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Proteomic and peptidomic profiling of spirulina-fortified probiotic powder formulations during in vitro digestion☆

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ABSTRACT

This study reports on the influence of lactic acid fermentation on the proteomic and peptidomic profiles of spirulina protein isolate (SPI)-fortified, freeze-dried powders containing living *Lacticaseibacillus rhamnosus* GG (LGG) cells during in vitro digestion. For comparison, powders fortified with whey protein isolate (WPI) and pea protein isolate (PPI) were also evaluated. Prior to freeze-drying, the powder precursors were either non-treated or fermented. Capillary SDS–PAGE electropherograms revealed a mild proteolytic effect due to fermentation. C-phycocyanin (SPI) and β-lactoglobulin (WPI) showed the highest resistance to pepsinolysis. All samples were responsive to pancreases, with fermented WPI showing the lowest responsiveness. Fermentation enhanced the degree of hydrolysis (DH) in gastric chymes, whereas in intestinal chymes, DH followed the order SPI > PPI > WPI, with fermentation showing no significant impact. A total of 6, 11, and 52 potential bioactive peptide sequences, associated with various beneficial activities, were identified in the SPI, PPI, and WPI digesta, respectively. The highest amino acid bioaccessibilities were observed for cysteine and methionine in SPI, isoleucine and arginine in PPI, and glycine in WPI. In conclusion, fortifying probiotic formulations with protein isolates offers secondary health benefits, stemming from the release of bioactive peptides and bioaccessible essential amino acids.

1. Introduction

Due to an increased awareness of consumers regarding the link of a healthy diet lifestyle and chronic degenerative diseases, like obesity, metabolic syndrome and type-II diabetes, the demand of new ingredients with a multi-faceted technological and bio-functional profile, is growing. Microalgal biopolymers (e.g. polysaccharides, proteins and lipids) and bioactive secondary metabolites (e.g., vitamins, carotenoids, polyphenols, chlorophylls, etc.) have a significance in the realm of nutrition [1–3]. One of the most prominent microalgal species is *Arthrospira platensis* commonly known as spirulina [4]. The utilisation of raw spirulina biomass or its bioactive fractions in the production of dietary supplements has gained recognition due to its potential prebiotic effects, immune system stimulation, anti-inflammatory and anti-tumor properties, neuroprotection, and alleviation of metabolic disorders [5].

Spirulina is composed of 60 to 70 % DM protein, depending on the life stage, cultivation conditions, extraction, isolation and drying methods employed [6]. Spirulina proteins consist mainly of the so-called phycobiliproteins, which are water soluble pigment – protein complexes. The most abundant phycobiliproteins in spirulina are phycocyanins, phycoerythrins and allophycocyanins (APC), depending on their composition and chromospheres content [7]. Additionally, the amino acid (AA) composition of spirulina comprises the whole range of essential amino acids (EAA) and has a major AA content in glutamic acid (Glu), alanine (Ala), arginine (Arg) and leucine (Leu) [4,8,9]. Interestingly, fermenting spirulina with *Lactobacillus plantarum* and *Bacillus subtilis* was reported to increase significantly its total EAA (TEAA) content [8]. Moreover, the fermentation of spirulina biomass with *Lactobacillus plantarum* significantly increased the content of histidine (His), lysine (Lys) and aspartic acid (Asp) but decreased the content of serine (Ser), Ala and Arg ([9]. As an asset to its well-balanced AA profile, the proteins found in spirulina are also highly digestible (75.5–83.9 %) [10].

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The protein digestibility can be improved by changes in the protein secondary structure, i.e., increased β-sheet and decreased α-helix formation, induced by processing such as heating, fermentation etc. [11–14]. In view of this, Niccolai et al. [15] demonstrated that fermenting spirulina biomass with *L. plantarum* enhanced the DH from 74.3 to 77.6 %.

Bioactive peptides can be classified into endogenous and exogenous ones. Endogenous peptides are produced by different cell types in the body in contrast to exogenous peptides, which are produced outside the body and enter the body from different sources, i.e., foods, nutraceuticals, and medications [16]. Due to their antimicrobial [17], antihypertensive [18], antioxidant [19], cholesterol lowering [20], immunomodulatory [21], anti-aging [22], metal chelating [23], antidiabetic [24], and opioid activity [25] potential, the application of bioactive peptides is focused on the implementation in food supplements, medicinal and cosmetical products. Regarding the production method of exogenous peptides, nowadays various methods like enzymatic hydrolysis, chemical digestion, microbial fermentation, recombinant production, and chemical synthesis, are applied, followed by a purification step of the peptide. Additionally, bioactive peptides can be released during the gastrointestinal digestion by enzymatic cleavage of proteins [16]. Several studies have pinpointed the potential antihypertensive (angiotensin converting enzyme (ACE) inhibiting), antioxidant and anti-diabetic (dipeptidyl peptidase IV (DPP-IV) inhibiting) activity of exogenous bioactive peptides derived from spirulina proteins [26–28]. In addition, An et al. [29] found immune-enhancing activities in peptide fractions obtained from spirulina fermented with L. *plantarum* and *B. subtilis*.

A steadily increasing number of studies have showcased the importance of lactic acid fermentation in the development of novel functional foods bestowing targeting techno-functional, sensory and health promoting properties [30]. While various strains of lactic acid bacteria have been effectively used to modify the techno-functional properties of spirulina, our current understanding of the bio-functional role of SPI in anhydrobiotic formulations is poorly studied. Building upon our previous research, which demonstrated the role of SPI in preserving the viability of LGG cells encapsulated in lyophilised carriers during processing, storage, and in vitro digestion [31], this study aims to explore the proteomes and peptidomes of SPI-fortified anhydrobiotic formulations following in vitro digestion and highlight their potential bioactivity. To achieve this, the anhydrobiotic formulations were either unfermented (NT) or fermented (F) by *Lacticaseibacillus rhamnosus GG* prior lyophilisation. For comparison purposes, probiotic powders fortified with WPI and PPI were tested.

2. Materials and methods

2.1. Materials

Dried spirulina biomass was purchased from Sevenhills Wholefoods (Sheffield, United Kingdom). PPI (NATURALYS S85 plus N) and WPI (PRODIET 90S) with a protein content of 85 % and 85.8 % wt. were kindly donated by Roquette (Lestrem, France) and Ingredia (Arras, France), respectively. Trehalose (99.4 % wt.) was purchased from Louis-François (Croissy-Beaubourg, France). LGG ATCC 53103 was purchased from VTT Technical Research Centre of Finland Ltd. (Espoo, Finland). SPI with a protein content of ~82 % wt. was isolated according to Fortuin et al. [31]. All other chemicals were of analytical grade and purchased from Sigma-Aldrich (Leuven, Belgium).

2.2. Preparation of the probiotic powders

The inoculation method as described in Hellebois et al. [32] was used for the preparation of the probiotic powder precursors. A LGG preculture was prepared by inoculating one microbead from the cryovials (Microbank 2D, Novolab, Geraardsbergen, Belgium) in 50 mL of MRS broth overnight at 37 °C. 100 μL of pre-culture were inoculated in 50 mL MRS broth for approximately 14 h at 37 °C followed by centrifugation (7000 *g*, 2 min). After centrifugation, the bacterial biomass pellet, obtained from the inoculated 50 mL broth, was washed twice with phosphate buffered saline (PBS) solution. 100 mL of the formulation prepared as described in Fortuin et al. [31] were inoculated with 4 pellets of LGG. Aliquots of 1 mL of the solution containing probiotics were transferred into 24-well cell culture plates (CELLSTAR, Greiner Bio-One, Frickenhausen, Germany) and either immediately frozen at −80 °C for 2 h (NT) or fermented at 37 °C until pH 4.5 ($t_{pH4.5}$ = 4 h, 90 min, 90 min for WPI, PPI and SPI, respectively) was reached and frozen afterwards (F). Finally, the samples were lyophilised (main stage: 0.120 mbar for 18 h; final stage: 0.010 mbar for 22 h) using a Martin Christ freeze-dryer (Alpha 2–4 LSC plus, Germany). The final microbial load of each protein solution was approximately 10 log CFU g^{-1}.

2.3. In vitro digestion protocol

The INFOGEST v2.0 static in vitro digestion protocol was implemented for simulating the oral, gastric and small intestinal phases of the probiotic powders [33]. Any modifications were implemented according to Fortuin et al. [31].

2.4. Capillary SDS-PAGE

The peptic cleavage of the proteins throughout in vitro gastrointestinal processing of the food matrices was investigated by means of capillary SDS-PAGE. The initial food matrix as well as the obtained gastric and intestinal chymes were solubilized using 2-mercaptoethanol. Moreover, the gastric chymes were diluted 1:1 with phosphate-buffered saline (PBS). The electropherograms were assessed using a Bioanalyzer 2100 and Protein 80 Chip Kit (Agilent Technologies, Santa Clara, CA, United States) according to the manufacturer's instructions. Agilent's 2100 Expert software was used for the reconstruction of the gel.

2.5. Degree of hydrolysis

An acidic hydrolysis was conducted for each system to ascertain the total AA content in the food matrix. Initially, 100 μL of the food matrix (used for in vitro digestion) were diluted to 1 mL using 6 M HCl. Subsequently, the samples underwent hydrolysis at 110 °C for a duration of 24 h in Pyrex tubes (Hach, Loveland, United States). After hydrolysis, the samples were neutralised by adding 1 mL of 6 M NaOH and then diluted to a final volume of 10 mL with MilliQ water.

The approach described by Halabi et al. [34] was employed to measure the primary amino groups in various samples, including the food matrix, gastric and intestinal chymes. The food matrix was not diluted. Gastric and small intestine samples were diluted 10 and 20 times, respectively. Subsequently, the gastrointestinal samples underwent a 5-min centrifugation at 10000 *g* and 4 °C. Afterwards, 20 μL of the supernatant were mixed with 150 μL of an o-phthalaldehyde (OPA) solution in an UV 96-well microplate (UV-Star Greiner, Germany). After incubating the samples for 2 min in darkness at room temperature, the absorbance was measured at 340 nm using a UV/VIS Spark 20 M microplate reader (Tecan, Männedorf, Switzerland).

The quantification of free AAs was carried out following the previously described method. The degree of protein hydrolysis was determined as follows (Eq. 1):

$$\mathrm{DH}\ (\%) = \frac{\mathrm{NH_{2\ digested}} - \mathrm{NH_{2\ FM}}}{\mathrm{NH_{2\ total}} - \mathrm{NH_{2\ FM}}} \times 100 \quad (1)$$

where DH is the degree of hydrolysis and NH_{2FM}, $NH_{2digested}$ and NH_{2total} denote the content of primary amino groups in the food matrix, the obtained digesta (gastric or intestine) and the acidic hydrolysed food matrix.

2.6. Proteomic and peptidomic profile using nano-LC-MS/MS

The determination of the peptidomic profile in the gastric and intestinal chymes was investigated by means of nano – liquid chromatography – mass spectrometry (nano-LC-MS/MS) as described in Hellebois et al., (2023) with slight modifications. Duplicate aliquots of 10 mL of gastric and intestinal chymes were centrifuged (10.000 rpm, 10 min, 4 °C) in Amicon tubes (Merck, Darmstadt, Germany) with a 10 kDa filter cut-off, followed by vacuum-drying (CentriVap, Labcono, Kansas City, MO, United States) aliquots of 500 μL. Afterwards, the sample was solubilized in 200 μL of 0.1 % *v*/v formic acid and different dilutions were performed prior to LC-MS injection. As described by Hellebois et al. [35] the separation of peptides was carried out two technical replicates per sample using a Nano LC-425 Eksigent system coupled with a TripleTOF 6600+ mass spectrometer (SCIEX, Darmstadt, Germany). Briefly, the first 30 ions with the highest intensity were selected for fragmentation in high sensitivity positive mode using the automatically adjusted system of rolling collision energy. MS scans were acquired over a mass range 150-1500 *m/z* with an accumulation time set at 250 ms. MS/MS scans were acquired over a mass range 100–1500 m/z with an accumulation time set at 50 ms.

