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Whey-based sports supplements: heat damage and protein breakdown after *in vitro* gastrointestinal digestion

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Highlights

- Four whey protein(WP)-based sports supplements (SPS) were *in vitro* digested
- Protein breakdown and amino acid release in digests were promoted by WP denaturation
- Peptides with bioactivities beneficial to sports activities released in SPS digest

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ABSTRACT

This study was aimed to evaluate the effect of heat damage on the release of total amino acids (AA), essential AA (EAA), branched-chain AA (BCAA) and bioactive peptides following *in vitro* static simulated gastrointestinal digestion (SGID) of four commercial whey-protein based sports supplements. The extent of protein glycation and denaturation was evaluated through the determination of the content of furosine and soluble whey proteins. The strongest protein breakdown (41.3 %) and the highest release of AA, EAA and BCAA (36.20, 27.78, and 11.30 g/100 g protein, respectively) was observed in the sports supplement characterised by the lowest (52.5 %) level of soluble whey proteins; whereas the protein glycation had a negligible impact on the studied parameters. The SGID also led to the release of several peptides with various reported bioactivities that may be beneficial to sports activity.

Keywords: whey-based sports supplements; *in vitro* static digestion; heat damage; protein breakdown; amino acids; bioactive peptides

1. Introduction

Bovine caseins (CN) and whey proteins (WP) are utilized, either alone or in combination, for manufacturing sports supplements (SPS). Whey proteins account for approximately 18% of the total protein content of bovine milk. Beta-lactoglobulin (LG) and α-lactalbumin (LA) are the predominant (45–55% and 15–25%, respectively), followed by immunoglobulins (10–15%), glycomacropeptide (10–15%, GMP, only in cheese sweet whey), bovine serum albumin (BSA, 10%), and other minor proteins (<1%) such as lactoferrin and lactoperoxidase (Goulding, Fox, & O'Mahony, 2020).

Different types of WP are commercially available, and they include WP concentrates (WPC), WP isolates (WPI) and WP hydrolysates (WPH). Whey protein concentrate and WPI are obtained from whey resulting from cheese-making process or acid casein production. The whey is submitted to heat treatment (pre-heating) to achieve the microbiological safety and then passed through ultrafiltration (UF) membranes to retain WP. Diafiltration of UF retentate is applied during manufacturing of WPC with high protein content (>60%) and WPI (>90%). After concentration (evaporation) and spray-drying of the retentate, WP powders with a protein content ranging from 35 to 95% are obtained. The amount of individual WP can vary as application of ion exchange chromatography can provide additional protein selectivity and enrichment in manufacturing of WPI. Hydrolysates are partially enzymatically hydrolyzed WPC or WPI and, therefore, they are characterized by presence of short peptides and free amino acids (AA) depending on the proteolysis extent they underwent. Generally, WPH designed for SPS contain moderately-to-highly (2–8%) hydrolysed WP (Foegeding, Luck and Vardhanabhuti, 2011).

Whey proteins in either native or hydrolyzed forms are used for formulating infant formulas, supplements for nutritional and therapeutic foods, and, historically, for bodybuilding and sports nutrition. In this regard, WPC, WPI and WPH largely predominate as protein source for SPS formulation (Couto Almeida, Guerra Monteiro, Reis Carneiro da Costa-Lima, Silveira Alvares, & Conte-Junior, 2015). Potential interesting nutritional and metabolic effects such as protein replenishment, increased muscle mass, weight loss, increased energy intake and improved performance as well as recovery justify the consumption of WP-based SPS (West, Abou Sawan, & Moore, 2017). Whey proteins have also the functions of improving immunity, anti-oxidation, alleviating fatigue, enhancing muscle exercise capacity, and strengthening bone quality (Zhao & Ashaolu, 2020). Whey proteins are high-quality proteins containing all essential amino acids (EAA), and they are relatively rich in the branched-chain AA (BCAA) (Brennan, Keerati & Bolster,2019). Besides, WP (mainly LA) are particularly rich in the AA Trp, a limiting factor in serotonin synthesis (Markus, Olivier, & de Haan, 2002), and in sulfur-containing AA involved in the synthesis of glutathione, a tripeptide characterized by high antioxidant activity (Parodi, 2001).

Sports supplements contain WPI and WPC with variable level of heat denaturation. Beta -lactoglobulin and LA are globular heat-sensitive proteins and, during processing of native whey, their structure can denature and aggregate due to the pre-heating treatment. In addition, this manufacturing step, together with concentration by evaporation and spray-drying, may result in protein glycation and protein cross-linking (Cattaneo, Masotti, & Pellegrino, 2012). The extent of the changes caused by heat treatment can affect the digestibility of WPC/WPI (Cattaneo, Stuknyte, Masotti, & De Noni, 2017) and consequently the release as well as bioavailability of AA and peptides in the gastrointestinal tract. This has a substantial nutritional relevance considering that WP are an excellent source of EAA and BCAA, which are paramount for their effectiveness in sports nutrition (Couto Almeida et al., 2015). Indeed, digestion of WP leads to an increase of the AA Ile, Leu and Val circulating in blood. These BCAA act as a substrate in protein synthesis and energy production. In combination with exercise, they lead to an increase in muscle mass, play a key role in neurotransmission processes and glucose metabolism, and help to improve symptoms of the syndrome of cachexia (Piestansky, Matuskova, Cizmarova, Olesova, & Mikus, 2021).

Whey proteins may be also an important source of bioactive peptides (BAPs) that are released during both manufacturing (enzymatic hydrolysis) and digestive processes. The effects of such BAPs have long been known and studied, and recently there has been increasing scientific evidence of their health benefits in sports nutrition (König, Kohl, Jerger, & Centner, 2021). Angiotensin-converting enzyme inhibitory (ACE-inhibitory), Dipeptidylpeptidase-IV (DPP-IV) inhibitory, cytomodulatory, antioxidant, anti-inflammatory, antimicrobial, opioid and mineral-binding peptides are the main reported functionalities (Madureira, Tavares, Gomes, Pintado, & Malcata, 2010). Some authors have also highlighted the positive effects of certain whey BAPs on vascular function, body composition and the improvement of endurance performance (Zhao, Chen, & Ashaolu, 2022). It has also been reported that peptides derived from WP can improve muscle regeneration processes (Lollo et al., 2014) and prevent sarcopenia (Cheah & Cheah, 2023).

