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sRAGE-binding and antimicrobial bioactivities of soy and pea protein after heating and *in vitro* infant digestion

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ABSTRACT

During infant formula production, proteins are always heated, potentially affecting their digestibility and the bioactivities of resulting peptides. Although plant proteins are a promising dairy alternative for infant formula, they remain understudied, necessitating further investigations. Therefore, this research aimed to fill this gap by assessing the impact of different heating modes on soy protein (SP) and pea protein (PP), focusing on glycation levels, peptide formation during *in vitro* infant digestion, and immune protection potential (sRAGE-binding and antimicrobial activities) of the resulting peptides. Consequently, dry heating led to increased glycation and glycated peptide production, particularly with higher glycation in PP than SP. Moreover, PP exhibited an overall stronger sRAGE-binding capacity than SP, regardless of heating and digestion conditions. Regarding antimicrobial activity, both SP and PP-derived peptides displayed reduced effectiveness against *Enterobacter cloacae* after dry heating. Additionally, *Staphylococcus epidermidis* was differently inhibited, where PP-derived peptides showed inherent inhibition. The primary determinant of sRAGE-binding and antimicrobial potential in digestion-derived peptides was the protein source. Subsequent bioinformatics analysis predicted 519 and 133 potential antimicrobial peptides in SP and PP, respectively. This study emphasises the importance of protein source for infant formula to ensure infant health.

1. Introduction

Plant-based infant formula (IF) has garnered significant attention as a dairy substitute, primarily due to its potential to alleviate cow's milk allergy. Amongst all, soy protein (SP) constitutes the primary protein source within plant-based IF, while a promising alternative, pea protein (PP), is also under consideration (Gorissen et al., 2018). Once infants consume IF, the dietary proteins within these formulas will be digested, leading to the release of peptides and free amino acids (AAs), to support infant growth and development. An important category is that of the bioactive peptides, which, apart from the primary nutritional role, possess a diverse range of potential bioactivities related to health, such as antioxidant, anti-inflammatory, and antimicrobial effects (Daliri, Oh, & Lee, 2017; Li, Dhordain, Hearn, Martin, & Bennett, 2023).

During IF production, external factors such as wet and dry heating conditions, as well as the presence of reducing sugars, can induce changes in protein structure, such as denaturation, aggregation, and glycation (i.e., the Maillard reaction). These structural modifications can subsequently affect the overall digestibility. Our previous studies showed that wet heating unfolded SP and PP, enhancing their accessibility to digestive enzymes and therefore facilitating infant digestion; conversely, dry heating hindered digestion due to the glycation-induced blockage of trypsin-cleavage sites at e.g., lysine residues (Tang, Wichers, & Hettinga, 2022, 2023). Consequently, the digestive kinetics and peptide patterns may vary, potentially influencing the bioactivity of the released peptides. However, this aspect remains largely unexplored and requires further investigation.

Developing infants have an immature digestive system, characterised by relatively lower enzyme activities and a higher gastric pH than that of adults (Bourlieu et al., 2014; Nguyen, Bhandari, Cichero, &

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Prakash, 2015). Furthermore, infant food is typically consumed in liquid form. Both these factors lead to the digestive process occurring predominantly in the intestinal phase rather than the oral and gastric phases, as well as restricted hydrolysis and different digestion kinetics compared to adults. Thus, more peptides rather than free AAs are produced during gastrointestinal digestion. Subsequently, these released peptides may enter the blood via the intestinal epithelial barrier to exert their potential physiological activity (Amigo & Hernández-Ledesma, 2020; Xue et al., 2021). However, the underdeveloped gut barrier in infants exhibits increased permeability, allowing for the absorption of larger molecules, e.g., peptides (Weström, Arévalo Sureda, Pierzynowska, Pierzynowski, & Pérez-Cano, 2020). Consequently, this combination of factors contributes to the release and absorption of a greater number of bioactive peptides in the infant, which can serve as functional molecules (Li et al., 2023).

Breastmilk naturally possesses antimicrobial and immune-stimulatory potential, protecting infants from various health risks. Such immune protection was also found in dairy-based IFs (Bonhomme et al., 2012; Brück, Graverholt, & Gibson, 2003; Raikos & Dassios, 2014). However, a notable gap exists in the understanding of their plant-based alternatives. In this study, two bioactivities related to immune protection of digestion-derived peptides of SP and PP were studied: the binding capacities to the soluble receptor for advanced glycation end products (sRAGE) as well as the antimicrobial potential.

The assessment of sRAGE-binding capacities of the peptides is particularly important for infants, as their developing immune and metabolic systems are sensitive to changes in cellular responses. RAGE is a multi-ligand receptor that regulates inflammatory responses, oxidative stress, and tissue damage, making it a key player in various physiological processes (Fritz, 2011; Yue, Song, Liu, Zhang, Yang, & Li, 2022). sRAGE is its soluble isoform that binds to the same ligands. Various ligands include advanced glycation end products (AGEs) formed by glycation, S100 protein, high-mobility group protein 1, and amyloid β protein, etc. Quintanilla-García et al. (2019) reported that higher sRAGE levels in neonatal circulation of neonates compared to maternal circulation, suggesting placental origin of the receptor and its possible role as a decoy protein for circulating AGEs. Therefore, assessing sRAGE-binding potential could offer valuable insights into the pro-inflammatory activity of food proteins and especially of the less studied peptides.

In addition to their sRAGE-binding properties, the antimicrobial capacity is also interesting to study. Antimicrobial peptides (AMPs) serve as a natural defence mechanism against potential threats. Infants are vulnerable to microbial infections. When released during digestion, these peptides act as a first line of defence within the infant's gastrointestinal tract (Benkerroum, 2010), endogenously preventing the colonization and proliferation of harmful microorganisms to maintain a healthy gut microbiota. Apart from that, those digestion-resistant AMPs, sourced from harmless and cost-effective origins, can be synthesised and introduced into infant foods to enhance their functional properties. Furthermore, glycated AMPs are also an emerging research field offering potential advantages in enhancing stability, targeting, pathogen specificity, microbicidal potency, and reducing microbial resistance (Bednarska, Wren, & Willcocks, 2017; Bellavita, Braccia, Galdiero, & Falanga, 2023).