For peptide identification, the raw data (.wiff file) were directly converted to .mgf file prior submitting to the search engine MASCOT (Matrix Science, London, UK) via Mascot Daemon interface (version 2.6.0, Matrix Science). The MS/MS files from the two technical replicates were merged into a single search. UniProt Cowmilk (25,682 sequences, released 27th October 2020), UniProt Arthrospira (29,131 sequences, released 24th February 2023) and UniProt Pisum (45,848 sequences, released 24th February 2023) databases were used for identifying the sequences of WPI, SPI and PPI, respectively.

For the investigation of the proteomic profile of the in vitro digested probiotic powders, the acquired data were imported into Progenesis QI for Proteomics software (version 4.2, Nonlinear Dynamics, Waters, Newcastle upon Tyne, UK). The peptides and proteins were identified searching the Bovin milk database on Uniprotkb (containing 331 sequences) for WPI. For SPI and PPI, the protein identification was refined with taxonomy *Arthrospira platensis* and *Pisum sativum* respectively in Progenesis QIP.

The following parameters were used to conduct the whole Mascot research: a peptide mass tolerance of 20 ppm for the precursor; 0.02 Da of fragment mass tolerance; no proteolytic cleavage specificity; no static modifications, no missed cleavages; Met oxidation, pyroGlu at the N-term Gln, and Ser/Thr phosphorylation as variable modifications.

The target decoy database with a false discovery rate at a peptide level of 1 % was used filter peptide spectrum matches and hence to eliminate false spectrum matches. Regarding the identification of the proteins, a Mascot-calculated confidence of 95 %, a minimum of two identified peptides per protein and at least one significant unique peptide as well as a protein score $\geq$ 50 and peptide score $\geq$ 20 were used to process the achieved data. Venn diagrams of the significant unique peptides were prepared with OriginLab 2019b. Potential bioactivities of the unique peptide sequences were investigated by comparison to the database of bioactive peptides obtained from Biopep [36]. In addition, an in-house database based on research findings was used for SPI and PPI. Peptide sequences obtained from WPI were also compared to the Milk bioactive peptide database [37,38]).

The ProteomeXchange Consortium via the PRIDE partner [39,40] repository was used to deposit the proteomic data. Data are available via ProteomeXchange with identifier PXD052301.

2.7. Amino acid bioaccessibility

The methyl-chloroformate (MCF) derivatisation method (see paragraph 2.6.2) was used for the determination of alanine (Ala), aspartic acid (Asp), cysteine (Cys), glutamic acid (Glu), glutamine (Gln), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe) proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr) and valine (Val). Asparagine (Asn) and cysteic acid (Cya) were determined by the trimethylsilyl (TMS) derivatisation method. Since Cys was not determined by the MCF method in each sample, the sum of Cys and Cya represents the total Cys content. The volatile esters present in the derivatised samples were identified by means of gas chromatography – mass spectrometry (GC (7890B) – MS (5977 A), Agilent Technologies, Santa Clara, US) equipped with a multipurpose autosampler (MPS, GERSTEL, Mühlheim, Germany). Arg content was determined using an enzymatic kit (Megazyme, K-LARGE 07/20).

2.7.1. Preparation of the hydrolysed food matrix and intestinal chymes

To determine the AA composition of the initial probiotic lyophilisates, duplicates of ~30 mg aliquots were hydrolysed with 1 mL of HCl 6 M for 24 h at 110 °C in Pyrex tubes (Hach, Loveland, United States) [41]. Prior to the hydrolysis, the samples were flushed with nitrogen to avoid oxidation of the AAs. For determining the AA's bioaccessibility in the intestinal digesta, aliquots of 10 mL after 120 min of intestinal digestion were centrifuged (10.000 rpm, 10 min, 4 °C) in Amicon tubes (Merck, Darmstadt, Germany) with a 10 kDa filter cut-off and stored at −80 °C until further use.

2.7.2. MCF derivatisation method

In order to transform the AAs into volatile esters, the MCF derivatisation method and GC–MS settings as described in Smart et al. [42] were used.

2.7.2.1. Sample preparation and derivatisation. Prior to the derivatisation, 50 μL of the acidic hydrolysates and 600 μL of both, alkaline hydrolysates and intestinal chymes, were mixed with 20 uL of internal standard (D4-alanine 10 mM) and vacuum-dried at 35 °C (CentriVap, Labcono, Kansas City, MO, United States). Afterwards, the dried samples were resuspended in 200 μL NaOH 1 M.

For the preparation of the standard curves, a standard mix of 18 AAs including Ala, Arg, Asp, Cys, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr and Val was used. The concentration of the standard curves ranged from 0.01 to 8 mM. A total volume of 200 μL of each standard concentration was prepared with NaOH 1 M and 20 μL of internal standard followed by the derivatisation.

The samples were derivatised as described in Smart et al. [42]. The derivatised samples were transferred into amber glass vials and closed with a cap prior injection into the GC–MS.

2.7.2.2. GC–MS analysis. One μL of sample was injected under pulsed splitless mode (1 bar for 1 min, 20 mL min^{-1} split flow after 1.01 min) into the GC–MS. The initial temperature of the GC oven was held at 45 °C for 2 min. Afterwards, the temperature was increased as follows: 1) at 9 °C min^{-1} to 180 °C hold for 5 min, 2) at 40 °C min^{-1} to 220 °C hold for 5 min, 3) at 40 °C min^{-1} to 240 °C hold for 11.5 min, 4) at 40 °C min^{-1} to 280 °C hold for 2 min, 5) at 40 °C min^{-1} to 300 °C hold for 1 min. The equilibration time before each analysis was 5 min. The He gas flow through the column (Agilent 1701) was kept constant at 1.0 mL min^{-1}. The inlet, interface and quadrupole temperature were 290, 250 and 200 °C, respectively.

2.7.2.3. Data analysis. Data analysis was conducted according to Aggio et al. [43]. To deconvolute and identify the peaks, the free Automated Mass Spectral Deconvolution and Identification System (AMDIS, http://www.amdis.net, V2.71) and the in-house library as provided in the Supplementary Data 1 and 2 of Smart et al. [42] were used. For the quantification of the AAs, R studio (R 4.2.3) with the Metab package (version 1.32.0) was used.

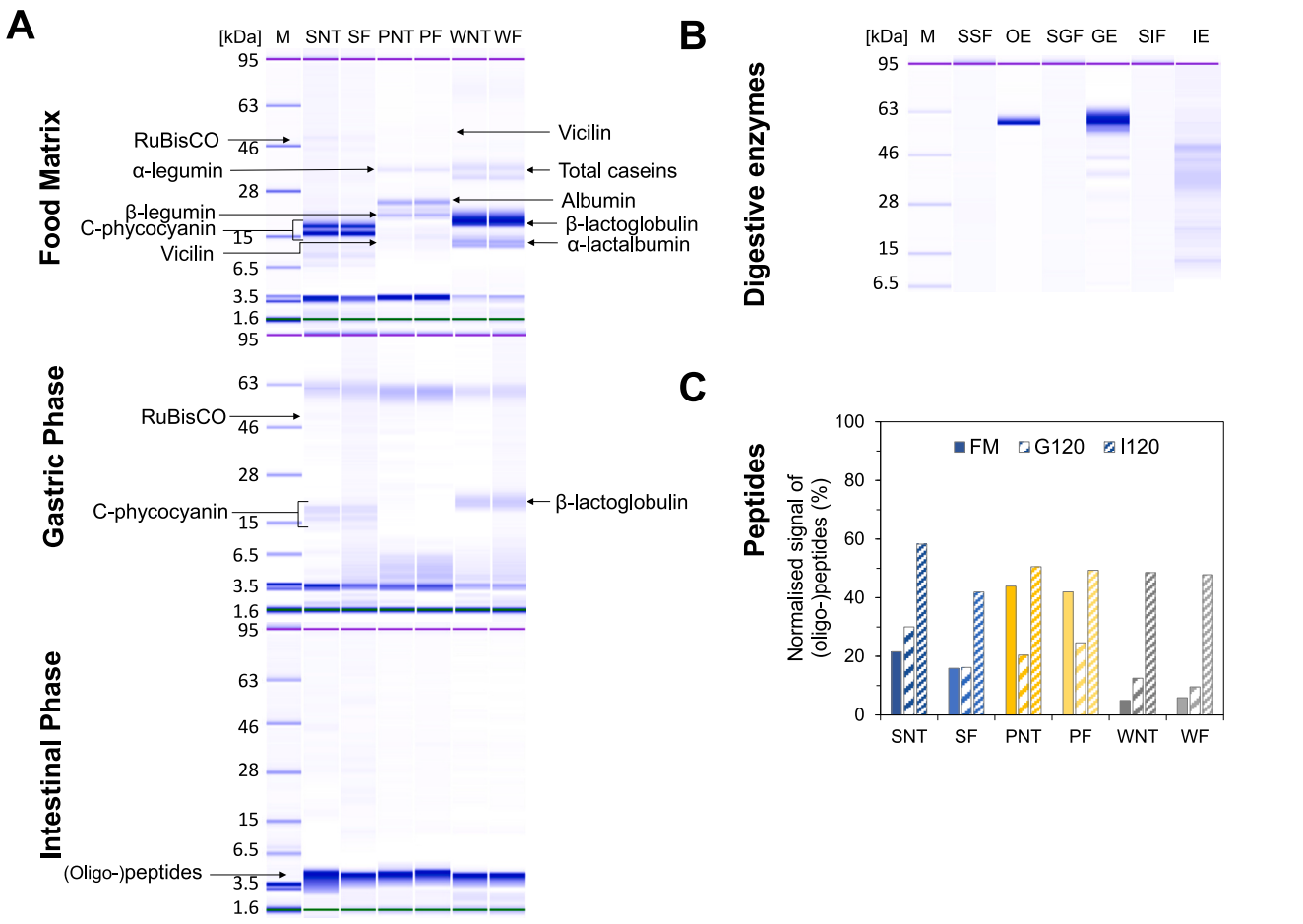

Fig. 1. Influence of protein composition (S = spirulina, P = pea, W = whey) and precursor treatment (NT = non-treated, F = fermented) on the SDS-PAGE electropherograms of the initial food matrix, gastric and intestinal chymes (A), SDS-PAGE electropherograms of simulated digestive fluids (SSF = simulated salivary fluid, SGF = simulated gastric fluid, SIF = simulated intestinal fluid) and digestive enzymes (OE = oral enzymes, GE = gastric enzymes, IE = intestinal enzymes) (B) and densitometric analysis of the bands corresponding to (oligo-) peptides (C). RuBisCO: Ribulose-1,5-bisphosphate carboxylase/oxygenase.

2.7.3. TMS derivatisation method

The TMS derivatisation method and GC–MS settings of Villas-Bôas et al. [44] were implemented for the quantification of Asn and Cya.

2.7.3.1. Sample preparation and derivatisation. The probiotic lyophilisates were hydrolysed as mentioned in 2.6.2.1. 200 μL of acidic hydrolysed samples and 600 μL of intestinal chymes mixed with 20 μL of intestinal standard (D4-Alanine 10 mM) were prepared for the analysis. 200 μL standards of Cya and Asn were prepared in the range of 0.1 to 5 mM including 20 μL of internal standard. The samples were vacuum-dried prior derivatisation (CentriVap, Labconco, Kansas City, USA). The dried samples were resuspended in 80 μL methoxyamine hydrochloride in pyridine (2 g 100 mL^{-1}) and incubated at 30 °C for 90 min at 750 rpm. After the incubation, the samples were mixed with 80 μL of *N*-methyl-N-(trimethylsilyl)trifluoroacetamide and incubated at 37 °C for 30 min at 750 rpm. The derivatised samples were transferred into amber GC–MS vials and kept at 4 °C until further use.

2.7.3.2. GC–MS analysis. One μL of derivatised sample was injected under split mode (25:1. 25 mL min^{-1}) into the GC–MS. The He glass flow through the column (Agilent 19091S-433UI, 30 m × 250 μm × 0.25 μm) was kept constant at 1 mL min^{-1}. The equilibration of the column lasted 6 min before each run. The initial temperature of the GC oven was held at 70 °C for 5 min. Afterwards, the temperature was increased as follows: 1) at 10 °C min^{-1} to 179 °C hold for 0 min, 2) at 0.5 °C min^{-1} to 180 °C hold for 2 min, 3) at 10 °C min^{-1} to 220 °C hold for 1 min, 4) at 2.5 °C min^{-1} to 265 °C hold for 1 min, 5) at 10 °C min^{-1} to 280 °C hold for 1 min and 6) at 10 °C min^{-1} to 300 °C hold for 0 min.