In general, the effects of proteins of different sources and AA composition have been the focus of sports-specific research for many years. To the best of our knowledge, the AA and peptide profiles of SPS after *in vitro* gastrointestinal digestion have not been evaluated so far, also in relation to heat damage of the WP they contain. Moreover, no studies reported the BAPs profile of digested WP-based SPS. Considering the potential physiological effects resulting from the consumption of SPS, the present study was undertaken to determine the heat damage suffered by four commercial WP-based SPS, and to assess the AA and (bio)peptides released after *in vitro* static simulated gastrointestinal digestion (SGID).

2. Materials and methods

2.1. Sports supplement samples

Four samples of WP-based powdered SPS were selected from a local sports food store; they were kept under refrigerated conditions and analyzed within one week. According to labels, SPS contained soy lecithin and WPI (samples S1, S2 and S3) or WPC (sample S4). The protein, lactose and water contents of SPS are reported in Table 1. These constituents were determined according to standards ISO 8968:2014, ISO 22662:2007 and ISO 5537:2004, respectively. The standard ISO 17997-1:2004 was adopted for the determination of the casein nitrogen content.

2.2. Evaluation of heat-damage of sports supplements

The content of furosine (FUR) was determined according to ISO 18329/IDF 2012 method. Briefly, a sample aliquot, corresponding to 50 mg of protein, was hydrolyzed (with 8 N HCl) at 110 °C for 23 h, and then submitted to solid phase extraction on a 500 mg C18 cartridge (Millipore, Milford, MA, USA). Ion Pairing – Reverse phase HPLC was performed on a C8 furosine-dedicated column (Alltech, Lokeren, Belgium) with the UV detection at 280 nm. The level of soluble WP was evaluated according to ISO 13875/IDF 2012 methods.

2.3. In vitro *static simulated gastrointestinal digestion of sports supplements*

Enzyme activity determination and *in vitro* SGID were performed according the INFOGEST static protocol as reported by Brodkorb et al. (2019). Salivary, gastric and intestinal simulated fluids (SSF, SGF and SIF, respectively) were prepared accordingly. In detail, SPS powders were dissolved in distilled water to the concentration (25–30 g/100 mL water) recommended by the manufacturer of SPS. Five mL of sample were supplemented with 5 mL SSF at pH 7.0 and kept for 2 min at 37 °C to reproduce the salivary phase of digestion. The gastric phase was performed by adding 10 mL SGF, porcine pepsin (2000 U/mL digest) and rabbit gastric lipase (60 U/mL) along with 1 M HCl to reach pH 3.0. The gastric phase was simulated at 37 °C for 2 h in slight stirring. Upon completion of the gastric phase, 20 mL SIF containing bile salts (20 mM) were aliquoted to each digest. Subsequently porcine pancreatin (8 × USP,100 U/mL trypsin activity) was added, and the intestinal phase was performed at 37 °C for 2 h at pH 7.0 (reached with 1 M NaOH) in slight stirring. At the end of the SGID, the samples were immediately frozen. The mineral salts for simulated fluid preparation, enzymes and bile salts were from Merck (Darmstadt, Germany). Each digestion was carried out in triplicate.

2.4. Determination of protein breakdown

Sports supplement powders were dissolved in distilled water to the concentration recommended by the manufacturer of SPS (as reported above) and ultrafiltered through a 3 kDa regenerated cellulose membrane in an Amicon stirred ultrafiltration cell (Merck, Darmstadt, Germany). The SPS digests were ultrafiltered in the same conditions as they are. The amount of the nitrogen (N) content was determined to assess the extent of protein breakdown before and at the end of SGID. The protein breakdown (as % of total N, TN) was calculated by Eq. (1):

$$((NSGID - Nb) – NSB)/TN \times 100 \quad (1)$$

where: NSGID, N (nitrogen) content of the UF (3 kDa) permeate of the samples after SGID; Nb, N content of the UF (3 kDa) permeate of the blank SGID sample (enzymes, bile salts and simulated digestive fluids) after SGID; NSB, N content of UF (3 kDa) permeate of the samples before SGID; TN, total N content of the samples.

The N content of each fraction was determined by Kjeldahl method according to the International standard ISO 8968–2014.

2.5. SDS-PAGE

SDS-PAGE of SPS before and after SGID was performed using 12% polyacrylamide gels. Before analysis samples were diluted 1:1 (v/v) with Tricine Sample Buffer (Bio-Rad Laboratories, Hercules, CA, USA) containing 2% 2-mercaptoethanol and heated at 95 °C for 5 min. Gels were run in TRIS/Tricine/SDS Running Buffer (Bio-Rad) on a Mini vertical electrophoresis unit (SE250, Hoefer, Holliston, MA, USA) at a constant voltage of 60 V. Standard proteins of LA and LG (Merck), Na caseinate (Fonterra, Auckland, New Zealand) and Spectra Multicolor Low Range Protein Ladder (1.7–40 kDa; Thermo Fisher Scientific, Pierce Biotechnology, Rockford, IL, USA) were run together with the samples. Finally, gels were stained with Coomassie Blue R-250.