To the best of our knowledge, previous research has primarily focused on the bioactive peptides in breastmilk and dairy proteins in the context of infant digestion (Raikos et al., 2014). However, limited studies have explored plant proteins, especially considering the heat treatment that IF proteins undergo, which can lead to structural modifications and variations in peptide bioactivities. Therefore, this study aimed to fill this gap by exploring the effect of different heating conditions on the immune protection potential, including sRAGE-binding and antimicrobial capacities, of peptides derived from SP and PP. To achieve this aim, soy and pea proteins were wet or dry-heated and then digested via a full-term infant *in vitro* digestion model system. The released peptides were identified by LC-MS/MS, and their sRAGE-binding and antimicrobial capacities were determined.

2. Materials and methods

The experimental design of this research is displayed in Fig. 1.

2.1. Materials and chemicals

Fresh soybean (*Glycine* max; protein content: 39.3 %, w/w) was obtained from Wageningen Plant Research, Lelystad, The Netherlands (courtesy ing. Ruud Timmer). Fresh pea (*Pisum sativum*; protein content: 23.3 %, w/w) was purchased from a local retailer (Brand name: HAK). Protein contents were determined by DUMAS Flash EA 1112 Protein analyser (Thermo Fisher Scientific, Waltham, Massachusetts), using a nitrogen-to-protein conversion factor of 5.7. Recombinant human sRAGE was purchased from R&D systems (Minneapolis, Minnesota). Goat anti-human IgG-HRP antibody was purchased from SouthernBiotech (Birmingham, Alabama). Enhanced K-Blue® TMB Substrate was purchased from NEOGEN (Lansing, Michigan). Ovalbumin (OVA) was purchased from InvivoGen (San Diego, California). β-Amyloid peptide 1–42 (AMB 1–42) was purchased from HelloBio (Bristol, UK). All the chemicals were obtained from Sigma-Aldrich (Saint Louis, Missouri) unless otherwise stated.

2.2. Sample preparation and heat treatment

Soy protein (SP) and pea protein (PP), with protein contents of 77.9 % and 78.8 % (w/w), respectively, were prepared and heat-treated according to previously established protocols with some modifications (Tang et al., 2022, 2023). Specifically, prior to heating, proteins were mixed with 4-fold glucose (w/w) to mimic plant-based IF. To induce considerable structural modifications, two heating conditions were chosen based on our previous studies (Tang et al., 2022, 2023). For wet heating (W), samples with a protein concentration of 1.2 % (w/v), mimicking IF, were heated in a waterbath at 85 °C for 30 min. For dry heating (D), powdered samples were first heated in a desiccator at 60 °C and a water activity of 0.6 for 48 h and then reconstituted in 10 mM PBS buffer to achieve 1.2 % protein (w/v). Samples containing 1.2 % protein (w/v) that were non-treated (NT) were used as controls. All these treatments were performed in independent duplicates.

2.3. Quantification of glycation markers

Lysine, furosine (Nε-2-furoylmethyllysine), CML (Nε-carboxymethyllysine), and CEL (Nε-carboxyethyllysine) of all samples were quantified by LC-MS/MS according to our previously established method (Tang et al., 2023). The final results were displayed as mg/g protein for lysine and furosine, and mg/100 g protein for CML and CEL.

2.4. Simulated in vitro infant digestion

Simulated infant digestion was conducted by using a full-term *in vitro* infant digestion model according to Ménard et al. (2018) with minor modifications. Briefly, all the sample mixtures were submitted to 60 min gastric digestion at pH 5.3 with 268 U/mL pepsin and 60 min intestinal digestion at pH 6.6 with 16 U/mL trypsin in pancreatin. Sampling was done at 10 min and 60 min of intestinal digestion (I10 and I60). The digestion process was stopped by immediately transferring samples into liquid nitrogen. Digests were filtered through 10 kDa cut-off filters to collect peptide fractions, which were kept at −20 °C until usage.

2.5. Peptidomics analysis

Peptide sequences and their glycation-related modifications were identified according to Dingess et al. (2017) and Zenker, Wichers, Tomassen, Boeren, De Jong, and Hettinga (2020) with modifications.

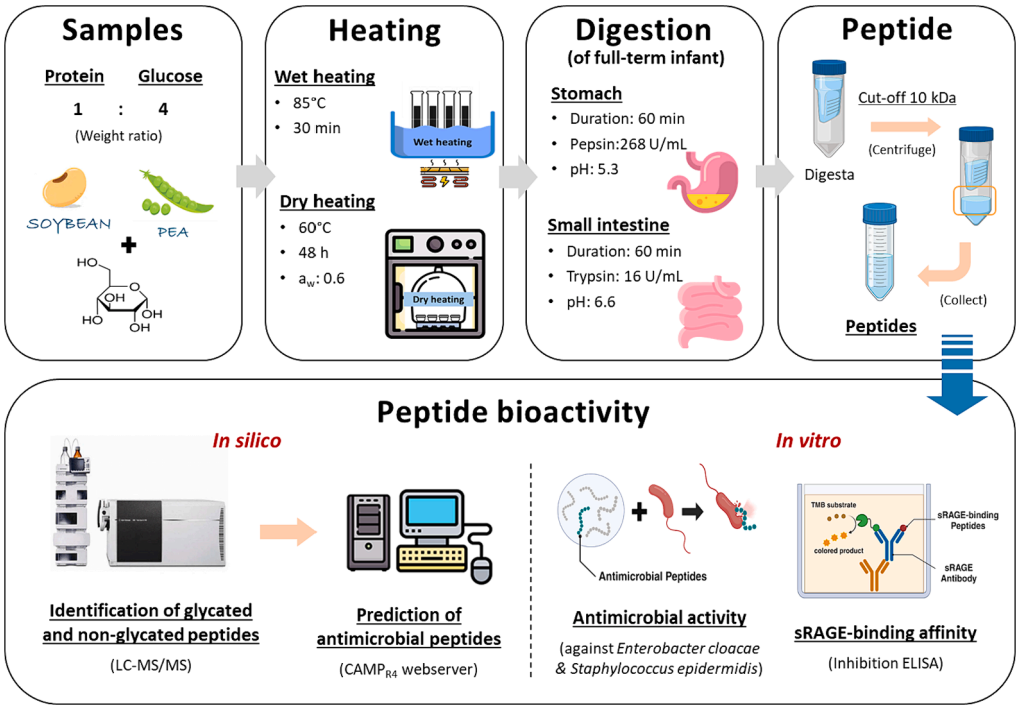

Fig. 1. The schematic overview of this research.