2.7.3.3. Data analysis. The identification of the peaks of the AAs was performed by using in-house MS library of standards. The peaks were quantified by using the height of the peaks. The data was normalised by the intestinal standard followed by calculation of the quantity.

2.8. Statistical analyses

The DH data were subject to two-way ANOVA followed by Tukey's post hoc means comparison test ($p < 0.05$). The nano-LC-MS/MS acquired proteomic datasets (16 samples and 96, 70 and 38 variables for WPI, PPI and SPI, respectively) were log_{10} transformed, auto-scaled and subject to Principal Components Analysis (PCA). Unsupervised PCA was employed to identify potential outliers according to the Hotelling's ellipse and leverage plot criteria. As a next step, the log transformed, mean centred and auto-scaled proteomic data were subject to partial least squares – discriminant analysis (PLS-DA) using the leave-one-out (LOO) cross-validation method to evaluate the performance of the models constructed and determine the optimal number of principle components (PCs) required to achieve the best classification. The protein accession numbers having a variable-importance-in-projection score, VIP > 1 were considered as significant. For the analysis of the peptidomes of the digesta, the datasets were log transformed and auto-scaled and then, subjected to hierarchical cluster analysis using the Euclidean distances and Ward's agglomeration methods based on rows (samples) and

columns (peptides relative concentration). Finally, the centred and auto-scaled peptidomic (amino acids bioaccessibility), colloidal (span and Brouckere particles size mean diameter in the gastrointestinal digesta), protein secondary structure (α-helix, β-sheet, and aggregated strands), and in vitro digestibility (SDS-PAGE densitometric analysis and degree of hydrolysis) were subject to hierarchical cluster analysis as above mentioned. ANOVA was conducted using Origin 2019b (OriginLab, Northampton, MA, USA), PLS-DA was performed using Unscrambler X (Camo, As, Norway) and hierarchical cluster analysis was carried out employing ClustVis web tool [45].

3. Results & discussion

3.1. Proteomic characterisation of the probiotic powders and their in vitro digesta

To assess the extent of bacterial, pepsin, and pancreatic/tryptic-induced protein cleavage, the initial, gastric, and intestinal phases of the SPI-, PPI-, and WPI-based probiotic powders were analysed using capillary SDS-PAGE (Fig. 1A). Two major bands at 18 and 16 kDa, attributed to the α- and β- subunits of C-phycocyanin (CPC) [46] and a less intense band around 54 kDa, indicating the presence of Ribulose-1,5-bisphosphat-carboxylase/−oxygenase (RuBisCO), were identified in spirulina-based food matrices. Consistent with previous studies [35], three major bands at 30–35, 18, and 14 kDa, associated with residual (α-, β-, and κ-) caseins, β-lactoglobulin (β-Lg), and α-lactalbumin (α-La), were detected. For the PPI-based probiotic powders, the bands detected at approximately 15–18, 50, 37, 25, and 20 kDa were attributed to the presence of vicilin, α-legumin, albumin, and β-legumin [47,48]. Notably, the LGG-mediated fermentation of the powder precursors did not significantly alter the protein cleavage induced by LGG, likely due to the relatively short duration of the fermentation process (4 h for WPI and approximately 90 min for PPI and SPI, respectively) compared to previous studies [49,50].

As expected, proteolytic activity in the simulated oral boluses (data not shown) was negligible, primarily due to the absence of proteases in the simulated salivary fluids [51]. For the SPI- and WPI-based probiotic powders, in vitro gastric digestion of the oral boluses led to a significant increase in the intensity of molecular weight bands associated with oligopeptides (i.e., <3.5–5 kDa) at the expense of bands corresponding to intact proteins (Fig. 1B). Densitometric analysis of the oligopeptide-associated bands (Fig. 1C) confirmed an increase in normalised intensities by 60 % to 150 %, with the most notable differences observed in the gastric digesta of the untreated probiotic powders. In WPI-based gastric digesta, β-Lg resisted pepsin-induced hydrolysis—unlike total caseins and α-La—with residual intact polypeptides averaging 69–87 %, consistent with previous studies [51,52]. In contrast to Minic et al. [53], who reported that CPCs are rapidly hydrolysed by pepsin to chromopeptides, both CPC subunits showed notable resistance to hydrolysis in the present study, with the normalised amount of residual intact CPC polypeptides accounting for 30–59 % in the gastric digesta from both non-treated and fermented SPI probiotic powders. This discrepancy may be attributed to differences in composition (e.g., the presence of maltodextrin) and processing (thermal treatment, fermentation, and freeze-drying) of the initial food matrix, as well as variations in the in vitro digestion protocols (i.e., INFOGEST standardised digestion model vs. pepsin digestion assay protocol) between the two studies.

In contrast to WPI and SPI digesta, a significant reduction in all distinctive bands—including the oligopeptide-related bands—was observed in the PPI-based gastric digesta, indicating the sensitivity of pea protein subunits to pepsin-mediated hydrolysis. However, densitometric analysis of the PPI SDS-PAGE electropherograms confirmed the presence of approximately 4–22 %, 1–10 %, 5–23 %, and 17 % residual intact α-legumin, albumin, β-legumin, and vicilin, respectively. Interestingly, the amount of residual intact proteins was higher in PNT compared to PF for each protein.

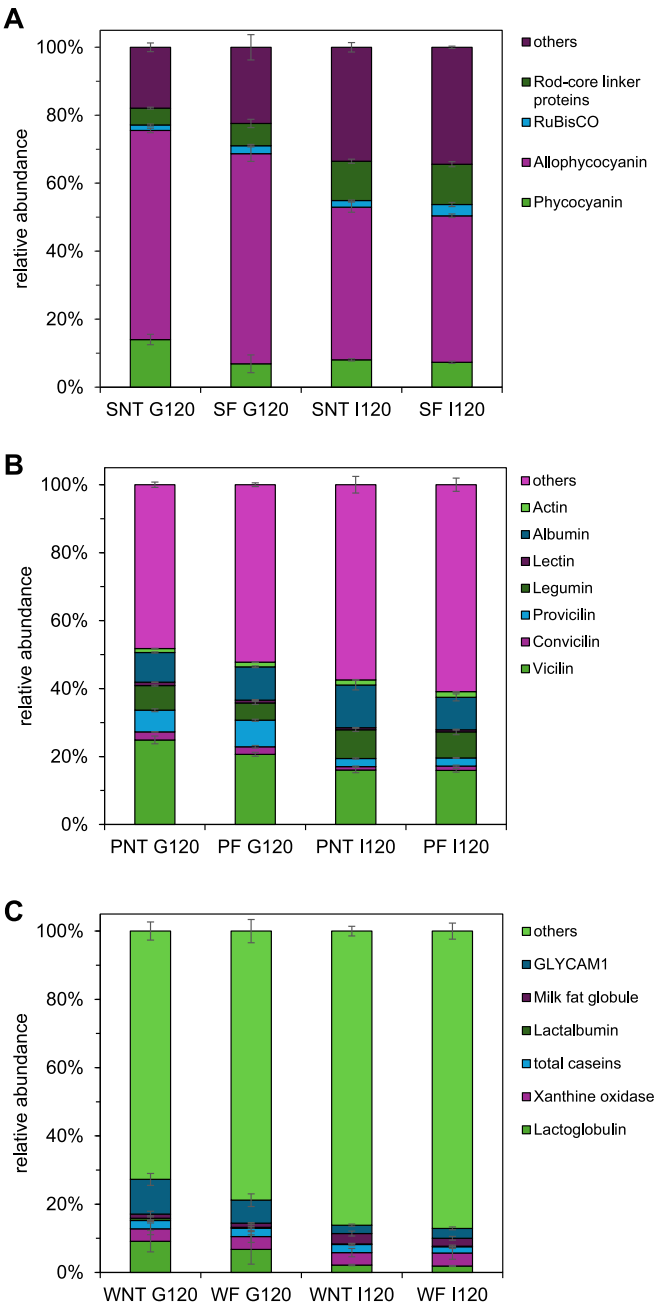

Fig. 2. Relative abundance of the proteins detected by nano-LC-MS/MS characterisation the gastric (G120) and intestinal (I120) digesta of the probiotic powders as influenced by their composition (S = spirulina (A), P = pea (B), W = whey (C)) and precursor treatment (NT = non-treated, F = fermented). RuBisCO: Ribulose-1,5-bisphosphate carboxylase/oxygenase, GLYCAM1: Glycosylation-dependent cell adhesion molecule-1.

Upon completion of simulated intestinal digestion, a substantial increase in the intensity of the oligopeptide-related bands (Mw < 3.5–5 kDa) was observed across all samples. As shown in Fig. 1C, the Mw < 3.5–5 kDa intensity increased substantially in the intestinal digesta, ranging from 41.1 % to 58.3 %, compared to the gastric digesta (9.6 % to 30 %). The Mw < 3.5–5 kDa intensity followed a descending order of SPI < PPI–WPI, aligning with the degree of hydrolysis as measured by OPA assay. The progressive cleavage of PPI and WPI subunits during intestinal processing has also been reported in previous studies [54,55]. It is well-documented that the secondary structure of proteins (i.e., prevalence of α-helix and β-sheet conformations) is closely linked to their susceptibility to peptic enzymes [56]. In view of this, SPI, which

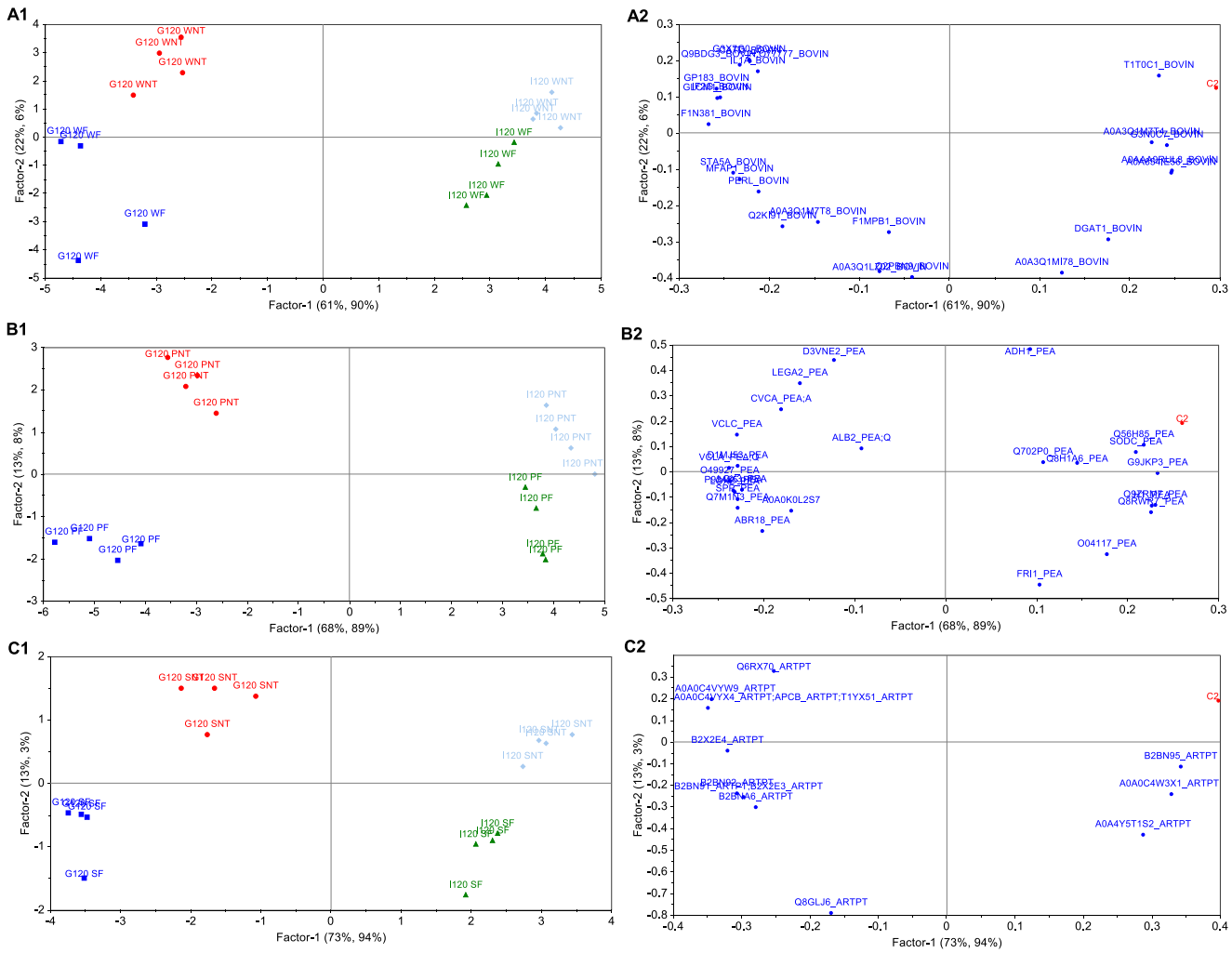

Fig. 3. Partial least squares regression – discriminant analysis (PLS-DA) of the proteomes of the gastrointestinal digesta of the probiotic powders as influenced by their protein composition (S = spirulina (A), P = pea (B), W = whey (C)) and precursor treatment (NT = non-treated, F = fermented). The significant accession numbers were selected according to the variable importance in projection criterion (VIP > 1). A complete description of the protein accession number is provided in Suppl. Table 1.

has a lower α-helix and higher β-sheet content compared to PPI and WPI [31], possesses a more open molecular structure and, consequently, greater steric accessibility to tryptic and pancreatic enzymes.

