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Effects of different satiety levels on the fate of soymilk protein in gastrointestinal digestion and antigenicity assessed by an *in vitro* dynamic gastrointestinal model

Shuting Qing,^a Qiuqin Zhang,^a Wei Li,^a Elham Azarpazhooh,^b Benjamin K. Simpson^c and Xin Rui[✉]^a

This study was designed to compare the effects of different satiety levels on the digestion and antigenicity of soymilk protein. The DIVRSD-II dynamic *in vitro* digestion model (DIVD) was employed to simulate different satiety degrees by changing the amount of food intake, namely full satiety (DIVD-FS), semi-satiety (DIVD-SS) and limited-satiety (DIVD-LS). A standardized static *in vitro* digestion method (SIVD) was used as a reference. Coupled with 60 min of gastric digestion and 120 min of intestinal digestion, the pH, particle size, soluble protein content, peptide content, gel electrophoresis, and antigenicity of soymilk digesta were monitored. The results showed that different satiety degrees altered soymilk protein digestive patterns in terms of gastric transit time, hydrolysis degree and antigenicity. The results of soluble protein content suggested that soymilk protein in DIVD-FS showed faster gastric transition than DIVD-SS and DIVD-LS. Gel electrophoresis and peptide content indicated that DIVD-FS digesta showed predominantly lower hydrolysis degrees in gastric and early intestinal stages. This further led to the persistence of major allergens at the early stage of intestinal digestion for DIVD-FS digesta. Soymilk protein digested using SIVD showed mostly intermediate values which indicated a good reference to the different satiety degrees of DIVD, although a descriptive result was obtained at early intestinal digestion. The results of this study showed for the first time the effect of different satiety degrees on hydrolysis kinetics, digestive degree and antigenicity of soy protein. The results suggested that the knowledge-base of soy protein digestibility depends on different satiety degrees and will be useful for guiding a healthy diet mode.

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1. Introduction

Soy is a good source of plant protein which has a high protein content (~40%) and a good biological value.¹ Soy protein has prominent nutritional value and processing function characteristics, and is widely used in the food industry. One of the most common ways to consume soy protein is processing soybeans into an emulsion-like foodstuff which is called soymilk. Through soaking, grinding, filtration, and heating, the predominant soy proteins are extracted and transferred into the soymilk emulsion. Macronutrients like proteins have to be released from

the food matrix to obtain the real benefit.² This process is usually achieved by the function of gastrointestinal digestion allowing degradation of soy protein into smaller molecules. Therefore, as a popular food matrix delivering soy protein, it is of vital importance to understand the digestive behavior of soy protein in the gastrointestinal tract in the form of soymilk.

Several authors conducted studies on soy protein digestibility. Paiva *et al.* carried out *in vivo* and *in vitro* static digestion and found that the protein digestibility of soymilk increased by approximately 15% due to the destruction of trypsin inhibitors after boiling.³ Another study investigated the particle characteristics of soymilk during static *in vitro* digestion and the results revealed a reduction in particle size and predominant release of amino acids and polypeptides after intestinal digestion.⁴ In addition, several studies focused on the changes of antigenicity of soy protein during gastrointestinal digestion because soy protein is one of the “big eight” allergens.^{5–7} Some studies confirmed that the change of antigenic properties of soy protein by enzymatic hydrolysis was mainly due to the change of 7S and 11S components.⁸ A study showed that

^aCollege of Food Science and Technology, Nanjing Agricultural University, Jiangsu Province, P R China. E-mail: ruix@njau.edu.cn; Fax: +86 2584399090; Tel: +86 15651661026

^bKhorasan Razavi Agricultural and Natural Resources Research and Education Center, AREEO, Mashhad, Iran

^cDepartment of Food Science and Agricultural Chemistry, Macdonald Campus, McGill University, 21, 111 Lakeshore Rd., Ste-Anne-de-Bellevue, QC, H9X 3V9, Canada