Specifically, peptide samples were purified through a solid phase extraction C18 column and were then analysed through LC-MS/MS by MaxQuant 2.2.0.0 with an "unspecific" digestion mode. Soy and pea protein databases were downloaded from UniProt (accessed June 2022, https://www.uniprot.org) for Andromeda searches with peptide lengths ranging from 8 to 25 AAs to minimize false identifications. Glycation-related modifications at lysine residues included hexose modification (+162 Da), CML modification (+58 Da), and CEL modification (+72 Da); as well as modifications at arginine residues included methylglyoxal hydroimidazolone (MG-H) modification (+54 Da) and glyoxal hydroimidazolone (G-H) modification (+40 Da). Only peptides detected in both duplicate digestions were considered valid. Peptides with a score $\geq$ 80 were included in the overall profiles and classified into non-glycated and glycated peptides. In addition, for the prediction of antimicrobial peptides, only the peptides with a score $\geq$ 100 were considered.

2.6. Inhibition sRAGE ELISA

Inhibition ELISA-based sRAGE binding assay was conducted following the method described by Teodorowicz et al. (2021) with some modifications. A transparent high-binding ELISA plate was coated with soy protein (G90) glycated under specific conditions (wet heated with glucose, 90 min, at 100 °C). This plate was incubated overnight at 4 °C, followed by washing with 0.05 % Tween-20 in 10 mM PBS. Washing was carried out after each step of the ELISA process. Afterward, the plate was blocked with 3 % BSA in 10 mM PBS for 1 h at room temperature. Simultaneously, sample solutions and sRAGE receptor were mixed in a 1:1 vol ratio in the dilution buffer (1.5 % BSA and 0.025 % Tween-20 in 10 mM PBS), resulting in final protein concentrations of 50 and 25 μg/mL, with the final sRAGE concentration reaching 1 μg/mL. The mixture of the sample/sRAGE solutions was then incubated on a NuncTM polystyrene plate for 1 h at 37 °C, followed by transfer to the ELISA plate for an additional 1-hour incubation at 37 °C. After the addition of goat anti-human IgG-HRP antibody, the plate was incubated for 30 min at room temperature. Color development was achieved by TMB solution and was stopped by adding 2 % HCl. Absorbance (Abs) was measured in duplicate at 450 nm with a reference wavelength of 620 nm, by a Filter Max F5 multi-mode microplate reader (Molecular Devices, San Jose, California). Results were manifested by the percentage of inhibition, with high inhibition (%) indicating high receptor binding affinity. Inhibition (%) was calculated by the following equation (1):

$$Inhibition(\%) = \frac{Abs_{sRAGE} - Abs_{sample}}{Abs_{sRAGE}} \quad (1)$$

where Abs_{sRAGE} represented the absorbance for sRAGE without samples, and Abs_{sample} represented the absorbance for the mixture of the sample/sRAGE solutions. All the absorbance measurements were corrected for the dilution buffer used as a blank control.

2.7. Surface hydrophobicity

Surface hydrophobicity of digestion-derived peptides was determined by 8-anilino-1-naphthalenesulfonic acid ammonium salt fluorescence assay (Tang et al., 2022), with a minor modification that the samples were diluted to 0.5 mg/mL in 10 mM PBS.

2.8. Culture of bacterial strains

Gram-negative *Enterobacter cloacae* (DSM 30054) and gram-positive *Staphylococcus epidermidis* (DSM 20044) were purchased from DSMZ (Göttingen, Germany) for use in the antimicrobial assay according to Xiong, Boeren, Vervoort, and Hettinga (2021). These two strains were cultured overnight in specific growth media and temperatures: nutrient broth medium at 30 °C for *Enterobacter cloacae*, and tryptic soy broth medium at 37 °C for *Staphylococcus epidermidis*. After culturing, the optical density (OD) of the bacterial solution was measured at a wavelength of 600 nm. Subsequently, the bacteria solutions were diluted with PFZ (peptone physiological salt solution; Tritium Microbiology, Eindhoven, The Netherlands) and plated onto their respective agar medium plates, and then cultured for 48 h. Only plates with 20–300 colonies were used for counting. The colony numbers obtained were correlated with their corresponding OD values to draw calibration curves. These curves were then used to calculate the bacterial concentrations needed

for subsequent inoculation. All the experimental procedures were conducted under sterile conditions.

2.9. Antimicrobial assay

Antimicrobial activity was measured based on the methods of Xiong et al. (2021) and Tidona et al. (2011) with some modifications. Briefly, peptide samples were diluted with 10 mM PBS to achieve a final nitrogen concentration of 0.2 mg/mL (based on DUMAS results), followed by sterile filtration using 0.2 μm filters. Subsequently, 135 μL of the diluted samples were transferred into a transparent 96-well plate. Following this, 15 μL of each bacterium solution was inoculated into the samples, resulting in a final bacterial concentration of approximately 10^5 CFU/mL. Positive controls involved replacing the sample solutions with broth medium, while the negative controls replaced the sample solutions with distilled water.

Peptide samples and bacteria were co-incubated at their respective optimal temperature for specific durations: 15 h for *Enterobacter cloacae* and 12 h for *Staphylococcus epidermidis*. The selection of these incubation times was informed based on our preliminary study, with the selected time point representing the end of exponential growth (data not shown). After incubation, the OD_{600} values were measured by SpectraMax ABS Plus microplate reader from Molecular Devices (San Jose, California). The final results were displayed as the bacterial growth rate, with a lower growth rate indicating higher antimicrobial activity. The experiment was conducted in duplicate under sterile conditions. Equation (2) was used to calculate the growth rate as follows:

$$Growthrate = \frac{OD(t) - OD(t_0)}{OD(t_0)} \quad (2)$$

where OD(t) referred to the OD values measured after incubation, and $OD(t_0)$ referred to the OD values measured before incubation.

2.10. Prediction of antimicrobial peptides

The peptides identified by LC-MS/MS were submitted to the $CAMP_{R4}$ webserver (accessed July 2023, https://www.camp.bicnirrh.res.in/predict/) to predict their potential as antimicrobial peptides with a random forest algorithm (Gawde et al., 2022). Prediction mode was set as "natural", and a threshold of 0.5 was selected.

2.11. Statistical analysis

GraphPad Prism 9.0 was used for data visualisation and unpaired *t*-test. IBM SPSS 25.0 was used for analysis by ANOVA with Duncan post-hoc test. Statistical significance was determined at $p < 0.05$.