The proteomes of the gastrointestinal digesta from the probiotic powders were determined by nano-LC-MS/MS and the relative abundancies of the proteins identified are illustrated in Fig. 2. In general, a total of 96, 70 and 38 proteins were detected in the digesta of the WPI, PPI and SPI fortified probiotic powders. No significant differences in the cumulative number of proteins were found when comparing the gastric and the intestinal digesta. Yet, a significant ($p < 0.05$) decline in the cumulative protein concentration in the intestinal digesta was observed for all protein isolates accounting for the 70.7, 72.6 and 64.5 % of the total protein amount quantified in the gastric digesta. GLYCAM1 and β-Lg were the most abundant proteins identified in the WPI-containing gastric digesta. In contrast, the relative abundance of total (α_{s1}-, α_{s2}-, β- and κ-) caseins and xanthine oxidase remained unaltered during the in vitro digestion process. In the PPI-containing digesta, vicilin, provicilin and convicilin were the most abundant protein subunits showing relatively low resistance to pancreases-mediated hydrolysis (from 22.8 to 16, 7.1 to 2.4 and 2.3 to 1.2 %, in gastric and intestinal phases, respectively) compared to the albumin, legumin and actin counterparts (from 9.3 to 11.1, 6.2 to 8 and 1.2 to 1.6 %, in gastric and intestinal phases, respectively). Phycocyanins and APCs underwent a substantial reduction in their relative abundancies in the intestinal phases (from 61.7 to 44 and 10.5 to 7.6 %, in gastric and intestinal phase, respectively) compared to RuBisCO and rod-core linker proteins - the latter refer to phycobilisome linker polypeptides involved in light-harvesting [57]. In agreement with the SDS-PAGE densitometric results, the relative abundance of the major protein subunits per type of protein isolate source was higher in the case of the non-treated probiotic powder gastric digesta (53.6 vs. 48.8 % for -NT and —F, respectively). Nevertheless, these differences were diminished in the case of the intestinal phases with values of 40.9 and 39.9 % for -NT and —F, respectively.

To assess the similarities and differences in the proteomes of the gastric and intestinal digesta of the probiotic powders - as influenced by the protein isolate type and the pre-fermentation step - the acquired nano-LC-MS/MS data matrices (comprising 96, 70 and 38 protein accession numbers in the case of WPI, PPI and SPI protein fortified powders, respectively) were subjected to partial least squares – discriminant analysis (PLS-DA) using the leave-one-out (LOO) cross-validation method to identify the significant accession numbers having a variable-importance-in-projection score, VIP > 1 (Suppl. Table 1). As detailed in Suppl. Table 1, a total of 23, 27 and 11 protein accession numbers were identified as significant in the case of WPI, PPI and SPI fortified probiotic powder gastrointestinal digesta. In agreement with the SDS-PAGE data, β-Lg (Q9BDG3), GLYCAM1 (GLCM1) and lipocalin

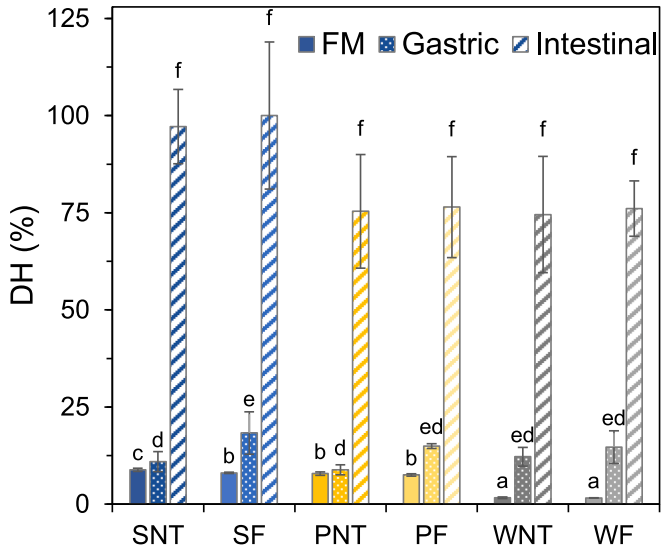

Fig. 4. Influence of protein composition (S = spirulina, P = pea, W = whey) and precursor treatment (NT = non-treated, F = fermented) on the degree of hydrolysis (DH) of probiotic powders during gastrointestinal in vitro digestion. [a-f] Different letters denote a significant difference according to Tukey's post hoc means comparison test ($p < 0.05$).

(G3X7G0) were among the strongest discrimination factors of the proteomes of the WPI-containing gastric digesta. On the other hand, β-casein (T1T0C1) and milk fat globule (MFG) proteins (A0AAA9RUL8) were significant proteomic markers of the intestinal digesta. In all cases, the digesta of the non-treated WPI based probiotic powders were associated with higher scores of the accession numbers corresponding to β-Lg, GLYCAM1, lipocalin, β-casein and MFG proteins. A very good discrimination of the proteomes of the digesta of the PPI fortified probiotic powders was achieved (Fig. 3B). Vicilin (D3VNE2), legumin A2 (LEGA2), convicilin (CVCA), vicilin (VCLC) and provicilin (VCLA) were significant discrimination factors for the digesta of the non-treated PPI-fortified probiotic powders. On the other hand, legumin L1 (Q7M1N3) and lectin (Q9ZRM7) were significant discriminators of the gastric digesta of the fermented PPI-fortified probiotic powders, whilst actin (P93485) was closely associated with their intestinal digesta counterparts. Phycocyanin α-subunit (Q6RX70), APC-B (A0A0C4VYX4) and CPC (A0A0C4W3X1) were prevalent in the gastric digesta SPI-based probiotic powders. On the other hand, three types of CPCs i.e., B2BN92, B2BN91 and B2BNA6 classified correctly the gastric phases of the fermented SPI-based probiotic powders. Finally, three fragments of phycocyanin α-subunit and CPC (A0A4Y5T1S2, A0A0C4W3X1 and B2BNA6, respectively) were significant classifiers of the intestinal digesta of the fermented SPI-based probiotic powders. Notably, RuBisCO did not confer any significant discrimination power as concerns the proteomes of the gastrointestinal digesta.

To provide insights into the extent of proteolysis under gastrointestinal conditions, the amount of free amines released per gram of protein was determined (Fig. 4). The DH rates in the undigested probiotic powders ranged from 8.0 to 8.9 %, 7.5–7.9 %, and 1.6–1.7 % for SPI, PPI, and WPI, respectively. The variations in the DH rates may be attributed to the selectivity of LGG's extracellular proteases for SPI and PPI over WPI. According to Kieliszek et al. [58], *Lacticaseibacillus* exhibits high selectivity for caseins over whey proteins, primarily due to the lower α-helix and β-sheet content in caseins. This open molecular structure increases protease accessibility compared to the more compact structure of whey proteins. Additionally, as demonstrated in our previous work [31], the secondary protein structures of SPI and PPI have lower α-helix and β-sheet content, potentially enhancing the proteolytic activity of LGG. Furthermore, the minimal difference in DH values

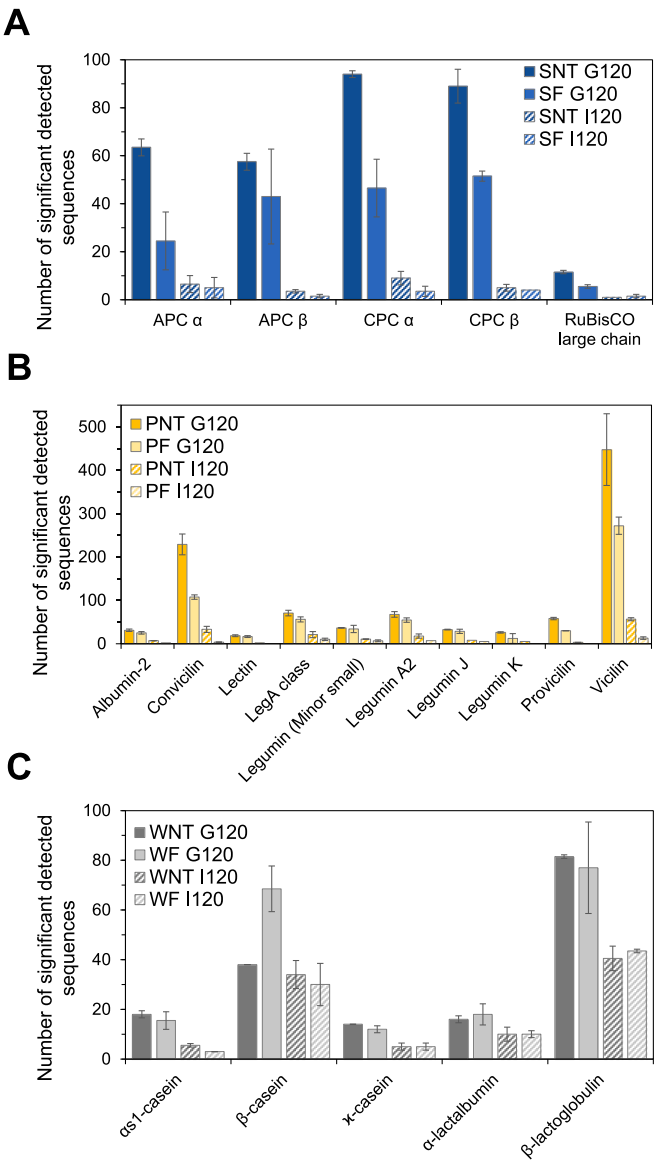

Fig. 5. Influence of protein composition (S = spirulina (A), P = pea (B), W = whey (C)) and precursor treatment (NT = non-treated, F = fermented) on the protein origin of detected peptide sequences in the gastrointestinal digesta of probiotic powders. Plain bars refer to gastric digesta (G120), and diagonal striped bars refer to intestinal digesta (I120). APC: Allophycocyanin, CPC: C-phycocyanin, RuBisCO: Ribulose-1,5-bisphosphate carboxylase/oxygenase, LegA: Legumin A.

between non-treated and fermented formulations could be due to the high LGG microbial load (~10 log CFU g^{-1}), the abundance of readily available carbon sources, and LGG's ability to cleave proteins and polypeptides in slightly acidic conditions (pH 6–6.5) in the early stages of lactic acid fermentation [59].