50–70 kDa protein bands disappear after 20 min of pepsin digestion and the antigenicity of soy protein decreases.⁹ In general, most studies conducted were focused on the changes of granules and bioactive substances during gastrointestinal digestion such as polypeptides, polyphenols, flavonoids *etc.*^{10,11} There are few studies that investigate the effect of different satiety conditions on the digestive and antigenic properties of soymilk, although it has been proved that different satiety levels have a great impact on the digestive characteristics of food.^{12–15} Previous studies mainly focused on *in vivo* animal experiments and found that a feeding ratio of 6.5–7.0% was optimum for the growth and feed utilization of *Labeo rohita*.¹³ The PER (protein efficiency rate) of grass carp (*Ctenopharyngodon idella*)¹⁴ and bagrid catfish (*Mystus nemurus*)¹⁵ decreased with the increase of the feeding ratio. Van Ham *et al.* studied the growth and food conversion rate of juvenile turbot under different satiety feeding conditions, and found that 65% of satiety feeding had the best conversion rate.¹⁶ In addition, some studies found that food consumption affects the gastrointestinal environment¹⁷ and dilutes the digestive juice *in vivo*,¹⁸ which further influence the digestive pH environment, enzyme concentration, and have an impact on the digestive characteristics of food.

Digestibility of protein in a complex food matrix can be evaluated by *in vitro* gastrointestinal digestion simulation (GIS). Static *in vitro* digestion has been well developed especially the “international consensus static *in vitro* digestion model” which has been co-elaborated in the framework of COST action INFOGEST by more than 200 scientists from 32 countries.¹⁹ This has provided a standardized protocol to evaluate digestibility of food components. However, due to some inherent defects of static *in vitro* digestion such as disconnected compartments, discontinuous injection of enzymes, and non-dynamic alternation of pH, deviations might occur when compared to the real digestive pattern.²⁰ Therefore, some dynamic gastrointestinal models have been developed in order to better simulate the real situation *in vivo*, which are usually equipped with continuous secretion of gastric and pancreatic juice, simulated gastric and intestinal emptying, and connected to an artificial stomach and intestine apparatus.²¹ One of the *in vitro* dynamic digestive systems named DIVRSD-II plus was developed and applied recently.²² The model is equipped with a silicone artificial stomach and intestine which exhibited physiological parameters very close to the *in vivo* rat digestion. Thickness of the gastric wall is uniform allowing stabilization of the mechanical force produced by gastric extrusion, thus improving the mixing degree of digestive juice and food.²³ Although DIVRSD-II plus is an apparatus established based on the rat gastrointestinal digestive system, a good metabolic similarity between rats and humans has long been known²² and confirmed by previous studies which compared the tendency of milk protein hydrolysis in an *in vivo* rat experiment and an *in vitro* human digestive system.^{24,25} This model has so far been successfully applied in the evaluation of digestibility of rice,²⁶ casein protein²³ and bioactive substances.²⁰

Therefore, in the current study, three different satiety levels were applied to a dynamic *in vitro* gastrointestinal system (DIVD), namely, full-satiety, semi-satiety and limited-satiety, and the results were compared to a static *in vitro* gastrointestinal system (SIVD). The different satiety levels that influence the digestion process of soymilk protein were monitored by analyzing the particle size, soluble protein content, peptide content, electrophoresis and antigenicity. To the best of our knowledge, the current study revealed for the first time the effects of different satiety degrees on hydrolysis kinetics, digestibility and antigenicity of soy proteins. It is also novel that an *in vitro* dynamic gastrointestinal digestive system was used to explore the influence of different satiety degrees on food digestion. Results of this study will help to fill the gap in the study of digestive properties and antigenicity of soymilk in dynamic and static simulated digestive systems, and preliminarily reveal the effects of different satiety levels on the changes of digestive kinetics and antigenicity of soymilk, which may also guide healthy diet habits.

2. Materials and methods

2.1 Materials and chemicals

Soybeans were purchased from a local supermarket in Nanjing, P. R. China. Gastric mucin (M2378), pepsin (P7125), bile (48305), and pancreatin (P7545) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). The molecular mass standard (15–150 kDa) was obtained from Sangon Biotech Co. (Shanghai, China). RIDASCREEN® FAST Soya test kits used for determining the soy antigenicity were from R-Biopharm AG (Darmstadt, Germany). All other chemical reagents used were of analytical grade and purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.2 Preparation of soymilk

The selected soybeans were cleaned and soaked overnight in distilled water at room temperature. The soaked soybeans were then rinsed and ground with 9-fold water to make a slurry. Filtration was then performed with a 200-mesh screen cloth to remove insoluble residues (okara). Raw soymilk was collected and was boiled while stirring at 95 °C for 5 min. Soymilk was then cooled and subjected to both static and dynamic *in vitro* gastrointestinal digestion.

