted using the "rolling ball" algorithm of 90 pixels. Within each gel, lanes were selected and converted into intensity plots. The area under the curve for the β-lactoglobulin band at 18.3 kDa in the intensity plot was calculated using the Gel Analyzer toolbox in ImageJ. Protein digestibility for each gel was calculated as follows:

$$Prot\ dig(\%) = \frac{\left(AUC_{Non-Dig} - AUC_{DTP}\right)}{AUC_{Non-Dig}} * 100\% \quad (2)$$

where $Prot\ dig(\%)$ represents the percent of the soluble β-lactoglobulin digestibility in percent, $AUC_{Non-Dig}$ represents the area under the curve in the intensity plot of β-lactoglobulin band for the non-digested sample (arbitrary units) and AUC_{DTP} represents the area under the curve in the intensity plot β-lactoglobulin band for each digestion time point (arbitrary units). Soluble β-lactoglobulin digestibility was calculated and compared to the non-digested sample within each gel to compensate for any difference in staining which might interfere with intensity values across different gels.

Soluble amino acid content using Ion-Exchange Chromatography

The soluble amino acid content using ion-exchange chromatography of the soluble fraction after sulfosalicylic acid (SSA) precipitation was analyzed for each treatment before and after gastric and small intestinal digestion according to Adibi and Mercer (1973)[35] with minor modifications. Aliquots of 1 mL were taken before or at the end of gastric and small intestinal digestion. Aliquots were heated at 95°C for 10 min. To precipitate the protein, SSA (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 2% (w/v). Samples were vortexed for 10 seconds and let to sit at room temperature for 15 min to increase precipitation. Samples were frozen overnight at -20°C and thawed the next day. After thawing, samples were centrifuged and the supernatant was subjected to hydrolysis with 6N hydrochloric acid with 1% phenol at 110°C for 24 hours under vacuum for measurement of total amino acid content as described in the Section Amino Acid Analysis. Total amino acid content was analyzed in duplicate for each treatment.

Peptide Analysis

Sample preparation for peptide analysis

Peptide analysis was performed on whey protein isolate solutions in water and cranberry juice (54 mg/mL) that were not processed or after long thermal processing (99°C for 5 min), as these samples showed the greatest differences in the soluble amino acid content. Aliquots of 1 mL were taken before digestion, at the end of gastric digestion (120 min) and at the end of small intestinal digestion (240 min) and heated at 95°C for 10 min. Samples were diluted in water to obtain a whey protein isolate concentration of 10 mg/mL. Peptides were extracted as described by Dallas et al. (2015)[36] with the following exceptions: at a volume ratio of 1:1, a solution of 200 g/L of trichloroacetic acid (TCA) was added to the samples and vortexed for 10 seconds. The samples were centrifuged at 2800 g for 30 min at 4°C and the supernatant was recovered. The supernatant containing the naturally occurring peptides was transferred to a new tube and was purified by microplate C18 (Glygen™ Corp., Columbia, MD, USA) solid phase extraction (SPE) as described previously[36]. Salts, sugars, and trichloroacetic acid were washed from the microplate with six column volumes of 1% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). Peptide solutions were dried, and the samples were re-dissolved in 25 µL 2% ACN/0.1% TFA for further analysis.

Peptide abundance determination

The appropriate amount of peptides were injected into the mass spectrometer, the peptide abundance was first estimated by using a fluorometric peptide assay (Pierce™ Quantitative Fluorometric Peptide Assay, Eugene, OR, USA). Samples taken before digestion and at the end of the small intestinal digestion were diluted in 2% ACN/0.1% TFA to obtain peptide abundance of 0.1 mg/mL and samples taken at the end of the gastric digestion were diluted in 2% ACN/0.1% TFA to a peptide abundance of 0.02 mg/mL. An aliquot of 10 µL was loaded to the Q-Exactive+, Easy 1200 UPLC liquid chromatographic (LC) column (Thermofisher scientific, Waltham, MA). Abundance of peptides were determined using mass-spectrometry [37].

Spectral analysis and peptide identification

Spectral analysis and identification of peptides were performed as described previously by Dallas et al. (2013)[38].

Functional peptide annotation

Peptide sequences identified in the samples were matched against an in-house milk bioactive peptide database search program, which compared the identified peptides with sequences that are known to be bioactive[39]. The peptides with a 100% match with the functional peptides were reported.

Antioxidant activity and Bioaccessibility

Antioxidant activity of cranberry juice (control, no protein added) and cranberry juice-protein solutions (27 or 54 mg/mL) was measured before and after simulated gastric and small intestinal digestion using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and Ferric Reducing Antioxidant Power (FRAP) methods. Trolox solutions from 0 to 1.6 mM were used as a standard curve to calculate the Trolox equivalent antioxidant activity of the samples (mM Trolox) (Gonzalez-Centeno et al., 2012). For each digestion, all samples were analysed in triplicate.

Antioxidant activity measured by FRAP and ABTS was used to calculate the gastric and small intestinal antioxidant bioaccessibility as follows[11]:

$$Bioaccessibility\ (\%) = \frac{AC_{\ digested}}{AC_{non-digested}} * 100\% \qquad (3)$$

where $Bioaccessibility$ is the gastric or small intestinal bioaccessibility calculated as a percentage, $AC_{\ digested}$ is the antioxidant activity measured by FRAP or ABTS after gastric or small intestinal digestion (mM Trolox), and $AC_{non-digested}$ is the antioxidant activity measured by FRAP or ABTS before digestion (mM Trolox).

Ferric Reducing Antioxidant Power (FRAP)

The antioxidant activity from the FRAP method was performed as described by Gonzalez-Centeno et al. (2012)[40] for 96-well microplates, with minor modifications. Three solutions were prepared: 0.01 M of 2,4,6-Tripyridyl-s-Triazine (TPTZ) in 0.04 M HCl, 0.03 M of $FeCl_3$ and acetate buffer (3.1 g/L of sodium acetate with 16 mL/L of glacial acetic acid mixed with water, pH 3.6). These solutions were mixed in a volume ratio of 1:1:10. 190 µL of the solution was transferred to each well in a 96-well microplate. The initial absorbance at 593 nm was read at 25°C for 5 min in a microplate reader (Synergy HTX, BioTek Instruments Inc., Winooski, VT, USA). 10 µL of sample or Trolox standard solution was added to each well. The mixture was incubated for 30 min at room temperature. The final absorbance was measured at 593 nm. The absorbance difference before and after incubation with the sample was used to determine the antioxidant activity

2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS)

The antioxidant activity by ABTS was performed as described by Re et al. (1999) with adaptations from Gonzalez-Centeno et al. (2012)[40] for 96-well microplates, with minor modifications. Briefly, a solution of the ABTS radical cation was prepared by reacting 7.5 mM ABTS and 2.5 mM potassium persulfate solutions (1:1, v:v). This solution reacted for 18 h at room

temperature (20 °C) in the dark and was used within 6 hours. Before analysis, 8 mL of the ABTS radical cation solution was diluted with EtOH/H2O (25:75, v:v). 190 μL of the diluted solution was transferred to each well in a 96-well microplate. The absorbance at 734 nm was read for 5 min at 25°C using a microplate reader (Synergy HTX, BioTek Instruments Inc., Winooski, VT, USA). A volume of 10 μL of sample or Trolox standard solution was then added to each well. The mixture was incubated for 30 min at room temperature and the final absorbance was measured at 734 nm.

Statistical Analysis

All statistical analysis was completed using SAS Enterprise 4.3 (Cary, NC, U.S.A.). Protein solubility was analyzed with a three-way analysis of variance (ANOVA) using a mixed model. The three fixed factors were: processing method (not processed, low thermal treatment, medium thermal treatment, long thermal treatment, or high-pressure processing), protein concentration (27 or 54 mg/mL) and solvent (cranberry juice or water). A three-way ANOVA using a mixed model with repeated measures was used to analyze differences in degree of protein hydrolysis and soluble β-lactoglobulin digestibility during digestion. The three fixed factors were: processing method, protein concentration and solvent. The repeated factor was digestion time, as samples from all digestion times were taken from the same test tube during digestion experiments. A two-way ANOVA using a mixed model with repeated measured was used to analyze differences in soluble amino acid content using ion-exchange chromatography. The factors were: processing method and solvent. The repeated factor was digestion phase, as samples from all digestion times were taken from the same test tube during digestion experiments.

To determine differences between the antioxidant bioaccessibility after gastric or small intestinal digestion of cranberry juice-protein solutions, a two-way ANOVA using a mixed model was utilized. The two fixed factors were processing method and protein concentration. Statistical significance was evaluated at $p < 0.05$. Where main effects were significant, the Tukey-Kramer test was utilized to evaluate differences between means. Results are shown as averages ± standard deviation.

Results and discussion

Amino Acid Analysis

The amino acid composition of the whey protein isolate was analyzed (Table 1) and was similar to previous studies on whey protein isolate[41 42]. The essential amino acid content of the whey protein isolate was 50 ± 0.32 g per 100 g of protein.

Protein Solubility

Protein solubility (Table 2) was significantly influenced by solvent ($p < 0.0001$), protein concentration ($p=0.0241$), processing ($p < 0.0001$), the interaction of solvent and protein concentration ($p=0.0099$), the interaction of protein concentration and processing ($p=0.0036$), and the solvent x protein concentration x processing interaction ($p=0.0221$).

Water-protein solutions had a significantly higher solubility (average 95.6 ± 2.1% dissolved, across all treatments) in comparison to cranberry juice-protein solutions (average 90.8 ± 4.0% dissolved, across all treatments). It should be noted that the true protein solubility in the cranberry juice-protein solution may be slightly higher than the reported values since the composition of the precipitate was not measured. It is possible that other insoluble aggregates such as polysaccharides also precipitated, resulting in slightly higher true protein solubility values[43].

The increased solubility of whey protein in water compared to cranberry juice might be related to the pH of the solutions. The pH of the water-protein solutions was 3.71 ± 0.19 (average across all solutions) and the pH of cranberry juice-protein solutions was 2.77 ± 0.09 (average across all solutions). pH can alter the protein net charge and promote conformational changes altering the accessibility of amino acid residues on exposed surfaces and therefore might have also affected the interaction between the whey protein and cranberry polyphenols[44].

The effect of cranberry juice on whey protein isolate solubility is consistent with previous reports that showed a decrease of protein solubility in the presence of polyphenol extracts or polyphenols in comparison to protein solubility in water[7, 45, 46]. The decrease in solubility of cranberry juice-protein solutions compared to water-protein solutions might also be related to the interactions between protein and components in the cranberry juice such as polyphenols. In solution, proteins can interact with polyphenols; this type of interaction can modify properties such as solubility, foam formation, thermal stability and aggregation[47].

The interaction between whey protein and the polyphenols present in the juice matrix might also have been affected by the mineral content of both whey protein and cranberry juice. Calcium and magnesium can interact with polyphenols and formed metalo-polyphenol complexes or associate primarily with whey proteins.[44] However, mineral content was not measured within the scope of this study, but these specific interactions are an area for future investigation.

In addition, viscosity and flow behaviour may impact the behaviour of the protein solutions, as previous studies have indicated that increased viscosity leads to decreased mass transport during digestion[48], and may impact polyphenol bioaccessibility and bioavailability.[49] Viscosity and flow behaviour were not measured within the scope of this study. However, it is hypothesized that flow behaviour played a limited role in the trends observed in the current study. Based on visual observation of protein solutions, the viscosity was similar to water or juice, in contrast to some commercial products that have significantly higher viscosity than water or juice alone.

Protein Digestibility

O-Phthalaldehyde (OPA) Assay

Protein degree of hydrolysis (Equation 1, Table 3) was significantly ($p < 0.0001$) influenced by solvent, protein concentration, digestion time, and the interactions of: solvent and digestion time; protein concentration and digestion time; solvent and protein concentration, solvent, protein concentration and digestion time; protein concentration, processing, and digestion time, and solvent; protein

concentration, processing, and digestion time (p=0.0057). Processing did not significantly influence the degree of hydrolysis (p > 0.05) (Supplementary figure 1).

The degree of hydrolysis after 120 min of gastric digestion and after 240 min of gastrointestinal digestion for the non-processed water-protein solutions were similar to previously reported results of degree of hydrolysis of whey protein isolate during gastrointestinal digestion[50, 51]. For example, after 120 min of gastric digestion, the degree of hydrolysis reported by Mat et al., (2018) was 3.7 ± 0.9%[51]. In the current study, the degree of hydrolysis after 120 min of gastric digestion for non-processed water-protein solutions was 3.3 ± 0.4% (average across both protein concentrations). Also, the degree of hydrolysis after 240 min of gastrointestinal digestion in the current study was 47.2 ± 4.3% (average for non-processed water-protein solutions after 120 min of gastric digestion across both protein concentrations) which is similar to the values reported by Mat et al., (2016) (52 ± 2% after 2 hours of gastric digestion and 2 hour of small intestinal digestion)[50].

Degree of hydrolysis was significantly influenced (p=0.0065) by solvent during the small intestinal phase (135-210 min) except for the last time point (240 min). At these time points, the water-protein solutions had a significantly higher degree of hydrolysis in comparison to the cranberry juice-protein solution. For example, after 150 min, water-protein solutions had 32.6 ± 5.1% degree of hydrolysis compared to 28.2 ± 2.6% for cranberry juice-protein solutions (average across all processing methods and protein concentrations) (Supplementary figure 1). These results follow the same trend as described by Stojadinovic et al. (2013), who reported that during simulated small intestinal digestion of β-lactoglobulin in the presence of coffee and cocoa polyphenols, the time to digest 50% of the protein was almost 2 times longer in comparison to the absence of coffee and cocoa polyphenols[8]. However, in the same study by Stojadinovic et al. (2013) a different effect was observed by the addition of green tea extract[8]. The addition of green tea extract decreased the time to digest 50% of β-lactoglobulin almost 3 times compared to absence of green tea extract. The difference in trend can be related to the difference in structure and composition of the polyphenols in each extract. Stojadinovic et al. (2013) related the effect of polyphenols on the degree of hydrolysis to the loss of stability of the β-lactoglobulin secondary structure in the presence of polyphenols at pH 7.2[8].