Following gastric processing, the DH rates remained relatively low, ranging from 9 % to 18 %, with higher rates observed in probiotic powders derived from fermented precursors (10.7 % for NT vs. 16.0 % for F). Although no significant differences ($p > 0.05$) were found regarding the protein source, a strong correlation was observed between gastric DH and the amount of residual intact protein classes in PPI and WPI. While protein secondary structure is typically linked to DH during digestion [60], no clear correlation was found between gastric DH and the prevalence of identified secondary structures (α-helix, β-sheet, aggregated strands), as was seen in the undigested probiotic powders ($r = -0.89$, $p < 0.05$). As expected, exposure of the gastric digesta to the

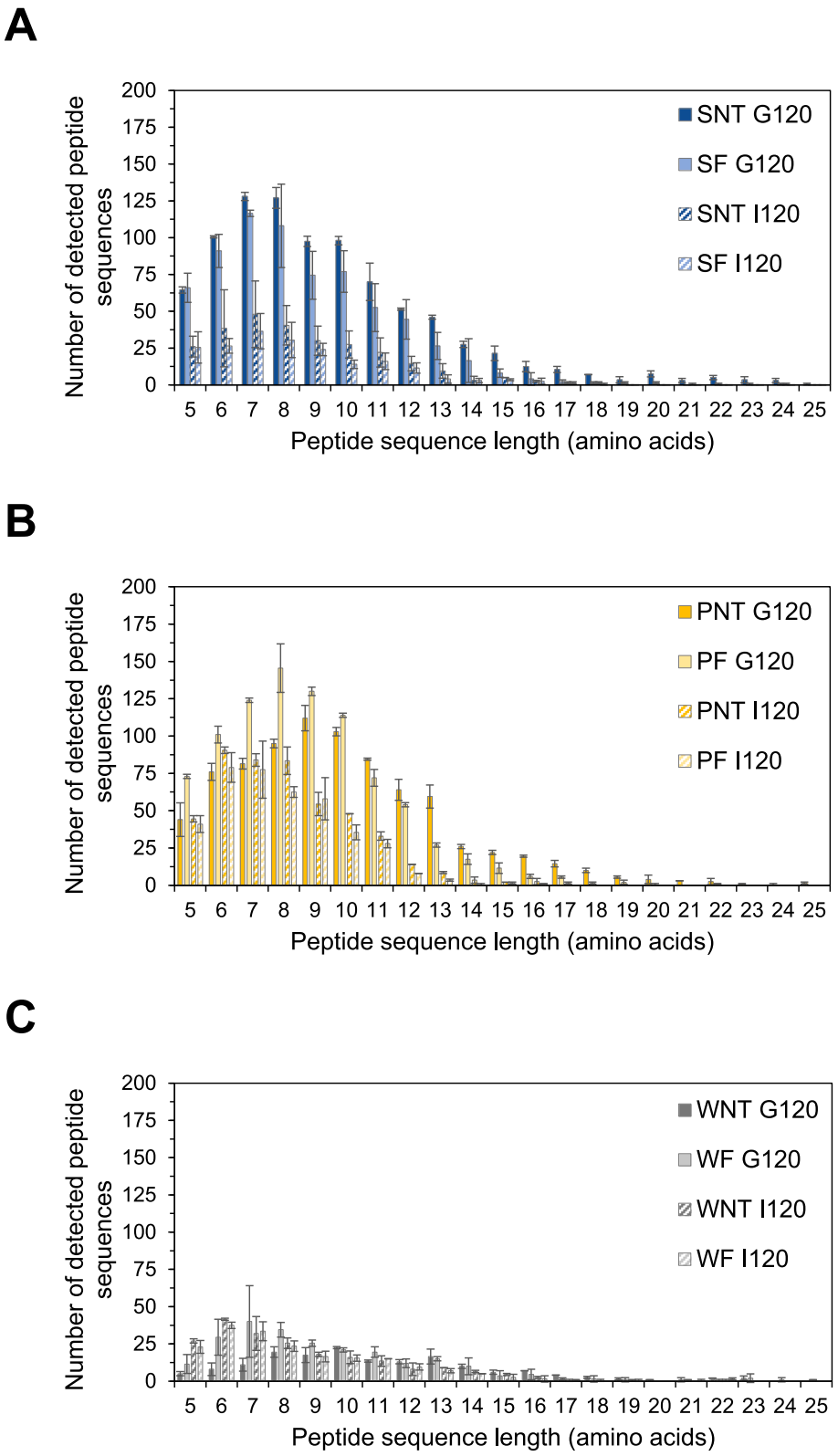

Fig. 6. Influence of protein composition (S = spirulina (A), P = pea (B), W = whey (C)) and precursor treatment (NT = non-treated, F = fermented) on the number of detected peptide sequences vs. their peptide sequence length (amino acid units) in the gastrointestinal digesta. Plain bars refer to gastric digesta (G120), and diagonal striped bars refer to intestinal digesta (I120).

jejunum environment led to a sharp increase in free amino acids, with average DH rates reaching approximately 99 %, 76 %, and 75 % for the SPI-, PPI-, and WPI-based intestinal digesta, respectively. Interestingly, neither protein type nor pre-fermentation significantly affected DH following intestinal digestion ($p > 0.05$).

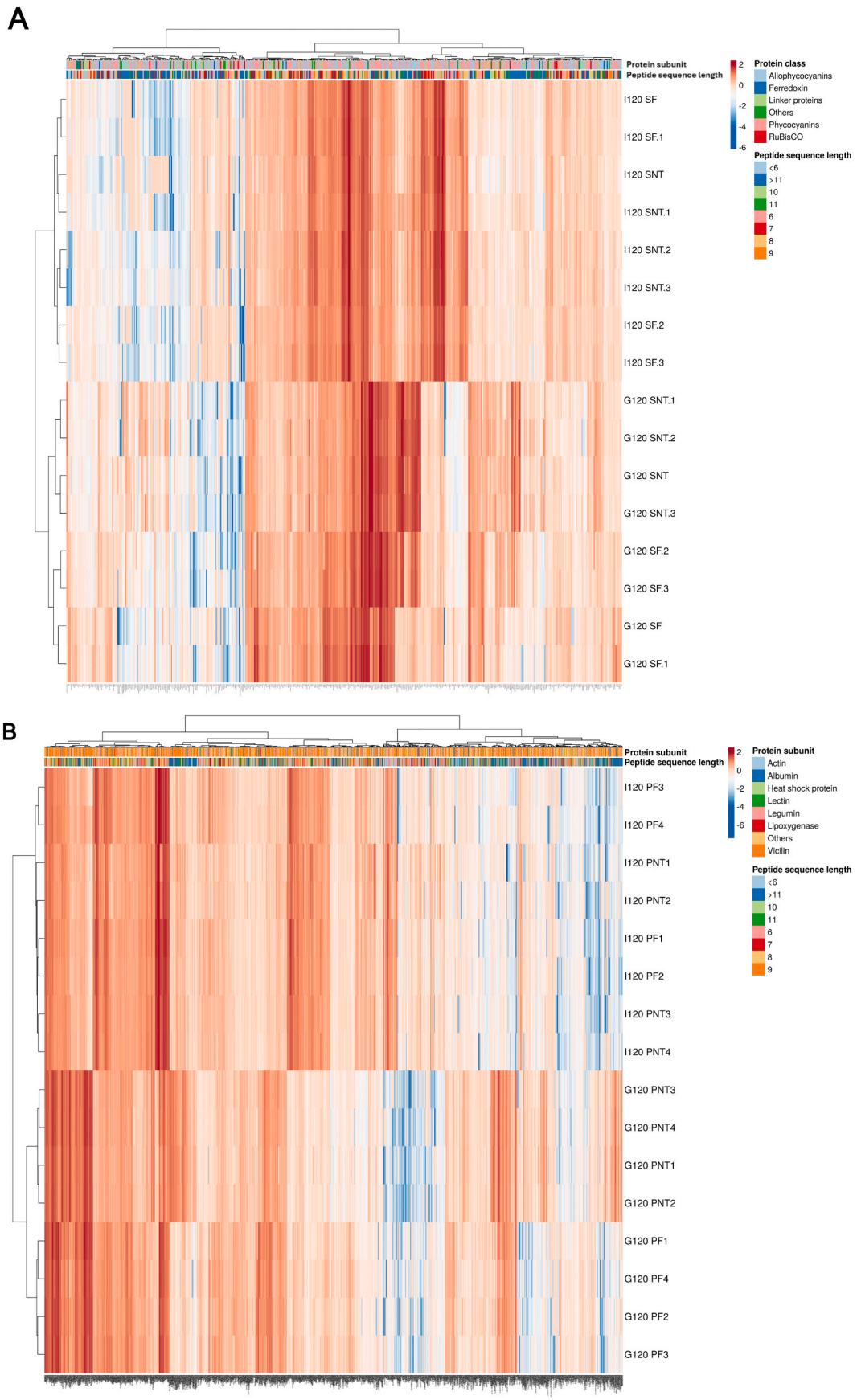

Fig. 7. Hierarchical cluster analysis heat map with dendrograms analysing the effect of precursor treatments (NT = non-treated, F = fermented) on the abundance of peptides, their corresponding peptide sequence lengths, and protein classes detected in the in vitro gastric (G120) and intestinal (I120) digesta of probiotic powders fortified with different protein isolates (S = spirulina (A), P = pea (B), and W = whey (C)).

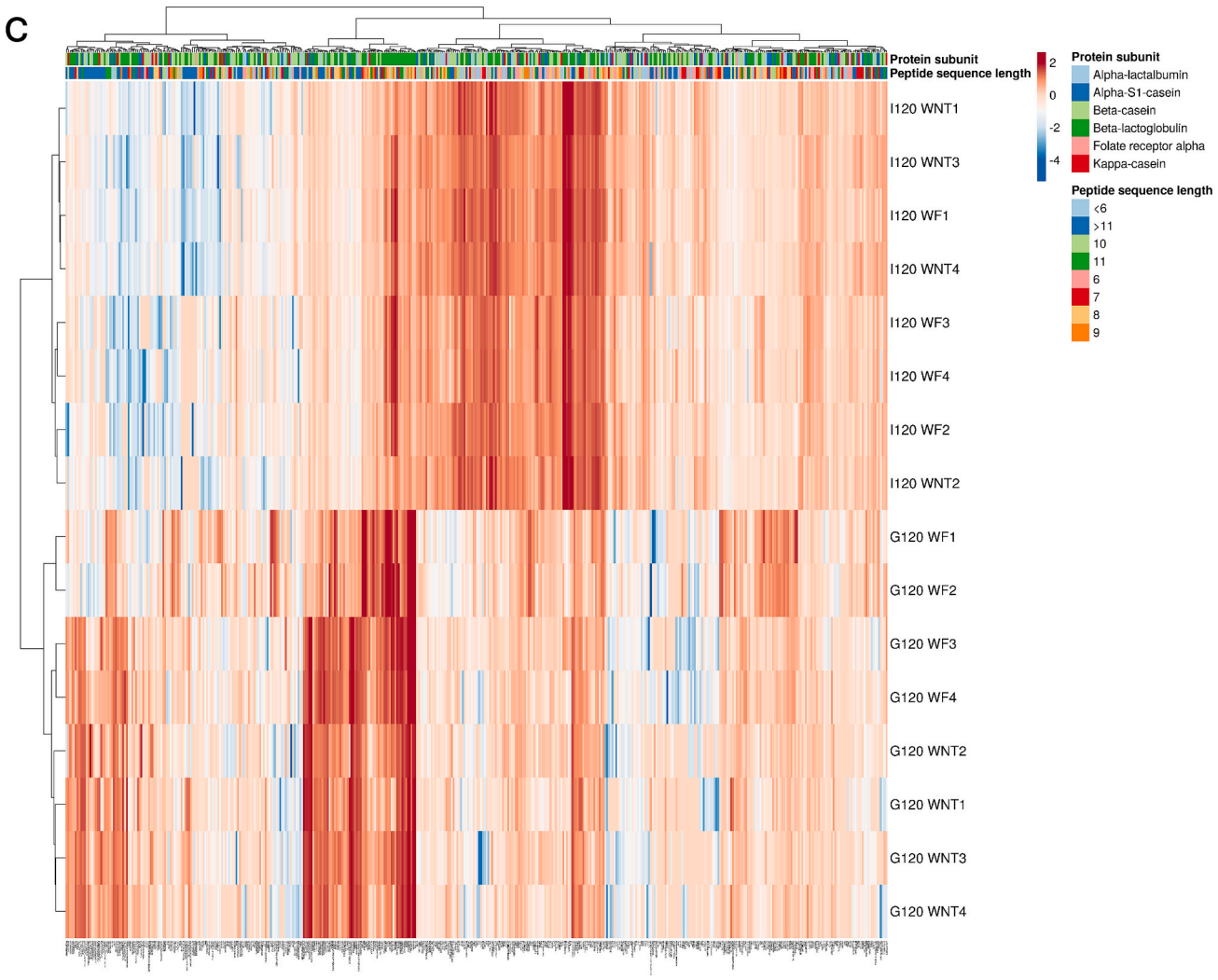

Fig. 7. (*continued*).