2.3 *In vitro* gastrointestinal digestion

Enzyme cocktails applied in both static and dynamic *in vitro* gastrointestinal digestion systems were identical and followed by previous studies.^{19,20} Simulated Salivary Fluid (SSF) contained α -amylase (75 U mL⁻¹), KCl (15.1 mmol L⁻¹), KH₂PO₄ (3.7 mmol L⁻¹), NaHCO₃ (13.6 mmol L⁻¹), MgCl₂(H₂O)₆ (0.15 mmol L⁻¹), and (NH₄)₂CO₃ (0.06 mmol L⁻¹) at pH 7.0. Simulated Gastric Fluid (SGF) was prepared by dissolving pepsin (0.27 g L⁻¹) and mucin (1.5 g L⁻¹) in simulated gastric fluid, which contained KCl (6.9 mmol L⁻¹), KH₂PO₄ (0.9 mmol L⁻¹), NaHCO₃ (25 mmol L⁻¹), NaCl (47.2 mmol L⁻¹), MgCl₂(H₂O)₆

(0.1 mmol L⁻¹), and (NH₄)₂CO₃ (0.5 mmol L⁻¹) and the pH was set to 1.6. Simulated Intestinal Fluid (SIF) was prepared by dissolving pancreatin (5.62 g L⁻¹) and bile salts (8.17 g L⁻¹) in simulated duodenal fluid, which contained KCl (6.8 mmol L⁻¹), KH₂PO₄ (0.8 mmol L⁻¹), NaHCO₃ (85 mmol L⁻¹), NaCl (38.4 mmol L⁻¹), MgCl₂(H₂O)₆ (0.33 mmol L⁻¹) and the pH was set to 7.0. A general process of operation applied in static and dynamic gastrointestinal digestion systems is summarised in Fig. 1 and detailed descriptions are provided in 2.3.1 and 2.3.2.

2.3.1 *In vitro* static gastrointestinal digestion. The static *in vitro* gastrointestinal digestion (SIVD) of soymilk was conducted according to the “standardised static *in vitro* digestion” protocol with minor modifications.¹⁹ Buccal digestion was conducted by mixing 3.5 mL of soymilk with SSF (1 : 1, v/v) and placed in a shaking water bath (SWB series, Biobase, Shandong, China) with 55 rpm at 37 °C for 2 min. Subsequently, gastric digestion was initiated by addition of SGF to the buccal digesta at a ratio of 1 : 1. Simulated gastric digestion was conducted in a shaking water bath at 37 °C, 55 rpm, for 1 h. The gastric digestion was terminated by adjusting the pH to 7.0 and an equal amount of SIF was added to the gastric digesta (1 : 1, v/v). The incubation was carried out at 37 °C, 150 rpm for 2 h. Eight samples were collected during the digestion, including soymilk without digestion (P0); after buccal digestion (P1), gastric digestion at 5 min, 30 min, and 60 min (P2-5, P2-30, and P2-60) and intestinal digestion at 5 min, 30 min, and 120 min (P3-5, P3-30, and P3-120) were carried out. The samples were immediately subjected to boiling temperature for 5 min to inactivate the enzyme. Three replicates were conducted.

