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Interaction of magnetic silica nanoparticles with food proteins during *in vitro* digestion

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ABSTRACT

The aim of the present work was to investigate the interaction of nanoparticles (NPs) with proteins during the digestion of selected food matrices. To accomplish this, magnetic silica NPs were added to skimmed milk powder (SMP), peanuts, and tofu, and the mixtures were subjected to *in vitro* digestion. The proteins and peptides present in the digesta, with and without NPs, and in the protein corona (PC) of NPs at the different digestion stages (oral, gastric, and intestinal) were characterised and identified with gel electrophoresis and mass spectrometry. Electrophoretic results revealed no differences in protein patterns between the control and NPs containing digesta at all digestion stages. At the end of simulated intestinal digestion, no intact food proteins were detectable, either in the food or on the NPs. At this stage, exclusively, digestive enzymes originating from pancreatin were present in the digesta and bound to NPs. Digestion-resistant peptides were detected in the digests of all studied food matrices, independently of NP presence. Moreover, on the PC of NPs digestion-resistant peptides, especially longer ones, were exclusively present after gastric and intestinal digestion stages from SMP and peanuts, but not from tofu, indicating that the PC composition was influenced by the food matrix.

1. Introduction

Over the past years there has been steady growth in the use of nanoparticles (NPs) in agriculture, cosmetics, and medicine due to their novel physico-chemical properties (e.g. their ability to improve solubility and protect active components during the production process (He, Deng, & Hwang, 2019); The NPs mainly used in the food industry can be classified according to their composition into two types: organic (e.g. lipid, protein, or carbohydrate) and inorganic (e.g. TiO2, SiO2, FeO, and Ag) NPs (McClements & Xiao, 2017). NPs are widely used as additives in the food industry to modify texture and colour, as well as to increase the shelf life of products (Bajpai et al., 2018; Dehnad, Mirzaei, Emam-Djomeh, Jafari, & Dadashi, 2014). They are also often unintentionally present in food as a result of processing during homogenisation, grinding, heat treatment, or packaging. In dairy products, NPs (mainly SiO2; E551, European designation) are added to modify texture and powder flow characteristics (McClements et al., 2016). Since October 2011, European Union (EU) legislation has required that nanomaterials used in the food and cosmetics industries be appropriately labelled as such (EU-2283, 2015).

NPs have a high surface-to-volume ratio, which allows them to interact very efficiently with food components, and it has been suggested that this interaction can affect the digestion and absorption of food in the digestive tract. The surface properties of NPs depend on several inherent factors, such as size, shape, and functional group, but also on external factors, such as the concentration and properties of various food components and environmental conditions (pH, temperature, etc. (McClements et al., 2016; McClements et al., 2017)). However, because all these factors change constantly along the intestinal tract during digestion, it is challenging to predict the interaction of NPs with food ingredients during the digestion process. The surfaces of NPs spontaneously interact with molecules (i.e. proteins and peptides) that are present in the NP surrounding medium, thereby forming a mostly protein rich layer around the NPs, being called the 'protein corona' (PC). The composition of the medium therefore has a great impact on the PC and can affect colloidal stability of NPs, initiate NP aggregation, and influence cellular interactions (Monteiro-Riviere, Samberg, Oldenburg, & Riviere, 2013; Moore et al., 2015). Very little is known about the influence of NPs' presence on proteolysis during the digestion of food. Di

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Abbreviations

NP	nanoparticle
SMP	skimmed milk powder

Silvio and colleagues (Di Silvio, Rigby, Bajka, Mackie, & Baldelli Bombelli, 2016) reported that some proteins of the corona were resistant to the duodenal digestion of bread, suggesting that proteins constituting part of the corona of NPs might be less accessible to the action of proteolytic enzymes in the digestive tract, which could result in an incomplete digestion process and the presence of partially hydrolysed proteins and peptides, which in turn could lead to allergic reactions or other health problems (McClements et al., 2016).

Studies on the toxicity and absorption of silver NPs in epithelial cells have shown that, in the absence of food, silver NPs tend to aggregate quickly during their passage through the digestive tract (Bove, Malvindi, & Sabella, 2017; Kästner, Lichtenstein, Lampen, & Thünemann, 2017; R. J. B. Peters et al., 2014); however, in the presence of food, NPs are protected from aggregation and remain present as such or as very small aggregates, which can facilitate their absorption by epithelial cells. Additionally, studies involving epithelial Caco-2 cells showed that NPs in the presence of food have greater cytotoxicity and are absorbed at a higher rate than NPs digested in the absence of food (Lichtenstein et al., 2015). Moreover, silica NPs exposed to cells in the absence of proteins adhere more strongly to the cell membrane and have higher internalisation efficiency than in a medium containing proteins (Lesniak et al., 2012). Other studies have shown that the PC can play a role in dispersing NPs (Bihari et al., 2008; Go, Bae, Kim, Yu, & Choi, 2017) or providing a steric stabilising layer against aggregation by particle contact (Walkey & Chan, 2012). It was previously reported that the surface chemistry of silver nanoparticles (AgNPs) and their digestion had an influence on the dissolution properties and uptake by the Caco-2/HT29-MTX monolayer (Abdelkhaliq, van der Zande, Undas, Peters, & Bouwmeester, 2020). The cellular Ag concentration decreased following exposure to AgNPs *after in vitro* digestion (IVD) compared to pristine NPs; therefore, although the cellular transport of AgNPs was limited ($P < 0.1$) for both pristine and digested AgNPs, it proved essential to take into consideration the interaction between NPs, foods, and the enzymatic activity in the gastrointestinal tract (DeLoid et al., 2017; McClements et al., 2016).

Regarding the above, the main objective of this study was to determine the mutual interaction and impact of NPs during the digestion of different foods. For this purpose, SMP, peanuts, and tofu were selected as model foods, representing different macronutrient compositions (protein, fat, and carbohydrate content), different origins (animal or plant), or previous associations with allergenic peptides. Magnetic silica NPs were chosen as a core material to allow for easy incubation and isolation of the NPs for surface analysis. To ensure that the NPs were comparable with commercially applied compounds, the magnetic cores were surface coated with a silica shell. The three selected foods were subjected to a static IVD process, using the INFOGEST protocol (Brodkorb et al., 2019). At the different stages of digestion (oral, gastric, and intestinal), the proteins and peptides present in the digested solutions and in the NPs' PC were identified and characterised using SDS gel electrophoresis and liquid chromatography–mass spectrometry (LC-MS).