Protein concentration significantly influenced (p < 0.0001) the degree of hydrolysis at 135 and 150 min small intestinal digestion. Protein solutions with 27 mg/mL whey protein isolate had a significantly higher degree of hydrolysis after 135 and 150 min small intestinal digestion (26.1 ± 3.6% and 32.9 ± 4.7%, respectively) in comparison to protein solutions with 54 mg/mL whey protein isolate (21.4 ± 3.2% and 27.9 ± 2.7%, respectively). The impact of protein concentration might be related to the higher enzyme/protein ratio in protein solution with 27 mg/mL in comparison to protein solution with 54 mg/mL[52].

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The proteins present in the whey protein isolate solutions before digestion (time 0) were identified according to their molecular weight compared to molecular weight standards used in each gel (Figure 1 & 2). The whey proteins identified were bovine serum albumin (66 kDa), dimer of bovine β-lactoglobulin (36.6 kDa), bovine β-lactoglobulin (18.3 kDa) and bovine α-lactalbumin (14.2 kDa)[53]. For all protein solutions, after 15 min of gastric digestion, serum albumin and α-lactalbumin were digested into small proteins or peptides which were no longer visible in the SDS-PAGE images (Figure 1 & 2). β-lactoglobulin was largely resistant to hydrolysis by pepsin during the gastric phase but was digested into small proteins or peptides that were no longer visible using SDS-PAGE after 15 min of small intestinal digestion (Figure 1 & 2). Rapid hydrolysis of α-lactalbumin and serum albumin after 15 min of gastric digestion has been reported during both *in vitro*[20, 54] and in vivo digestion[55], which is consistent with the observations in the current study. The resistance of β-lactoglobulin to pepsin hydrolysis has also been reported by several authors, and the trends are consistent with the current study[8, 56]. The β-lactoglobulin susceptibility to small intestinal digestion has also been observed during *in vitro* digestion[19].

Overall, water and cranberry-juice-protein solutions showed different protein breakdown profiles during digestion. The difference in protein breakdown profile between water and cranberry juice-protein solutions was more visible as the temperature of thermal processing increased (Figure 1 & 2). Differences can be observed in the molecular weight of the bands formed during *in vitro* digestion, which may indicate that β-lactoglobulin hydrolysis products are influenced by the solvent and type of processing before *in vitro* digestion.

Soluble β-lactoglobulin digestibility (Equation 2, Table 4), quantified during from SDS-PAGE image analysis was significantly (p < 0.05) influenced by the solvent, processing, digestion time, and the interactions between: solvent and processing; solvent and digestion time; protein concentration, solvent, and digestion time; solvent, processing, and digestion time; and solvent, protein concentration, processing, and digestion time. Soluble β-lactoglobulin had a significantly lower (p < 0.0001) resistance to pepsin hydrolysis during gastric digestion (15-120 min) for samples after long thermal processing compared to the other treatments. After 120 min of gastric digestion, protein solutions that were not processed and after high-pressure, low, and medium thermal processing had an average of 18 ± 7.2 % soluble β-lactoglobulin digestibility compared to 60 ± 25.0 % for protein solutions after long thermal processing (average across both protein concentrations and types of solvent).

An increase in susceptibility of β-lactoglobulin to hydrolysis during *in vitro* digestion after thermal treatment has been reported in the literature in milk[19], whey protein[20], and β-lactoglobulin[18]. In these studies, soluble β-lactoglobulin digestibility was dependent on the severity of the treatment and the source of protein. The increase in soluble β-

lactoglobulin digestibility due to severe thermal treatment (99 °C for 5 min) in the current study can be related to β-lactoglobulin denaturation during prolonged thermal treatment. Pepsin is a protease which preferentially cleaves amino acids with aromatic or aliphatic side chains[6]. β-lactoglobulin has a globular structure with hydrophobic amino acids buried inside the structure. Heating causes unfolding, making the hydrophobic amino acids more accessible for hydrolysis by pepsin[6, 18].

It should also be noted that the protein hydrolysis in the current study might also be impacted by the pH of the gastric juice used in the current study (pH =1.8 fasted state), since it is a lower pH than used in some in vitro models (pH=3.0 fed state)[57]. The lower pH used in this study after the first hour of gastric digestion (compared to a static pH of 3 as recommended by Brodkorb et al., 2019[57]) might have increased the protein hydrolysis as a result of the higher pepsin activity anticipated at pH 2 compared to pH 3.

The solvent (cranberry juice or water) did not significantly influence ($p > 0.05$) soluble β-lactoglobulin digestibility during the gastric phase for non-processed, high-pressure, low, and medium thermal processing (Table 4). After the long thermal treatment, water-protein solutions had a significantly higher ($p < 0.001$) soluble β-lactoglobulin digestibility during the gastric phase compared to cranberry juice-protein solutions. After 15 min of gastric digestion, water-protein solutions that underwent long thermal treatment had 47 ± 8.0 % soluble β-lactoglobulin digestibility compared to 24 ± 3.4 % for cranberry juice-protein solutions with the same treatment (Supplementary figure 1).

The effect of the solvent on protein digestibility during gastrointestinal digestion has been reported previously using fruit extracts[6-8] and single polyphenols that are abundant in fruit extracts and juices [58]. The effect of the solvent on protein digestibility depends on the type of protein, type of protein-polyphenol interaction (covalent or non-covalent), and the types of polyphenols present in the extract or juice. Previous studies have reported decreased in pepsin hydrolysis of milk and whey proteins due to the presence of tea or fruit extracts as well as single polyphenols[6, 8]. The decrease in protein hydrolysis may be related to the binding of polyphenols to amino acids which are at cleavage sites of the gastrointestinal enzymes. It has been previously demonstrated that the protein parameters that predict the non-covalent binding of β-lactoglobulin to polyphenols are the number of proline and aliphatic residues [59]. When denaturation of β-lactoglobulin occurs, hydrophobic amino acids are exposed and the number of possible hydrophobic interaction with polyphenols between β-lactoglobulin and polyphenols increase[60]. The preferential cleavage of amino acids by pepsin are amino acids with aromatic or aliphatic side chains[6]. Solutions processed through other thermal and high-pressure treatments did not show significant differences between water and cranberry juice. The difference in effect of solvent in the less severe thermal and high pressure cranberry juice-protein solutions may be related to their lack or partial denaturation of β-lactoglobulin, hampering the hydrophobic interaction of whey proteins with cranberry juice polyphenols[58].

Soluble amino acid content using Ion-Exchange Chromatography

Cation-exchange chromatography enabled separation and quantification of amino acids from acid-hydrolyzed peptides and free amino acids in individual samples. The soluble amino acid content using ion-exchange chromatography (Figure 3A) was significantly ($p < 0.05$) influenced by all the main effects and their interactions. The soluble amino acid content using ion-exchange chromatography significantly increased ($p < 0.0001$) during simulated digestion in both water and cranberry juice-protein solutions. This increase is likely related to the protein breakdown due to pepsin and pancreatin activity which resulted in the formation of soluble peptides[19]. The influence of processing method on the soluble amino acid content using ion-exchange chromatography was dependent on the digestion phase. During gastric digestion, processing method significantly ($p < 0.05$) influenced soluble amino acid content. However, at the end of the small intestinal phase, processing method did not have a significant influence ($p > 0.05$) on the soluble amino acid content. The influence of processing on whey protein isolate solutions during the gastric phase might be related to the increase of susceptibility of whey proteins to pepsin hydrolysis after thermal treatment, since whey protein such as β-lactoglobulin, in their natural structure are resistant to pepsin hydrolysis [18, 20]. These results align with the trend observed in soluble β-lactoglobulin digestibility for which samples that underwent long thermal processing were significantly ($p < 0.0001$) more susceptible to pepsin hydrolysis during gastric digestion (15-120 min).

However, during the small intestinal phase, enzymes present in pancreatin such trypsin and chymotrypsin, are capable to hydrolyze whey proteins regardless of whether they are processed or not[61].

The soluble amino acid concentration in each of the samples after gastric and small intestinal digestion is shown in Table 1. The amount of each amino acid in the soluble phase after digestion was similar across processing treatments and in either juice or water-protein solutions. This indicates that the soluble amino acids released as a result of the protein hydrolysis were similar across all treatments. This lack of differences would suggest minimal impact on protein digestibility scores, such as the protein digestibility corrected amino acid score (PDCAAS). Determination of the impact of thermal treatment and beverage system on PDCAAS and protein digestibility in vivo is an area recommended for future study.

In order to quantify the relative magnitude of the changes during gastrointestinal digestion, (as the initial values for each solution were significantly different), soluble amino acid content after digestion was normalized with the initial value for each sample and compared in Figure 3B. The normalized soluble amino acid content using ion-exchange chromatography was significantly influenced by solvent, processing method, digestion phase, and their interactions ($p < 0.001$).

Water-protein solutions had a significantly higher ($p < 0.001$) normalized soluble amino acid content compared to the

cranberry juice-protein solutions (Figure 3B). For example, the normalized soluble amino acid content of water-protein solutions after the small intestinal phase was 2.4 ± 1.0 times higher than the non-digested sample, while cranberry juice-protein solutions increased only 1.8 ± 0.5 times compared to the non-digested sample. The lower rate of normalized soluble amino acid content of water-protein solutions compared to cranberry juice-protein solutions might be related to the interaction of whey proteins with the polyphenols present in the cranberry juice. Polyphenols can bind and precipitate digestive enzymes such as α-amylase, pepsin, trypsin and consequently inhibit their activity[13]. Proanthocyanidins, the most abundant type of polyphenol in cranberry juice[62], has been related to decreased in activity of pancreatic α-amylase, lipase, and trypsin[12]. However, proanthocyanidins effect depends on their level of polymerization and the concentration of the polyphenols present. Another possible explanation of the higher rate of normalized soluble amino acid content of water-protein solutions compared to cranberry juice-protein solutions might be the interaction of peptides formed during the small intestinal digestion with the polyphenols present in the cranberry juice, which may cause their precipitation during the sample processing[56].

The long thermal treatment had the greatest increase in normalized soluble amino acid content during digestion, with an average increase of 2.4 ± 1.2 (average across both solvents and digestion phases).

The trends observed here are similar to those described above and quantified by the SDS-PAGE method, which were similar to previously reported results[6, 8, 18, 20]. It is difficult to compare these results with previous studies, as this is the first report of soluble amino acid content using ion-exchange chromatography of whey protein isolate solutions that underwent in vitro gastrointestinal digestion.

Peptide analysis

Peptide sequences in water and cranberry juice-protein solutions were identified at different stages of digestion by Orbitrap mass spectrometry. The peptide sequences are shown in Supplementary Table 1.

Peptide identification by LC–Orbitrap MS/MS

In the present study, high-resolution Orbitrap mass spectrometry and related peptidomic techniques were utilized to identify peptides in selected samples before and after in vitro digestion.

The statistically significant differences ($p < 0.001$) between the long thermal processing and the rest of other protein solutions evidenced by the SDS-PAGE analysis suggested that more severe thermal processing affected protein breakdown after in-vitro digestion of whey protein isolate in water and cranberry juice solutions. As a result, the treatments that were not processed or after long thermal processing (99°C for 5 min) were selected for further analysis of peptides.

The number of peptides identified varied between 80 peptides in the water-protein solution that underwent long thermal processing after small intestinal digestion and 2055 peptides in the water-protein solution that was not processed and did not undergo in vitro digestion (Figure 4). Studies employing similar analytical techniques reported an average peptide count of 1421 ± 42 peptides for kefir samples, which included the added proteolytic activity of kefir microorganisms[37] and an average of 1732 peptides in a set of 10 commercial dairy products, including 8 milk-based and 2 yoghurt samples[63]. Another study on validating the static in-vitro digestion protocol published by Minekus et al., 2014[29] towards in vivo data confirmed that the harmonized in vitro protein digestion of skim milk powder, correlated with in vivo gastric samples ($r = 0.8$) and intestinal in vitro digestion correlated best with in vivo samples collected from the median jejunum ($r = 0.57$) in pigs. Protein hydrolysis at different levels was analysed by various analytical methods, including mass spectrometry[64, 65]. The apparently lower peptide numbers identified in the present study, as compared to the above referenced studies, could be attributed to the samples undergoing in vitro digestion resulting in wide difference between the lowest and highest number of peptides. These findings illustrate the capability of high-throughput mass spectrometry platforms in identifying a multitude of peptides for peptidomic profiling.

Even though the product used in this study was whey protein isolate, the majority of peptides identified in the starting material originated from caseins (Table 5). This finding is not surprising when considering that caseins' rheomorphic structure renders them more prone to hydrolysis by endogenous milk enzymes as well as by the enzymes in starters cultures used in cheese making[38]. It has been previously demonstrated that a high number of naturally occurring peptides from caseins were found in whey permeate, a co-product of whey protein isolate production[66]. It is well-known that globular proteins found in whey, such as β-lactoglobulin, are intrinsically more resistant to enzymatic digestion. Nonetheless, it is demonstrated here that peptides derived from β-lactoglobulin dominated over casein-derived peptides during the more advanced stages of in vitro digestion (Table 5). Figure 3A shows that in the gastric phase, the abundance of peptides in the long thermal processing sample led to a higher degree of hydrolysis of whey proteins. This hypothesis is supported by the results from Table 5, which showed 608 peptides from whey proteins vs 252 peptides from caseins (average across both solvents and processing methods) during gastric phase. Also, a current study supports the hypothesis that heat treatment strongly influences β-lactoglobulin degradation during the gastric phase[19].