2.6. Determination of free amino acids by HILIC UPLC/HR-MS

In vitro digested samples of SPS were centrifuged 10,000 x *g* for 10 min and the obtained supernatant was filtered through a 3 kDa MWCO Omega polyethersulfone UF membrane in a Nanosep Advance device (Pall, Life Sciences, Port 266 Washington, NY, USA). An Acquity UPLC module (Waters, Milford, MA, USA) coupled to the Q Exactive high-resolution mass spectrometer (HR-MS, Thermo Fisher Scientific, San Jose, CA, USA) was used to quantify free AA in the digests. Ten µL of sample (diluted 10:90 with eluent B) were separated with an Acquity UPLC (Waters, Milford, MA, USA) on an Accucore 150 Amide HILIC column (150×2.1 mm, 2.6 µm) (Thermo Fisher Scientific) kept at 30 °C. Eluents were 90/10 (v/v) water/200 mM aqueous ammonium formate at pH 2.8 (eluent A) and 90/10 (v/v) acetonitrile/200 mM aqueous ammonium formate at pH 2.8 (eluent B). Mobile phases were prepared as described by Park et al. (2019). For the UPLC separation, a linear elution gradient was applied (100% to 85% of eluent B in 8 min) at a flow rate of 0.4 mL/min, followed by a column wash and re-equilibration (run-to-run time 20 min). The LC eluate was analyzed by HR-MS on a Q Exactive instrument (Thermo Fisher Scientific) interfaced through a HESI-II probe for electrospray ionization (Thermo Fisher Scientific). The ion source and interface conditions were: spray voltage 2500 V (positive polarity); probe heater temperature 280 °C; ion transfer tube temperature 350 °C; S-lens RF level 50; sheath gas pressure 35 psig; auxiliary gas pressure 15 psig. Mass spectra were acquired over the *m/z* range 50–500 in positive polarity in a Full MS mode. The resolution was set at 70,000. The automatic gain control (AGC) target was 3×10^6, and maximum ion injection time was 200 ms. Identification and quantification of AA was conducted using the Xcalibur software (v3.0, Thermo Fisher Scientific). Peak areas were calculated from extracted ion chromatograms of each AA protonated ions, with 3 ppm mass tolerance. Analyses were performed in triplicate and the results were expressed as means ± standard deviation. Serial dilutions of single AA were prepared as external standards (5 points calibration curve).

2.7. Determination of peptides in sports supplement digests by reverse-phase UPLC/HR-MS/MS

Five µL of 10-kDa-ultrafiltered [Omega polyethersulfone UF membrane in a Nanosep Advance device (Pall Life Sciences)] samples were separated with an Acquity UPLC module (Waters, Milford, MA, USA) on an Aeris PEPTIDE XB-C18 column (150×2.1mm, 1.7µm) (Phenomenex, Torrance, CA, USA) kept at 50 °C. Eluents were: 0.1% (v/v) formic acid (FA) in MilliQ-treated water (solvent A) and 0.1% (v/v) FA in acetonitrile (solvent B). For the UPLC separation, a linear elution gradient was applied (2% to 55% of solvent B in 35min) at a flow rate of 0.3mL/min. The LC eluate was analysed by UPLC/HR-MS/MS on a Q Exactive instrument (Thermo Fisher Scientific, San Jose, CA, USA) interfaced through a HESI-II probe for electrospray ionization (Thermo Fisher Scientific). The ion source and interface conditions were set as previously reported (Cattaneo et al., 2017). The LC eluate was analyzed by MS using full scan and data dependent tandem MS analysis (ddMS^2) of the ten most intense ions (Top10). Mass spectra were acquired over m/z range 100–1500; the ten most intense 1^+–8^+-charged ions detected in each spectrum underwent HCD fragmentation (ddMS^2, data dependent scan acquisition mode). The resolution was set at 70000 and 17500 for full scan and ddMS^2 scan types, respectively. The AGC targets were 1×10^5, and maximum ion injection times were 110ms.

High-resolution mass spectrometry data were processed and peptides were identified with Proteome Discoverer v1.4 software (Thermo Fisher Scientific). Automatic peak detection was performed with a setting of a signal-to-noise ratio of 4. Peptide sequences were identified from MS/MS spectra using SequestHT algorithm against the protein database of *Bos taurus* (UniProt taxon ID 9913). A non-specific enzyme cleavage pattern was defined, and 12 missed cleavage sites (maximum allowed for the algorithm) were set. Phosphorylation of Ser and Thr, deamidation of Asp, Gln and Arg, oxidation of Met and cyclisation of an N-terminal Gln to pyro-Glu were selected as dynamic modifications. Mass error tolerance for precursor ions was 5 ppm and for fragment ions was 0.02 Da. A strict false discovery rate of peptide identification was set (FDR=0.01). Two digests (obtained in a two consecutive days) of the same sample were used for the UPLC/HR-MS/MS analysis by performing double injections of each. The resulting four data processing outputs were merged to obtain multiconsensus reports with an absolute number of peptides that was found in any sample.

Peptides identified in all samples were examined for homology using the Milk Bioactive Peptide Database (Species *Bos taurus*, updated January 15, 2024) (Nielsen et al., 2023). The search was performed setting the following parameters: “100” for similarity threshold, “identity” for AA scoring matrix and “yes” for getting extra output.

2.8. Statistical analysis

In order to study significant differences between the variables, analysis of variance (ANOVA) was performed, with a confidence interval of 95% ($p < 0.05$). The statistical difference was determined using the Daniel’s XL Toolbox addin for Microsoft Excel, version 7.3.4 (Daniel Kraus, Würzburg, Germany; www.xltoolbox.net).

3. Results and discussion

3.1. Gross composition of sports supplements

Whey protein isolate and WPC are extensively used for applications in which proteins play a dominant nutritional and functional role such as for manufacturing of SPS. In this study, we considered four commercial products of WP-based SPS, named S1, S2, S3 and S4. We firstly analyzed SPS for protein, lactose and water contents (Table 1). The determined values overlapped those reported in literature for similar SPS. For instance, Sánchez-Oliver, Contreras-Calderón, Puya-Braza, & Guerra-Hernández (2018) analyzed 14 and 3 samples of protein powder containing only WPC or WPI, respectively. The mean sugar and protein contents of the former were 10.9 and 80.8 g/100 g, respectively, while the lowest sugar content was 0.9 g/100 g. In WPI, the sugar level ranged from <1.0 to 2.3 g/100 g, and that of proteins from 84.5 to 94.6 g/100 g. Overall, these values, and those of the SPS considered in the present study, reflect the different processing conditions adopted for manufacturing of WPC and WPI.