3. Results and discussion

3.1. Level of glycation

Lysine, furosine, CML, and CEL are important glycation markers to evaluate the level and stage of glycation after heating of SP and PP. This is due to the covalent reaction between lysine residues and reducing sugars, leading to the formation of furosine at the early stage and CML and CEL at the advanced stage of glycation. It is reported that glycation tends to be more intense when reactants are dry heated in a powdered form at a water activity of 0.6–0.8, especially under conditions of increased heating temperature and duration (de Oliveira, Coimbra, de Oliveira, Zuñiga, & Rojas, 2016; Fanni, Hardy, & Parmentier, 1999; Kutzli, Weiss, & Gibis, 2021; Schong & Famelart, 2017). As thus expected, dry heating contributed more to glycation compared to wet heating. The contents of glycation markers are shown in Fig. 2.

Regarding lysine, the initial lysine content of SP before heating was 84.2 mg/g protein. After wet heating, it decreased by ~ 22 %; while after dry heating, it decreased by ~ 47 %. For PP, its initial lysine content was the higher at 101.7 mg/g protein, which decreased by ~ 8 % and 49 % after wet and dry heating, respectively. Interestingly, despite PP having a higher initial lysine content than SP, after dry heating, their lysine levels became comparable. In terms of furosine contents, only a slight increase was observed in the wet-heated (W) SP and PP samples compared to the non-treated (NT) samples, by 1.4 and 1.9 mg/g protein for SP and PP, respectively. However, after dry heating, the furosine contents of SP and PP sharply increased by ~ 46 mg/g protein. As for CML, compared to the NT samples, after wet heating, no increase was found in the CML contents for SP and only a slight 2-fold increase was observed for PP. Moreover, in the dry-heated SP and PP samples, the contents of CML were ~12 and 48-fold higher than those of the NT samples, respectively. Likewise, the CEL contents show similar trends compared to CML. Only slight increases were found after wet heating, of around 2-fold. After dry heating, their contents were approximately 9 and 16 times higher than the NT samples, respectively.

Taken together, dry heating contributed more to glycation than wet heating, and glycation of PP was more extensive than SP. To our knowledge, this study is the first to simultaneously report the glycation levels of SP and PP in the presence of glucose and compare different heating conditions. Notably, dry heating-induced glycation could result in decreased digestibility and lysine bioavailability (Li, Ye, & Singh, 2021). This could potentially lead to inadequate supply of this essential amino acid and subsequent nutritional deficiencies, affecting infant growth. Therefore, it is suggested to use limited dry heating for IF manufacturing.

3.2. Identification of glycated peptides in the digests

Peptides released after 10 min (I10) and 60 min (I60) of intestinal

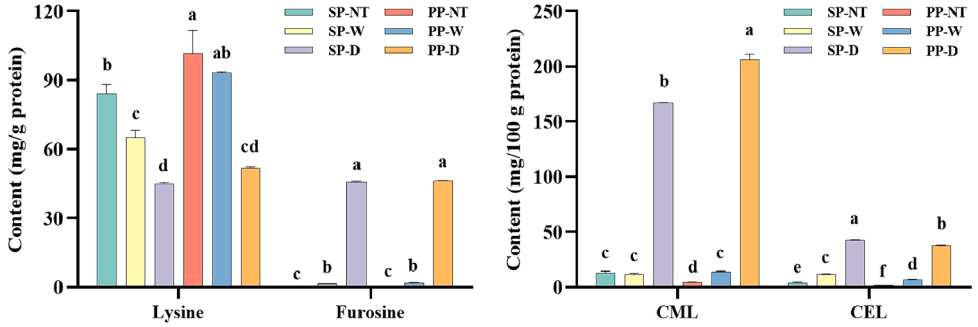

Fig. 2. Contents of glycation markers—lysine, furosine, CML, and CEL of non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP). Error bars represent standard deviations of duplicates. Statistical differences were analysed with ANOVA and Duncan post-hoc test. Different letters above the bars represent significant differences ($p < 0.05$).

digestion were analysed by LC-MS/MS. The subsequent data analysis separated the glycated from non-glycated peptides. Results are presented in Fig. 3, showing the relative intensity and number of glycated peptides to the total peptides detected. Intensity refers to the cumulative abundance of peptides, while number represents the diversity of peptides.

In general, a trend of increases in both the relative intensity and number of glycated peptides was found after wet and especially dry heating. However, as digestion progressed from I10 to I60, no clear further changes were found. Specifically, by the end of intestinal digestion (I60), the wet-heated (W) SP and PP exhibited only minor increases compared to the NT samples. In the dry-heated (D) samples, the relative intensities of SP and PP increased to ~ 28 % and 29 %, respectively; for the relative numbers, the percentages were ~ 10 % and 20 %, respectively. These results indicate that wet, and particularly, dry heating contributed to glycation and thus the formation of glycated peptides. In addition, PP is more sensitive to dry heating, as manifested by the higher levels of relative counts than that of SP. These findings are in agreement with the results of the level of glycation in section 3.1 (Fig. 2).

Further analysis of the modification types within the glycated peptides is displayed in Fig. 3 C&D. A total of 5 different types of glycation-related modifications were measured, including modifications at lysine (K) residues (Hexose, CML, and CEL modifications), as well as modifications at arginine (R) residues (MG-H and G-H modifications). Specifically, the K modifications were the dominant modification types among all the differently-treated samples, accounting for more than 80 % of the glycated peptides for both SP and PP. Notably, hexose-modified peptides were the most abundant modifications. Specifically for the D samples, which were glycated to the largest extent, around 80 % of glycated peptides were hexose-based in SP, while for PP, nearly 95 % of them were hexose-based. As for the R modifications, both MG-H and G-H-modified peptides were found in SP. However, in the PP-derived glycated peptides, only MG-H modification was observed. Overall, lysine-based glycation was the main type of glycation for both SP and PP of peptides formed during digestion. Notably, some glycated peptides can be resistant to digestion and potentially possess antimicrobial capacity, potentially promoting infant health, which will be further explored in section 3.4.2.

3.3. sRAGE-binding affinity

The sRAGE-binding affinity (% inhibition) of the SP and PP samples before and after digestion upon wet and dry heating was measured by sRAGE inhibition ELISA, with a protein concentration of 25 μg/mL (Fig. 4). Before digestion, both SP and PP samples exhibited significantly high binding affinity (>50 % inhibition) to sRAGE, regardless of whether they were heated or not. The NT sample for SP exhibited the highest inhibition, whereas for PP, the W sample did so. Furthermore, both the SP-D and PP-D samples had the lowest inhibition, at ~ 50 % and 53 %, respectively. Notably, under the same heating conditions, PP exhibited higher inhibition compared to SP. This phenomenon was also found in the samples with a higher protein concentration of 50 μg/mL (Figure S1).