2. Materials and methods

2.1. Samples

Partially skimmed milk powder (EMMI, CH), with 30 g/100 g protein content, and skimmed milk powder (SMP; Rapilait, Migros, CH), with 32.86 g/100 g protein content, were purchased at a local store, along with peanuts (country of origin, United States; protein content 31.98 g/100 g). Tofu (Proteix; protein content 16.07 g/100 g) was obtained from Agroscope (Changins, Switzerland). Total nitrogen was determined using the Kjeldahl method according to ISO-8968-3 (ISO 8968–3, 2007) and multiplied by a factor of 6.25 to obtain the amount of crude protein in the food.

2.2. Reagents

All reagents were purchased from Sigma Aldrich, except for rabbit gastric extract (RGE25; Lipolytec, Marseille, France). The enzymes used for static IVD were amylase (A1031), porcine pepsin (P7012), porcine pancreatin (P7545), trypsin (T0303), and bile extract (B8631).

2.2.1. Magnetic silica nanoparticle synthesis

Magnetic-core shell silica nanoparticles were synthesised and characterised at the Adolphe Merkle Institute (Fribourg, Switzerland), adapting the protocol of (R. Peters et al., 2012). The magnetic core particles were synthesised following the well-known protocol for the co-precipitation of ferrous and ferric ion solutions. Briefly, aqueous $FeCl_3$ (1 mol/L, 20 mL) and 5 mL of $FeSO_4$ (2 mol/L) in HCl (2 mol/L, 5 mL) were added to 250 mL of NH_4OH (0.7 mol/L) and stirred mechanically at 800 rpm for 30 min. The formed compound was allowed to precipitate and subsequently redispersed in 50 mL of distilled water. Ten mL of tetramethylammonium hydroxide solution (1 mol/L) were added and stirred mechanically at 800 rpm for 30 min, and this process of precipitation and redispersion was repeated three times. Finally, 85 mL of distilled water was added up to a final volume of 250 mL. The magnetic-core NPs were further purified by dispersing 4 mL of the magnetic core suspension in 40 mL of water, centrifuged, and redispersed in pure water (100 mL).

In the second step, the magnetic core NPs were surface functionalised with a silica shell. Tetraethyl orthosilicate (TEOS; 2 mL of TEOS in 30 mL of EtOH) was added to a mixture of 6.22 mL of the previously washed magnetic core suspension, 4.85 mL of NH_4OH solution (28 g/100 mL), 28.8 mL of H_2O, and 27.5 mL of EtOH and mechanically stirred at 800 rpm at room temperature (RT) for 4 h. The formed particles were centrifuged and magnetically washed to remove uncoated silica and finally redispersed in 100 mL of pure water.

Particle size distribution was determined at a concentration of 0.0025 g/100 mL (estimated at 4.25×10^6 NPs/mL) of dispersion, using transmission electron microscopy (TEM) (Supplementary Fig. 1a) and the diameter was estimated at 404 ± 286 nm as shown in the size histogram ($n = 89$) $\pm$ standard deviation (R. Peters et al., 2012) (Supplementary Fig. 1b).

2.3. Sample preparation for in vitro digestion

Before *in vitro* digestion, the peanuts and tofu were ground to a particles size of 2–3 mm to simulate mastication, using an OMNI Prep Homogenizer (LabForce, Nunningen, Switzerland). The foods were suspended in water to achieve a final protein concentration of 0.04 g protein/mL (w/w) and magnetic silica NPs were added to obtain a final concentration of 0.5 g/100 mL (w/w) in the diluted samples with an estimated number of 8.5×10^8 NPs/mL. Samples without NPs were used as controls. The samples were stirred at RT for 1 h and stored at 4 °C for 16 h prior to digestion.

2.4. In vitro digestion

The INFOGEST static IVD model (Brodkorb et al., 2019), with small modifications, was used to compare the digestion of the diluted food samples with and without NPs. The enzyme activities and bile concentration were measured prior to the digestion experiment using the assays described in the harmonized protocol (Minekus et al., 2014). Enzyme activities were 51.5 U/mg for Amylase, 27.9 U/mg for lipase in and

386.1 U/mg of pepsin in Rabbit gastric extract (Lypolytech, Marseille, France), 2595 U/mg for pepsin, and 3.2 U/mg of trypsin activity for pancreatin. Prior to the addition, pancreatin was centrifuged at 3000 *g* at 4 °C for 3 min to remove undissolved particles. Loss of activity was assessed, and trypsin was added to reach the final trypsin activity of 100 mU/mL that is required for digestion. In brief, 1 mL of the diluted sample was mixed with 1 mL386. of simulated salivary fluid (SSF; pH 7, 37 °C) containing amylase (300 U/mL of digesta) and incubated for 2 min. Two mL of simulated gastric fluid (SGF; pH 3, 37 °C) containing pepsin (2000 U/mL of digesta) and lipase (RGE25, 60 U/mL) were then added and incubated for 120 min. Subsequently, 4 mL of simulated intestinal fluid (SIF; pH 7, 37 °C) containing pancreatin, trypsin (100 U trypsin activity/mL of digesta), and bile (10 mmol/L of digesta) were added and incubated for 120 min. The entire digestion protocol was performed at 37 °C under constant gentle mixing on a rotating wheel. Digestion was stopped after 120 min of gastric digestion by increasing the pH to 7 with NaOH (1 mol/L) and after 120 min of intestinal phase by using the protease inhibitor 4-(2 aminoethyl) benzensulfonylfluorid (AEBSF, Pefabloc®, 500 mmol/L; Roche, Basel, Switzerland). Immediately after stopping the digestion, all the samples were snap-frozen in liquid nitrogen.

For the analyses, samples of oral (2 mL), gastric (4 mL), and intestinal (8 mL) digests, with and without NPs, were thawed. Protein corona (PC-NP) complexes present in the samples were isolated with a magnet, washed three times with phosphate buffered saline (PBS), and centrifuged (8000 x *g* at 4 °C for 10 min). Proteins and peptides from the digests and PC were characterised using SDS-PAGE and LC-MS, and all experiments were carried out in triplicate.