3.2. Determination of the protein profile of sports supplements

We assessed the protein profile of SPS by SDS-PAGE (Fig. 1), and revealed the presence of a significant amount (25.3 g/100 g) of CN, not declared on the label, in sample S1. In WP-based SPS the presence of low CN amount and CN-derived peptides can be justified. Indeed, whey (generally deriving from cheese manufacture process) used as a raw material for SPS production contains small amount of CN according to the adopted cheese-making technology. These are CN (sub-)micelles that remain in the WP powders even after UF and/or diafiltration processes. On the other hand, the presence of high concentrations of CN, such as in sample S1, is likely attributable to addition of caseinates or milk protein concentrate powders.

3.3. Heat-damage of sports supplements

We assessed the heat-damage through the determination of furosine and soluble WP. The former index evaluates the extent of Maillard reaction (MR) induced in food by heat load, and in dairy products it refers to the amount of lactulosyllysine (ε-*N*-(furoylmethyl)-l-lysine), i.e. the Amadori compound formed during the initial phase of MR upon the reaction of Lys and lactose (Rufián-Henares, Delgado-Andrade, Jimenez-Perez, & Morales, 2007). Generally, very low levels of lactose are present in WPI, as the diafiltration process applied in their production process eliminates soluble components, lactose among them. Nonetheless, the removal of lactose is highly variable, and it is influenced by the production process, pH and washing steps (diafiltration) the product undergoes (Cattaneo et. al., 2012).

Low amounts of lactose were present in samples S1, S2 and S3, whereas the highest level was detected in sample S4 (Table 1). Reducing sugars can react with WP during heating, spray-drying and storage of SPS, thus enhancing MR development. The FUR levels of samples S1, S2 and S3 were 158 ± 5, 176 ± 8 and 125 ± 6 mg/100 g protein, respectively. The highest level (354 ± 11 mg/100g protein) characterized sample S4, which also presented the highest lactose content (Table 1). The FUR values of the studied SPS were within the range provided by Rufián-Henares et al. (2007), who assessed the heat damage of different commercial SPS based on WPI or WPC as protein ingredients. In detail, the FUR level ranged from 2.8 to 1125.7 mg/100 g protein, and it was usually lower in samples containing mainly WPI, and with an average value of 150 mg/100 g of protein. The same authors suggested a reference FUR value of 70 mg/100 g protein as a quality marker of dried milk-protein ingredients used in SPS formulation. Sánchez-Oliver et al. (2018) obtained similar results since they found three times less FUR in SPS containing WPI than in SPS containing WPC (181 vs 464 mg/100 g protein, on average).

Heat denaturation of WP proceeds according to time/temperature ranges characteristic for each protein. Denaturation of LA and LG begins at about 70 °C. The undenatured (native soluble) forms of WP can be quantified and their level is considered a reliable index for assessing the heat damage of WPC and WPI (Wouters, Boeve, Dams, & Joye, 2022). In this regard, very similar content of soluble WP characterized samples S1, S3 and S4 (73.6, 72.3 and 77.3% of total WP, respectively). Contrarily, sample S2 showed the lowest content (52.5% total WP). Overall, these values were not directly related to the FUR values. This fact is likely due to the different activation energy, and hence kinetics, that characterizes MR and WP denaturation, which can differently develop during the different (pre-)heating and spray-drying steps adopted for WPI and WPC manufacturing (Cattaneo et. al., 2012).

3.4. Protein breakdown of digested sports supplements

The heat treatments in presence of reducing sugars applied in WPC and WPI manufacturing may induce protein denaturation, aggregation, glycation and cross-linking. These phenomena can modify the protein structure altering the enzymatic protein breakdown, and thus the kinetics of peptide and AA release during gastrointestinal digestion (Rutherfurd and

Moughan, 2012). Therefore, it is important to consider enzymatic degradability of WP when the nutritional properties of WP-based SPS are compared. As it is known, native LG is not digested by pepsin, since the specific cleavage sites for the enzymes are generally buried in the protein hydrophobic core. On the contrary, LG unfolding upon heating causes loss of tertiary structure and an increase in digestibility as the protein is more accessible to digestive enzymes (Singh, Øiseth, Lundin, & Day, 2014). The results that we obtained from the evaluation of the protein breakdown support these findings. Indeed, sample S2 presented the highest level of protein breakdown (41.3 ± 2.1 % of total N) along with the lowest content of soluble (undenatured) WP (52.5% total WP). We determined the lowest level of protein breakdown (33.7 ± 1.1 %) in sample S4 along with the highest content of soluble WP (77.3% total WP). The samples S1 and S3 overlapped for protein breakdown (36.9 ± 1.9 and 36.6 ± 1.4 % of total N, respectively) and WP denaturation (73.6 and 72.3% total WP, respectively). Different authors reported a reduction of protein hydrolysis during *in vitro* digestion of glycated (Chevalier, Chobert, Popineau, Nicolas, & Haertl, 2001) or cross-linked proteins via MR (Gerrad et al.,2012). The data obtained in this study show that the effect of MR on protein breakdown during SGID appears to be less pronounced than that observed for protein denaturation. The protein breakdown of sample S4 was only slightly lower than that of the other samples, although it showed more than double FUR value.

3.5. Free amino acid profile of digested sports supplements

Prompt degradability of WP is important for SPS, since it results in a great increase in postprandial plasma AA availability and thereby further stimulated postprandial muscle protein synthesis (Zhao & Asholou, 2020). The total free AA content in sample S2 overcame those of the other samples (Table 2). About 36% and 20–25% of WP were degraded to free AA in sample S2 and the other samples, respectively. Sample S2 presented the lowest level of soluble WP (52.5% total WP) and the highest level of protein breakdown (41.3 ± 2.1 % of total N).