The number of peptides of whey protein isolate solutions that were either non-processed or underwent long thermal processing decreased as digestion progressed (Figure 4). A lower number of peptides identified in juice protein solutions (1209 versus 1169 peptides) compared to water protein solution (2055 versus 1899 peptides) before digestion could be attributed to the interaction of polyphenols with peptides[60, 67], as the polyphenols might interact with peptides from whey proteins resulting into their precipitation during the sample processing[67] (Figure 4). Thus, binding of peptides and polyphenols in juice-protein solutions rendered them unextractable for peptidomic analysis affecting the total number of identified peptides enlisted in Supplementary Table

2. For example, ACE-inhibitory peptides from αs1 casein (amino acid sequence 201-212)[68], ß casein (amino acid sequence 208-217)[69], αs1 casein (amino acid sequence 16-24)[70] and an antimicrobial and immunomodulatory peptide from β-lactoglobulin (amino acid sequence 100–107)[71] were missing in juice protein solutions, but were found exclusively in water-protein solutions before digestion, possibly affected by polyphenol-peptide interaction.

Complementing a higher number of peptides in water and juice protein solutions before digestion (>1000 peptides; Figure 4), the soluble amino acid content (representing peptides and free amino acids) demonstrated (Figure 3A) that such peptides are found in low amounts and proteins are then further degraded during in vitro digestion. However, the average number of peptides across both solvents and processing methods decreased from gastric (856 peptides) to intestinal digestion (88 peptides). The decrease in the number of peptides is the result of increased hydrolysis into di- and tripeptides and amino acids during gastric and small intestinal digestion of proteins. As evidenced by the literature on peptidomics[72, 73], the current inability of measuring short peptides is a limitation of the technique. Further method development by the scientific community will be necessary in order to enable quantification of the lower molecular weight peptides.

During in vitro digestion, the difference between the number of peptides of water and cranberry juice-protein solutions was small, suggesting that the number of peptides were not affected by digestion. Although, during gastric phase, a higher number of peptides observed in juice protein solution which did not undergo processing compared to the juice protein that underwent long thermal treatment (1077 peptides vs 643 peptides) could be attributed to the binding of small oligomers of polyphenols such as proanthocyanidins[12, 62] to digestive enzymes such as α-amylase, trypsin[12] and pepsin[13], leading to precipitation and inhibition of their activity. This possibly explains the lower number of peptides in thermally treated juice-protein solution. Thermal treatment such as long thermal processing might lead to break-down of polymers and oligomers of medium and high molecular weight to small oligomers, which could bind and inhibits the activity of digestive enzymes in the juice-protein solutions that underwent long thermal processing.

Through utilization of various techniques for quantifying protein digestion in the current study, it is possible to gain a more complete understanding of the digestion process, within the limitations of each analytical method. For example, the SDS-PAGE images of water and cranberry juice protein solutions that were not processed showed similar protein profiles during the gastric phase (Figure 1 & 2) which aligns with the results that the soluble β-lactoglobulin digestibility (%) was not significantly different ($p > 0.05$) between water and cranberry juice (Table 4). However, water-protein solutions that underwent thermal processing showed a different protein profile and significantly lower resistance to pepsin hydrolysis ($p < 0.001$) during gastric digestion compared to cranberry juice-protein solutions. Similar results were observed during the quantification of soluble amino acid using ion-exchange chromatography (Figure 3A), in which water-protein solutions that underwent long thermal processing had relatively higher soluble amino acid content (48.3 ± 0.034) compared to cranberry juice-protein solutions with the same type of processing (40.9 ± 2.2), although these values were not significantly different. Similarly, the peptide count was 699 and 643 peptides for the water and cranberry juice-protein solutions after long thermal processing, respectively.

The water and cranberry-juice-protein solutions showed similar SDS-PAGE protein breakdown profiles during small intestinal digestion. After 240 min, whey protein isolate was digested into small proteins or peptides that were no longer visible using SDS-PAGE, therefore we cannot resolve differences in peptides based on SDS-PAGE. Peptides that are present in the sample vary in molecular weight range, so they do not resolve as a band even if they are present (Figure 1 & 2). Complementary information was obtained from the peptidomic profiling which presented 81 peptides. Considering that peptides below 5 amino acid length are not measured[74], due to limitation of mass spectrometry as explained above, it is reasonable to assume an intrinsic underestimate in the peptide numbers for the samples obtained at the end of small intestinal digestion.

The peptide profiles reported in the current study are similar to previous studies that have identified bioactive peptides in whey protein concentrates, isolates, and related dairy fractions (Supplementary Table 2). A dipeptidyl peptidase- IV inhibitory peptide from α-lactalbumin (amino acid sequence 123-136) identified in a study on pepsin-treated whey protein isolate[75] was also identified in all 4 samples subjected to gastric digestion. The presence of this peptide in the gastric digests in present study could be a result of hydrolysis by pepsin, an enzyme present during gastric digestion. Another dipeptidyl peptidase- IV inhibitory peptide from α-lactalbumin with amino acid sequence 123-129[76] was unique to water and cranberry juice-protein solutions that underwent long thermal processing and gastric digestion, suggesting its formation could be due to changes in protein structure during the long-thermal treatment followed by pepsin hydrolysis during digestion. A recent publication on α-lactalbumin-based Maillard reaction products (MRPs) have shown to exhibit increased surface hydrophobicity and antioxidant capacity, compared to the unprocessed α-lactalbumin[77]. In contrast, an immunomodulatory peptide from β-lactoglobulin (amino acid sequence 158–164)[78, 79] was unique to water-protein solution that had not undergone long-thermal treatment and digestion, suggesting that this could be a naturally occurring peptide.

A previous study on plant protease-hydrolyzed whey protein reported presence of an ACE-inhibitory peptide from β-lactoglobulin (amino acid sequence 49-58) in the 3 kDa fraction of whey protein concentrate[80]. This same peptide was also identified in all 4 samples subjected to gastric digestion in the current study. Similarly, a previous study analysed nanofiltration retentate and permeate of whey protein tryptic hydrolysate. A peptide from β-lactoglobulin (amino acid sequence 141–151) identified in the nanofiltration retentate, as reported by Demers-Mathieu et al. (2013)[71] was also found in all 4 samples subjected to small intestinal digestion, suggesting

that trypsin, a predominant intestinal enzyme, could be responsible for generating this peptide. In the cited study, the authors also noted that a peptide from β-lactoglobulin (amino acid sequence 141–151) was an effective antimicrobial against *Listeria, Staphylococcus aureus* and *Escherichia coli*. In the current study, this antimicrobial peptide was also found in water and cranberry juice-protein solutions before digestion. A peptide from β-casein (123-128) identified in bovine milk-based formulas after in vitro digestion with pepsin and pancreatin (which contains trypsin)[81] was also present in both the samples that were not processed and that underwent long thermal processing during intestinal digestion, suggesting that trypsin could be responsible for generating this peptide. An antimicrobial peptide from αs1 casein (amino acid sequence 95-117) identified in a previous study[82] was also identified in water and cranberry juice-protein solutions that underwent long thermal processing before digestion. These peptides could have been potentially generated during industrial processing of whey protein isolate and are likely increased during small intestinal digestion due to the presence of trypsin.

Antioxidant Bioaccessibility

Antioxidant Bioaccessibility by FRAP

Gastric antioxidant bioaccessibility from the FRAP method (Equation 3; Figure 5 A1) was significantly ($p < 0.001$) influenced by the protein concentration, processing method, and their interaction. Overall, the addition of 27 and 54 mg/mL of whey protein isolate significantly ($p < 0.0001$) increased the gastric antioxidant bioaccessibility in cranberry juice determined by the FRAP method (101.8 ± 11.2 %) compared to cranberry juice without protein (86.9 ± 9.7 %). However, the specific amount of whey protein isolate (27 or 54 mg/mL) added to the cranberry juice-protein solutions did not significantly affect ($p > 0.05$) the gastric antioxidant bioaccessibility. To determine the impact of whey protein on the bioaccessibility from the FRAP method, antioxidant activity by FRAP of water-protein solutions with 0, 27 and 54 mg/mL were measured before and during in vitro gastrointestinal digestion (data not shown). All water-protein solutions had an antioxidant capacity by FRAP that was not significantly different from 0 ($p>0.05$). Since the FRAP method does not directly measure the protein capacity to donate electrons[83], the increase in antioxidant activity in cranberry juice protein solutions by FRAP from non-digested to after gastric digestion might indicate a protective effect of whey protein isolate to cranberry antioxidant compounds during gastric digestion. A protective effect of proteins on antioxidant compounds may have facilitated the slight increase in antioxidant activity during the gastric phase compared to the antioxidant activity of the cranberry juice without protein (0 mg/mL).

Small intestinal antioxidant bioaccessibility determined by the FRAP method (Figure 5 B1) was not significantly ($p > 0.05$) influenced by protein concentration, processing or their interactions.

The lack of significance of processing method on small intestinal antioxidant bioaccessibility is similar to results observed in a previous study. Mennah-Govela & Bornhorst (2017) measured the small intestinal antioxidant bioaccessibility of orange juice that was not processed and after thermal (99°C for 10s, 85°C for 1, 5 and 15 min) and high-pressure processing (600 MPa for 4 min). In this study, the small intestinal antioxidant bioaccessibility by FRAP was not significantly influenced ($p < 0.05$) by orange variety, processing method or their interaction[11]. Although the type of juice was different (orange juice) compared to one used in the current study (cranberry juice) a similar trend with the relationship to processing method was observed. This might indicate that even though the antioxidants and polyphenols present in cranberry juice changed during processing, their capacity to donate electrons at the end of the small intestinal digestion was only slightly affected.

Antioxidant Bioaccessibility by ABTS

Gastric antioxidant bioaccessibility from the ABTS method (Figure 5 A2) was significantly ($p < 0.0001$) influenced by protein concentration, processing method, and their interaction. Cranberry juice-protein solutions that underwent long thermal processing had a significantly higher ($p=0.03$) gastric antioxidant bioaccessibility from ABTS (116.1 ± 11.0 %) compared to cranberry juice-protein solutions that were not processed or underwent other processing methods (81.5 ± 11.8 %) (Figure 5 A2). However, cranberry juice samples without protein (0 mg/mL) that underwent long thermal processing had a significantly lower ($p=0.03$) gastric antioxidant bioaccessibility by the ABTS method (63.2 ± 14.0 %) compared to cranberry juice that was not processed or that underwent high-pressure processing or low thermal processing (88.7 ± 7.5 %).

The effect of processing on the gastric and small intestinal antioxidant bioaccessibility by ABTS was dependent on protein concentration. The significantly lower ($p=0.03$) gastric antioxidant bioaccessibility measured by ABTS of cranberry juice without protein after long thermal processing (63.2 ± 14.0 %) might be related to the severe thermal treatment, which may have changed the thermally-sensitive polyphenols and antioxidant structures present in the solution during gastric digestion and decreased their antioxidant activity and bioaccessibility[84]. Conversely, cranberry juice-protein solutions (27 and 54 mg/mL) that underwent long thermal processing (99°C for 5 min) had a significantly higher ($p < 0.05$) gastric antioxidant bioaccessibility by ABTS (116.1 ± 11.0 %) compared to the non-processed cranberry juice-protein solutions or those that underwent other types of processing (81.5 ± 11.8 %). The increase of gastric antioxidant bioaccessibility of cranberry juice-protein solutions that underwent long thermal processing might be attributed to increased products of protein hydrolysis, as well as exposure of amino acids with antioxidant activity measurable by ABTS during digestion, such as tryptophan, tyrosine, cysteine, phenilalanine and histidine[85]. An increase in whey protein isolate antioxidant activity after in vitro digestion in samples with increases in thermal treatment has been reported previously [86]. This may be due to protein hydrolysis products that react with ABTS cations, increasing antioxidant activity. In the current study, cranberry juice-protein solutions that underwent the long thermal treatment had a significantly

higher soluble protein digestibility compared to the other cranberry juice-protein solution treatments at the end of the gastric phase. The increased number of protein digestion products in these samples may have reacted with ABTS cations, influencing the gastric antioxidant bioaccessibility. Antioxidant activity of whey protein (27 and 54 mg/mL) dissolved in water were measured to demostrate the interaction of ABTS cations with protein in the absence of cranberry polyphenols.

Small intestinal antioxidant bioaccessibility by the ABTS method was significantly influenced by the protein concentration ($p < 0.0001$) and the interaction of protein concentration and processing method ($p=0.0164$). The small intestinal antioxidant bioaccessibility by the ABTS method ($295.2 \pm 71.1\%$, average of cranberry juice-protein solutions with 27 and 54 mg/mL of whey protein isolate across all processing methods) was significantly greater than cranberry juice without protein (95.2 ± 43.4 %, average across all processing methods). The relationship between the increase of antioxidant activity by ABTS with the hydrolysis of whey proteins might also explain the significant increase ($p < 0.0001$) of small intestinal antioxidant bioaccessibility of cranberry juice-protein solutions (27 and 54 mg/mL) compared to cranberry juice without protein (0 mg/mL). Therefore in cranberry juice protein solutions, ABTS method likely captures both the antioxidant activity of both the cranberry juice and the protein. Interaction of whey protein and milk products after digestion with ABTS cations has been previosuly reported[86]. Another possible explanation of the increase of antioxidant activity by ABTS during the small intestinal phase might be related to the dependance of the ABTS reagent to the pH of the sample, increasing its capacity with higher pH value[87]. The initial pH of the cranberry juices tested ranged between 2.77 ± 0.09. At the end of the gastric phase, the pH of digested cranberry juice-protein decreased to pH 2.06 ± 0.01, however at the end of the small intestinal phase the digested juice increased to up to pH 7.11 ± 0.09. Some studies have suggested that the changes of pH during digestion might affect the ABTS reaction due to its lack of buffering capacity[87]. However, a previous study by Mennah-Govela & Bornhorst (2017) that used the same methodology as in the current study measured the antioxidant capacity of L-ascorbic solutions before and after gastrointestinal digestion, both at the pH of the intestinal fluids (~7.2) as well as at the initial pH of the sample (~3.0). In this study, they observed that there were no significant differences ($p > 0.05$) between the pH of the sample on the antioxidant activity from the ABTS assay[11]. Due the similarity of the methods used, it is hypothesized that the increases in antioxidant capacity and the resulting antioxidant bioaccessibility after digestion were due to the digestion of the cranberry juice and proteins, and not only due to sample pH.