2.5. Scanning electron microscopy (SEM) with energy dispersive X-ray spectroscopy (EDX)

For SEM, SMP from EMMI was prepared according to a protocol developed in-house, in which the organic matrix was digested. The residual undigested dispersion was applied to carbon tape and allowed to dry overnight. The samples were analysed on a Mira3 LM (Tescan, Brno, Czech Republic) field emission scanning electron microscope (FE-SEM) using a secondary electron (SE) detector and an In-Beam detector for high resolution images.

The samples were measured using an energy dispersive X-ray spectrometer from EDAX, equipped with a lithium-doped silicon detector, and analysed using EDAX Genesis software.

2.6. ζ-potential measurement

Magnetic silica NPs were incubated in PBS and SMP at a concentration of 5 g/100 g (w/w) at 4 °C, overnight. Afterwards, the NPs were magnetically trapped, washed three times with PBS, and redispersed separately in PBS, SSF, SGF, or SIF without digestive enzymes. Diluted 0.1 g/100 mL (w/w) samples were equilibrated at 37 °C for 2 h prior to measurements in order to ensure the stability during the time of *in vitro* digestion.

ζ-potential measurements were carried out on a 90Plus Parallel Phase Analysis Light Scattering (PALS, Brookhaven Instruments Corporation, NY, United States) equipped with a platinum electrode. The ζ-potentials were derived from the electrophoretic mobility using the Smoluchowski method of data processing (10 cycles). The viscosity, refractive index, and dielectric constant of pure water were used to characterise the solvent.

2.7. SDS-PAGE

SDS-PAGE under reducing conditions was performed as previously described (Egger et al., 2016). Samples (digests with and without NPs and PC-NP) were loaded on gel after normalisation according to their original protein concentrations. Samples were mixed with sample buffer (350 mmol/L Tris-HCl, pH 6.8, 10 g/100 mL SDS, 100 mmol/L DTT, and 50 mL/100 mL glycerol) and separated using SDS-PAGE (15 g/100 mL polyacrylamide). Molecular weight markers (Benchmark™, Invitrogen) were included in each gel, and the gels were stained with Coomassie Blue (Kang, Gho, Suh, & Kang, 2002).

2.8. Peptide mass fingerprinting

Protein identifications were performed using peptide mass fingerprinting (Egger, Ménard, & Portmann, 2018; Saraswathy & Ramalingam, 2011; Sousa, Portmann, Dubois, Recio, & Egger, 2020). Briefly, protein bands were manually excised from the polyacrylamide gel and washed three times, alternating between 100 μL destain buffer (ammonium bicarbonate 25 mmol/L, acetonitrile 50 mL/100 mL v/v) and 100 μL digestion buffer (ammonium bicarbonate 25 mmol/L). The gel pieces were then digested with 2 μL trypsin (4 mg/L) in 20 μL of digestion buffer at 37 °C, overnight. After tryptic in-gel digestion, the peptides were separated using high-performance liquid chromatography (HPLC; Rheos 2200, Flux Instruments, Switzerland) equipped with an XTerra MS C18 column (3.5 mm, 1.0 mm 3150 mm; ThermoFisher Scientific, Reinach, Switzerland). The HPLC was directly coupled to a linear ion trap mass spectrometer (LTQ; ThermoFisher Scientific, Reinach, Switzerland) using an electron spray ionisation interface. Protein identifications were performed by submitting the fragmentation data to the Mascot search engine (Matrix Science, London, UK) using UniProt (February 2018) with the following search parameters: enzyme, trypsin; maximum miscleavages, 1; peptide and MS/MS tolerance, 0.8; variable modifications, deamidated (NQ) Gln → pyro-GLU (N-term Q) and oxidation (M); significance threshold, $P < 0.05$; ion score cut-off, 20. Identifications were manually validated according to the following criteria: protein score above 40, peptide score above 25, identification of at least two different peptides, and identification of at least three consecutive fragmentation ions per peptide.

2.9. Peptide identification using liquid chromatography and mass spectrometry (LC-MS)

Prior to the analysis, control and NP digests were filtered through Amicon Ultra filter units (30K; Millipore, Darmstadt, Germany) and centrifuged at 13,000×*g* at 4 °C for 10 min. Peptides from the corona of isolated NPs were extracted with 75/25 mL/100 mL v/v acetonitrile/H2O during 10 min and centrifuged at 8000×*g* at 4 °C for 10 min. Supernatants were then taken for analysis.

MS analysis for peptide identification was performed as previously described (Egger et al., 2016, 2018, 2019; Kopf-Bolanz et al., 2012). Briefly, peptides were separated using the LC-MS equipment mentioned above. The signals of an $m\,z^{-1}$ between 100 and 1300 were measured in multiple overlapping mass windows. The MSMS spectra of the overlapping mass windows were merged, and peptides were identified with a Mascot search (Matrix Science) using a home-built database containing all typical proteins of different species for each food matrix analysed. Peptides from main milk proteins (β-casein, α_{s1}-casein, α_{s2}-casein, κ-casein, and β-lactoglobulin (BLG)), and main proteins from peanuts (arachin 6, arachin Ara h3, allergen Ara h1, and conarachin) and tofu (glycinin G1, glycinin G2 (GLYG2), β-conglycinin α1 (GLCA1); β-conglycinin α and trypsin inhibitor A), were monitored. The minimum criteria for peptide identification was an ion score of 20 being assigned as the most probable identity. If the identification was questionable, the results were manually verified by checking the fragmentation pattern of the protein (peptide coverage and fragmentation pattern). Peptides were identified in triplicates and single identifications were not included in the results. To visualise the peptide abundance, individual amino acids within the identified peptides from the above-mentioned proteins were summed up and numbers were displayed along the protein sequence.