Generally, athletes take advantage of WPC and WPI because protein hydrolysis provides biologically important AA. In this regard, EAA in SPS are primarily responsible for stimulation of muscle protein synthesis following ingestion (Cuthbertson et al., 2005). Essential AA also help regulating several important metabolic pathways of immune cells, from cell response to oxidative stress and anti-inflammatory response, all of which are essential for an optimal immune function and for recovery after physical training (Cruzat, Krause, & Newsholme, 2014). In the present study, EAA accounted for 70–77% of total free AA in all digested SPS (Table 2). The highest and the lowest contents of EAA were recovered in digests of samples S2 and S4, respectively (Table 2). Bovine LA and LG contain approximately 23–28% of BCAA. The total free BCAA content of the sample S2 almost doubled that of the other SPS samples (Table 2), while BCAA represented 25–31% of total free AA released in all digested samples. In detail, Leu accounted for about 71–81% of BCAA. This AA mostly acts in stimulating muscle protein synthesis and in decreasing protein degradation (Jäger et al., 2017). It has been suggested that intake of 1–3 g Leu can maximize muscle protein synthesis in active and trained individuals (Jäger et al., 2017). By considering the protein content of the studied SPS and their recommended dosage (25–30 g), they would provide about 1.2–2.5 g Leu per serving. In addition to Leu content, it has been suggested that the rapidity of protein digestion and thus the peak plasma concentration of Leu is important for achieving maximal muscle protein recovery (Phillips, 2014).

In digested sample S2, we determined greater amounts of other EAA, namely Met, Thr and Lys, and the highest content (8.4 g/100 g protein) of non-EAA (Table 2). This sample also presented the highest content (17.2 g/100 g protein) of Phe, Trp, Tyr and Leu potentially causing bitter taste. In comparison, the SPS digests S1, S3 and S4 possessed 13.9, 12.2 and 9.9 g/100 g protein, respectively. The release of Trp in digests was quite similar for samples S1, S2 and S3 (Table 2). Tryptophan is a precursor of serotonin, which is known to suppress food intake and promote satiety (Halford, Boyland, Lawton, Blundell, & Harrold, 2011). Alpha-lactalbumin is easily and quickly ingested, having a higher content of Trp in comparison to LG (6% vs 2.6%), and thus is considered a source of this AA (Markus et al., 2002). Overall, the highest content of both free EAA and free BCAA characterized the digested sample S2.

Summing-up, the higher was the degree of protein breakdown the larger was the release of total AA, BCAA and EAA (Fig. 2).

The data obtained overlap those observed for the protein breakdown and confirm the enhancing effect of whey protein denaturation and the negligible role of the measured level of glycation on the release of amino acids. This evidence is justified by the fact that AA can be considered as the final product of protein digestion.

3.6. Peptide profile of digested sports supplements

We determined the sequences of peptides by UPLC/HR-MS/MS and identified a total of 527, 501, 362 and 386 unique peptides in digests of samples S1, S2, S3 and S4, respectively (Table 3).

On average, the species deriving from β-CN and LG represented more than half of the detected peptides (Table 3). As previously reported, the occurrence of CN-derived peptides in digested WPC and WPI can be explained by the presence in the whey of both casein micelles or submicelles that are not retained in the coagulum during cheese-making or acid casein manufacturing. More specifically, presence of β-CN peptides was likely related to the hydrolysis of proteose peptones, which derived from the plasminolysis of β-CN and were retained during UF. Peptides deriving from κ-CN could also arise from GMP deriving from rennet coagulation of cheese milk. Other research studies also reported contamination with CN (peptides) partially hydrolyzed WP (Wada & Lönnerdal, 2015). Overall, LG released the highest number of peptides in digests with exception of sample S1, which contained CN. This resulted in the highest number of peptides being released from CN. About half of the peptides equally released from WP and CN in digested samples S2 and S4. In sample S3, peptides from WP largely prevailed on CN peptides.

Overall, the detected peptides showed a MW from 396 to 3201 Da, and their distribution within the range <0.5 to 3.5 kDa was very similar among SPS digests (Fig. 3). Those with MW in the range 0.5–1 kDa largely prevailed, followed by peptides sized 1.0–1.5 kDa. It should be noted that MS/MS data processing algorithm did not allow to reveal di- and tri-peptides.

Among the detected peptides, 134, 157, 28 and 33 were distinctive to digests of samples S1, S2, S3 and S4, respectively (Fig. 4). Contrarily, the digests shared 99 CN-derived and 128 WP-derived peptides (Fig. 4).

Among the common peptides, 33 were ascribed to BAPs (Table 4) by searching against the Milk Bioactive Peptide Database (Nielsen et al., 2023). Contrarily, 18, 5 and 2 BAPs were identified only in digests of samples S1, S2 and S3, respectively when matching sequences against the same Database (Table 4). Two-thirds of BAPs released from CN in a digest of sample S1.

As reported by König et al. (2021), there is scientific evidence for positive effects of certain BAPs in sports nutrition and performance. In this regard, most of the common or distinctive BAPs found in digests may be relevant, as they have been reported to elicit ACE-inhibitory, DPP-IV-inhibitory, antimicrobial and antioxidant properties (Table 4) (Zhao et al., 2020).