Conclusions

The influence of processing on protein solubility of cranberry juice and water-whey protein isolate solutions was dependent on the severity of the thermal treatment. Higher temperatures and longer thermal treatments resulted in lower whey protein isolate solubility. The solvent in which whey protein isolate was dissolved also influenced protein solubility, where the solubility of whey protein isolate in water was greater compared to cranberry juice. Processing method and solvent significantly influenced whey protein isolate digestibility. Whey protein isolate dissolved in water had a significantly higher degree of hydrolysis and soluble amino acid content using ion-exchange chromatography during small intestinal digestion. However, the specific influence of processing on protein digestibility depended on the severity of the thermal treatment. Water-protein solutions only had higher protein digestibility during the gastric phase after long thermal processing (99°C for 5 min) compared to cranberry juice-protein solutions. The slightly lower peptide count measured for cranberry protein solutions compared with whey protein isolate in water could be due to interaction of polyphenols with peptides. The influence of processing on antioxidant bioaccessibility of cranberry juice with whey protein isolate depended on the measurement method. Antioxidant bioaccessibility by ABTS was significantly influenced by the presence of protein due to the capacity of ABTS cations to react with protein hydrolysis products. In contrast, antioxidant bioaccessibility by FRAP significantly increased in cranberry juice with the addition of 27 and 54 mg/mL of whey protein isolate, although the FRAP method does not take into account changes due to protein digestion. This trend might indicate a protective effect of whey protein isolate on cranberry antioxidant compounds during gastric digestion. The information from this study can be utilized to optimize the processing and formulation of high-protein juice products to increase both the protein digestibility and antioxidant bioaccessibility after digestion.

Conflicts of interest

There are no conflicts to declare

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1. Z. He, B. Yuan, M. Zeng, G. Tao and J. Chen, Effect of simulated processing on the antioxidant capacity and in vitro protein digestion of fruit juice-milk beverage model systems, *Food Chem*, 2015, **175**, 457-464.
2. F. J. Barba, C. Cortés, M. J. Esteve and A. Frígola, Study of Antioxidant Capacity and Quality Parameters in An Orange Juice–Milk Beverage After High-Pressure Processing Treatment, *Food and Bioprocess Technology*, 2011, **5**, 2222-2232.
3. T. F. Wegrzyn, J. M. Farr, D. C. Hunter, J. Au, M. W. Wohlers, M. A. Skinner, R. A. Stanley and D. Sun-Waterhouse, Stability of antioxidants in an apple polyphenol-milk model system, *Food Chem*, 2008, **109**, 310-318.
4. D. C. Whitcomb and M. E. Lowe, Human pancreatic digestive enzymes, *Dig Dis Sci*, 2007, **52**, 1-17.
5. J. L. Slavin and B. Lloyd, Health benefits of fruits and vegetables, *Adv Nutr*, 2012, **3**, 506-516.
6. S. Lamothe, N. Azimy, L. Bazinet, C. Couillard and M. Britten, Interaction of green tea polyphenols with dairy matrices in a simulated gastrointestinal environment, *Food Funct*, 2014, **5**, 2621-2631.
7. N. J. Plundrich, B. L. White, L. L. Dean, J. P. Davis, E. A. Foegeding and M. A. Lila, Stability and immunogenicity of hypoallergenic peanut protein-polyphenol complexes during in vitro pepsin digestion, *Food Funct*, 2015, **6**, 2145-2154.
8. M. Stojadinovic, J. Radosavljevic, J. Ognjenovic, J. Vesic, I. Prodic, D. Stanic-Vucinic and T. Cirkovic Velickovic, Binding affinity between dietary polyphenols and beta-lactoglobulin negatively correlates with the protein susceptibility to digestion and total antioxidant activity of complexes formed, *Food Chem*, 2013, **136**, 1263-1271.
9. Z. He, Y. Tao, M. Zeng, S. Zhang, G. Tao, F. Qin and J. Chen, High pressure homogenization processing, thermal treatment and milk matrix affect in vitro bioaccessibility of phenolics in apple, grape and orange juice to different extents, *Food Chem*, 2016, **200**, 107-116.
10. R. Apak, S. Gorinstein, V. Böhm, K. M. Schaich, M. Özyürek and K. Güçlü, Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report), *Pure and Applied Chemistry*, 2013, **85**, 957-998.
11. Y. A. Mennah-Govela and G. M. Bornhorst, Fresh-Squeezed Orange Juice Properties Before and During In Vitro Digestion as Influenced by Orange Variety and Processing Method, *J Food Sci*, 2017, **82**, 2438-2447.
12. A. A.-O. Vazquez-Flores, A. A.-O. Martinez-Gonzalez, E. A.-O. Alvarez-Parrilla, G. A.-O. Díaz-Sánchez Á, L. A.-O. X. de la Rosa, G. A. González-Aguilar and C. A.-O. Aguilar, Proanthocyanidins with a Low Degree of Polymerization are Good Inhibitors of Digestive Enzymes Because of their Ability to form Specific Interactions: A Hypothesis, 2018.
13. Q. He, Y. Lv and K. Yao, Effects of tea polyphenols on the activities of α-amylase, pepsin, trypsin and lipase, *Food Chemistry*, 2007, **101**, 1178-1182.
14. A. Cilla, A. Alegria, B. de Ancos, C. Sanchez-Moreno, M. P. Cano, L. Plaza, G. Clemente, M. J. Lagarda and R. Barbera, Bioaccessibility of tocopherols, carotenoids, and ascorbic acid from milk- and soy-based fruit beverages: influence of food matrix and processing, *J Agric Food Chem*, 2012, **60**, 7282-7290.
15. M. J. Rodriguez-Roque, M. A. Rojas-Grau, P. Elez-Martinez and O. Martin-Belloso, Changes in vitamin C, phenolic, and carotenoid profiles throughout in vitro gastrointestinal digestion of a blended fruit juice, *J Agric Food Chem*, 2013, **61**, 1859-1867.
16. A. Patras, N. P. Brunton, C. O'Donnell and B. K. Tiwari, Effect of thermal processing on anthocyanin stability in foods; mechanisms and kinetics of degradation, *Trends in Food Science & Technology*, 2010, **21**, 3-11.
17. B. K. Tiwari, C. P. O'Donnell and P. J. Cullen, Effect of non thermal processing technologies on the anthocyanin content of fruit juices, *Trends in Food Science & Technology*, 2009, **20**, 137-145.
18. M. R. Peram, S. M. Loveday, A. Ye and H. Singh, In vitro gastric digestion of heat-induced aggregates of beta-lactoglobulin, *J Dairy Sci*, 2013, **96**, 63-74.
19. K. A. Kopf-Bolanz, F. Schwander, M. Gijs, G. Vergères, R. Portmann and L. Egger, Impact of milk processing on the generation of peptides during digestion, *International Dairy Journal*, 2014, **35**, 130-138.
20. S. B. Kim, K. S. Ki, M. A. Khan, W. S. Lee, H. J. Lee, B. S. Ahn and H. S. Kim, Peptic and tryptic hydrolysis of native and heated whey protein to reduce its antigenicity, *J Dairy Sci*, 2007, **90**, 4043-4050.
21. E. Pappas and K. M. Schaich, Phytochemicals of cranberries and cranberry products: characterization, potential health effects, and processing stability, *Crit Rev Food Sci Nutr*, 2009, **49**, 741-781.
22. B. L. White, L. R. Howard and R. L. Prior, Impact of different stages of juice processing on the anthocyanin, flavonol, and procyanidin contents of cranberries, *J Agric Food Chem*, 2011, **59**, 4692-4698.
23. S. C. Walpole, D. Prieto-Merino, P. Edwards, J. Cleland, G. Stevens and I. Roberts, The weight of nations: an estimation of adult human biomass, *BMC Public Health*, 2012, **12**, 439.
24. F. D. Macchi, F. J. Shen, R. G. Keck and R. J. Harris, *Amino Acid Analysis Protocol*, Humana Press, 2000.
25. C. E. LaClair and M. R. Etzel, Ingredients and pH are key to clear beverages that contain whey protein, *J Food Sci*, 2010, **75**, C21-27.
26. A. International, *Official methods of analysis of AOAC International.*, AOAC International, 1995.
27. G. M. Bornhorst and R. P. Singh, Kinetics of in Vitro Bread Bolus Digestion with Varying Oral and Gastric Digestion Parameters, *Food Biophysics*, 2013, **8**, 50-59.
28. M. J. Roman, B. J. Burri and R. P. Singh, Release and bioaccessibility of beta-carotene from fortified almond butter during in vitro digestion, *J Agric Food Chem*, 2012, **60**, 9659-9666.
29. M. Minekus, M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carriere, R. Boutrou, M. Corredig, D. Dupont, C. Dufour, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, S. Marze, D. J. McClements, O. Menard, I. Recio, C. N. Santos, R. P. Singh, G. E. Vegarud, M. S. Wickham, W. Weitschies and A. Brodkorb, A standardised static in vitro digestion method suitable for food - an international consensus, *Food Funct*, 2014, **5**, 1113-1124.
30. F. C. Church, D. H. Porter, G. L. Catignani and H. E. Swaisgood, An o-phthalaldehyde spectrophotometric assay for proteinases, *Anal Biochem*, 1985, **146**, 343-348.
31. P. M. Nielsen, D. Petersen and C. Dambmann, Improved Method for Determining Food Protein Degree of

Hydrolysis., *Food Chemistry and Toxicology*, 2001, **66**, 642–646.

32. R. A. Inglingstad, T. G. Devold, E. K. Eriksen, H. Holm, M. Jacobsen, K. H. Liland, E. O. Rukke and G. E. Vegarud, Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes, *Dairy Science & Technology*, 2010, **90**, 549-563.
33. G. M. Bornhorst, K. C. Drechsler, C. A. Montoya, S. M. Rutherfurd, P. J. Moughan and R. P. Singh, Gastric protein hydrolysis of raw and roasted almonds in the growing pig, *Food Chem*, 2016, **211**, 502-508.
34. C. Swackhamer, Z. Zhang, A. Y. Taha and G. M. Bornhorst, Fatty acid bioaccessibility and structural breakdown from in vitro digestion of almond particles, *Food Funct*, 2019, **10**, 5174-5187.
35. S. A. Adibi and D. W. Mercer, Protein digestion in human intestine as reflected in luminal, mucosal, and plasma amino acid concentrations after meals, *J Clin Invest*, 1973, **52**, 1586-1594.
36. D. C. Dallas, C. J. Smink, R. C. Robinson, T. Tian, A. Guerrero, E. A. Parker, J. T. Smilowitz, K. A. Hettinga, M. A. Underwood, C. B. Lebrilla, J. B. German and D. Barile, Endogenous human milk peptide release is greater after preterm birth than term birth, *J Nutr*, 2015, **145**, 425-433.
37. D. C. Dallas, F. Citerne, T. Tian, V. L. Silva, K. M. Kalanetra, S. A. Frese, R. C. Robinson, D. A. Mills and D. Barile, Peptidomic analysis reveals proteolytic activity of kefir microorganisms on bovine milk proteins, *Food Chem*, 2016, **197**, 273-284.
38. D. C. Dallas, A. Guerrero, N. Khaldi, P. A. Castillo, W. F. Martin, J. T. Smilowitz, C. L. Bevins, D. Barile, J. B. German and C. B. Lebrilla, Extensive in vivo human milk peptidomics reveals specific proteolysis yielding protective antimicrobial peptides, *J Proteome Res*, 2013, **12**, 2295-2304.
39. S. D. Nielsen, R. L. Beverly, Y. Qu and D. C. Dallas, Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization, *Food Chem*, 2017, **232**, 673-682.
40. M. R. Gonzalez-Centeno, M. Jourdes, A. Femenia, S. Simal, C. Rossello and P. L. Teissedre, Proanthocyanidin composition and antioxidant potential of the stem winemaking byproducts from 10 different grape varieties (Vitis vinifera L.), *J Agric Food Chem*, 2012, **60**, 11850-11858.
41. R. Sinha, C. Radha, J. Prakash and P. Kaul, Whey protein hydrolysate: Functional properties, nutritional quality and utilization in beverage formulation, *Food Chemistry*, 2007, **101**, 1484-1491.
42. M. Greenwood, D. S. Kalman and J. Antonio, *Nutritional Supplements in Sports and Exercise*, Springer Berlin Heidelberg, 2008.
43. A.-S. Stübler, U. Lesmes, V. Heinz, C. Rauh, A. Shpigelman and K. Aganovic, Digestibility, antioxidative activity and stability of plant protein-rich products after processing and formulation with polyphenol-rich juices: kale and kale–strawberry as a model, *European Food Research and Technology*, 2019, **245**, 2499-2514.
44. S. Moser, M. Chegeni, O. G. Jones, A. Liceaga and M. G. Ferruzzi, The effect of milk proteins on the bioaccessibility of green tea flavan-3-ols, *Food Research International*, 2014, **66**, 297-305.
45. M. von Staszewski, F. L. Jara, A. L. T. G. Ruiz, R. J. Jagus, J. E. Carvalho and A. M. R. Pilosof, Nanocomplex formation between β-lactoglobulin or caseinomacropeptide and green tea polyphenols: Impact on protein gelation and polyphenols antiproliferative activity, *Journal of Functional Foods*, 2012, **4**, 800-809.
46. M. Schneider, D. Esposito, M. A. Lila and E. A. Foegeding, Formation of whey protein-polyphenol meso-structures as a natural means of creating functional particles, *Food Funct*, 2016, **7**, 1306-1318.
47. K. Brudzynski and L. Maldonado-Alvarez, Polyphenol-Protein Complexes and Their Consequences for the Redox Activity, Structure and Function of Honey. A Current View and New Hypothesis – a Review, *Polish Journal of Food and Nutrition Sciences*, 2015, **65**, 71-80.
48. A. Tharakan, I. T. Norton, P. J. Fryer and S. Bakalis, Mass transfer and nutrient absorption in a simulated model of small intestine, *J Food Sci*, 2010, **75**, E339-346.
49. T. Bohn, Dietary factors affecting polyphenol bioavailability, *Nutr Rev*, 2014, **72**, 429-452.
50. D. J. L. Mat, S. Le Feunteun, C. Michon and I. Souchon, In vitro digestion of foods using pH-stat and the INFOGEST protocol: Impact of matrix structure on digestion kinetics of macronutrients, proteins and lipids, *Food Research International*, 2016, **88**, 226-233.
51. D. J. L. Mat, T. Cattenoz, I. Souchon, C. Michon and S. Le Feunteun, Monitoring protein hydrolysis by pepsin using pH-stat: In vitro gastric digestions in static and dynamic pH conditions, *Food Chem*, 2018, **239**, 268-275.
52. G. Shu, B. Zhang, Q. Zhang, H. Wan and H. Li, Effect of Temperature, pH, Enzyme to Substrate Ratio, Substrate Concentration and Time on the Antioxidative Activity of Hydrolysates from Goat Milk Casein by Alcalase *Acta Universitatis Cibiniensis. Series E: Food Technology*, 2016, **20**.
53. A. Grácia-Juliá, M. René, M. Cortés-Muñoz, L. Picart, T. López-Pedemonte, D. Chevalier and E. Dumay, Effect of dynamic high pressure on whey protein aggregation: A comparison with the effect of continuous short-time thermal treatments, *Food Hydrocolloids*, 2008, **22**, 1014-1032.
54. S. Zhang, Z. Zhang and B. Vardhanabhuti, Effect of charge density of polysaccharides on self-assembled intragastric gelation of whey protein/polysaccharide under simulated gastric conditions, *Food Funct*, 2014, **5**, 1829-1838.
55. L. M. Sullivan, J. J. Kehoe, L. Barry, M. J. Buckley, F. Shanahan, K. H. Mok and A. Brodkorb, Gastric digestion of alpha-lactalbumin in adult human subjects using capsule endoscopy and nasogastric tube sampling, *Br J Nutr*, 2014, **112**, 638-646.
56. G. Picariello, P. Ferranti, O. Fierro, G. Mamone, S. Caira, A. Di Luccia, S. Monica and F. Addeo, Peptides surviving the simulated gastrointestinal digestion of milk proteins: biological and toxicological implications, *J Chromatogr B Analyt Technol Biomed Life Sci*, 2010, **878**, 295-308.
57. A. Brodkorb, L. Egger, M. Alminger, P. Alvito, R. Assuncao, S. Ballance, T. Bohn, C. Bourlieu-Lacanal, R. Boutrou, F. Carriere, A. Clemente, M. Corredig, D. Dupont, C. Dufour, C. Edwards, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. R. Mackie, C.