2.3.2 *In vitro* dynamic gastrointestinal digestion. *In vitro* dynamic gastrointestinal digestion (DIVD) was performed in a

mechanized soft rat gastrointestinal model (Bionic Rat Model II+, Nantong Dong-Concept New Material Technology Ltd, Jiangsu Province, China) composed of dynamic gastric and duodenal parts (Fig. 1). Operation of the system and preparation of the artificial digestive juices were conducted according to the previous studies with slight changes.^{20,23}

Soy milk was firstly subjected to *in vitro* buccal digestion by addition and then thoroughly mixed with an equal volume of SSF. The oral digesta was then fed to the stomach through the model esophagus. A volume of 0.6 mL of SGF was pre-added to the artificial rat stomach to mimic the real situation of the rat digestive system *in vivo*. Since the artificial rat stomach has a volume of six mL, six, three, and one mL of soymilk buccal digesta was later fed into the artificial rat stomach model to simulate the state of full-satiety (DIVD-FS), semi-satiety (DIVD-SS) and limited-satiety (DIVD-LS). During the entire digestion process, the system was kept at 37 °C. Gastric digestion lasted for 60 min and the intestinal phase lasted for 120 min. Artificial gastric juice was delivered continuously by a syringe pump connected to the stomach model through four silicone tubes. The mean rates of gastric juice secretion were 52 μL min⁻¹. *In vivo* movement of the rat stomach was mimicked by pressing the silicone stomach model against a compression plate and a rolling extrusion plate at a rate of 3 compressions and 12 extrusions per minute, respectively. Gastric digesta passed through the artificial pylorus to initiate duodenal digestion, where artificial intestinal fluid was continuously delivered by a syringe pump with the injection rate set as 52 μL min⁻¹. The artificial duodenal was compressed by 6 sets of rolling extrusion plates to mimic the *in vivo* movement. The rolling extrusion plate was set at a rate of 36 extrusions per minute. The ratios SGF : sample and SIF : sample in

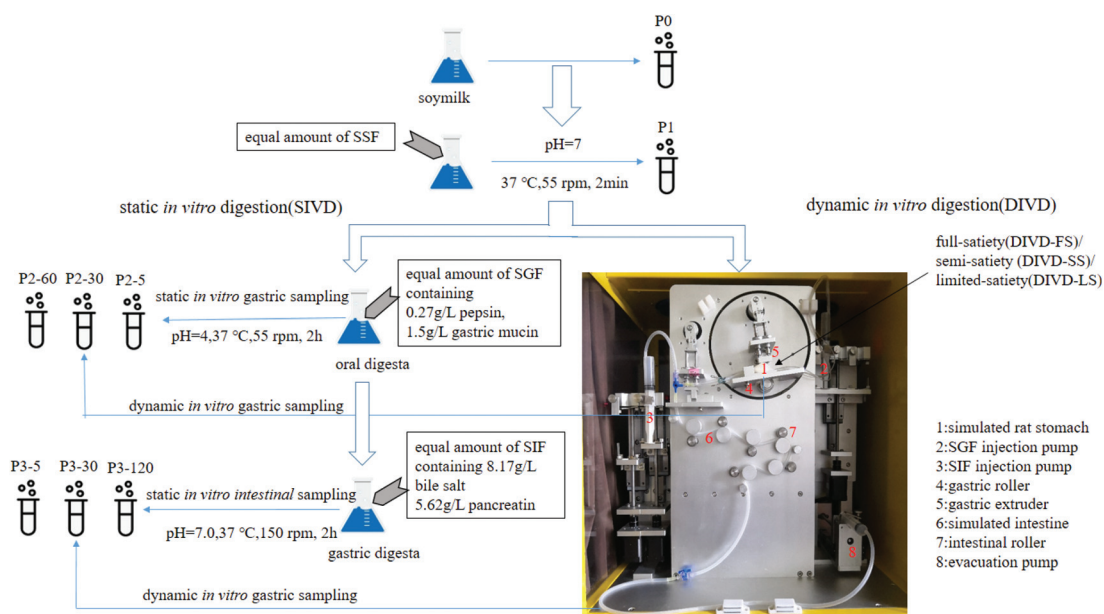


Fig. 1 A schematic diagram of static *in vitro* gastrointestinal simulated digestion (SIVD) and dynamic *in vitro* gastrointestinal simulated digestion with different satiety degrees: full-satiety (DIVD-FS), semi-satiety (DIVD-SS), and limited-satiety (DIVD-LS).

DIVD are varied according to the sample volume. The final volume attained in the gastric step of DIVD-FS, DIVD-SS, and DIVD-LS was 9.72 mL, 6.72 mL, and 4.72 mL, respectively, whereas the final volume attained in the intestinal step of DIVD-FS, DIVD-SS, and DIVD-LS was 19.08 mL, 16.08 mL, and 14.08 mL, respectively.