Angiotensin-converting enzyme-inhibitory peptides represented the main class of BAPs identified in SPS digests. It is known that some BAPs can improve endothelial function by suppressing ACE and influencing performance (Ballard et al., 2013). Moreover, BAPs are increasingly considered as a crucial factor for regulating muscle protein turnover (Lollo et al., 2014). We found the peptides YQEPVLGPVR and LNVPGEIVE from β-CN in all SPS digests, and they, together with the WP-derived peptides VSLPEW (from LA), IIAEK, IPAVFK VLDTDYK, ALPMH, GLDIQK, LDIQK and ALPMHIR (from LG), were reported to exhibit strong ACE-inhibitory activity (Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000). The presence of the antihypertensive peptide ALPMHIR was revealed in all digests. This peptide was previously found by the other investigators to be absorbed through the mucosal side of Caco-2 cell monolayers in an Ussing chamber and to reach the serosal side undegraded (Vermeirssen et al., 2002). Other ACE-inhibitory peptides (VAGTWY from LG and LHLPLPL, KVLPVPQ, NIPPLTQTPV from β-CN) were present only in digests S1, S2and S4 (Table 3). The species YKVPQL (from α_{S1}-CN), AMKPW (from α_{S2}-CN), NIPPLTQTPVVVPPFLQ (from β-CN), PGPIPN (from β-CN) and LDTDY (from LG) were present only in sample S1. Interestingly, other research studies found these CN-derived peptides in hydrolysates of milk and milk proteins produced by fermentation with (proteinase of) lactic acid bacteria (Hayes et al., 2007).

The peptides PFPGPIPN, YPFPGPIPN and VYPFPGPIPN from β-CN are known as multi-functional BAPs with ACE-inhibitory, antioxidant, DPP-IV-inhibitory and opioid agonist activities (Eisele, Stressler, Kranz, & Fischer, 2013). The species YPFPGPIPN and VYPFPGPIPN were reported to survive *in vitro* SGID (Tagliazucchi, Helal, Verzelloni, & Conte, 2016). They were also found in human gastrointestinal tract (Boutrou et al., 2013). Also, the peptides TTMPLW and DAYPSGAW from α_{S1}-CN can inhibit ACE (Pihlanto-Leppälä, Rokka, & Korhonen, 1998). The antihypertensive activity of the former peptide was also demonstrated *in vivo* when administered to rats (Maruyama, Mitachi, Aawaya, Kurono, Tomizuka, & Suzuki, 1987). Otte, Shalaby, Zakora, & Nielsen (2007) described the peptide VSLPEW, found in an enzymatic hydrolysate of LA, as a potent ACE-inhibitor. This peptide was present in all our studied digests.

During SGID, CN and WP can also release antioxidant peptides, which protect from the oxidative damage and the onset of oxidative diseases (Tagliazucchi et al., 2016). Bioactive peptides potentially eliciting antioxidant properties generally possess hydrophobic AA (Pro, Leu, Phe, Trp, Tyr and Met) at their C-terminus, thus making the peptides capable to bind free radicals (Tavares et al., 2011). In addition, AA with aromatic residues (His and Pro) can donate electrons to charged radicals and potentially prevent oxidative stress, which can damage cellular constituents without suitable scavenging and clearance of reactive oxygen species. Among the antioxidant BAPs, we found the peptide YFYPEL in the digest of sample S1. This peptide is a fragment released form α_{S1}-CN, and it has free radical scavenging effects (Suetsuna, Ukeda, & Ochi, 2000). As reported by Corrochano, Buckin, Kelly, & Giblin (2018), also peptides from WP have shown to possess antioxidant potential. The sequence TPEVDDEALEK from LG is an iron-binding peptide (Cruz-Huerta et al., 2016) that we found in all our studied digested samples. This peptide also elicits antimicrobial activity (Mann et al., 2015). Tavares et al. (2011) discovered highly potent peptides from LA in WPC hydrolysates, DKVGINYW among them. This peptide demonstrated to not resist the SGID, despite the derived hydrolyzed smaller peptides yet kept ACE-inhibitory effects (Tavares et al., 2011). Accordingly, we found the truncated form DKVGINY in all digests of the studied SPS. The SGID of sample S1 released the peptide SRYPSY (known as casoxin-6) from κ-CN, which can act as an antioxidant. Similarly, the peptide EAMAPK from β-CN showed antioxidant activities by inhibiting the release of reactive oxygen species and increasing the antioxidant response (Pepe et al., 2016). The peptides VLPVPQK and AVPYPQR from β-CN and KVLPVPQ from β-CN also possess a strong antioxidant potential (Mann et al., 2015; Mada et al., 2017). Besides other bioactivities, the peptides VAGTWY and YVEELKPTPEGDL from LG exert antioxidant effects (Power, Fernández, Norris, Riera, & FitzGerald, 2014). We found this last species in all studied digests of SPS.

In vitro SGID of SPS led to the release of some neuropeptides from β-CN, mainly related to pro-casomorphins, which act as opioid peptide agonists by playing a crucial role in the response to pain and stress (Tyagi, Banan-Mwine Daliri, Kwami Ofosu, Yeon, & Oh, 2020). We found the peptide YPVEPF (neocasomorphin-6) in all digests, and despite it does not possess an aromatic amino acid in the N-terminal sequence, as in case of β-casomorphin, there is evidence that it contributes to onset of opioid activity (Jinsmaa & Yoshikawa, 1999). As stated, the SGID of sample S1 released the antioxidant peptide SRYPSY from κ-CN. This neuropeptide elicits opioid antagonistic properties by selectively binding to μ- and κ-receptors and not to δ-receptors (Yoshikawa, Tani, & Chiba, 1988). Beside ACE-inhibitory activity, LHLPLPL from β-CN (found in digests S1 and S2) is known as prolyl-oligopeptidase inhibitor and, therefore, involved in cognitive and neurological diseases (Myöhänen, García-Horsman, Tenorio-Laranga, & Männistö, 2009).

Antimicrobial activity has been ascribed to other BAPs found in all our studied digests. For instance, the peptide SDIPNPIGSENSEK generated from bovine α_{s1}-CN was reported to show antibacterial activity against the pathogens *Enterobacter sakazakii* and *Escherichia coli* (Hayes, Ross, Fitzgerald, Hill, & Stanton, 2006). Similarly, the peptide VQVTSTAV from κ-CN is known for its activity against *Escherichia coli* and *Listeria innocua* (López-Expósito, Minervini, Amigo, & Recio, 2006), while the peptides IDALNENK and VLVLDTDYK (from LG) inhibit *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* (Demers-Mathieu et al., 2012).