Martins, S. Marze, D. J. McClements, O. Menard, M. Minekus, R. Portmann, C. N. Santos, I. Souchon, R. P. Singh, G. E. Vegarud, M. S. J. Wickham, W. Weitschies and I. Recio, INFOGEST static in vitro simulation of gastrointestinal food digestion, *Nat Protoc*, 2019, **14**, 991-1014.
58. H. M. Rawel, J. Kroll and U. C. Hohl, Model studies on reactions of plant phenols with whey proteins, *Nahrung*, 2001, **45**, 72-81.
59. K. Nagy, M. C. Courtet-Compondu, G. Williamson, S. Rezzi, M. Kussmann and A. Rytz, Non-covalent binding of proteins to polyphenols correlates with their amino acid sequence, *Food Chem*, 2012, **132**, 1333-1339.
60. A. Shpigelman, G. Israeli and Y. D. Livney, Thermally-induced protein–polyphenol co-assemblies: beta lactoglobulin-based nanocomplexes as protective nanovehicles for EGCG, *Food Hydrocolloids*, 2010, **24**, 735-743.
61. T. T. P. Nguyen, B. Bhandari, J. Cichero and S. Prakash, Gastrointestinal digestion of dairy and soy proteins in infant formulas: An in vitro study, *Food Res Int*, 2015, **76**, 348-358.
62. J. B. Blumberg, T. A. Camesano, A. Cassidy, P. Kris-Etherton, A. Howell, C. Manach, L. M. Ostertag, H. Sies, A. Skulas-Ray and J. A. Vita, Cranberries and their bioactive constituents in human health, *Adv Nutr*, 2013, **4**, 618-632.
63. M. Bhattacharya, J. Salcedo, R. C. Robinson, B. M. Henrick and D. Barile, Peptidomic and glycomic profiling of commercial dairy products: identification, quantification and potential bioactivities, *NPJ Sci Food*, 2019, **3**, 4.
64. L. Egger, P. Schlegel, C. Baumann, H. Stoffers, D. Guggisberg, C. Brugger, D. Durr, P. Stoll, G. Vergeres and R. Portmann, Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion, *Food Res Int*, 2017, **102**, 567-574.
65. L. Egger, P. Schlegel, C. Baumann, H. Stoffers, D. Guggisberg, C. Brugger, D. Durr, P. Stoll, G. Vergeres and R. Portmann, Mass spectrometry data of in vitro and in vivo pig digestion of skim milk powder, *Data Brief*, 2018, **21**, 911-917.
66. D. C. Dallas, A. Guerrero, E. A. Parker, L. A. Garay, A. Bhandari, C. B. Lebrilla, D. Barile and J. B. German, Peptidomic profile of milk of Holstein cows at peak lactation, *J Agric Food Chem*, 2014, **62**, 58-65.
67. A. J. Charlton, N. J. Baxter, M. L. Khan, A. J. Moir, E. Haslam, A. P. Davies and M. P. Williamson, Polyphenol/peptide binding and precipitation, *J Agric Food Chem*, 2002, **50**, 1593-1601.
68. M. Hayes, C. Stanton, H. Slattery, O. O'Sullivan, C. Hill, G. F. Fitzgerald and R. P. Ross, Casein fermentate of Lactobacillus animalis DPC6134 contains a range of novel propeptide angiotensin-converting enzyme inhibitors, *Appl Environ Microbiol*, 2007, **73**, 4658-4667.
69. Y. Lu, S. Govindasamy-Lucey and J. A. Lucey, Angiotensin-I-converting enzyme-inhibitory peptides in commercial Wisconsin Cheddar cheeses of different ages, *J Dairy Sci*, 2016, **99**, 41-52.
70. T. Saito, T. Nakamura, H. Kitazawa, Y. Kawai and T. Itoh, Isolation and Structural Analysis of Antihypertensive Peptides That Exist Naturally in Gouda Cheese, *Journal of Dairy Science*, 2000, **83**, 1434-1440.
71. V. Demers-Mathieu, S. F. Gauthier, M. Britten, I. Fliss, G. Robitaille and J. Jean, Antibacterial activity of peptides extracted from tryptic hydrolyzate of whey protein by nanofiltration, *International Dairy Journal*, 2013, **28**, 94-101.
72. J. A. Gomez-Ruiz, M. Ramos and I. Recio, Identification and formation of angiotensin-converting enzyme-inhibitory peptides in Manchego cheese by high-performance liquid chromatography-tandem mass spectrometry, *J Chromatogr A*, 2004, **1054**, 269-277.
73. M. Lund and Y. Ardo, Purification and identification of water-soluble phosphopeptides from cheese using Fe(III) affinity chromatography and mass spectrometry, *J Agric Food Chem*, 2004, **52**, 6616-6622.
74. V. R. Koskinen, P. A. Emery, D. M. Creasy and J. S. Cottrell, Hierarchical clustering of shotgun proteomics data, *Mol Cell Proteomics*, 2011, **10**, M110 003822.
75. I. M. Lacroix and E. C. Li-Chan, Isolation and characterization of peptides with dipeptidyl peptidase-IV inhibitory activity from pepsin-treated bovine whey proteins, *Peptides*, 2014, **54**, 39-48.
76. I. M. E. Lacroix, G. Meng, I. W. Y. Cheung and E. C. Y. Li-Chan, Do whey protein-derived peptides have dual dipeptidyl-peptidase IV and angiotensin I-converting enzyme inhibitory activities?, *Journal of Functional Foods*, 2016, **21**, 87-96.
77. Y. Joubran, A. Moscovici, R. Portmann and U. Lesmes, Implications of the Maillard reaction on bovine alpha-lactalbumin and its proteolysis during in vitro infant digestion, *Food Funct*, 2017, **8**, 2295-2308.
78. A. Yamada, T. Sakurai, D. Ochi, E. Mitsuyama, K. Yamauchi and F. Abe, Antihypertensive effect of the bovine casein-derived peptide Met-Lys-Pro, *Food Chem*, 2015, **172**, 441-446.
79. M. M. Mullally, H. Meisel and R. J. FitzGerald, Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine beta-lactoglobulin, *FEBS Lett*, 1997, **402**, 99-101.
80. T. Tavares, M. Contreras Mdel, M. Amorim, M. Pintado, I. Recio and F. X. Malcata, Novel whey-derived peptides with inhibitory effect against angiotensin-converting enzyme: in vitro effect and stability to gastrointestinal enzymes, *Peptides*, 2011, **32**, 1013-1019.
81. B. Hernández-Ledesma, A. Quirós, L. Amigo and I. Recio, Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin, *International Dairy Journal*, 2007, **17**, 42-49.
82. Y. Liu, J. Eichler and M. Pischetsrieder, Virtual screening of a milk peptide database for the identification of food-derived antimicrobial peptides, *Mol Nutr Food Res*, 2015, **59**, 2243-2254.
83. L. Cerretani and A. Bendini, *Rapid Assays to Evaluate the Antioxidant Capacity of Phenols in Virgin Olive Oil*, Academic Press, Inc., San Diego, USA, 2010.
84. M. J. Rodríguez-Roque, B. de Ancos, C. Sánchez-Moreno, M. P. Cano, P. Elez-Martínez and O. Martín-Belloso, Impact of food matrix and processing on the in vitro bioaccessibility of vitamin C, phenolic compounds, and hydrophilic antioxidant activity from fruit juice-based beverages, *Journal of Functional Foods*, 2015, **14**, 33-43.
85. J. Chen, H. Lindmark-Månsson, L. Gorton and B. Åkesson, Antioxidant capacity of bovine milk as assayed by spectrophotometric and amperometric methods, *International Dairy Journal*, 2003, **13**, 927-935.

86. M. Goudarzi, A. Madadlou, M. E. Mousavi and Z. Emam-Djomeh, Optimized preparation of ACE-inhibitory and antioxidative whey protein hydrolysate using response surface method, *Dairy Science & Technology*, 2012, **92**, 641-653.

87. D. Tagliazucchi, E. Verzelloni, D. Bertolini and A. Conte, In vitro bio-accessibility and antioxidant activity of grape polyphenols, *Food Chemistry*, 2010, **120**, 599-606.