Samples were collected at the end of both the gastric phase and intestinal phase at identical time intervals as SIVD. Three replicates were conducted.

2.4 pH measurement

A pH meter (Schoot Lab 850, Mainz, Germany) was used for pH determination.

2.5 Average particle size

The average particle size was determined by using a Zetasizer Nano-ZS90 equipment (Malvern Instruments, Malvern, UK) by using the dynamic light scattering technique. Prior to the measurement, each sample was diluted 50 times with deionized water and then 1.2 mL of the diluted sample was added to the cuvette for determination. The average particle size was recorded and three replicates were conducted. The results were characterized in the form of mean particle size $\pm$ standard deviation.

2.6 Soluble protein content

Quantification of the soluble protein content was based on the Bradford assay.²⁷ The sample was diluted to the appropriate concentration and was mixed with five-fold Coomassie Brilliant Blue G-250 solution and incubated for 5 min at room temperature. The absorbance at 595 nm was then recorded by using a microplate reader (VersaMax ELISA Microplate Reader, Molecular Devices, USA). Bovine serum albumin was taken as a standard. In order to avoid the dilution effect of the digestive fluid, the results were adjusted to the quantity of soluble protein (mg) per initial volume of ingested soymilk (mL). Three replicates were conducted.

2.7 Peptide content

Peptide content was determined by using the *O*-phthaldialdehyde (OPA) assay according to a previously reported protocol.²⁸ OPA solution (200 mL) consisted of 100 mL of 100 mM borax, 10 mL of 20% (w/w) sodium dodecyl sulfate, 4 mL of 40 mg mL⁻¹ of *o*-phthaldialdehyde solution (OPA, dissolved in methanol), and 400 μ L of β -mercaptoethanol. Before measurement, each sample was mixed with 10% of trichloroacetic acid (TCA) in a ratio of 1 : 1 to allow precipitation of proteins and the supernatants were collected by centrifugation at 12 000 rpm for 10 min. Fifty μ L of supernatant was mixed with 2 mL of OPA solution and was incubated for 2 min. The absorbance was measured by using a UV spectrometer (U-4100, Hitachi Ltd, Japan) at 340 nm. The final results were adjusted to the quantity of peptide content (mg) per initial volume of ingested soymilk (mL) to avoid the dilution effect in the gastrointestinal digestion. Different concentrations of tryptone were used as standards.

2.8 Electrophoresis

Protein degradation during *in vitro* gastrointestinal digestion was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (in the presence of β -mercaptoethanol) by using a Bio-rad apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A stacking gel of 4% and a separating gel of 12% were applied. The running voltage was set at 60 V for the stacking gel and 120 V for the separating gel. A molecular mass standard (15–150 kDa) was adopted as a marker. The gels were dyed with 0.1% (w/v) Coomassie Brilliant Blue R-250 and then scanned with Image Scanner III (GE Healthcare Biosciences, Uppsala, Sweden).

2.9 Antigenicity

The antigenicity of soymilk digesta was evaluated based on the sandwich enzyme-linked immunosorbent assay by using the RIDASCREEN® FAST Soya ELISA test kit.^{29,30} Samples were first diluted with deionized water to ensure that their protein content was within the range of 7–13 mg kg⁻¹ soybean. One hundred μ L of the prepared sample were added into primary antibody-coated microtiter plate wells and incubated at room temperature for 10 min. The excess proteins were removed by washing twice with PBST solution (containing 0.05% Tween 20, pH 7.0). One hundred μ L of horseradish peroxidase-coupled secondary antibody were successively added and reacted at room temperature for another 10 min. The microtiter plate was washed twice before 100 μ L of substrate solution were added and then incubated at room temperature in the dark for 10 min. The reaction was terminated by addition of 100 μ L of stop solution. Within 10 min the absorbance at 450 nm was measured by using a microplate reader (VersaMax ELISA Microplate Reader, Molecular Devices, USA). The antigen concentration in the sample was calculated according to a standard curve. Three replicates were conducted for each determination.