Regarding the sRAGE-binding mechanisms before digestion, various factors could contribute to it, including surface hydrophobicity, aggregation, glycation-induced AGE formation, and β-sheet structures (Fritz, 2011; Liu, Teodorowicz, Wichers, van Boekel, & Hettinga, 2016; Sugihara et al., 2012; H. E. Zenker et al., 2020). All these factors involve RAGE recognising the negative charge and/or hydrophobic domains on its ligands (Bongarzone, Savickas, Luzi, & Gee, 2017; Deane, 2012). For SP and PP, both contain more than 30 % β-sheet structures (Shevkani, Singh, Chen, Kaur, & Yu, 2019). In addition, research reported that the surface charge of SP and PP were comparable, but the surface hydrophobicity of PP was considerably higher than SP (Karaca, Low, & Nickerson, 2011). Thus, in this study, before digestion, the general higher sRAGE binding capacity of PP than SP was more due to the higher level of surface hydrophobicity of PP. Furthermore, the reduced inhibition observed in dry-heated proteins could be attributed to a) reduced surface hydrophobicity by the formation of hydrophilic AGEs on the outer surface of SP and PP, and b) the increased covalent aggregation by glycation leading to the burial of sRAGE recognising sites. The related results were shown in our previous study (Tang et al., 2023). In other words, despite that AGEs are the main ligands for RAGE, their contribution to binding is limited, potentially even hindering it. On the contrary, the high surface hydrophobicity facilitated the interactions of SP and PP with RAGE.

After digestion at I10 and I60, the binding affinity of the peptide fractions was sharply reduced to less than 18 % (Fig. 4), due to RAGE primarily recognising complex conformational structures, rather than specific amino acid sequences (Liu et al., 2016). This decline suggests

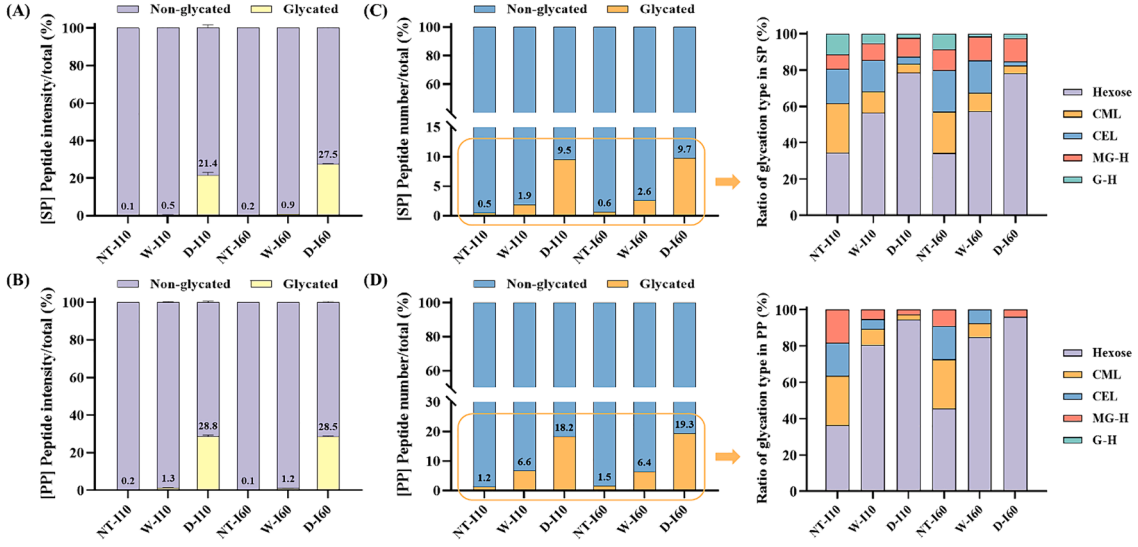

Fig. 3. (A&B) Relative intensity of glycated peptides to the total peptides in each sample. (C&D) Relative number of glycated peptides and the ratio of glycation types within those peptides. Peptides were fractionated from non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP), after *in vitro* infant digestion. I10 and I60 represent the samples after 10 min and 60 min of intestinal digestion, respectively.

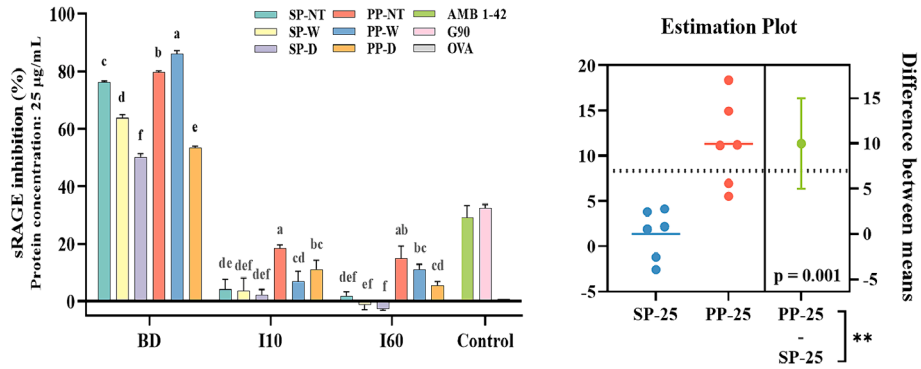

Fig. 4. sRAGE inhibition ELISA of non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP), before and after *in vitro* infant digestion, using a protein concentration of 25 μg/mL. BD refers to the samples before digestion. I10 and I60 represent the samples after 10 min and 60 min of intestinal digestion, respectively. β-Amyloid peptide 1–42 (AMB 1–42) and soy protein glycated with glucose for 90 min at 100 °C (G90) were used as positive controls, while ovalbumin (OVA) was used as a negative control. Error bars represent standard deviations of duplicates. Statistical differences of samples before and after digestion were analysed with ANOVA and Duncan post-hoc test. Different letters in black (for proteins before digestion) and in grey (for peptides) above the bars represent significant differences within the group ($p < 0.05$). Statistical differences between peptide groups of SP and PP were analysed with unpaired *t*-test: *$p < 0.05$ and **$p < 0.01$.