3.2. Peptidomic profile

The peptidome (0.5–3 kDa) of the gastrointestinal digesta of the probiotic powders was analysed to investigate potential health benefits associated with the release of bioactive peptides. Two methods were employed to present the peptidomic data: (a) illustrating the average number of detected peptide sequences and their originating proteins (Fig. 5) and (b) illustrating the average number of detected peptide sequences in relation to their amino acid sequence length (Fig. 6). In addition, hierarchical cluster analysis heat maps (Fig. 7) were constructed for revealing the relative concentration weighted differences in the peptidomes of the gastrointestinal digesta whereas Venn diagrams were constructed to illustrate shared peptide sequences within a single protein type (Fig. 8). As expected, the majority of the identified peptide sequences in the SPI-based gastrointestinal digesta were associated with phycocyanins (subunits α- and β-), APC (subunits α- and β-), linker proteins (CpcI, CpcH) and ribulose-1,5-bisphosphate (RuBisCO) proteins (Figs. 5A & 7A). Most of the peptide sequences found in the gastrointestinal digesta of PPI originated from vicilin, convicilin, and legumins (A2, J and K) (Fig. 5B & 8B). Actins, albumins, heat shock proteins, lectins and lipoxygenase (LOX) originated peptides were detected at lower concentrations (Fig. 8B). In the case of the WPI-containing digesta, the β-casein and β-Lg derived peptides were the most prevalent followed by lesser amounts of peptides originating from the hydrolysis of α-La, α_{s1}-casein and κ-casein (Fig. 5C & Fig. 8C). The significant amounts of total caseins (α_{s1}-, β-, and κ-casein) in the gastrointestinal digesta indicated the presence of impurities in the WPI powder [32]. Except for WPI, the fermentation of the lyophilisate precursors was associated with a significantly lower number of unique peptide sequences in both gastric and intestinal digesta. This observation is consistent with the higher DH rates observed in the gastric digesta of the fermented probiotic powders.

As shown in Fig. 6, the peptide sequences detected in the digesta of the probiotic powders were composed of 5–25 amino acid (AA) monomers, with the majority ranging from 7 to 10 AAs in the gastric phase and 6–7 AAs in the intestinal phase. Interestingly, fewer peptide sequences were released from WPI (Fig. 6C) compared to SPI (Fig. 6A) and PPI (Fig. 6B). In the case of the PPI fortified probiotic powders, fermentation led to a significant reduction in the number and relative concentration of the peptides detected in the gastric digesta, which aligns with the OPA assay findings (Figs. 6 & 7). As for the SPI-based digesta, fermentation led to a significant decrease in the relative concentration of the peptides composed of at least 12 AA monomers. In both cases, the peptides comprising 6–10 AAs were the most prevalent in terms of number and relative concentration. Although fermentation led to an increase in the number of the peptide sequences identified in the gastrointestinal digesta, their relative concentration remained rather unaltered when comparing the WF and WNT digesta systems (Fig. 7C). Mixed effects of the pepsin and proteases mediated hydrolysis on the peptides relative concentration were observed (Fig. 6C & 7C). In

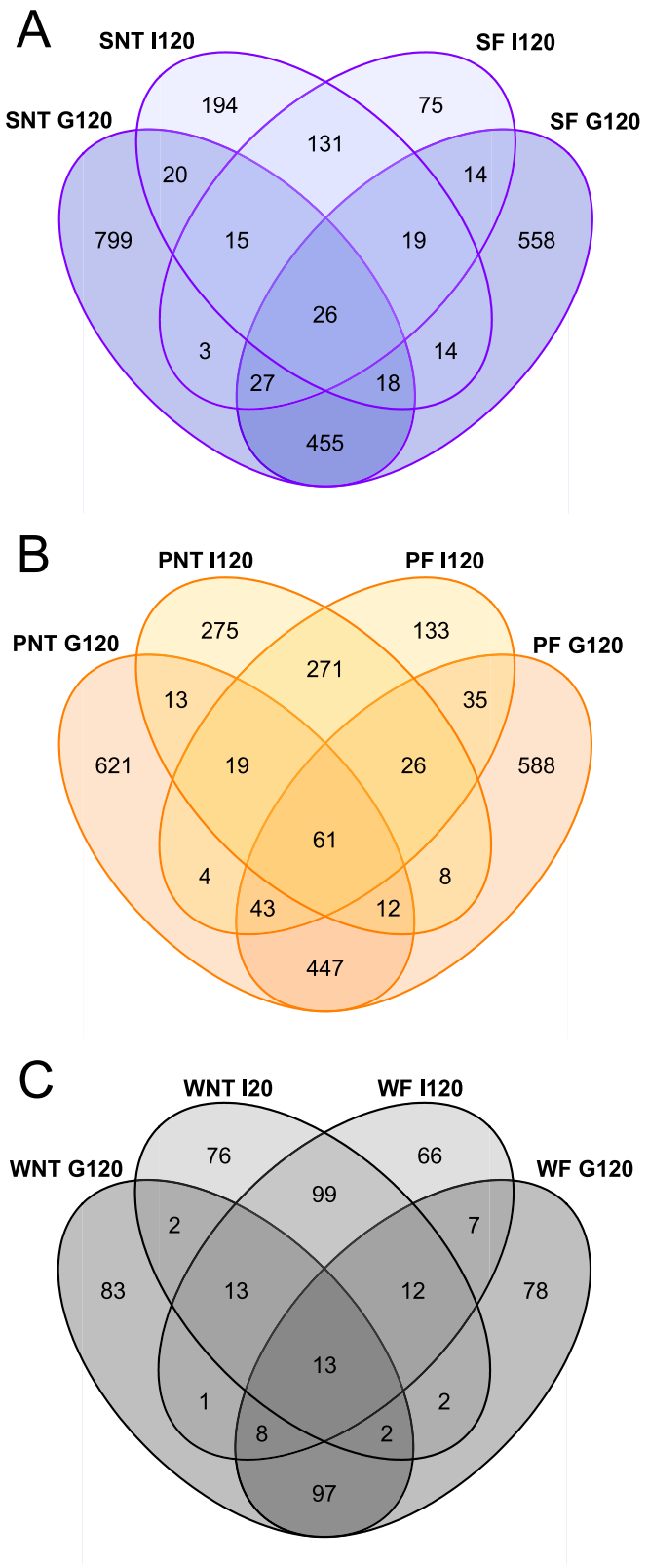

Fig. 8. Venn diagrams illustrating the common peptide sequences found in the gastric (G120) and intestinal (I120) digesta of probiotic powders prepared with different protein isolates (S = spirulina (A), P = pea (B), W = whey (C)) and precursor treatments (NT = non-treated, F = fermented).

general, a substantial increase in the concentration of peptides comprising 5–9 AAs detected in the intestinal digesta was found (Fig. 7C), which is mainly attributed to the depletion of the intestinal digesta in peptides comprising >11 AA monomers.

Venn diagrams illustrated the common peptide sequences in the gastrointestinal digesta of the probiotic powders (Fig. 8). Approximately 1247, 1259, and 313 peptide sequences were detected in the gastric digesta of SPI (Fig. 8A), PPI (Fig. 8B), and WPI-based (Fig. 8C) samples, respectively. In the intestinal digesta, 373, 638, and 281 unique peptide sequences were detected in the SPI, PPI, and WPI-based chymes of the probiotic powders, respectively. Notably, nearly twice as many unique peptide sequences were found in non-fermented gastric digesta compared to fermented samples, suggesting that fermentation enhanced the diversity of proteases (except pepsin, tryptic, and pancreatic enzymes) in the gastrointestinal digesta, facilitating untargeted peptide hydrolysis.

Table 1 summarises the identification of 6, 11, and 52 potential bioactive peptide sequences in the gastrointestinal digesta of SPI, PPI, and WPI-based probiotic powders, respectively. Most bioactive peptides were associated with potential DPP-IV and ACE inhibitory activities. Other potential bioactivities included antioxidative, zinc-binding, antimicrobial, and osteoanabolic effects, particularly in WPI and PPI-based gastrointestinal digesta. Regarding protein origin, most bioactive peptide sequences were derived from APC and CPC in SPI, vicilin, legumin, and albumin-2 in PPI, and β-Lg and β-casein in WPI-based probiotic powders. More bioactive peptide sequences were detected in the gastric and intestinal digesta of non-treated samples compared to fermented ones. In contrast, pre-fermented probiotic powders based on PPI and WPI yielded the majority of identified bioactive peptide sequences in the gastrointestinal chymes.

Regarding the potential bioactive peptide sequences found in SPI-based samples, none of the peptides identified in the gastric digesta were resistant to cleavage in the intestine. In contrast, four potential bioactive peptide sequences (416LAFPGSAQE424, 385LAFPGSS391, 273LPNYNSRA280, and 54TETWNPNHPEL64) were identified in PF, which were resistant to pancreas – induced cleavage. Notably, ^{54}TETWNPNHPEL^{64} was the only bioactive peptide sequence present in each gastrointestinal digesta of the PPI-based probiotic powders. It can be hypothesised that ^{416}LAFPGSAQE^{424} and ^{385}LAFPGSS^{391} might be cleaved into shorter potential bioactive peptide sequences such as LAF, FP, or AF during in vitro digestion, potentially exerting new bioactivities, such as antioxidative effects and ACE inhibition [61,62]. Due to the jack-knifing of 5 AAs used for data analysis, verifying this hypothesis is not possible.

Four potential bioactive peptide sequences (^{143}AYFYPEL^{149}, ^{56}LNVPGEIVE^{64}, ^{141}TPEVDDEALEK^{151} and ^{58}YVEELKPTPEGDL^{70}) resistant to gastrointestinal in vitro digestion were found in the digesta of WPI-based probiotic powders. Interestingly, potential bioactive peptide sequences such as ^{143}AYFYPEL^{149}, ^{59}VEELKPTPEGDLEIL^{73}, ^{145}VENLHLPLPLL^{155}, ^{195}SDIPNPIGSENSEK^{208}, ^{58}YVEELKPTPEGDL^{70} in the WPI-based digesta partially aligned with other bioactive peptide sequences found in the chymes. Comparing shorter bioactive peptide sequences (2–6 AAs), partial alignments were found across detected peptide sequences in each sample post gastrointestinal digestion (Suppl. Material Excel file). The majority of these partially overlapping potential bioactive peptides were found in the gastric chymes of the SPI- and PPI-based samples (734 and 663, respectively).

3.3. Initial total amino acid (AA) composition and bioaccessibility

Except for Try, which degraded under acid hydrolysis conditions, the AA composition of the undigested probiotic powders is displayed in Suppl. Table 2. The AA composition of these powders was characterised by a predominantly higher total non-essential amino acids (TNEAAs) content (37.7–43.3 %) compared to total essential amino acids (TEAAs) (17.8–22.6 %). The fermentation of the precursors was associated with a

Table 1
Potential bioactive peptides derived from probiotic powders prepared with different protein isolates during in vitro gastrointestinal digestion (S = spirulina, P = pea, W = whey; NT = non-treated, F = fermented; G120 = after 120 min of gastric digestion, I120 = after 120 min of intestinal digestion).