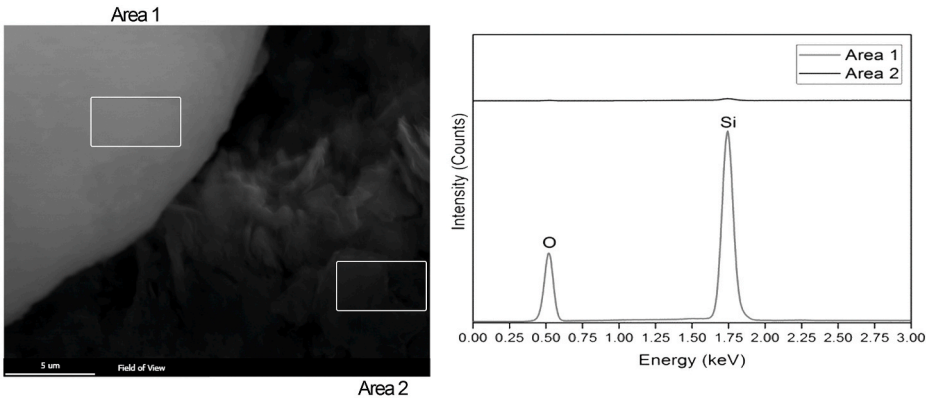

Fig. 1. Scanning electron microscopy micrograph coupled to x- ray spectroscopy of silica nanoparticles in skim milk powder. Left: Scanning electron microscopy (SEM) micrograph of unlabelled silica nanoparticles present in commercial partially skimmed milk powder. Right: Energy dispersive X-ray spectroscopy (EDX) line scan with silicon (Si) Kα signal and oxygen (O) Kα signal measured along the section indicated in a). Area 1 contains silicon and oxygen, and in Area 2, the carbon grid is used as a background.

Table 1
ζ-potential of magnetic silica nanoparticles in solution and food matrix.

	PBS-NP	SMP-NP
	ζ-potential (mV)	ζ-potential (mV)
PBS (pH **7.2**)	−27 ± 1	−12 ± 1
SSF (pH **7**)	−50 ± 1	−27 ± 1
SGF (pH **3**)	0 ± 2	10 ± 2
SIF (pH **7**)	−32 ± 2	−17 ± 2

ζ-potential of magnetic silica nanoparticles (NPs) resuspended in phosphate buffered saline (PBS) and in skimmed milk powder (SMP), added to PBS, simulated salivary fluid (SSF), simulated gastric fluid (SGF), or simulated intestinal fluid (SIF) without the addition of digestive enzymes.

3. Results and discussion

In the first step, the presence of NPs was observed in several commercial dairy products, using SEM coupled with EDX, indicating that NPs are indeed used in the dairy industry. While the ingredients are always declared on food packaging, it is often not specified whether they are present in the form of nanoparticles or not. Fig. 1 shows, as an example, the presence of silica NPs in partially skimmed milk powder being declared as silicium oxide on the corresponding food package.

3.1. ζ-potential of magnetic NPs in simulated gastrointestinal fluids

Magnetic silica NPs were suspended in SMP or PBS at 4 °C overnight for PC formation. Table 1 shows the ζ-potential (Zp) of the resuspended NPs added to the PBS, SSF, SGF, and SIF solutions in the absence of digestive enzymes. In all the solutions, the NPs had a negative Zp, except in SGF, in which both the PBS-NP and the SMP-NP had a Zp of 0 and + 10, respectively. This value can be explained by the protonation of the silanol groups on the NPs' surface at a pH of 3 of the gastric solution, ionic strength, and concentration, as others have observed (Lowry et al., 2016; Milosevic et al., 2017; Sharma, Jaiswal, Duffy, & Jaiswal, 2019). An increase in Zp in NPs resuspended in SGF also agreed with the results found by Di Silvio and colleagues (Di Silvio, Rigby, Bajka, Mackie, & BaldelliBombelli, 2016).

3.2. Proteolysis of food matrices during in vitro digestion in the presence or absence of NPs

The digests of SMP, peanuts, and tofu were analysed in the presence and absence of magnetic silica NPs to characterise their protein and peptide compositions using SDS-PAGE and LC-MS. Protein patterns obtained by SDS-PAGE from *in vitro* digested SMP, peanuts, and tofu, with (+) and without (−) NP solutions and PC, are shown in Fig. 2. Proteins labelled with a number were identified with LC-MS (Table 2).

Samples from SMP in SSF with and without NPs, and from the PC eluted from the NPs, showed the same protein profiles as undigested SMP (Fig. 2a, lanes 2–4). The main milk proteins (α-, β-, κ-casein, BLG, and α-lactalbumin) can be seen in the mentioned lanes, as previously reported (Egger et al., 2016). In the PC, it seemed that κ-casein and α-lactalbumin had a higher affinity with NPs than with other main proteins (lane 4). In the gastric digests (Fig. 2a, lanes 5–7), caseins and α-lactalbumin disappeared, whereas BLG was clearly resistant to the action of pepsin (lanes 5 and 6), as previously demonstrated (Kopf-Bolanz et al., 2014). This protein was also present, to a lesser extent, in the PC (lane 7). The upper protein band corresponded to the pepsin added to the SGF; however, neither BLG nor any other milk protein could be identified in the digests and PC at the end of the intestinal digestion stage, and the remaining bands in the digesta and the PC corresponded to enzymes added to the SIF (Fig. 2a, lanes 8–10). Previous studies in our laboratory have confirmed this hydrolysis pattern for milk proteins after gastric and intestinal IVD (Egger et al., 2016, 2019).

Regarding the results of the simulated digestion of peanuts (Fig. 2b), no differences were found between solutions with and without NPs in the digestion phases studied. In peanuts, the PC of NPs eluted after the oral phase showed a similar protein pattern to that of the control and NP oral digests (Fig. 2b, lanes 2–4). Additionally, the protein profiles of the control and NP gastric digests (lanes 5 and 6) were similar to those of oral digests, indicating that these peanut proteins were resistant to the action of pepsin, as previously observed by Kopper and colleagues (Kopper et al., 2004). Moreover, in the PC for this step (lane 7), several weak bands were visible, among which two more intense bands were observed, which were identified as pepsin and Arachin 6 (Table 2). As indicated for the SMP, no peanut protein was identified in the digests or the PC after the intestinal digestion (lanes 8–10); the proteins identified referred to enzymes of pancreatin (bands 25–28, Table 2).

The protein patterns for the simulated digestion of tofu are shown in Fig. 2c. Similar protein profiles were found in undigested control and NP solutions and in the PC (lanes 2–4). These proteins were identified as the major soy protein allergens β-conglycinin and glycinin (Table 2). After gastric digestion, the same proteins were visible in the digests and the eluted PC, albeit with less intensity in the NPs' corona (lanes 5–7). It has previously been reported that β-conglycinin present in tofu can remain present after 120 min of pepsin digestion, potentially inducing allergenic symptoms in susceptible subjects (Adachi et al., 2009). In the gastric phase of the tofu digestion, a band with a low molecular weight (band 33) was identified as β-conglycinin (Table 2), which could be a cleavage product of the intact protein. In all the intestinal bands (Fig. 2c, lanes 8–10), only proteins from the digestive juices were identified (bands 34–38).