2.10 Statistical analysis

One-way analysis of variance (ANOVA) and Duncan's multiple comparison tests were used to determine significant differences among means ($P < 0.05$) by using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1 pH changes during digestion

Fig. 2 shows the changes in the pH of soymilk digesta collected from respectively static *in vitro* digestion (SIVD), dynamic *in vitro* digestion full-satiety (DIVD-FS), DIVD semi-satiety (DIVD-SS), and DIVD limited-satiety (DIVD-LS). Undigested soymilk (P0) demonstrated a pH value of 6.3 but it slightly increased to 7.0 after buccal digestion (P1). Subsequently, gastric digesta collected from SIVD demonstrated a rapid drop in pH and then it remained constant throughout the gastric stage (P2), whereas samples collected from DIVD showed dynamic changes in their pH values, depending on the

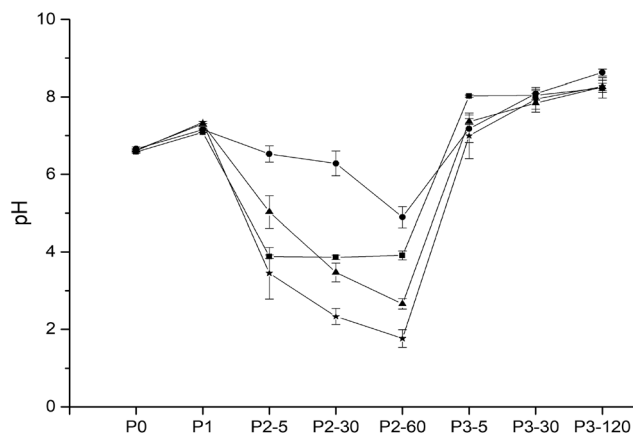


Fig. 2 pH curves of soymilk subjected to SIVD, DIVD-FS, DIVD-SS, and DIVD-LS: SIVD (square), DIVD-FS (circular), DIVD-SS (triangle), and DIVD-LS (star). P0-before digestion; P1-after buccal digestion; P2-5, P2-30, and P2-60 represent gastric digestion at 5 min, 30 min, and 60 min, respectively; P3-5, P3-30, and P3-120 represent intestinal digestion at 5 min, 30 min, and 120 min respectively.

different satiety degrees. Generally, DIVD-FS had the highest pH (4.89–6.53) during gastric digestion followed by DIVD-SS (2.66–5.03) and then DIVD-LS (1.76–3.45) in the descending order. In the intestinal digestion phase (P3), the pH of all the samples increased significantly ($P < 0.05$) and stabilized at 7.5 ± 1.0 during the 120 min of intestinal digestion stage. This indicated that the soymilk ingestion quantity or varied digestive models had little effect on the pH of the intestinal environment.

3.2 Particle size

Fig. 3 shows the change of the average particle size of soymilk digesta at different gastrointestinal digestion periods. The

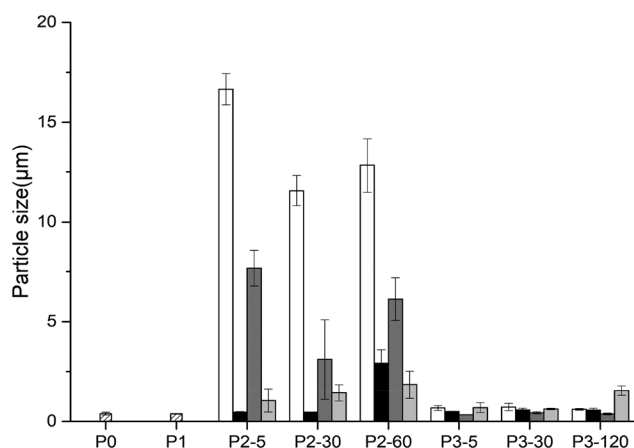


Fig. 3 Particle size of soymilk during static and dynamic *in vitro* digestion: SIVD (white bars); DIVD-FS (black bars); DIVD-SS (dark gray bars); DIVD-LS (light gray bars). P0: before digestion; P1-after buccal digestion; P2-5, P2-30, and P2-60: during gastric digestion at 5 min, 30 min, and 60 min, respectively; P3-5, P3-30, and P3-120: during intestinal digestion at 5 min, 30 min, and 120 min, respectively.