Potential bioactive peptide sequence	Protein	Bioactivity (IC_{50} (mg mL^{-1}))	G120		I120		References
			SNT	SF	SNT	SF	
^{44}ITSNASTIVSNAAR^{57}	β-CPC	DPP-IV inhibitory (1.0)	✓				[27]
^{7}SIVNADAEAR^{16}	α-APC	DPP-IV inhibitory (1.0)	✓	✓			[27]
^{455}IAENAGENGAVVAER^{470}	60 kDa chaperonin	DPP-IV inhibitory (0.1) ACE inhibitory (0.28)	✓				[26]
^{17}YLSPGELDR^{25}	α-APC	DPP-IV inhibitory (0.1) ACE inhibitory (0.28)			✓		[26]
^{135}EVTAGLVGADAGK^{147}	β-CPC	DPP-IV inhibitory (0.1) ACE inhibitory (0.28)			✓	✓	[26]
^{87}VDMVVGAVPK^{96}	Thioredoxin	DPP-IV inhibitory (0.1) ACE inhibitory (0.28)			✓	✓	[26]

Potential bioactive peptide sequence	Protein	Bioactivity (IC_{50} (μm))	G120		I120		References
			PNT	PF	PNT	PF	
^{343}ADLYNPR^{349}	Legumin J	antioxidative			✓		[69]
^{444}AKSLSDRFSY^{453}	LegA class	up-regulation ACE2 (antihypertensive)	✓	✓			[70]
^{40}ALEPDHR^{46}	Legumin (Minor small)	antioxidative			✓		[71]
^{109}FPFFEGT^{105}	Albumin 2	DPP-IV inhibitory (208)	✓	✓			[72]
^{105}IADMFP^{110}	Albumin-2	DPP-IV inhibitory (254)			✓		[72]
^{142}IAIPPGIPYW^{151}	Legumin (Minor small)	DPP-IV inhibitory (12)		✓			[73]
^{416}LAFPGSAQE^{424}	Vicilin	DPP-IV inhibitory (224)	✓	✓		✓	[72]
^{385}LAFPGSS^{391}	Vicilin	DPP-IV inhibitory (87)		✓	✓	✓	[72]
^{273}LPNYNSRA^{280}	Vicilin	DPP-IV inhibitory (> 500)	✓	✓		✓	[72]
^{380}SAEHGSLH^{387}	LegA class	antioxidative			✓	✓	[71]
^{54}TETWNPNHPEL^{64}	Legumin	antioxidative	✓	✓	✓	✓	[71]

[1]Potential bioactive peptide sequence	Protein	Bioactivity (IC_{50} (μm))	G120		I120		References
			WNT	WF	WNT	WF	
^{143}AYFYPE^{148}	α_{s1}-Cas	ACE inhibitory (106), antioxidative			✓		[74,75]
^{143}AYFYPEL^{149}	α_{s1}-Cas	ACE inhibitory (261), antioxidative (7)	✓	✓	✓	✓	[76,77]
^{33}DAQSAPLRVY^{42}	β-Lg	ACE inhibitory (12)	✓	✓			[78]
^{157}DAYPSGAW^{164}	α_{s1}-Cas	ACE inhibitory (98)		✓			[79]
^{112}DTDYKKY^{118}	β-Lg	Zinc binding peptide		✓			[]
^{99}EDVPSER^{105}	α_{s1}-Cas	osteoanabolic, antioxidative			✓	✓	[81,82]
^{90}EKTKIPAVF^{98}	β-Lg	Zinc binding peptide		✓			[80]
^{61}ELKPTPEGDLEIL^{73}	β-Lg	Zinc binding peptide	✓		✓	✓	[80]
^{83}FQSEEQQQTEDELQDK^{98}	β-Cas	Promote calcium uptake			✓	✓	[83,84]
^{145}FYPEL^{149}	α_{s1}-Cas	antioxidative, ACE inhibitory (81)		✓	✓		[76,85]
^{149}HLPLP^{153}	β-Cas	ACE inhibitory (41)			✓	✓	[86]
^{149}HLPLPL^{154}	β-Cas	PEP inhibitory (10, antiamnestic)			✓		[87]
^{21}INNQFLPYPY^{30}	κ-Cas	DPP-IV inhibitory (40)	✓	✓			[88]
^{89}IVQNNDSTEYGLF^{101}	α-La	DPP-IV inhibitory (337)	✓				[84]
^{90}KTKIPAVF^{98}	β-Lg	Zinc binding peptide	✓				[80]
^{26}LDIQKVAGTW^{35}	β-Lg	ACE-inhibitory (21)	✓	✓			[89]
^{148}LHLPLP^{153}	β-Cas	ACE inhibitory (3)				✓	[86]
^{148}LHLPLPL^{154}	β-Cas	ACE inhibitory (433)		✓		✓	[90]
^{156}LKALPMH^{162}	β-Lg	DPP-IV inhibitory (193), ACE inhibitory (11)		✓			[89]
^{62}LKPTPEGDL^{70}	β-Lg	DPP-IV inhibitory (45)			✓		[83,84]
^{62}LKPTPEGDLE^{71}	β-Lg	DPP-IV inhibitory (42)	✓	✓		✓	[89]
^{62}LKPTPEGDLEIL^{73}	β-Lg	DPP-IV inhibitory (57)	✓	✓			[84]
^{56}LNVPGEIVE^{64}	β-Cas	ACE inhibitory (300)	✓	✓	✓	✓	[91]
^{137}LVRTPEVDDE^{147}	β-Lg	Zinc binding peptide	✓	✓			[80]
^{108}LVYPFPGPI^{116}	β-Cas	ACE inhibitory (180.2)		✓	✓	✓	[92]
^{106}MAIPPK^{111}	κ-Cas	ACE inhibitory (6, 33 % inhibition)			✓	✓	[93]
^{73}NIPPLTQTPV^{82}	β-Cas	ACE inhibitory (1798)				✓	[91]
^{147}NLHLPLPLL^{155}	β-Cas	anticancer, ACE inhibitory (15)	✓	✓			[94]
^{61}PFPGPIPN^{68}	β-Cas	ACE inhibitory (13)			✓		[95]
^{86}PQNIPPL^{92}	β-Cas	DPP-IV inhibitory (1500)		✓			[96]

(*continued on next page*)

Table 1 (*continued*)

[1]Potential bioactive peptide sequence	Protein	Bioactivity (IC_{50} (μm))	G120		I120		References
			WNT	WF	WNT	WF	
[131]PVVVPPFLQPE[141]	β-Cas	antimicrobial		✓	✓	✓	[97]
[183]RDMPIQ[188]	β-Cas	antioxidative			✓		[98]
[90]RYLGYL[95]	α_{s1}-Cas	opioid; anticancer		✓			[99,100]
[195]SDIPNPIGSE[204]	α_{s1}-Cas	antidiabetic		✓			[101]
[195]SDIPNPIGSENSEK[208]	α_{s1}-Cas	antibacterial			✓	✓	[102]
[33]SRYPSY[38]	κ-Cas	opioid antagonist	✓				[103]
[141]TPEVDDEALEK[151]	β-Lg	DPP-IV inhibitory (320), antimicrobial	✓	✓	✓	✓	[104]
[77]TTFHTSGY[84]	α-La	ACE inhibitory (142)	✓	✓			[105]
[15]VAGTW[19]	β-Lg	ACE inhibitory (534)	✓				[106]
[59]VEELKPTPEGDLEIL[73]	β-Lg	Zinc binding peptide	✓		✓	✓	[80]I
[145]VENLHLPLPLL[155]	β-Cas	anticancer, ACE inhibitory (15)	✓	✓			[94]
[139]VESTVATL[146]	κ-Cas	antibacterial	✓	✓			[107]
[94]VLDTDYK[100]	β-Lg	ACE inhibitory (946)			✓	✓	[106]
[162]VQVTSTAV[169]	κ-Cas	antibacterial	✓	✓			[107]
[139]VRTPEVDDE[147]	β-Lg	Zinc binding peptide	✓		✓	✓	[80]
[109]VYPFPGPI[116]	β-Cas	PEP inhibitory (antiamnestic,110)		✓	✓		[87]
[193]WMHQPHQPLPPTVM[206]	β-Cas	Zinc binding peptide		✓			[80]
[144]YFYPEL[149]	α_{s1}-Cas	antioxidative	✓	✓		✓	[85]
[118]YKVPQL[124]	α_{s1}-Cas	ACE inhibitory (22)	✓	✓			[108]
[193]YQEPVL[198]	β-Cas	ACE inhibitory (8)		✓			[109]
[58]YVEEL[63]	β-Lg	antioxidative, osteoanabolic		✓		✓	[110,111]
[58]YVEELKPTPEGDL[70]	β-Lg	antioxidative	✓	✓	✓	✓	[112]

CPC: C-phycocyanin, APC: Allophycocyanin, DPP-IV: Dipeptidylpeptidase-IV, ACE: Angiotensin-converting enzyme, LegA: LeguminA, Cas: Casein, β-Lg: β-Lactoglobulin, α-La: α-Lactalbumin.

significantly ($p < 0.05$) higher amount of TNEAAs and TEAAs compared to their untreated counterparts (60.0 % vs. 50.3 % and 32.9 % vs. 24.7 %, respectively). The WPI-based probiotic powders exhibited the highest concentration of TEAAs (63.4 %) compared to the PPI- and SPI-based analogues (53.4 % and 43.8 %, respectively). Minor differences in the prevalence of free AAs in the probiotic powders, influenced by the protein composition, were found. In the SPI- and PPI-based formulations, His was the most abundant amino acid, followed by Lys and Leu. In contrast, in the WPI-based analogues, Lys had the highest content, followed by Leu and His. Regarding the NEAAs, Asp was the predominantly found across all formulations (6.0–6.6 %), followed by Cys, Glu, and Ala in SPI; Cys, Glu, and Gln in PPI; and Glu, Gln, and Cys in the WPI-based powders. Our findings are generally consistent with those reported by Auer et al. [63] and Becker [10] on PPI and spirulina biomass, respectively.

Following in vitro gastrointestinal digestion, a significant reduction in the TEAAs (9.3–14.3 %) and TNEAAs (6.8–15.8 %) content was observed in the intestinal digesta (Suppl. Table 2). This reduction could be attributed to the metabolic activity of live LGG cells during the in vitro simulated intestinal transit, with TVCs ranging from 7.7 to 9.0 log CFU g^{-1} in the jejunum digesta after 2 h [31]. The utilisation of free AAs as a primary nitrogen source by lactic acid bacteria is well documented. In this context, Sun et al., [64] reported that the free AA dependent growth rate (%) of LGG in bovine skim milk followed the order: Thr < Cys < Arg < Ile < Phe < Tyr < Leu < Pro < Lys < Asp < Asn < Met. Additionally, the nitrogen source itself (i.e., type of protein or protein hydrolysates) has been shown to influence the depletion or synthesis patterns of free AAs in Lactobacillus strains [65]. Consistent with the findings in undigested probiotic powders, the TEAAs content in the intestinal digesta followed a descending order of WPI < PPI < SPI. Notably, no significant differences in the TEAAs and TNEAAs content were observed between the untreated and fermented probiotic powder digesta, with values of 11.8 % vs. 11.0 % and 12.9 % vs. 10.3 %, respectively. This similarity could be attributed to the minimal differences in LGG survival rates throughout the gastrointestinal digestion between the non-treated and fermented probiotic formulations.

As illustrated in Fig. 9, the bioaccessibility of TEAAs and TNEAAs ranged from 17.7 to 39.4 % and 45.1 to 68.7 %, respectively (Suppl. Material Table 3). Fermentation of the powder precursors led to a significant decrease in the bioaccessibility of both TEAAs and TNEAAs. Notably, the bioaccessibility of TEAAs followed the descending order: WPI > PPI > SPI. Specifically, the SPI-based powders exhibited the highest bioaccessibility for Cys (91.3–93.0 % for SNT and SF, respectively) and Met (57.6–60.2 % for SNT and SF, respectively), while PPI demonstrated the highest bioaccessibility for Ile (56.2–32.0 % for PNT and PF, respectively) and Arg (469.5–372.9 % for PNT and PF, respectively). WPI exhibited the highest bioaccessibility for Gly (56.7–62.7 % for WNT and WF, respectively). Previous studies have highlighted the inextricability between free AAs bioaccessibility and the protein composition of the food matrix. For instance, Roux et al. [66] reported that PPI- and WPI-based infant food formulas had the highest bioaccessibility rates for Tyr and Lys (~87 % and 70 %, respectively). However, to our knowledge, no studies have reported on the bioaccessibility of AAs from microalgal protein isolates. Interestingly, the Arg content in the intestinal digesta was 2- to 5-fold higher than in the undigested probiotic powders, suggesting its de novo production by the bacteria, likely through the pyrimidine biosynthetic pathway [67] or via the catabolism of other AAs such as Ala, Glu, and Asp [68], as these AAs were significantly depleted. In alignment with this, Bao et al. [8] reported a substantial increase (up to 265 %) in free Arg following the fermentation of spirulina biomass with *B. subtilis*, *L. plantarum*, and L. *acidophilus*.