At the end of intestinal digestion, no intact proteins from any of the three selected matrices were identified either in the digests, independently of the presence of NPs, or in the eluted PC samples (Table 2). All the bands identified at this point were components of pancreatin added

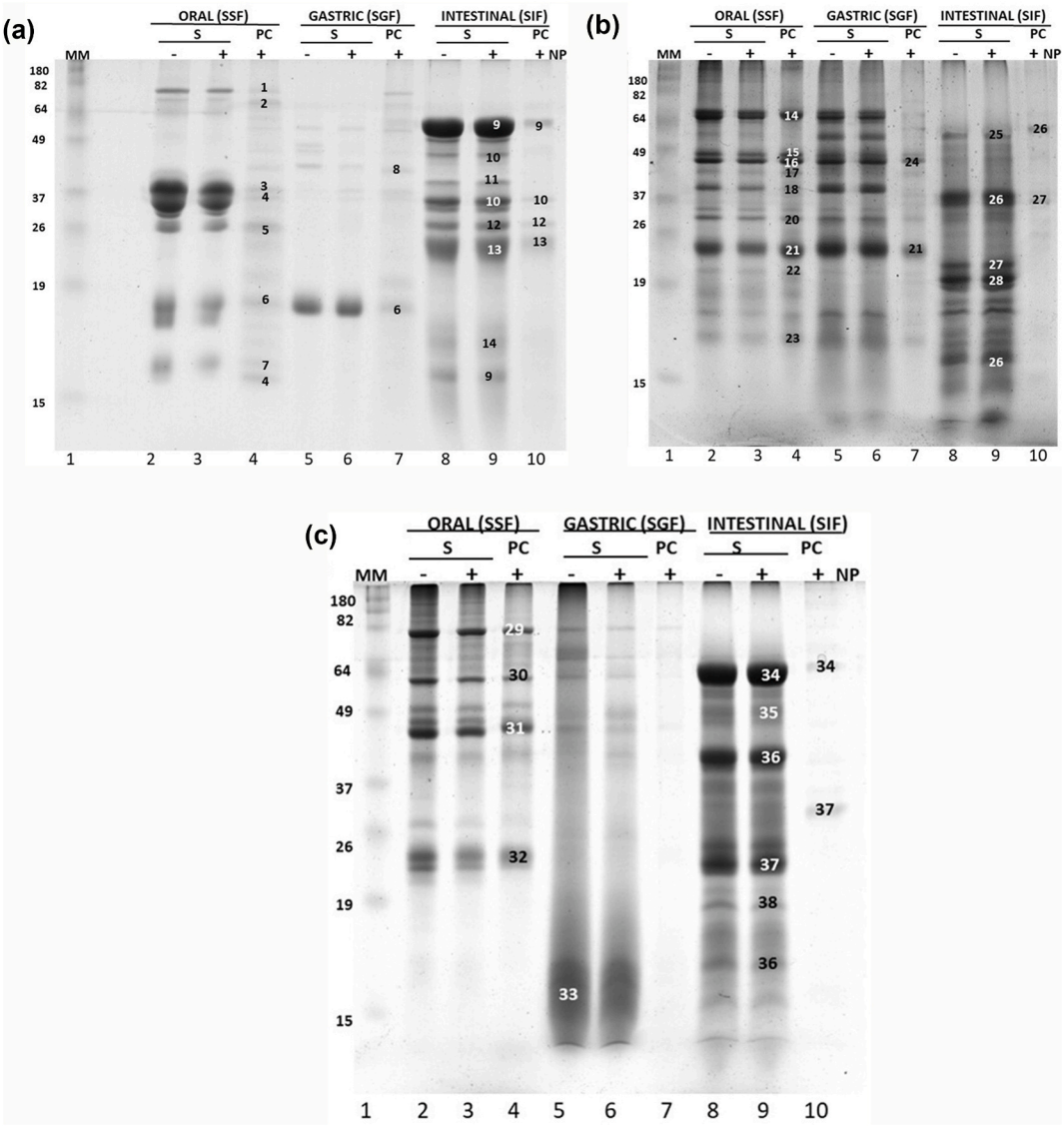

Fig. 2. Protein identifications from SMP, Tofu and Peanut before and after each digestion step. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins from a) skimmed milk powder, b) peanuts, and c) tofu. Solutions (S) without (−) and with (+) nanoparticles (NP) and protein corona extract (PC) are shown after oral, gastric, and intestinal digestion. A MW marker is shown in the first lane (MM). Labelled protein bands (1–38) were identified as listed in Table 2.

Table 2
Protein identifications from SMP, Peanut, and Tofu, before and during digestion.

Band	Skimmed milk powder	Band	Peanut	Band	Tofu
1	Lactotransferrin	14	Allergen Arah 1	29	β-conglicinin α
2	BSA	15	Arachin 3	30	β-conglicinin β
3	αs1-casein	16	Arachin 6	31	Glicinin G1
4	β-casein	17	Main allergen Arah 1	32	Glicinin G2
5	K-casein	18	Conarachin	33	β-conglicinin α
6	β-lactogobulin	19	Arachin Arah3 isoform	34	Pancreatic α-amylase
7	α-lactoalbumin	20	Arachin Arah 2	35	Carboxypeptidase B
8	Pepsin A	21	Arachin 6	36	Carboxypeptidase A
9	Pancreatic α-amylase	22	Arachin 3	37	Trypsin
10	Carboxypeptidase A	23	Conglutinin	38	Chymotrypsin
11	Carboxypeptidase B	24	Pepsin A		
12	Chymotrypsin	25	Pancreatic α-amylase		
13	Trypsin	26	Carboxypeptidase A		
14	Pancreatic α-amylase	27	Trypsin		
		28	Chymotrypsin		

Proteins from SDS-PAGE identified by LC-MS. Protein bands as labelled in Fig. 2: a) skimmed milk powder, b) peanuts, and c) tofu.