that digestion can lower the binding capacity of proteins to sRAGE, leading to the hypothesis that this reduction may play a role in regulating the risk of inflammatory processes linked to conditions such as diabetes, immune disorders, and chronic inflammation. Generally, the inhibitions for all the SP samples ranged from no inhibition (-3%) to about 4 %, whereas for PP, the inhibitions were between ~ 6 % and 18 %. Thus, similar to the undigested samples, digestion-derived PP samples had significantly higher inhibition than SP, at both protein concentrations of 25 μg/mL ($p = 0.001$) and 50 μg/mL ($p = 0.017$) (Fig. 4&S1). To be specific, for SP, no significant differences in inhibition were found among the samples from different heating and digestion conditions. While for PP, significances were observed between different heating conditions, with the NT samples exhibiting the highest inhibitions. After dry heating, the inhibitions dropped by ~ 7 % at I10 and ~ 9 % at I60. Moreover, the inhibition percentages between I10 and I60 were not significantly different. This might be explained by digestion occurring mostly during the initial 10 min of intestinal digestion (data not shown), during which period most peptides formed and displayed their potential sRAGE-binding capacities.

As mentioned above, surface hydrophobicity played a crucial role in sRAGE-binding before digestion. However, when considering the peptides derived from digestion, it appears that surface hydrophobicity was no longer a significant factor. As shown in Figure S2, the surface hydrophobicity of all the peptide samples did not show significant differences among them. Nevertheless, the overall inhibition of SP was lower than that of PP. Thus, it was probably the inherent protein characteristics that governed the binding affinity of peptides to sRAGE after digestion. Variations in protein structure and the AA sequences contributed to the different digestive selectivity, resulting in diverse peptide compositions between SP and PP, and consequently, distinct sRAGE-binding affinities. This phenomenon was primarily linked to the primary sequence composition of the peptides. Due to aggregation also promoting sRAGE binding, another potential reason is that some peptides themselves may self-aggregate, leading to sRAGE binding (Bellesia & Shea, 2007).

In summary, the undigested samples exhibited significantly higher sRAGE-binding affinities, compared to the digested peptides. Moreover, PP demonstrated a higher overall inhibition than SP, both before and after digestion. In addition, dry heating-induced glycation did not increase the binding capacity to sRAGE, but rather diminished it to some extent. Notably, after digestion, sRAGE binding to the samples seemed more closely related to the inherent protein characteristics rather than variations in heating conditions and digestion times. Ultimately, the differences in sRAGE-binding capacities of peptides were primarily attributed to the source of proteins.

3.4. Antimicrobial property

3.4.1. Antimicrobial activity of peptides

Antimicrobial activity of SP and PP-derived peptides was assessed based on the bacterial growth rate, where a low growth rate indicates a high capacity to inhibit the growth of microorganisms. Fig. 5 shows the growth rates of *Enterobacter cloacae* and *Staphylococcus epidermidis* in each sample.

In relation to *Enterobacter cloacae* (Fig. 5A), wet heating had minimal effects on bacterial growth; dry-heated protein samples did not inhibit bacterial growth but rather facilitated it, resulting in a growth rate surpassing even the positive control. The growth rate of *Enterobacter cloacae* in the presence of the dry-heated SP increased significantly by ~ 32 % and 28 % at I10 and I60, respectively, compared to the NT samples; for PP, it increased by ~ 54 % and 59 %, respectively. Reasons might be, firstly, members of the Enterobacteriaceae family possess the inherent capability to ferment sugars, and they also previously exhibited an enhanced growth response when stimulated by glycated proteins compared to non-glycated ones (Dominika, Arjan, Karyn, & Henryk, 2011; Singh, Sharma, & Nara, 2015). Secondly, the process of digestion enzymatically transformed intact proteins into peptides, enabling them to be more easily absorbed, transported, and utilised by microorganisms. Consequently, *Enterobacter cloacae* could effectively use glycated peptides. Furthermore, under the same heating conditions, no notable differences in growth rate were observed between I10 and I60, due to the high intestinal digestibility within the initial 10 min leading to the formation of the majority of peptides at I10 with only minor changes between I10 and I60. For *Staphylococcus epidermidis* (Fig. 5B), the SP and PP samples behaved differently regarding their growth rate. For SP, the NT samples were associated with the highest growth rate, and heating decreased bacterial growth to some extent. After dry heating, the growth rate at I10 declined by ~ 37 %, whereas after wet heating, it decreased by ~ 41 % at I60. For PP, the growth rates were all at a comparable level, irrespective of the extent to which the PP samples were heated and digested. Furthermore, similar to *Enterobacter cloacae*, from I10 to I60, the growth rates of *Staphylococcus epidermidis* were not significantly different. More importantly, the overall growth rate of *Staphylococcus epidermidis* in PP was significantly lower than in SP ($p = 0.0001$). The divergent primary AA compositions could be an inherent factor driving the microbial behaviour, similar to sRAGE-binding.

To conclude, *Enterobacter cloacae* displayed no specific preference for SP and PP-based peptide samples, and dry heating did not inhibit its