Digestion Phase	**Non-digested**	Gastric								Intestinal							
Solvent	N/A	Cranberry Juice				Water				Cranberry Juice				Water			
Processing	NP	NP	Low	Medium	Long	NP	Low	Medium	Long	NP	Low	Medium	Long	NP	Low	Medium	Long
Cysteic acid	0.1 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.6± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.8± 0.1	0.5 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
Asx	11.0 ± 0.1	3.9 ± 0.1	4.1 ± 0.4	4.4 ± 0.1	4.5 ± 0.1	4.1 ± 0.1	4.9 ± 0.2	4.9 ± 0.5	5.5 ± 0.1	5.4 ± 0.1	5.2 ± 0.3	5.4 ± 0.2	5.3 ± 0.3	5.7 ± 0.1	5.4 ± 0.3	5.3 ± 0.3	5.6 ± 0.1
Threonine	4.7 ± 0.1	1.6 ± 0.1	1.7 ± 0.2	1.7 ± 0.2	1.9 ± 0.0	1.7 ± 0.0	2.0 ± 0.1	2.1 ± 0.2	2.3 ± 0.0	2.3 ± 0.1	2.2 ± 0.2	2.3 ± 0.1	2.2 ± 0.2	2.4 ± 0.1	2.3 ± 0.2	2.3 ± 0.1	2.4 ± 0.1
Serine	3.5 ± 0.1	1.2 ± 0.0	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.0	1.4 ± 0.0	1.5 ± 0.1	1.5 ± 0.1	1.7 ± 0.0	1.7 ± 0.1	1.6 ± 0.2	1.7 ± 0.1	1.6 ± 0.2	1.8 ± 0.1	1.7 ± 0.2	1.7 ± 0.1	1.8 ± 0.0
Glx	16.9 ± 0.2	5.7 ± 0.3	6.1 ± 0.6	6.2 ± 0.6	6.8 ± 0.2	6.1 ± 0.0	7.4 ± 0.3	7.4 ± 1.0	8.6 ± 0.1	8.3 ± 0.1	8.1 ± 0.4	8.3 ± 0.2	8.2 ± 0.5	9.1 ± 0.2	8.6 ± 0.6	8.0 ± 0.6	8.7 ± 0.0
Proline	4.6 ± 0.0	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.2	1.8 ± 0.0	1.6 ± 0.0	2.0 ± 0.1	2.0 ± 0.2	2.2 ± 0.0	2.0 ± 0.0	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	2.2 ± 0.0	2.1 ± 0.1	2.0 ± 0.1	2.2 ± 0.1
Glycine	1.5 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.0	0.6 ± 0.1	0.6 ± 0.2	0.7 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	0.8 ± 0.1
Alanine	5.7 ± 0.2	2.0 ± 0.1	2.2 ± 0.2	2.3 ± 0.2	2.4 ± 0.0	2.2 ± 0.0	2.6 ± 0.1	2.6 ± 0.2	2.9 ± 0.1	2.8 ± 0.1	2.7 ± 0.2	2.8 ± 0.1	2.7 ± 0.1	3.0 ± 0.1	2.9 ± 0.1	2.82 ± 0.2	3.0 ± 0.1
Cysteine	1.1 ± 0.16	0.3 ± 0.1	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.3 ± 0.4	0.5 ± 0.4	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.8 ± 0.1	0.5 ± 0.4	0.7 ± 0.1	0.7 ± 0.1
Valine	5.0 ± 0.0	1.8 ± 0.0	1.9 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	1.9 ± 0.1	2.3 ± 0.06	2.3 ± 0.2	2.6 ± 0.0	2.4 ± 0.0	2.4 ± 0.1	2.5 ± 0.0	2.4 ± 0.1	2.7 ± 0.0	2.6 ± 0.0	2.5 ± 0.1	2.6 ± 0.2
Methionine	1.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.4± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.0
Isoleucine	5.6 ± 0.1	2.0 ± 0.1	2.1 ± 0.2	2.2 ± 0.2	2.3 ± 0.0	2.1 ± 0.0	2.4 ± 0.1	2.5 ± 0.3	2.8 ± 0.1	2.6 ± 0.0	2.6 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	2.8 ± 0.1	2.7 ± 0.0	2.7 ± 0.1	2.8 ± 0.1
Leucine	13.8 ± 0.1	4.7 ± 0.1	5.0 ± 0.5	5.2 ± 0.4	5.6 ± 0.1	5.1 ± 0.1	6.1 ± 0.2	6.2 ± 0.6	6.9 ± 0.1	6.7 ± 0.1	6.5 ± 0.3	6.7 ± 0.2	6.6 ± 0.4	7.1 ± 0.2	6.8 ± 0.3	6.7 ± 0.3	7.0 ± 0.2
Tyrosine	3.6 ± 0.0	1.2 ± 0.0	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.0	1.3 ± 0.1	1.6 ± 0.0	1.6 ± 0.1	1.8 ± 0.0	1.7 ± 0.0	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	2.7 ± 1.5	1.8 ± 0.1
Phenylalanine	13.5 ± 0.0	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.0	1.2 ± 0.0	1.5 ± 0.1	1.6 ± 0.2	1.8 ± 0.0	1.7 ± 0.0	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.0
Histidine	1.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.8 ± 0.1	0.9 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	0.8 ± 0.0	1.3 ± 0.7	0.9 ± 0.1
Lysine	10.9 ± 0.1	3.6 ± 0.1	3.9 ± 0.3	4.1 ± 0.4	4.4 ± 0.1	3.8 ± 0.0	4.8 ± 0.2	4.9 ± 0.5	5.5 ± 0.1	5.3 ± 0.0	5.2 ± 0.2	5.3 ± 0.1	5.2 ± 0.2	5.6 ± 0.1	5.4 ± 0.2	2.7 ± 3.6	5.6 ± 0.3
Tryptophan	3.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Arginine	2.5 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.1 ± 0.0	1.1 ± 0.1	1.3 ± 0.0	1.1 ± 0.1	1.0 ± 0.01	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1

Table 1. Average g of amino acids per 100 g of protein in the whey protein isolate used in the current study before digestion (Non-digested), and the soluble fraction of amino acids of water and cranberry juice-protein solutions with 54 mg/mL of whey protein isolate after the following processing treatments: non-processed (NP), high-pressure processing (HPP, 600 MPa for 4 min) and thermal processing: 85°C for 1 min (Low), 99°C for 10 s (Medium) and 99°C for 5 min (Long).Asx: aspartic acid (Asp) and asparagine (Asn); Glx: glutamic acid (Glu) and glutamine (Gln); cysteic acid caused by the oxidation of cysteine during acid hydrolysis. Results are averages (n = 2) ± standard deviation.

Solvent	Protein concentration (mg/mL)	Processing Method	Solubility (% dissolved)
Cranberry Juice	54	NP	93.3 ± 1.5^{abc}
		HPP	95.7 ± 0.6^{abc}
		Low	94.3 ± 0.6^{abc}
		Medium	92 ± 2.0^{abcd}
		Long	84 ± 5.3^{e}
	27	NP	91.3 ± 2.9^{bcd}
		HPP	92 ± 1.0^{bcde}
		Low	86 ± 1.0^{de}
		Medium	89.7 ± 2.1^{cde}
		Long	89.3 ± 2.3^{cde}
Water	54	NP	97 ± 1.0^{a}
		HPP	97.3 ± 2.1^{a}
		Low	95.7 ± 0.6^{abc}
		Medium	95.3 ± 0.6^{abc}
		Long	92 ± 2.6^{abcd}
	27	NP	96.3 ± 0.6^{ab}
		HPP	98 ± 1.0^{a}
		Low	95.3 ± 0.6^{abc}
		Medium	95.7 ± 0.6^{abc}
		Long	93 ± 1.0^{abc}

Table 2. Protein solubility (%) of water and cranberry juice-protein solutions with 27 and 54 mg/mL of whey protein isolate after the following processing treatments: non-processed (NP), high-pressure processing (HPP, 600 MPa for 4 min) and thermal processing: 85°C for 1 min (Low), 99°C for 10 s (Medium) and 99°C for 5 min (Long). Values are given as averages (n = 3) ± standard deviation. Different letters within the protein solubility (% dissolved) column (between solvent, protein concentration and processing treatment) represent means that are significantly different ($p < 0.05$) across all values.

Solvent	Protein concentration (mg/mL)	Type of processing	Digestion time (min)												
			5	15	30	45	60	90	120	135	150	165	180	210	240
Water	54	NP	0 ± 0	1 ± 1	2 ± 0	2 ± 0	2 ± 1	2 ± 0	3 ± 0	20 ± 3	27 ± 6	34 ± 2	39 ± 1	41 ± 3	46 ± 3
		HPP	1 ± 1	2 ± 1	3 ± 1	3 ± 1	3 ± 1	5 ± 0	5 ± 0	20 ± 1	29 ± 2	34 ± 1	35 ± 0	40 ± 2	40 ± 2
		Low	1 ± 0	1 ± 1	2 ± 1	2 ± 1	3 ± 2	4 ± 0	5 ± 0	22 ± 3	30 ± 2	34 ± 2	38 ± 3	40 ± 4	40 ± 1
		Medium	1 ± 0	2 ± 1	3 ± 0	3 ± 0	4 ± 1	4 ± 1	5 ± 2	23 ± 3	28 ± 2	33 ± 1	37 ± 5	40 ± 0	40 ± 2
		Long	-1 ± 1	1 ± 1	3 ± 2	3 ± 2	3 ± 1	7 ± 1	12 ± 5	25 ± 2	29 ± 2	32 ± 2	36 ± 4	38 ± 3	38 ± 3
	27	NP	1 ± 2	1 ± 2	2 ± 1	2 ± 0	2 ± 0	3 ± 1	3 ± 1	30 ± 2	38 ± 2	38 ± 1	42 ± 1	48 ± 4	49 ± 5
		HPP	1 ± 1	2 ± 1	3 ± 1	4 ± 1	4 ± 0	5 ± 0	5 ± 1	26 ± 1	35 ± 3	35 ± 2	41 ± 3	44 ± 2	43 ± 1
		Low	3 ± 1	2 ± 1	3 ± 1	3 ± 1	3 ± 2	5 ± 1	5 ± 1	27 ± 4	37 ± 4	38 ± 3	43 ± 5	48 ± 3	46 ± 4
		Medium	2 ± 1	3 ± 0	4 ± 0	4 ± 1	5 ± 1	6 ± 1	7 ± 1	29 ± 1	38 ± 5	38 ± 6	39 ± 6	43 ± 6	44 ± 1
		Long	0 ± 0	1 ± 0	2 ± 1	2 ± 2	4 ± 2	9 ± 3	11 ± 4	29 ± 3	35 ± 1	39 ± 1	41 ± 4	48 ± 6	41 ± 2
Cranberry Juice	54	NP	0 ± 1	1 ± 0	1 ± 0	2 ± 0	2 ± 1	2 ± 1	2 ± 1	19 ± 1	25 ± 0	33 ± 2	36 ± 4	44 ± 5	46 ± 9
		HPP	0 ± 1	1 ± 1	2 ± 0	2 ± 0	3 ± 0	3 ± 0	3 ± 0	21 ± 4	28 ± 4	32 ± 1	33 ± 3	37 ± 2	37 ± 2
		Low	1 ± 0	2 ± 0	2 ± 0	3 ± 1	3 ± 0	3 ± 0	4 ± 0	19 ± 4	28 ± 2	34 ± 0	36 ± 4	37 ± 4	44 ± 2
		Medium	1 ± 0	2 ± 1	2 ± 0	3 ± 1	3 ± 0	4 ± 1	4 ± 1	20 ± 0	27 ± 2	34 ± 1	39 ± 3	45 ± 7	48 ± 8
		Long	1 ± 1	3 ± 1	5 ± 1	5 ± 2	5 ± 1	6 ± 2	7 ± 1	26 ± 3	28 ± 4	33 ± 5	38 ± 5	38 ± 2	42 ± 5
	27	NP	1 ± 1	2 ± 1	2 ± 0	3 ± 0	3 ± 0	4 ± 0	4 ± 0	27 ± 4	30 ± 3	32 ± 3	40 ± 4	36 ± 2	47 ± 5
		HPP	1 ± 0	2 ± 0	3 ± 1	2 ± 1	4 ± 0	4 ± 0	4 ± 1	23 ± 2	30 ± 1	33 ± 3	35 ± 0	41 ± 2	41 ± 2
		Low	2 ± 2	2 ± 0	3 ± 1	3 ± 1	3 ± 0	4 ± 1	4 ± 1	27 ± 3	30 ± 3	33 ± 1	34 ± 4	39 ± 6	41 ± 6
		Medium	3 ± 0	2 ± 2	4 ± 1	4 ± 0	4 ± 2	4 ± 1	5 ± 0	20 ± 1	28 ± 2	28 ± 1	29 ± 3	32 ± 1	32 ± 1
		Long	1 ± 1	3 ± 1	4 ± 0	4 ± 2	6 ± 1	7 ± 0	7 ± 1	23 ± 1	27 ± 3	32 ± 4	35 ± 9	39 ± 3	38 ± 3

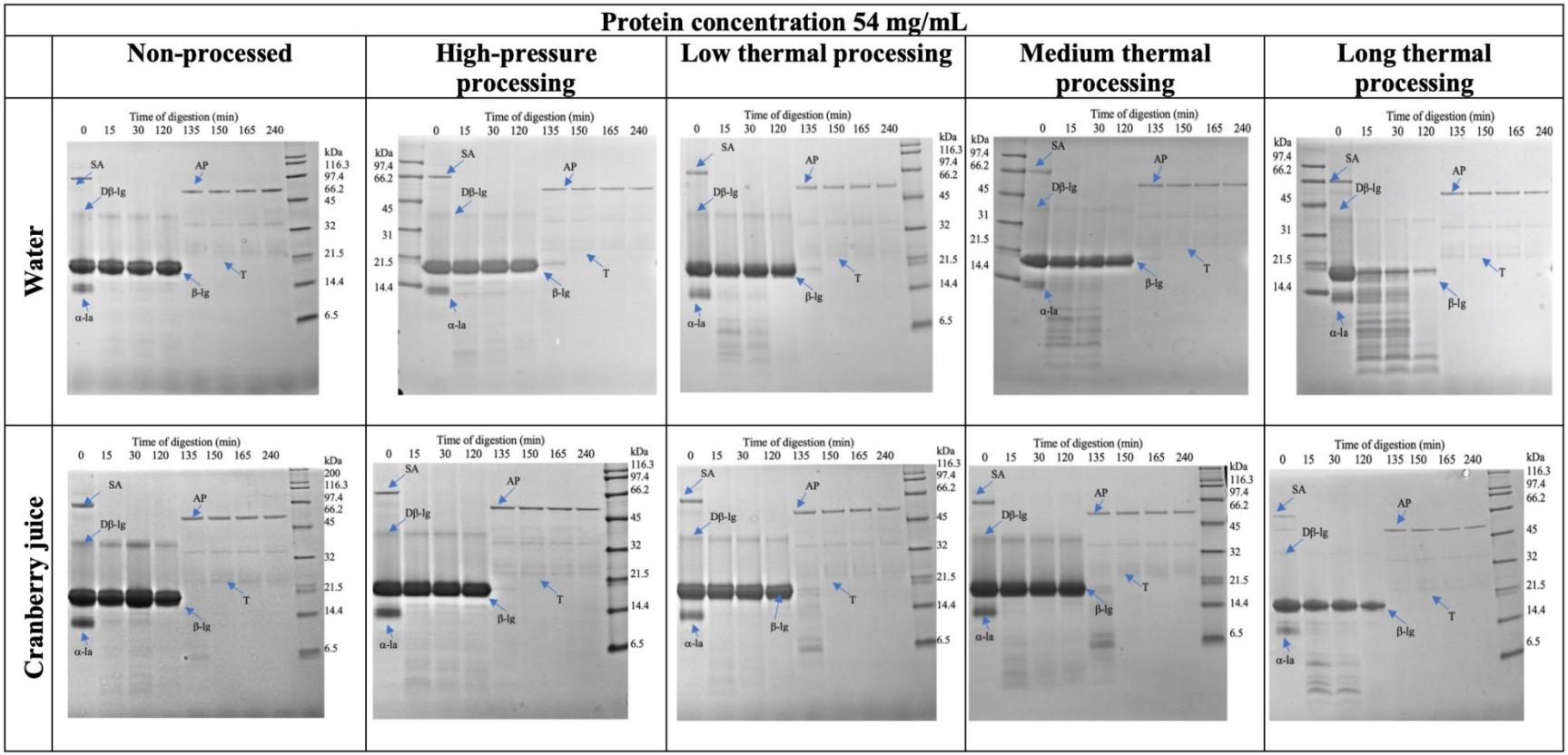
Table *3*. Degree of hydrolysis (%) of water and cranberry juice protein solutions with 27 and 54 mg/mL of whey protein isolate with the following processing treatments: non-processed (NP), high-pressure processing (HPP, 600 MPa for 4 min) and thermal processing: 85°C for 1 min (Low), 99°C for 10 s (Medium) and) 99°C for 5 min (Long). Values are given as averages (n = 3) ± standard deviation of the mean