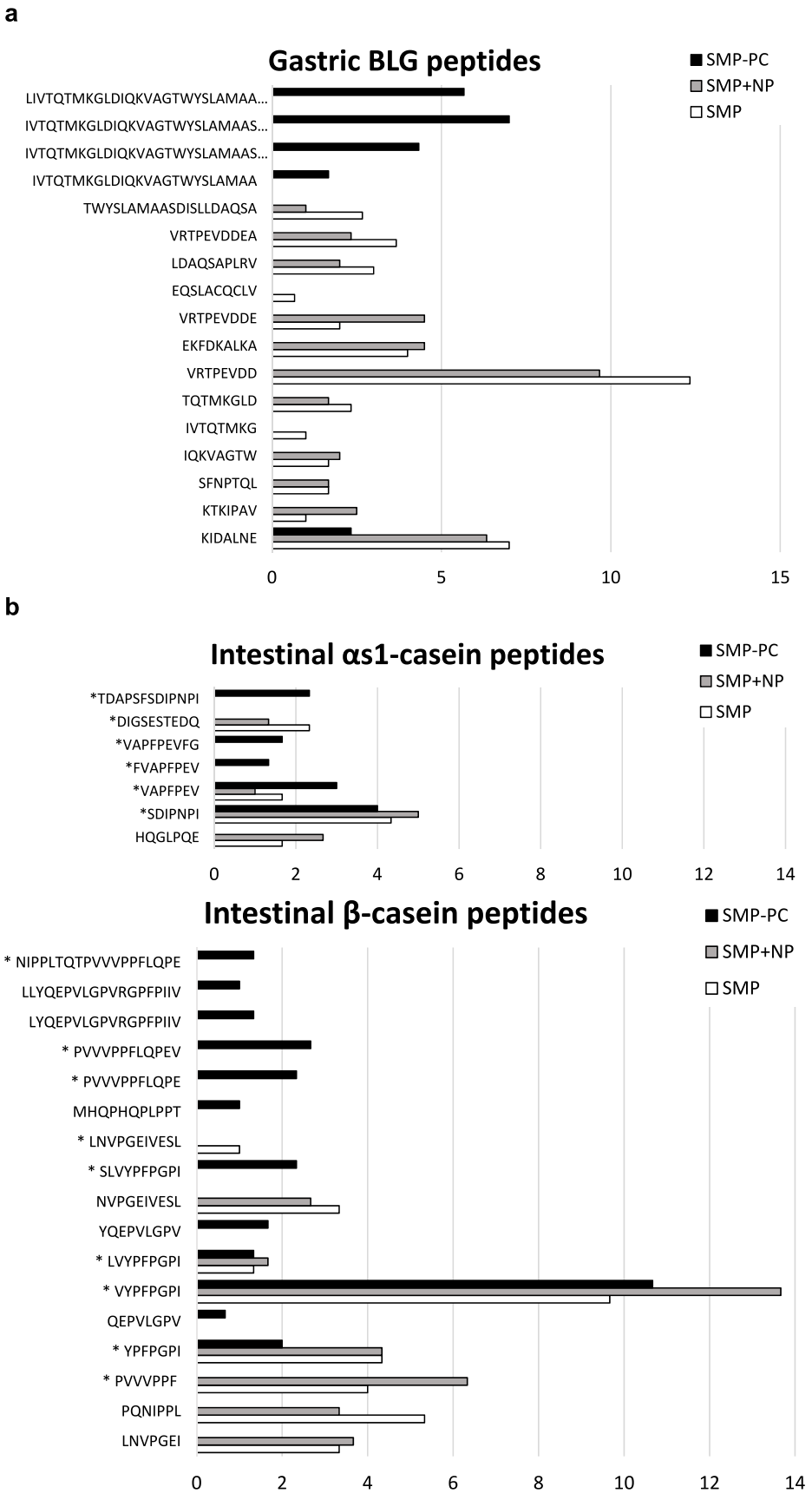

Fig. 3. Digestion resistant peptides after gastric and intestinal *in vitro* digestion of skim milk powder. β-Lactoglobulin peptides identified by mass spectrometry at the end of the gastric phase of *in vitro* digestion of SMP: SMP digests with and without nanoparticle solution (SMP+NP) and PC released from the NPs (SMP-PC). The x-axis provides an indication of the number of times each peptide was detected in the MS spectra a). Peptides from αs1-casein and β-casein at the end of the intestinal phase of *in vitro* digestion of SMP: SMP digests with and without nanoparticle solution (SMP+NP) and PC released from the NPs (SMP-PC). Peptides labelled with '*' were reported as IgE binding epitopes from milk proteins in transient and persistent allergic patients (Monaci et al., 2006). The x-axis provides an indication of the number of times each peptide was detected in the MS spectra, b).