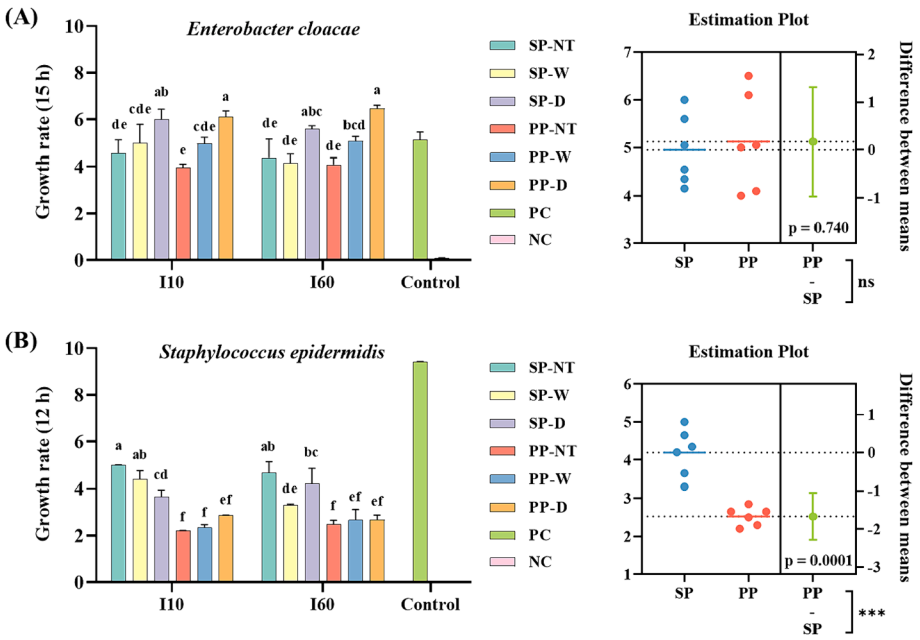

Fig. 5. Growth rates of (A) *Enterobacter cloacae* and (B) *Staphylococcus epidermidis* of non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP), after *in vitro* infant digestion. I10 and I60 represent the samples after 10 min and 60 min of intestinal digestion, respectively. PC refers to the positive controls replacing the sample solutions with broth medium. NC refers to the negative controls replacing the sample solutions with distilled water. Error bars represent standard deviations of duplicates. Statistical differences were analysed with ANOVA and Duncan post-hoc test. Different letters above the bars represent significant differences ($p < 0.05$). Statistical differences between peptide groups of SP and PP were analysed with unpaired *t*-test: ns $p > 0.05$ and ***$p < 0.001$.

growth but instead facilitated it. For *Staphylococcus epidermidis*, heating lowered its growth rate in the SP digests, with PP samples significantly inhibiting bacterial growth, independent of heating, when compared to SP. In addition, the extension of digestion time did not influence the bacterial growth of both SP and PP. These results highlight the differential responses of bacterial strains to peptide samples derived from digestion of different protein sources and heating conditions, suggesting potential implications for their antimicrobial activities.

3.4.2. Prediction of antimicrobial peptides

Antimicrobial peptides (AMPs) formed endogenously during digestion can support immunity and defence against microbial infections, and hold the potential to be valuable in infant foods. To identify the AMPs in the digests for samples with different heating and digestion conditions, the online prediction tool $CAMP_{R4}$ was used. As a result, 519 peptides were found to be AMPs in SP, with 9 of them being glycated. For PP, 133 distinct AMPs were predicted, including 6 that were glycated. All of these glycated peptides were formed based on the modifications at their lysine (K) residues. Moreover, at the end of intestinal digestion (I60), the SP samples had 361 AMPs for NT, 208 for W, and 120 for D, while the PP samples had 76 AMPs for NT, 70 for W, and 34 for D. For a complete list of all identified AMPs, please refer to Table S1&S2. The AMP probability score reflects the likelihood of a peptide having antimicrobial capabilities. For SP, a total of 103 peptides had a score over 0.6. Among them, peptide "KIGGIGTVPVGRVETGVLKPGM" (A0A0R0FER5, aa 243–264) achieved the highest score of 0.91. However, in the case of PP, only 13 peptides had a score above 0.6, with the peptide "VVIIPAGHPVA" (Q9M3X6, aa 513–523) having the highest score of 0.68. Furthermore, as shown in Fig. 6, the D samples exhibited the highest abundance of glycated AMPs: approximately 5.6 % at I10 and 3.3 % at I60 for SP, and respectively 10 % and 11.8 % for PP. The W samples showed a relatively lower abundance, while the NT samples contained the least. This trend was similar to the percentage of glycated peptides to the total peptide numbers (Fig. 3).

Given the above-mentioned potential benefits of glycated AMPs, we conducted a focused analysis of 9 glycated AMPs derived from SP and 6 from PP. Their detailed information and intensity changes are illustrated in Table 1. The highest probability score of 0.67 was found to be peptide

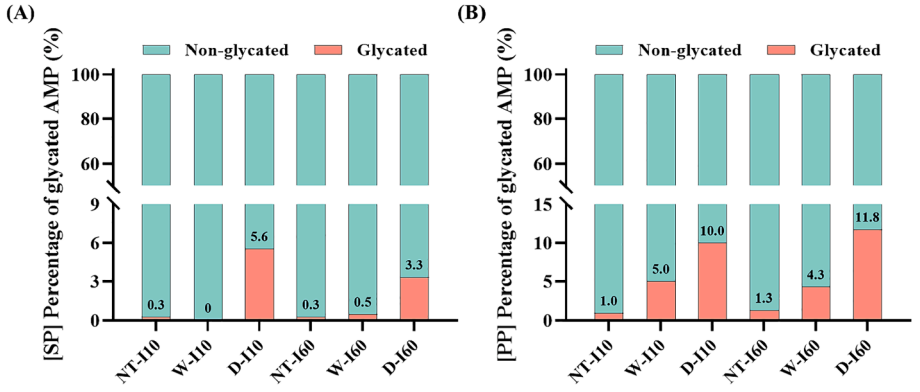

Fig. 6. Count ratio of predicted non-glycated and glycated antimicrobial peptides (AMPs) in the digests of non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP). I10 and I60 represent the samples after 10 min and 60 min of intestinal digestion, respectively.

Table 1
List of potential glycated antimicrobial peptides (AMP) of non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP), after *in vitro* infant digestion. Glycation sites at lysine (K) or arginine (R) residues were highlighted in bold and underlined. I10 and I60 represent the samples after 10 min and 60 min of intestinal digestion, respectively.

#	Glycated AMP of SP	AMP Probability	Glycation Type (Site)	Protein Group	Protein ID	Position	Length	Peptide Intensity					
								NT-I10	W-I10	D-I10	NT-I60	W-I60	D-I60
1	IAQG**K**GALGV	0.62	Hexose (K)	Glycinin G4	P02858	94–103	10	0	0	2.93×10^8	0	0	0
2	**K**GNVGGLIGTGL	0.54	Hexose (K)	Lipoxygenase	I1M596	41–52	12	0	0	3.06×10^7	0	0	1.67×10^7
3	NFNNPT**R**S**K**NP	0.5	Hexose (K); G-H (R)	Eukaryotic translation initiation factor 3 subunit D	I1MEK0	56–66	11	0	0	0	0	0	2.06×10^8
4	N**K**LQGKIA	0.55	CML (K)	Uncharacterized protein	A0A0R0I4F6	38–45	8	2.57×10^7	0	0	3.24×10^7	0	0
5	NVDNVFEDPDGL**K**	0.53	Hexose (K)	Beta-amylase	Q42795	25–37	13	0	0	0	0	1.75×10^6	1.41×10^8
6	TVGIGGPVGTG**K**T	0.67	Hexose (K)	Ni-binding urease accessory protein UreG	Q9XGS2	85–97	13	0	0	4.15×10^7	0	0	2.77×10^7
7	VETGVL**K**PGMVV	0.54	Hexose (K)	Elongation factor 1-alpha	A0A0R0FER5	255–266	12	0	0	4.44×10^7	0	0	0
8	VETGVV**K**PGMVV	0.55	Hexose (K)	Elongation factor 1-alpha	A0A0R0JTV5	255–266	12	0	0	5.65×10^7	0	0	0
9	VGSVEVG**K**LAD	0.5	Hexose (K)	Urease	I1K3K3	693–703	11	0	0	1.27×10^8	0	0	0