We also revealed DPP-IV inhibitor peptides in SPS digests. Didpeptidylpeptidase-IV is known to inactivate gut-derived hormones that play crucial roles in retarding gastric emptying and modulating appetite (Lim & Brubaker, 2006). In detail, the peptides TPEVDDEALEK, LKPTPEGDL and LKPTPEGDLE from LG inhibit the DPP-IV activity; and we found them in all digests. However, the species LKPTPEGDLEIL was present only in digest of sample S2 (Lacroix & Li-Chan, 2014).

Finally, other BAPs (FQSEEQQQTEDELQDK, VLPVPQ, LIVTQTMK) that we identified in digests have been reported to show activities such as increase of calcium uptake, cholesterol regulation, cellular growth (Cao et al., 2017).

Differently from what observed for the protein breakdown and the release of AA, the BAPs qualitative profile was similar among samples S2, S3 and S4. The main difference related to the release of BAPs in sample S1 which is linked to the presence of casein in the formulation.

Overall, several BAPs eliciting potential physiological benefits characterized the digests of our studied SPS. Nonetheless, according to literature present in Milk Bioactive Peptide Database (Nielsen et al., 2023), most of these possible effects have been assessed *in vitro* using synthesized peptides or peptide fractions purified from fermented dairy products and enzymatic hydrolysate of milk proteins.

4. Conclusions

The consumption of different whey protein-based sports supplements is important for achieving specific health benefits, especially in sports nutrition. In this regard, many components derived from milk proteins, especially from WP, are under study for their ability to elicit these effects. The present research demonstrated that, during *in vitro* SGID, the studied commercial SPS released AA, EAA, BCAA, and BAPs which could be potentially helpful for muscle growth and repair, strength, recovery, performance and other physiological and metabolic functions. The release of these molecules related to the extent of protein breakdown during SGID, and it was affected by the denaturation degree the WP underwent during processing. The obtained results are of paramount importance since they might open up a new perspective in the way the functionality of sports supplements is perceived by both the manufacturers and end users. Although these molecules present a great health potential, the scientific evidence is still limited to *in vitro* assays and more investigations are required to ascertain their potential health outcomes *in vivo*. Another issue that deserves further study is the nutritional role of the presence of CN in WP-based SPS, particularly in the case of unexpected CN concentrations resulting from the addition of CN-based powders.

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CRediT authorship contribution statement

Stefano Cattaneo: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft, Writing - review & editing. Ivano De Noni: Conceptualization, Methodology, Data curation, Supervision, Writing - original draft, Writing - review & editing. Fabio Masotti, Milda Stuknytė, Irene Da Costa: Investigation, Formal analysis, Data curation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure captions (Preference for color: online only)

Fig. 1. Protein profile of sports supplements S1, S2, S3 and S4 before and after *in vitro* static gastrointestinal digestion (SGID). STD, standard solution of sodium caseinate (CN), α-lactalbumin (LA), β-lactoglobulin (LG), Low Range Protein Ladder (1.7–40 kDa).

Fig. 2. Protein breakdown (%), free amino acids (AA), essential AA (EAA) and branched-chain AA (BCAA) (as g/100 g protein) quantified in digests of sports supplements S1, S2, S3 and S4. Lower-case letters indicate significant ($p < 0.05$) differences among samples.

Fig. 3. Molecular weight distribution of peptides detected in sports supplement digests S1, S2, S3 and S4.

Fig. 4. Venn diagrams, representing numbers of common peptides from caseins (left) or whey proteins (right) revealed among digests of sports supplements S1, S2, S3 and S4.

Table 1. Gross composition (g/100 g ± SD) of commercial sports supplements.

Sports supplement	WP source	Proteins	Lactose	Water
S1	WPI	88.0±0.4^{a}	0.6±0.1^{a}	3.3±0.2^{a}
S2	WPI	89.5±0.3^{b}	0.1±0.0^{b}	3.8±0.1^{b}
S3	WPI	91.2±0.4^{c}	0.1±0.0^{b}	3.2±0.1^{a}
S4	WPC	86.0±0.5^{d}	2.9±0.1^{c}	4.5±0.3^{c}

^{a,b,c,d}Data were expressed as mean ± standard deviation; means in the same column with different superscript letters are significantly different ($p < 0.05$).

Table 2. Free amino acid content of sports supplement *in vitro* digests (values were expressed as g per 100 g of protein ± SD).

Amino	Sports supplement

acid	**S1**	**S2**	**S3**	**S4**
Trp	2.05±0.21	2.26±0.23	2.10±0.39	1.55±0.18
Phe	4.04±1.24	4.47±0.34	3.51±0.63	2.53±0.47
Leu	5.29±1.20	8.18±0.72	5.30±1.03	3.84±0.83
Ile	0.54±1.00	1.60±1.04	0.56±0.71	0.50±0.60
Met	0.49±0.33	1.51±0.11	0.37±0.32	0.32±0.10
Tyr	2.51±0.81	2.25±0.26	1.91±0.70	1.94±0.13
Val	0.71±0.33	1.52±0.12	0.97±0.14	1.10±0.31
Pro	0.19±0.00	0.23±0.01	0.18±0.00	0.21±0.00
Ala	0.33±0.13	0.84±0.17	0.38±0.22	0.38±0.10
Thr	0.34±0.05	0.97±0.10	0.34±0.10	0.45±0.13
Gly	0.23±0.09	0.40±0.05	0.28±0.03	0.30±0.06
Glu	0.74±0.10	0.92±0.15	0.62±0.10	0.73±0.12
Ser	0.13±0.05	0.28±0.10	0.07±0.03	0.14±0.07
Gln	0.23±0.05	0.32±0.10	0.14±0.04	0.18±0.05
Asn	0.28±0.05	0.68±0.20	0.20±0.00	0.22±0.02
Asp	0.25±0.06	0.35±0.01	0.21±0.00	0.27±0.01
Arg	2.00±0.20	2.15±0.08	1.50±0.06	1.70±0.20

His	0.25±0.00	0.46±0.09	0.18±0.00	0.20±0.01
Lys	5.33±1.13	6.81±1.14	4.80±1.20	3.38±1.15
Total BCAA	6.54	11.30	6.83	5.44
Total EAA	19.04	27.78	18.13	13.87
Total	25.93	36.20	23.62	19.94

EAA, essential amino acids; BCAA, branched-chain amino acids

Table 3. Number of unique peptides revealed in digested sports supplements, listed according to their parent proteins.