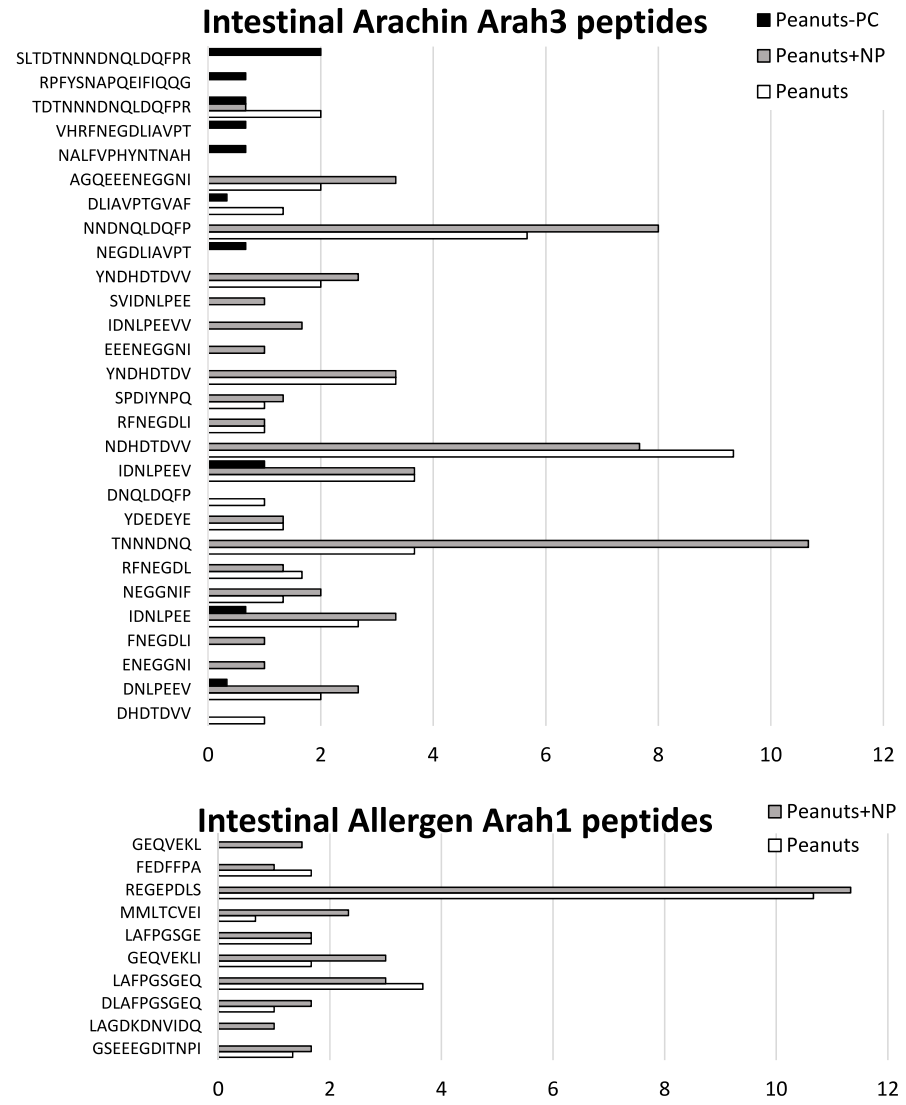

Fig. 4. Digestion resistant peptides after intestinal *in vitro* digestion of Peanut. Arachin Arah3 and Arachin Arah1 peptides at the end of intestinal phase of *in vitro* digestion of peanuts: peanut digests with and without nanoparticle solution (Peanut+NP) and PC released from the NPs (peanut-PC). The x-axis provides an indication of the number of times each peptide was detected in the MS spectra.

to the SIF, similar to Coreas and colleagues (Coreas, Cao, DeLoid, Demokritou, & Zhong, 2020) observations. The absence of intact food proteins in intestinal digesta agreed with previous studies on the IVD of milk (Egger et al., 2016, 2017, 2018, 2019). Over all, a decrease in protein or peptide size was observed on the PC along the digestive process, which was in agreement with earlier reported results (Walczak et al., 2015). In general, oral, gastric, and intestinal solutions showed no differences in the dependence of presence or absence of SiO_2 NPs, and the eluted proteins from corona also showed a similar protein pattern with a lower intensity. In contrast to our results, Cao and colleagues reported that gastric *in vitro* hydrolysis of caseins was reduced in presence of NPs, compared to casein alone (Cao et al., 2019). The reasons for this could be divers, such as the smaller NPs, the differences in the *in vitro* digestion protocol, or the digestion of isolated caseins.

Di Silvio et al. (2016) reported the presence of a non-hydrolysed bread protein in the PC of NPs isolated at the end of simulated digestion. In their study, however, the enzymatic activity of the SIF was somewhat lower compared to the conditions used in these experiments, since the activity lost during the centrifugation to remove the insoluble fraction of pancreatin was not compensated for by adding trypsin. Additionally, the researchers did not state whether this protein was also present in the control and NP digests.

The fact that uncleaved proteins remained anchored to the corona of the NPs during digestion may be a concern, since some of these NPs could translocate or be absorbed by the intestinal epithelium and as well modulate cellular uptake in dependence of the proteins present in the PC (Ritz et al., 2015). Moreover, the presence of allergenic peptides on the PC could induce an increase in cellular uptake as previously demonstrated for gluten peptides (Mancuso et al., 2021). Several authors have shown that undigested or digested food-grade NPs can cause translocation and cytotoxic effects in cells (Bohmert et al., 2014; Bove et al., 2017; Brun et al., 2014; Hou et al., 2019; Jia, Wang, Zhou, & Sun, 2017); however, most of those studies ignored the food matrix and gastrointestinal tract effects. Other studies have reported that the interaction between food and NPs prevents NP aggregation and may alter NPs' physicochemical properties (Kästner et al., 2017). Moreover, the *in vivo* or *in vitro* digestion of NPs in the presence of food leads to further modifications, which may promote their uptake by the intestinal epithelium cells and increase their potential toxicity (Di Silvio et al., 2016; Lichtenstein et al., 2015).

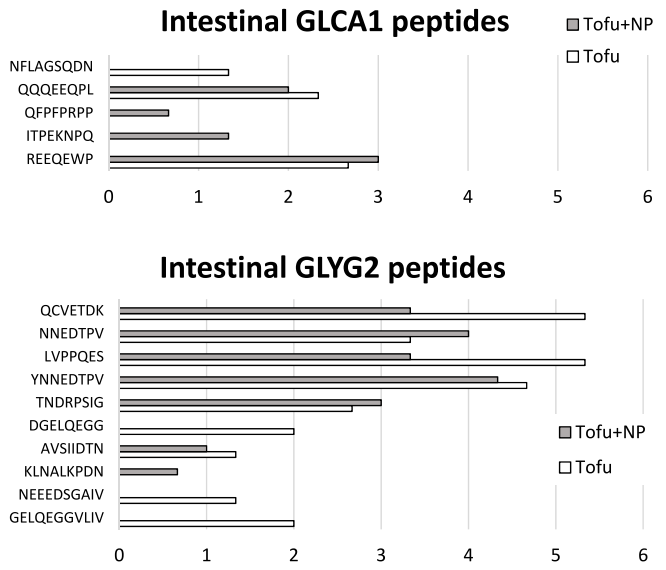

Fig. 5. Digestion resistant peptides after intestinal *in vitro* digestion of Tofu β-conglycinin α subunit 1 (GLCA1) and Glycinin G2 (GLYG2) peptides at the end of the intestinal phase of *in vitro* digestion of tofu: tofu digests with and without nanoparticle solution (Tofu+NP) and PC released from the NPs (tofu-PC). The x-axis provides an indication of the number of times each peptide was detected in the MS spectra.

3.3. Peptide release during digestion

The release of peptides during digestion from the major proteins of SMP, peanuts, and tofu was analysed with mass spectrometry using a specific protein database for each food. The peptide patterns of the gastric and intestinal digestion endpoints, with and without NPs, and those released from the PC of NPs were compared.