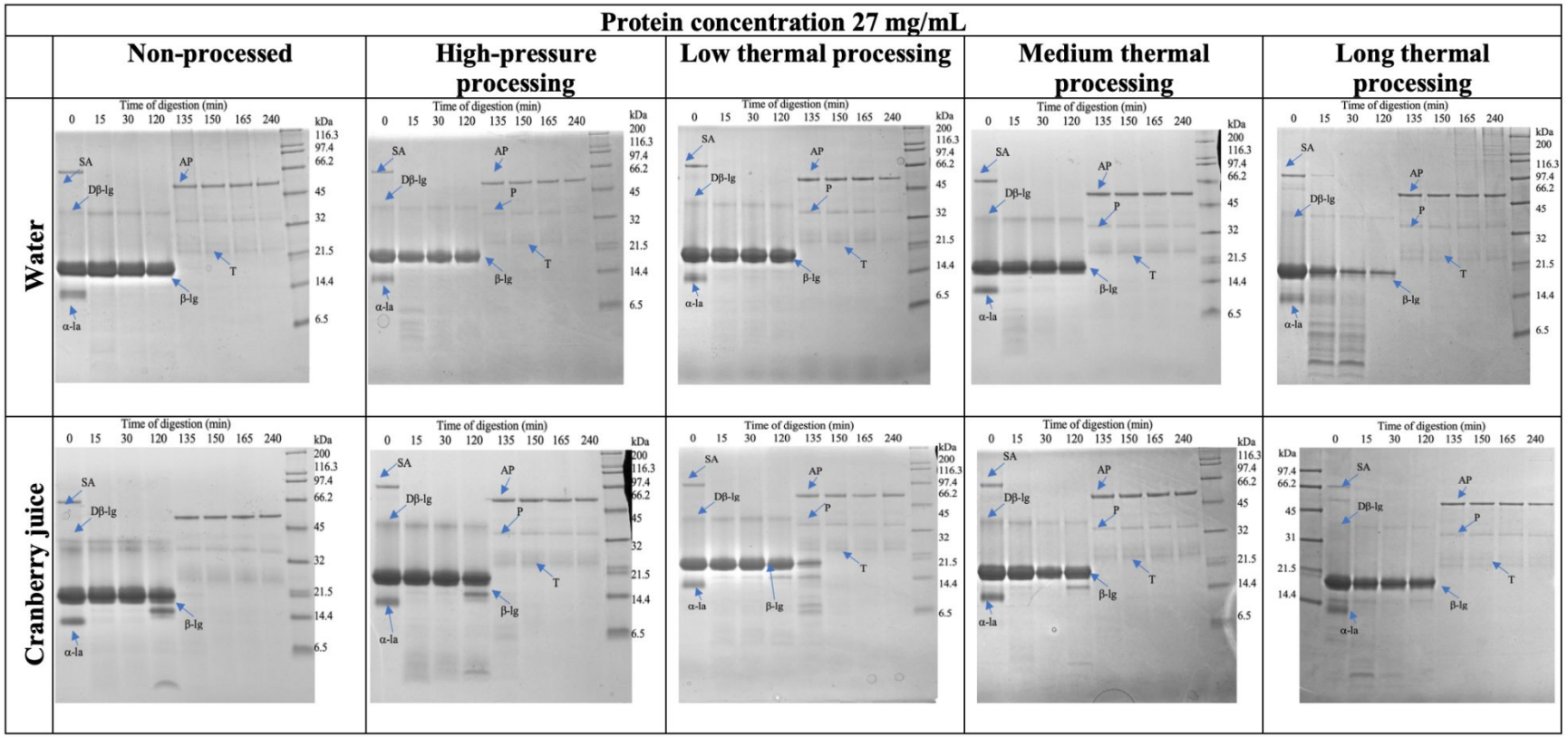
Solvent	Protein conc. (mg/mL)	Type of processing	Digestion time (min)						
			Gastric digestion			Small intestinal digestion			
			15	30	120	135	150	165	240
Water	54	NP	2 ± 1.0 ^{f}	4 ± 5.5 ^{ef}	7 ± 5.2 ^{f}	91 ± 8.8 ^{ab}	97 ± 5.3	99 ± 1.7	98 ± 1.7
		HPP	2 ± 1.8 ^{f}	2 ± 2.5 ^{f}	13 ± 8.7 ^{ef}	89 ± 4.7 ^{ab}	99 ± 0.3	97 ± 2.7	91 ± 8.3
		Low	13 ± 9.2 ^{bcdef}	16 ± 5.2 ^{bcdef}	23 ± 5.1 ^{bcdef}	95 ± 1.0 ^{ab}	96 ± 3.9	96 ± 2.4	97 ± 1.5
		Medium	12 ± 4.4 ^{bcdef}	11 ± 4.9 ^{bcdef}	19 ± 2.5 ^{cdef}	91 ± 3.7 ^{ab}	93 ± 2.3	91 ± 4.6	87 ± 9.2
		Long	45 ± 5.5 ^{a}	63 ± 3.1 ^{a}	84 ± 3.9 ^{a}	97 ± 2.8 ^{a}	99 ± 0.9	97 ± 1.5	96 ± 0.9
	27	NP	-6 ± 7.6 ^{f}	5 ± 2.3 ^{ef}	14 ± 1.2 ^{ef}	90 ± 2.4 ^{ab}	94 ± 1.7	97 ± 2.3	97 ± 2.6
		HPP	9 ± 6.9 ^{bcdef}	11 ± 8.3 ^{bcdef}	25 ± 7.3 ^{bcdef}	89 ± 4.7 ^{ab}	93 ± 6.4	95 ± 4.2	94 ± 6.4
		Low	10 ± 5.6 ^{bcdef}	7 ± 4.6 ^{bcdef}	17 ± 1.8 ^{cdef}	96 ± 2.4 ^{ab}	97 ± 0.3	98 ± 0.7	99 ± 0.1
		Medium	8 ± 3.7 ^{bcdef}	6 ± 3.9 ^{def}	12 ± 3.2 ^{ef}	98 ± 1.1 ^{a}	97 ± 2.8	97 ± 3.3	99 ± 1.2
		Long	49 ± 10.8 ^{a}	63 ± 5.5 ^{a}	81 ± 3.3 ^{a}	98 ± 1.0 ^{a}	98 ± 0.3	98 ± 0.4	99 ± 1.5
Cranberry Juice	54	NP	2 ± 3.9 ^{ef}	3 ± 6.4 ^{ef}	16 ± 2.6 ^{cdef}	86 ± 8.6 ^{ab}	92 ± 5.5	93 ± 6.5	94 ± 7.2
		HPP	6 ± 0.8 ^{def}	8 ± 1.4 ^{def}	14 ± 0.4 ^{ef}	87 ± 11.4 ^{ab}	97 ± 2.2	97 ± 1.9	98 ± 1.1
		Low	7 ± 3.9 ^{cdef}	8 ± 2.9 ^{def}	16 ± 5.4 ^{cdef}	89 ± 5.1 ^{ab}	99 ± 0.7	99 ± 0.5	99 ± 0.7
		Medium	11 ± 4.2 ^{bcdef}	16 ± 2.3 ^{bcdef}	22 ± 6.8 ^{bcdef}	89 ± 3.1 ^{ab}	99 ± 0.7	98 ± 3.0	98 ± 2.7
		Long	22 ± 3.1 ^{bcd}	24 ± 6.1 ^{bcd}	34 ± 17.3 ^{bcd}	93 ± 4.9 ^{ab}	93 ± 3.8	94 ± 3.6	96 ± 0.9
	27	NP	2 ± 3.8 ^{f}	3 ± 7.8 ^{ef}	15 ± 6.8 ^{ef}	77 ± 22.9 ^{b}	92 ± 7.3	91 ± 8.9	93 ± 8.3
		HPP	8 ± 2.0 ^{bcdef}	9 ± 2.4 ^{def}	21 ± 2.7 ^{bcdef}	93 ± 3.4 ^{ab}	97 ± 2.1	99 ± 0.5	98 ± 0.7
		Low	10 ± 4.1 ^{bcdef}	9 ± 5.3 ^{cdef}	25 ± 8.4 ^{bcdef}	87 ± 13.7 ^{ab}	96 ± 1.6	97 ± 2.8	96 ± 3.9
		Medium	11 ± 3.2 ^{bcdef}	21 ± 13.9 ^{bcde}	31 ± 3.1 ^{bcde}	92 ± 1.8 ^{ab}	97 ± 1.5	95 ± 2.8	97 ± 3.0
		Long	26 ± 2.6 ^{b}	28 ± 6.8 ^{b}	40 ± 6.3 ^{b}	91 ± 8.6 ^{ab}	92 ± 7.6	95 ± 0.5	95 ± 3.5

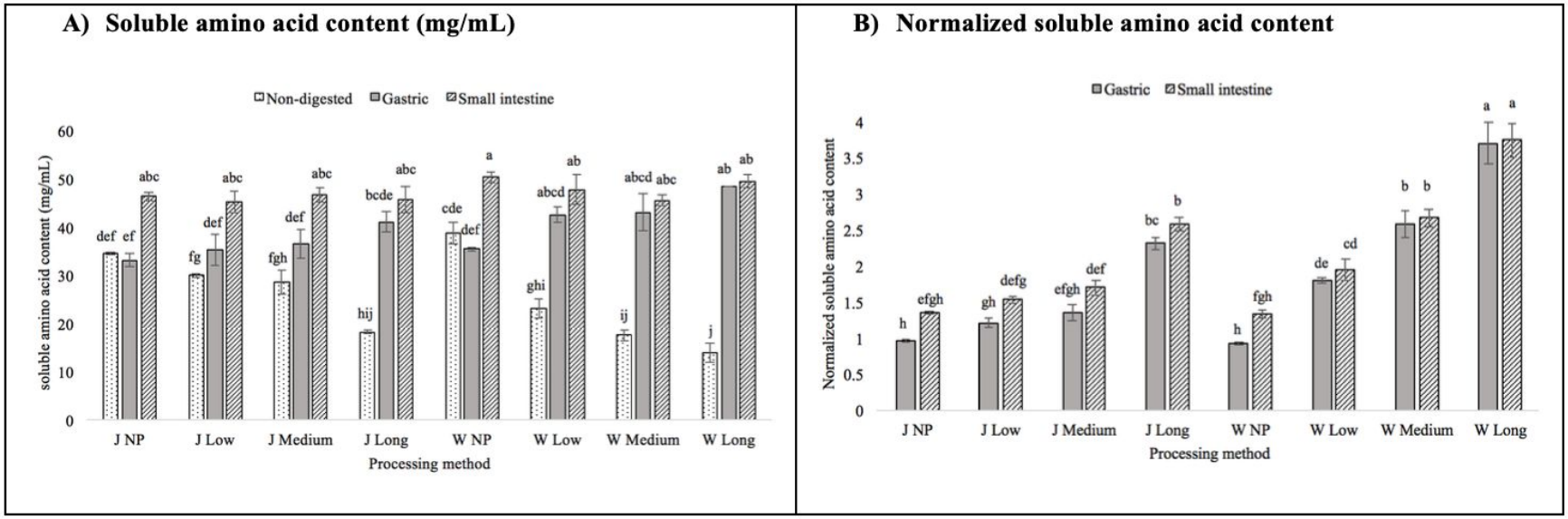
Table *4.* Soluble β-lactoglobulin digestibility (%) of whey protein isolate (27 and 54 mg/mL) in water and cranberry juice before digestion (0 min), during gastric digestion (15-120 min) and during small intestinal digestion (135-240 min) for samples that were non-processed (NP), high-pressure processing (HPP, 600 MPa for 4 min) and thermal processing: 85°C for 1 min (Low), 99°C for 10 s (Medium) and) 99°C for 5 min (Long). Values are expressed as averages (n=3) ± standard deviation. Different letters within each digestion time (each column) represent means that are statistically different ($p < 0.05$). No letters in the same row indicate that there is no significant difference.