Parent protein	Sports supplement			
	S1	**S2**	**S3**	**S4**
β-CN	153	115	74	92
κ-CN	64	91	34	40
α_{S1}-CN	70	28	13	27
α_{S2}-CN	32	16	11	22
LA	39	47	41	32
LG	116	143	138	120
BSA	42	47	46	48
Lactoferrin	11	14	5	5
Total	527	501	362	386

Table 4. Bioactive peptides revealed in digested sports supplements according to their potential biological effect as inferable from the Milk Bioactive Peptide Database (species *Bos taurus*, updated January 15, 2024) (Nielsen et al., 2023).

Parent protein	**Peptide sequence**	**Presence in SPS digest**	**Potential biological activity**															
			ACE-inhibitory	Antimicrobial	Antioxidant	DPP-IVInhibitory	Opioid	Increase mucin secretion	Antithrombotic	Immunomodulatory	Antiapoptotic	Bradykinin-potentiating	Osteoanabolic	Wound healing	Increase cellular growth	Cholesterol regulation	Catepsin inhibitory	Bradykinin potetntiating
α_{S1}-CN	SDIPNPIGSENSEK	S1, S2, S3, S4		x														
	TTMPLW	S1	x	x														
	YKVPQL	S1	x															
	YFYPEL	S1	x		x		x											
	VAPFPE	S1														x		
	DIGSESTEDQAMEDIK	S1											x					
	DAYPSGAW	S1, S4	x															
α_{S2}-CN	AMKPW	S1	x															
	VPITPTL	S1, S4				x												
	NMAINPSK	S1, S3, S4	x															
β-CN	EMPFPK	S1, S2, S3, S4	x	x				x				x						
	VYPFPGPIPN	S1, S2, S3, S4	x		x													
	VLPVPQ	S1, S2, S3, S4														x		
	PFPGPIPN	S1, S2, S3, S4																
	VLPVPQK	S1, S2, S3, S4	x	x	x						x		x	x				
	AVPYPQR	S1, S2, S3, S4	x	x	x													

Parent protein	Peptide sequence	Presence in SPS digest	Potential biological activity															
	YQEPVLGPVR	S1, S2, S3, S4	x		x				x	x								
	YQEPVL	S1, S2, S3, S4	x															
	VENLHLPLPLL	S1, S2, S3, S4	x															
	NLHLPLPLL	S1, S2, S3, S4	x															
	LNVPGEIVE	S1, S2, S3, S4	x															
	EAMAPK	S1, S2, S3, S4		x														
	YPVEPF	S1, S2, S3, S4	x	x	x		x	x										
	FQSEEQQQTEDELQDK	S1, S2, S3, S4											x					
	NIPPLTQTPVVVPPFLQ	S1	x															
	KVLPVPQ	S1	x							x								
	DVENLHLPLPL	S1		x														
	KVLPVPQK	S1			x					x								
	LYQEPVLGPVR	S1	x							x								
	GPFPI	S1															x	
	YPVEPFTE	S1																x
	VKEAMAPK	S1		x	x													
	PGPIPN	S1	x							x								
	IPPLTQT	S2				x												
	NIPPLTQTPV	S2	x															
	YPFPGIPN	S2			x	x												

Parent protein	Peptide sequence	Presence in SPS digest	Potential biological activity															
	LHLPLPL	S1, S2	x															
κ-CN	VQVTSTAV	S1, S2, S3, S4		x														
	FFSDK	S1		x														
	SRYPSY	S1					x											
	STVATL	S1, S2		x														
	LPYPY	S1, S4	x			x												
LA	VSLPEW	S1, S2, S3, S4	x															
	DKVGINY	S1, S2, S3, S4	x															
	EQLTK	S2		x														
	YGGVSLPEW	S3	x															
LG	IPAVFK	S1, S2, S3, S4	x	x		x									x			
	ALPMHIR	S1, S2, S3, S4	x												x			
	IIAEK	S1, S2, S3, S4	x													x		
	GLDIQK	S1, S2, S3, S4	x													x		
	ALPMH	S1, S2, S3, S4	x													x		
	VLDTDY	S1, S2, S3, S4	x			x												
	VLDTDYK	S1, S2, S3, S4	x															
	LDIQK	S1, S2, S3, S4	x															
	LKPTPEGDLE	S1, S2, S3, S4				x												
	LIVTQTMK	S1, S2, S3, S4													x			

Parent protein	Peptide sequence	Presence in SPS digest	Potential biological activity															
	YVEELKPTPEGDL	S1, S2, S3, S4			x													
	LKPTPEGDL	S1, S2, S3, S4				x												
	IDALNENK	S1, S2, S3, S4		x											x			
	VLVLDTDYK	S1, S2, S3, S4		x		x												
	TPEVDDEALEK	S1, S2, S3, S4		x		x												
	LKPTPEGDLEIL	S2				x												
	LDTDY	S1	x															
	LSFNPTQ	S3	x															
	ALPM	S1, S4	x															
	VAGTWY	S2, S4	x	x	x	x									x			
	IPAVF	S1, S2, S3	x			x												