To gain deeper insight into the association between protein digestibility and AA profiles in the gastrointestinal digesta of probiotic powders and their physicochemical, structure conformational, and microbiological properties (as previously measured in our work [31]), the generated data were log transformed, auto-scaled, mean centred and subjected to Principal Components Analysis (PCA). According to the obtained PCA biplot (Fig. 10), all probiotic powder samples were well-discriminated based on their protein composition and the pre-fermentation of the precursors. Notably, the PPI and SPI-based probiotic powders showed a higher affinity compared to the WPI counterparts. Regarding the impact of the secondary structure conformation of the protein isolates, we detected a straightforward correlation of the amount of α-helix (positive) and β-sheet (negative) structures with the bioaccessibility of the TEAAs. On the other hand, we observed a strong correlation of the amount of aggregated strands with the bioaccessibility of the TNEAAs. From a colloidal perspective, the protein aggregates

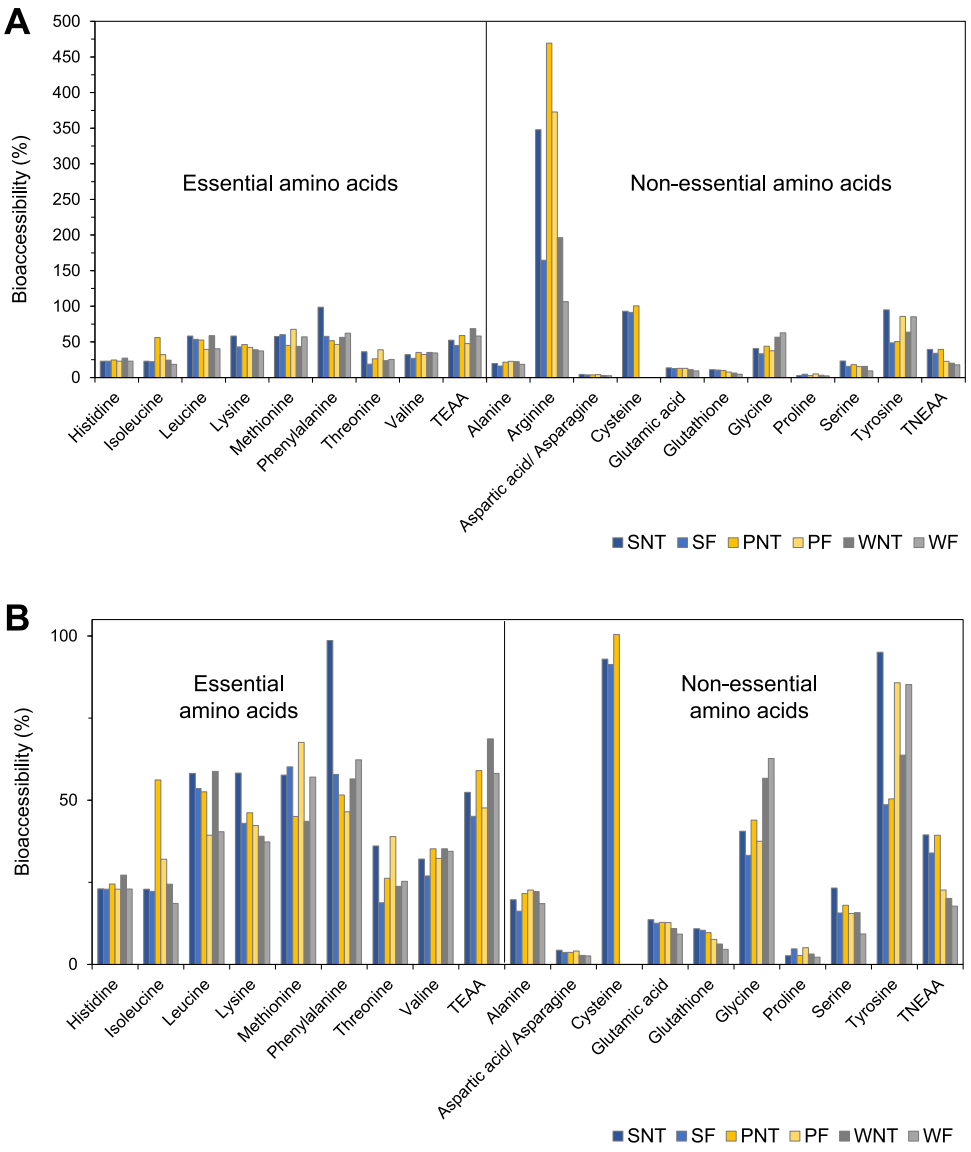

Fig. 9. Bioaccessibilities of total essential amino acids (TEAA), total non-essential amino acids (TNEAA), and amino acid monomers after intestinal digestion of probiotic powders prepared with different protein isolates (S = spirulina, P = pea, W = whey) and precursor treatments (NT = non-treated, F = fermented).

polydispersity (span) in the gastric digesta was impactful on the TEAAs and TNEAAs bioaccessibilities as well as the degree of hydrolysis achieved at the end of the in vitro intestinal digestion. That implies that the presence of different protein aggregate populations favours the accessibility of peptic enzymes to the protein cleavage sites. On the other hand, no clear associations between the mean size ($d_{4,3}$) of the protein aggregates, digestibility and TEAAs and TNEAAs bioaccessibilities were observed. Finally, changes in LGG TVC during gastrointestinal transit primarily affected the residual intact protein subunits, largely due to the ability of LGG to produce extracellular proteases, which synergistically contribute to the activity of peptic enzymes.

4. Conclusions

In this study, we investigated the potential secondary health benefits of protein isolate-fortified (SPI, PPI, and WPI) probiotic powders, specifically focusing on the release of bioactive peptides and free AAs. Fermentation of the lyophilised precursors resulted in only a mild proteolytic effect, as confirmed by densitometric analysis of capillary SDS–PAGE electropherograms of the undigested probiotic powders. Proteomic characterisation of the gastric digesta revealed that CPC and β-Lg (in SPI- and WPI-fortified powders) exhibited the highest resistance to pepsin hydrolysis compared to other polypeptide subunits, such as α-La, κ-caseins, legumins, and convicilin. This resistance to pepsinolysis was attributed primarily to the secondary structural conformation of the polypeptide subunits. In contrast, all polypeptide subunits in SPI, PPI, and WPI formulations underwent significant pancreatic cleavage. According to the OPA assay, the in vitro digestibility rate of the probiotic powders at the end of simulated gastrointestinal transit followed the order: SPI > PPI > WPI. Peptidomic screening of the gastric and intestinal digesta enabled the identification of 6, 11, and 52 potential bioactive peptide sequences in the digesta of the SPI, PPI, and WPI formulations, respectively. With the exception of Arg, a substantial decrease in the net amount of free essential and non-essential AAs was observed at the end of gastrointestinal digestion, likely due to metabolic utilisation by LGG. In conclusion, the inclusion of protein isolates in probiotic powder formulations offers tangible supplementary health benefits by enhancing LGG bioactivity through the release of potentially bioactive peptides and bioaccessible free essential AAs.

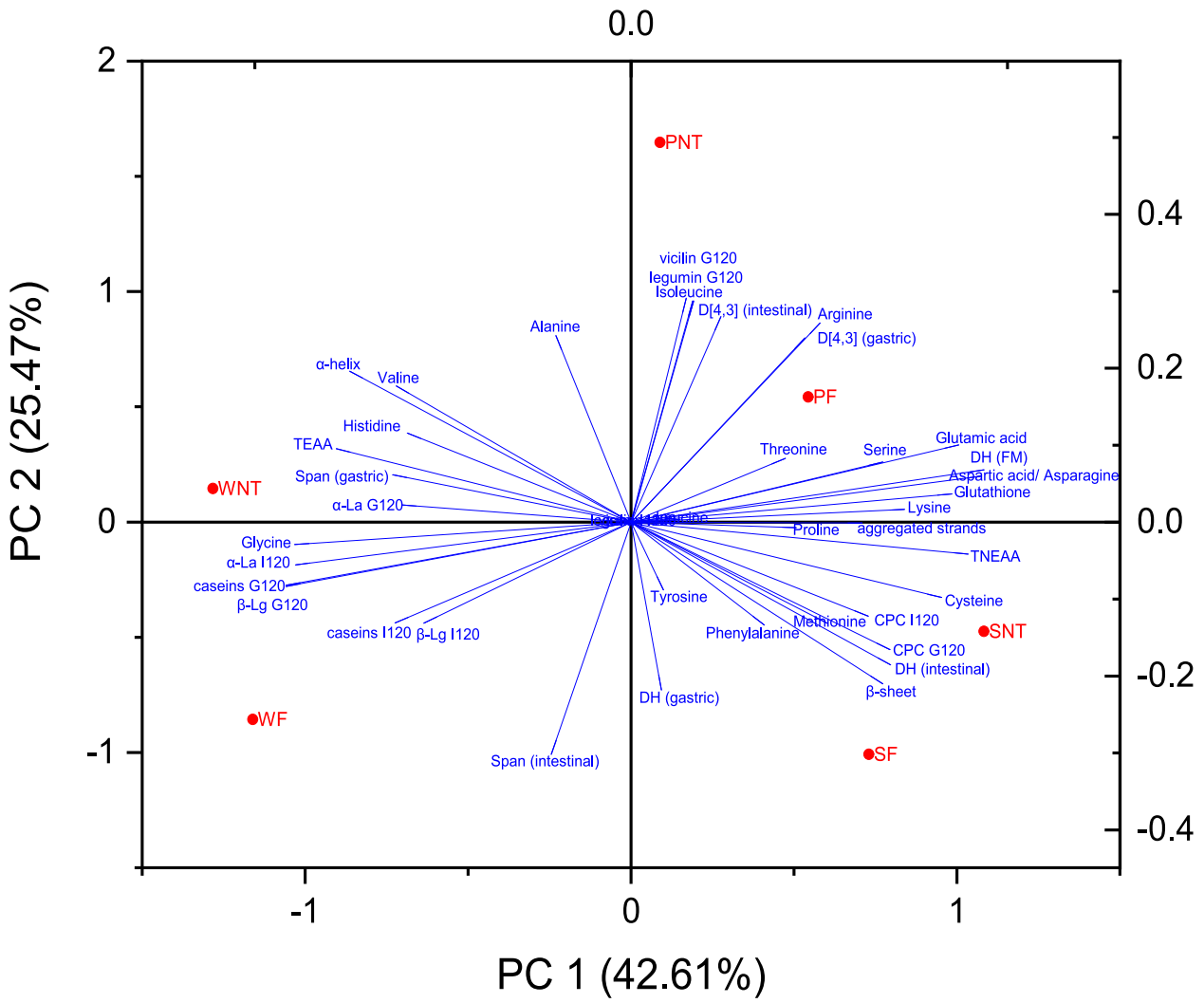

Fig. 10. Principal component analysis (PCA) for the effect of different proteins (S = spirulina, P = pea, W = whey) and precursor treatments (NT = non-treated, F = fermented) of probiotic powders on the degree of hydrolysis (DH), span and de Brouckere particle size mean diameter (D [3,4]) of the gastric (G120) and intestinal (I120) digesta, total essential amino acids (TEAA) and total non-essential amino acids (TNEAA) after intestinal in vitro digestion, as well as the protein secondary structure (α-helix, β-sheet, and aggregated strands). CPC: C-phycocyanin, β-Lg: β-lactoglobulin, α-La: α-Lactalbumin.

CRediT authorship contribution statement

Jennyfer Fortuin: Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Céline C. Leclercq:** Writing – review & editing, Investigation, Formal analysis. **Marcus Iken:** Writing – review & editing. **Silas G. Villas-Boas:** Writing – review & editing, Investigation. **Christos Soukoulis:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

PM International AG had no influence on the study design, data collection and analysis, decision to publish, or preparation of the manuscript. LIST affiliated authors declare that the research was conducted in the absence of any financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.140432.

Data availability

Data will be made available on request.

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