Figs. 3–5 and Supplemental Figs. 2 and 3 show the relative prevalence of peptides derived from different food proteins. In the gastric phase of digestion, many peptides from the studied proteins were found in the three analysed food matrices. In general, the peptide profiles of the major proteins were similar in the gastric digests independently of the presence of NPs; however, some specific corona-bound peptides were found, such as in SMP where, following the gastric digestion, some peptides from the N-terminal section of BLG were released from the NPs' PC, but were not present in the SMP and the SMP+NP solutions (Fig. 3a). These results strongly suggest that the NPs' corona protected the mentioned BLG peptides from cleavage by gastric pepsin. One of these BLG-derived peptides attached to the corona (LIVTQTMKGLDIQKVA) has been identified as an IgE binding epitope in transiently and persistently allergenic patients (Monaci, Tregoat, van Hengel, & Anklam, 2006).

The peptides of the main milk proteins were determined at the intestinal digestion endpoint. Concerning BLG, a few peptides were detected in the intestinal SMP digests, but no BLG peptides were found in the SMP+NP digest or in the PC released from NPs (not shown); however, many peptides from β-casein and αs1-casein (the two major milk proteins) were found in the digests and were also attached to the PC (Fig. 3b). Interestingly, several peptides were exclusively released from the PC that were not detected in the SMP and SMP+NP solutions. As indicated above, these peptides bound to the corona seemed to be less accessible to the action of the intestinal proteolytic enzymes. The peptides (a part of it or the whole peptide) from β-casein, labelled (*) in Fig. 3b, have been reported in the literature as allergenic (Monaci et al., 2006). The casomorphin peptides (SLVYPFPGPI, LVYPFPGPI, VYPFPGPI, and YPFPGPI) were found to resist cleavage, being present in relatively high abundance in the digests and attached to the corona. Of these, YPFPGPI (β-casomorphin-7 (BCM7)) has been associated with an increased risk of certain diseases, such as autism, cardiovascular diseases, and type I diabetes, but sound evidence for a causative link is lacking (EFSA, 2009). The release of BCM7 from β-casein only occurs in genetic A1 variants of bovine milk. The peptides released from αs1-casein at the end of the simulated digestion are shown in Fig. 3b. Some peptides were specifically bound to the NPs' PC. Peptides labelled (*) are fragments of recognised allergenic peptides (Monaci et al., 2006), and the HQGLPQE and VAPFPEV fragments were previously reported as digestion resistant by Egger and colleagues (Egger et al., 2018).

Regarding peanuts, many peptides from the studied proteins were found in the digests and in the corona after gastric digestion. As an example, peptides that were identified as fragments of one of the major proteins (arachin arah3) are shown in Supplemental Fig. 2. Notably, the longest peptides were found only in the PC, suggesting again that they were less accessible to cleavage by gastric pepsin. Fig. 4 shows the peptides released from arachin arah3 and from the allergen arah1 after intestinal digestion. Concerning arachin arah3, many peptides were still present at the end of the simulated digestion in the control and NP solutions, and some were evident in the PC. Several of the longest chain peptides were only released from the PC and not detected in the peanut and peanut+NP solutions. Of these, fragment RPFYSNAPQEIFIQQG merits attention because it was reported as a marker peptide for detection of this major allergen (Heick, Fischer, Kerbach, Tamm, & Popping, 2011); however, some peptides from the allergen arah1 were found in the peanut and peanut+NP solutions after intestinal digestion, several of which are recognised marker peptides of this allergen in foods (Heick et al., 2011). No peptide from allergen arah1 was released from the NPs' corona at the end of the intestinal digestion of peanuts. The peptides appearing from the mentioned proteins at the end of the simulated digestion were somewhat different from those reported by (Di Stasio et al., 2017). The identified fragments DLAFPFGSGEQ and YDDEYE from arah1 and arah3, respectively, corresponded to the predicted IgE binding regions (Di Stasio et al., 2017).

Finally, the peptides found in the gastric and intestinal digests, and in the PC from two of the major allergenic proteins (GLCA1 and GLYG2) of tofu, are shown in Fig. 5 and Supplemental Fig. 3. It can be seen that both proteins gave rise to many peptides in the tofu and tofu+NP digests after gastric digestion. Additionally, several peptides of these proteins remained at the end of intestinal digestion in these solutions, many of which have been used as markers for the detection and quantification of allergenic soy proteins in foods (Gomaa & Boye, 2015; Planque et al., 2016). However, no peptides from the mentioned proteins of tofu were released from the PC of NPs after gastric and intestinal digestion, which could be explained by a lower abundance of each individual peptide and the sensitivity of the method.

Altogether, these results provide more insights into the dynamics of protein digestion in the presence of NPs and thereby contribute to the assessment of the potential health impact of food-derived NPs.

4. Conclusions

In our study, no differences in protein hydrolysis levels and peptide patterns were observed between digests performed in the presence or absence of NPs for the three studied food matrices, indicating that the digestion process was not influenced by NPs. However, proteins detected in the PC were the ones mainly present in the digesta at the time of sample collection, which was observed by the constant change in composition of the PC depending on the surrounding medium. No intact food proteins were visible at the endpoint of digestion either in the digests or on the NPs' PC. After the gastric and intestinal digestion phases, many peptides were found in the PC and in the digests with and without NPs, for the studied food matrices, except the PC of NPs added to tofu, where no peptides were identified. Some digestion-resistant peptides from SMP and peanuts, especially ones with longer sequences, were found exclusively in the PC but not in the digests. Moreover, peptides with allergenic function were found in PC and digests of SMP and

peanuts. Furthermore, casomorphin peptides arising from β-casein remained in the digests or bound to the NPs' PC at the endpoint of the digestion. The persistence of NP-bound peptides with a potential bioactive or allergenic function throughout digestion could induce a potential risk and may cause health problems due to translocation or uptake of NPs in the intestinal epithelium. Future studies are needed to investigate the cellular uptake of various NPs added to different food types to better understand and predict these health risks.

CRediT authorship contribution statement

Maria del Carmen Martín-Hernández: Designed the research, Conducted the research, Formal analysis, Wrote the paper and had primary responsibility for the final content. **David Burnand:** Designed the research, Conducted the research, Formal analysis. **Corinne Jud:** Designed the research. **Reto Portmann:** Conducted the research, Formal analysis. **Lotti Egger:** Designed the research, Conducted the research, Formal analysis, Wrote the paper and had primary responsibility for the final content, All authors have read and approved the final manuscript.

Declaration of competing interest

All authors have read and approved the final manuscript. The authors declare that they have no conflicts of interest. The project received no additional funding.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.112303.

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