#	**Glycated AMP of PP**	**AMP Probability**	**Glycation Type (Site)**	**Protein Group**	**Protein ID**	**Position**	**Length**	**Peptide Intensity**					
								NT-I10	**W-I10**	**D-I10**	**NT-I60**	**W-I60**	**D-I60**
1	FNTDYEEIE**K**	0.5	Hexose (K)	Vicilin	P13918	186–195	10	0	1.06×10^7	8.99×10^7	0	4.10×10^6	1.45×10^7
2	GDAI**K**LPAGTI	0.52	Hexose (K)	Vicilin 47 k	Q43626	118–128	11	0	2.13×10^6	1.98×10^7	0	9.87×10^4	6.07×10^5
3	GDAI**K**LPAGTI	0.52	CML (K)	Vicilin 47 k	Q43626	118–128	11	3.66×10^6	1.22×10^7	0	1.45×10^6	0	0
4	IVTVTEG**K**GDFE	0.53	Hexose (K)	Vicilin 47 k	D3VNE0	309–320	12	0	2.20×10^7	5.26×10^8	0	4.50×10^6	2.82×10^7
5	IVTVTEG**K**GDFEL	0.53	Hexose (K)	Vicilin 47 k	D3VNE0	309–321	13	0	1.07×10^6	1.71×10^7	0	0	0
6	LNNNQNL**K**PL	0.53	Hexose (K)	Seed linoleate 9S-lipoxygenase-3	P09918	460–469	10	0	0	1.69×10^8	0	0	8.49×10^7

"TVGIGGPVGTGKT" in SP. On the other hand, in PP, peptides "IVTVTEGKGDFE", "IVTVTEGKGDFEL", and "LNNNQNLKPL" all shared an equal highest score of 0.53. The underlined AAs refer to the glycation sites. Notably, the hexose (K) modification was the dominant modification type amongst all the glycated AMPs. Besides, the SP peptide "NFNNPTRSKNP" had two modifications: G-H (R) modification and hexose (K) modification. An intriguing discovery was the identification of CML-modified peptides "NKLQGKIA" (SP) and "GDAIKLPAGTI" (PP), which were not generated from the D samples but were already present in the NT samples.

When comparing the various intensities of glycated AMPs after heating and digestion, only the 2 above-mentioned CML-modified peptides were present in the NT samples at both I10 and I60. Furthermore, as digestion progressed from I10 to I60, a decreasing trend in intensity was observed for nearly all the glycated AMPs, with some even becoming undetectable at I60. This was due to the further degradation of the AMPs during digestion. In contrast, only 2 SP-derived peptides showed detectable intensities at I60, whereas they were non-detectable at I10. Specifically, the intensity of peptide "NFNNPTRSKNP" was 2.0×10^8 at D-I60. As for peptide "NVDNVFEDPDGLK", its intensity was 1.8×10^6 at W-I60 and 1.4×10^8 at D-I60. The increase in intensity at I60 might be attributed to the further degradation of larger peptides or the intact protein, leading to the formation of peptides possessing antimicrobial properties.

4. Conclusion

The heating of both SP and PP samples, particularly through dry heating, led to increased glycation levels and subsequent formation of glycated peptides during digestion. PP exhibited a higher degree of

glycation compared to SP after heating. Furthermore, considering the negative effect of glycation on digestibility and amino acid bioavailability, IF producers are recommended to use limited dry heating to avoid glycation.

Moreover, PP consistently demonstrated higher sRAGE-binding affinity than SP, regardless of heating and digestion phase. This difference between SP and PP was expected to be related to their inherent physicochemical characteristics. Regarding antibacterial properties, dry heating resulted in reduced antimicrobial activity against *Enterobacter cloacae*, in both SP and PP. However, the response of SP and PP-derived peptides differed for *Staphylococcus epidermidis*. Specifically, heating increased the antimicrobial activity of SP, while PP exhibited intrinsic inhibitory effects on its growth, independent of heating. Therefore, the protein source is the primary factor shaping the sRAGE-binding and antimicrobial potentials of digestion-derived peptides. This emphasizes the significance of selecting suitable protein sources in IF, as they could play an important role in promoting health and combatting against infections during infancy. A notable limitation of this study is the narrow focus on only two strains—*Enterobacter cloacae* and *Staphylococcus epidermidis*—to assess the antimicrobial activity of digests. Given the potential for various effects on other bacteria, fungi, or even viruses, future studies should broaden the microbial range to gain a more comprehensive understanding of the peptides' antimicrobial properties. Furthermore, the immune modulation capabilities of the peptides cannot be fully represented by sRAGE alone, necessitating further molecular and cellular explorations, including T cell activation and antibody (e.g., IgE) binding.

Subsequent bioinformatics analysis predicted 519 potential SP-based AMPs, 9 of which were glycated. Similarly, for PP, a total of 133 AMPs were predicted, with 6 that were glycated. AMPs have gained recognition as a promising avenue for developing therapeutic agents to combat microbial infections. Given this context, an intriguing subsequent step could involve synthesizing the predicted AMPs to evaluate both their antimicrobial capacity and their resilience against digestion. Moreover, a deeper exploration into the analysis of glycated AMPs could enhance the comprehensiveness of our investigation. Afterwards, these validated AMPs could be possibly introduced into IF to support infant health.

CRediT authorship contribution statement

Jiaying Tang: Conceptualization, Formal analysis, Methodology, Software, Visualization, Writing – original draft. **Malgorzata Teodorowicz:** Data curation, Methodology, Software, Writing – review & editing. **Sjef Boeren:** Formal analysis, Methodology, Software, Writing – review & editing. **Harry J. Wichers:** Conceptualization, Supervision, Writing – review & editing. **Kasper A. Hettinga:** Conceptualization, Software, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114224.

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