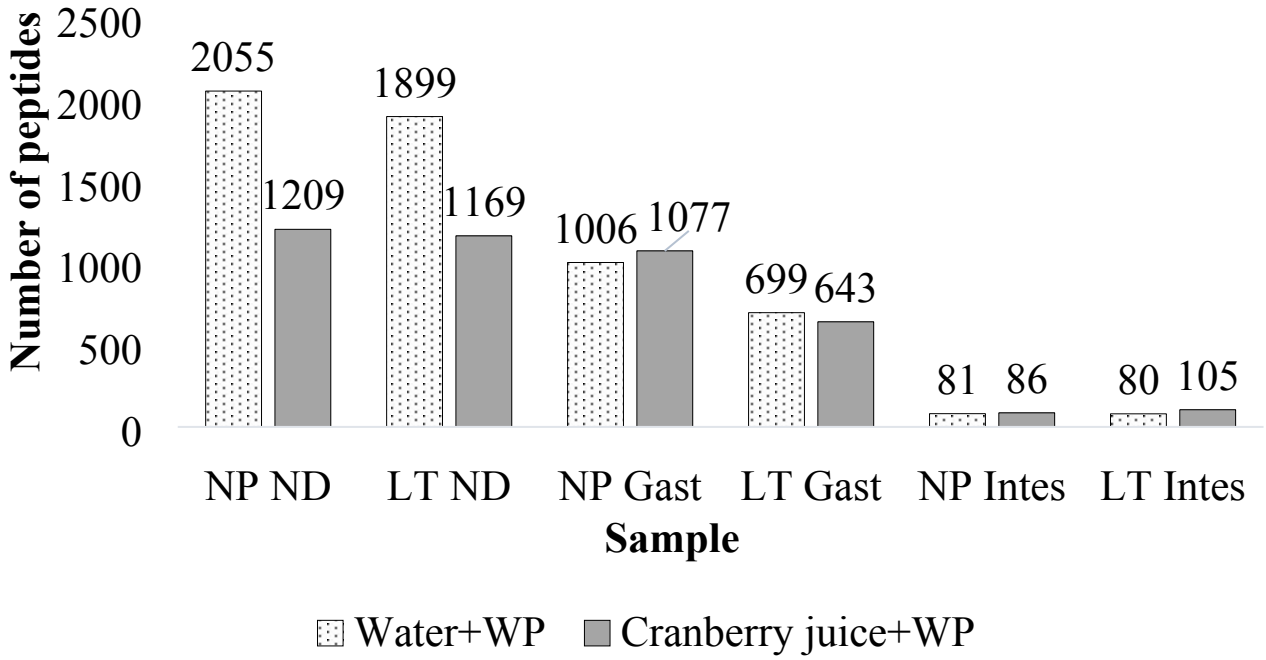
Samples	Peptides originating from casiens	Peptides originating from whey proteins
W NP ND	1247	808
J NP ND	611	598
W LT ND	1058	841
J LT ND	556	613
W NP Gast	313	693
J NP Gast	335	742
W LT Gast	185	514
J LT Gast	176	467
W NP Intes	16	65
J NP Intes	17	69
W LT Intes	10	70
J LT Intes	12	93

Table 5. Number of peptides originating from caseins and whey proteins in whey protein (WP) isolate (54 mg/mL) in water and cranberry juice non-processed (NP), not-digested (ND); and long-thermal processing (LT) at the end of the gastric (Gast) and small intestinal digestion (Intes).


Protein concentration 54 mg/mL
Non-processed
High-pressure processing
Low thermal processing
Medium thermal processing
Long thermal processing
Water
Cranberry juice
Time of digestion (min)
0 15 30 120 135 150 165 240
kDa
SA
AP
Dβ-lg
β-lg
α-la
T


Protein concentration 27 mg/mL
Non-processed
High-pressure processing
Low thermal processing
Medium thermal processing
Long thermal processing
Water
Cranberry juice
Time of digestion (min)
0 15 30 120 135 150 165 240
kDa
SA
Dβ-lg
AP
P
T
β-lg
α-la


A) Soluble amino acid content (mg/mL)
Non-digested
Gastric
Small intestine
soluble amino acid content (mg/mL)
Processing method
J NP
J Low
J Medium
J Long
W NP
W Low
W Medium
W Long
B) Normalized soluble amino acid content
Gastric
Small intestine
Normalized soluble amino acid content
Processing method


Number of peptides
2500
2000
1500
1000
500
0
2055
1209
1899
1169
1006
1077
699
643
81
86
80
105
NP ND
LT ND
NP Gast
LT Gast
NP Intes
LT Intes
Sample
Water+WP
Cranberry juice+WP

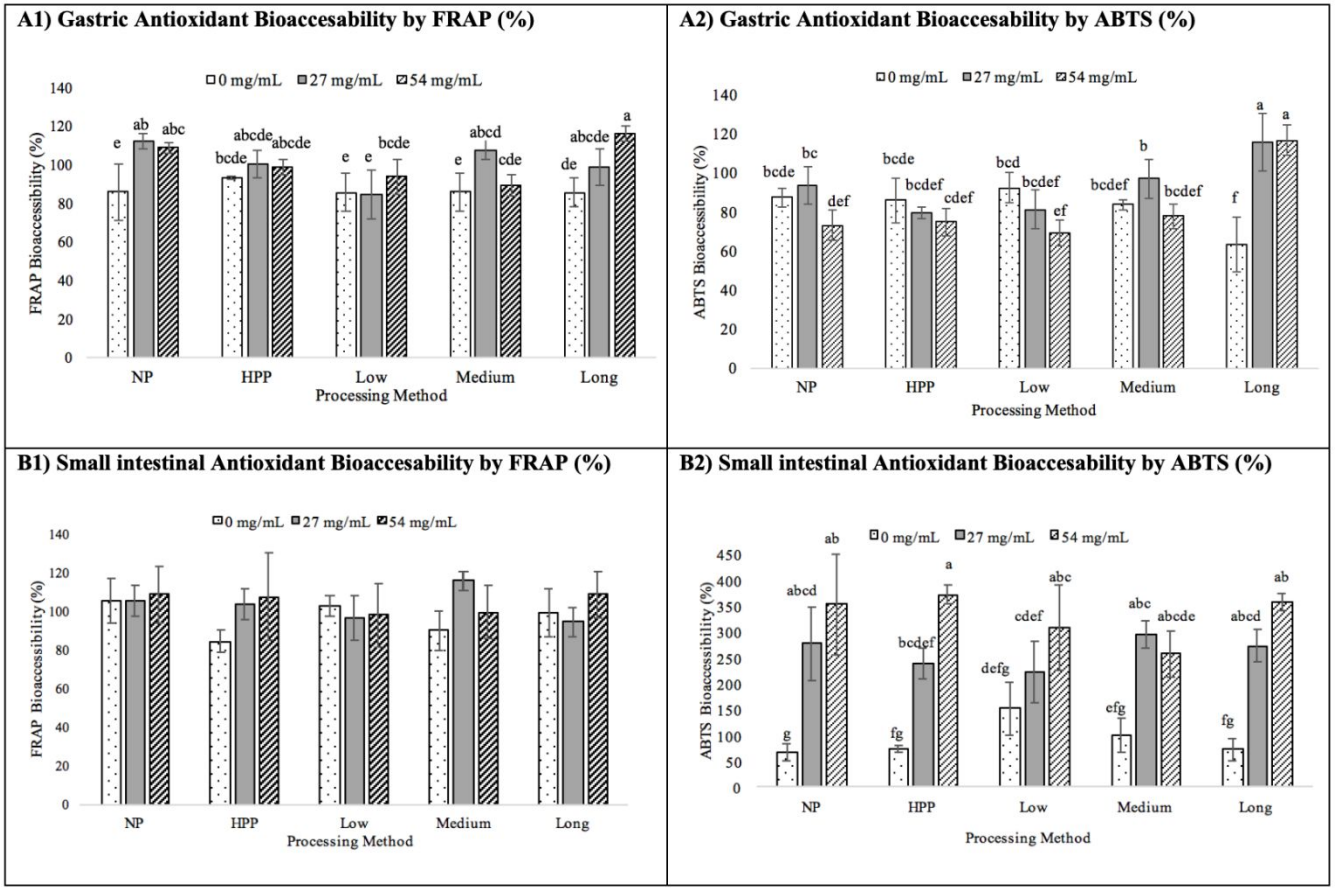

A1) Gastric Antioxidant Bioaccesability by FRAP (%)
0 mg/mL 27 mg/mL 54 mg/mL
FRAP Bioaccessibility (%)
Processing Method
NP HPP Low Medium Long
A2) Gastric Antioxidant Bioaccesability by ABTS (%)
ABTS Bioaccessibility (%)
B1) Small intestinal Antioxidant Bioaccesability by FRAP (%)
B2) Small intestinal Antioxidant Bioaccesability by ABTS (%)

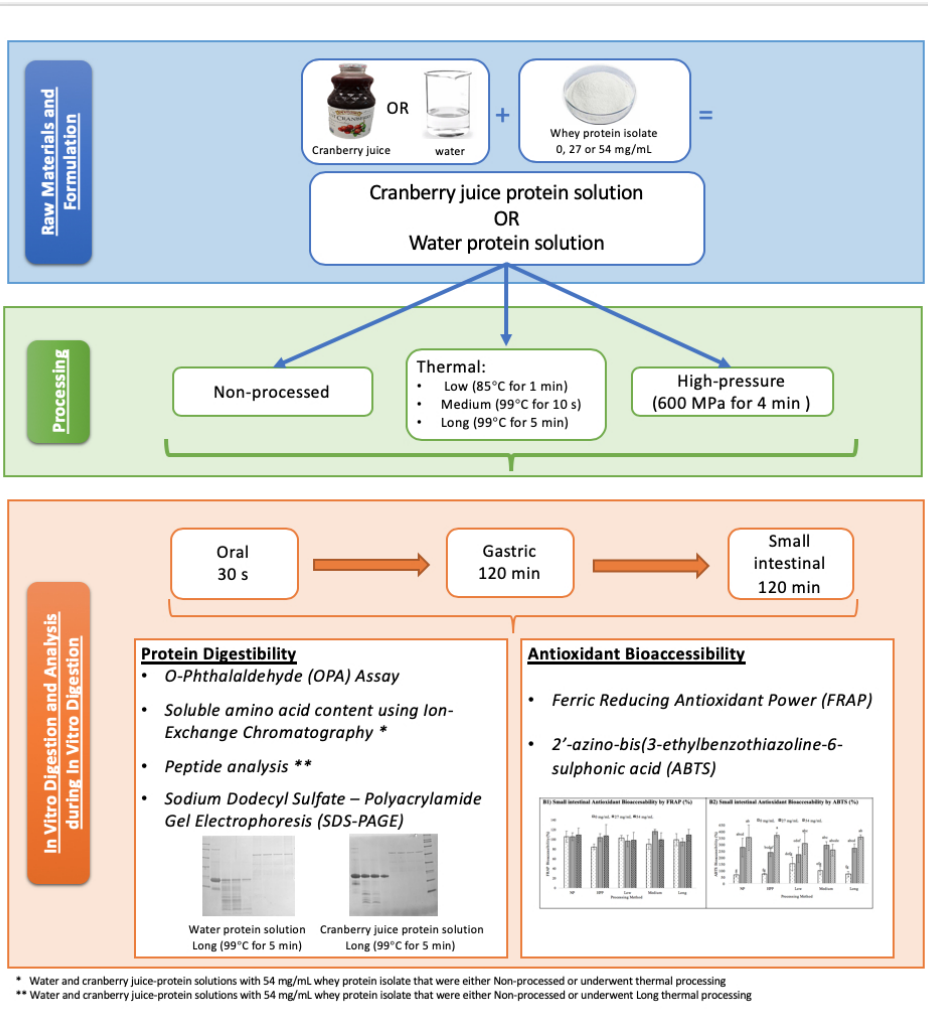
Figure 1. SDS-PAGE protein profiles from whey protein isolate (54 mg/mL) in water and cranberry juice that underwent different processing treatments that was not digested (0 min) and after in vitro gastric (15-120 min) and small intestinal digestion (135-240 min). For both solvents, the processing treatments are: non-processed, high-pressure processing (HPP, 600 MPa for 4 min), low thermal processing (85°C for 1 min), medium thermal processing (99°C for 10 s) and long thermal processing (99°C for 5 min). 20μL was loaded to each well (20 μg protein/well). In each gel, significant protein bands are denoted as follows: SA is serum albumin (66 kDa); Dβ-lg is the dimer of β-lactoglobulin; β-lg is β-lactoglobulin (18.3 kDa); α-la is α-lactalbumin (14.2 kDa); AP is amylase from porcine pancreatin (54 kDa) and T is trypsin (23.3 kDa).

Figure 2. SDS-PAGE protein profiles from whey protein isolate (27 mg/mL) in water and cranberry juice that underwent different processing treatments that was not digested (0 min) and after in vitro gastric (15-120 min) and small intestinal digestion (135-240 min). For both solvents, the processing treatments are: non-processed, high-pressure processing (HPP, 600 MPa for 4 min), low thermal processing (85°C for 1 min), medium thermal processing (99°C for 10 s) and long thermal processing (99°C for 5 min). 20μL was loaded to each well (20 μg protein/well). In each gel, significant protein bands are denoted as follows: SA is serum albumin (66 kDa); Dβ-lg is the dimer of β-lactoglobulin; β-lg is β-lactoglobulin (18.3 kDa); α-la is α-lactalbumin (14.2 kDa); AP is amylase from porcine pancreatin (54 kDa) and T is trypsin (23.3 kDa).

Figure 3. (A) Soluble amino acid content using ion-exchange chromatography (mg/mL) and (B) normalized soluble amino acid content of water (W) and cranberry juice (J) protein solutions with 54 mg/mL whey protein isolate with the following processing treatments: non-processed (NP) and thermal processing: 85°C for 1 min (Low), 99°C for 10 s (Medium) and) 99°C for 5 min (Long). Values are given as averages (n = 2) ± standard deviation of the mean. Different letters within the figure represent means that are statistically different ($p < 0.05$) between processing methods, solvent and digestion phase.

Figure 4. Number of peptides in whey protein (WP) isolate (54 mg/mL) in water and cranberry juice that were either non-processed (NP), or underwent long-thermal processing (LT) either not digested (ND), at the end of the gastric digestion (Gast), or at the end of small intestinal digestion (Intes).

Figure 5. Gastric (A) and small intestinal (B) antioxidant bioaccessibility by FRAP (%, 1) and ABTS (%, 2) of cranberry juice-protein solutions with 0 (no protein), 27 and 54 mg/mL of whey protein isolate with the following processing treatments: not processed (NP), high-pressure processing (HPP, 600 MPa for 4 min) and thermal processing: 85°C for 1 min (Low), 99°C for 10 s (Medium) and 99°C for 5 min (Long). Values are given as averages (n=4) ± standard deviation of the mean. Different letters within in each graph represent means that are significantly different ($p<0.05$) between processing treatments and protein concentration. No letters in the same graph indicate that there are no significant differences between processing treatments and protein concentration. Note that the y-axis in B2 has a greater scale (0-450 % vs. 0-140%) compared to the other panes to show the increases in ABTS bioaccessibility after small intestinal digestion.



* Water and cranberry juice-protein solutions with 54 mg/mL whey protein isolate that were either Non-processed or underwent thermal processing
** Water and cranberry juice-protein solutions with 54 mg/mL whey protein isolate that were either Non-processed or underwent Long thermal processing