undigested soymilk sample showed an average particle size of $0.39 \mu\text{m}$ and a zeta potential of -21.30 mV . No significant changes were observed ($P > 0.05$) in the mean particle size after buccal digestion. Gastric digestion greatly promoted the average particle size especially for SIVD samples which fluctuated between 12 and $16 \mu\text{m}$. This observation was in accordance with the previous findings.³¹ Compared to SIVD, all samples from DIVD possessed significantly lower average particle sizes, in which DIVD-SS showed a higher value than the other two trails. This might be due to the fact that the pH of the DIVD-SS gastric digesta was near the isoelectric point of the soy protein unlike those obtained from DIVD-FS and DIVD-LS. The drop of pH near the isoelectric point of a protein facilitate charge neutralization and reduction of the repulsive force between proteins, which further cause protein aggregation and precipitation to form large protein aggregates.³² In addition, a decrease (SIVD and DIVD-SS) or increase (DIVD-FS and DIVD-LS) in average particle size was observed for soymilk gastric digesta subjected to different gastrointestinal models. The decrease of particle size might be due to the enzymatic dissociation of soy protein, which led to the degradation of some exposed hydrophobic areas by pepsin and the formation of soluble fragments.³³ However, in the case of DIVD-LS, the increment of particle size might be related to electrostatic exclusion. It has been reported that the structure of soy protein would decompose or even expand at pH 1.5 to 2.0.³⁴ In DIVD-LS, the pH of digested samples gradually decreased to less than 2.0 with the progress of gastric digestion which might led to the unfolding of the structure of soy proteins, such as glycinin (11S protein),³² and resulted in the increase of particle size.

The average particle size of all the samples collected during the intestinal stage dropped to approximately $0.3\text{--}0.6 \mu\text{m}$. There was no significant difference between the four groups of samples during the entire intestinal stage, except a slightly higher value obtained in the DIVD-LS ($P < 0.05$) at the end of intestinal digestion (P3-120). This probably corresponded to the neutral intestinal condition. The particle size of the sample might further influence the digestibility of proteins which could be shown by the following results of soluble protein and peptide content evaluation.

3.3 Soluble protein

Fig. 4 shows the changes in the soluble protein content with respect to different gastrointestinal digestion times. The soluble protein content obtained at P0 and P1 showed similar results with values ranging from 30 to 36 mg mL^{-1} . Gastric digestion allowed a sudden decrease of soluble protein content especially in the chyme of SIVD, DIVD-SS, and DIVD-LS (P2-5, Fig. 4). This observation was mainly due to the acidic environment in the stomach which led to the unfolding and exposure of hydrophobic groups from the inner part of proteins.³⁵ Similar observations were reported by Lakemond *et al.*³⁶ A steady increment in soluble protein content was observed for the three samples, namely, SIVD, DIVD-SS, and DIVD-LS, throughout the whole gastric period. In addition to

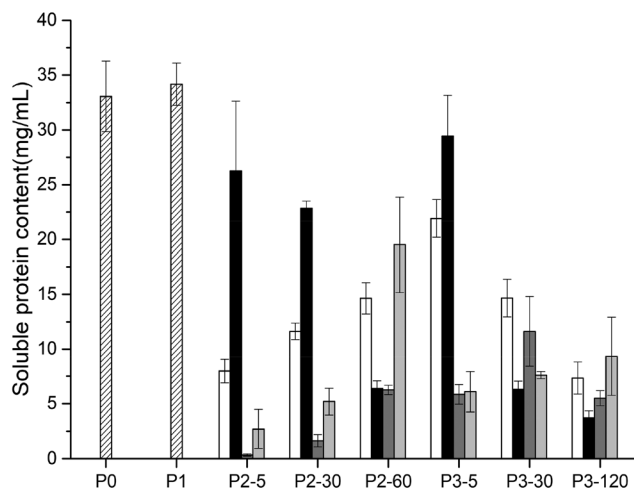


Fig. 4 Soluble protein content of soymilk during static and dynamic *in vitro* digestion: SIVD (white bars), DIVD-FS (black bars), DIVD-SS (dark gray bars), and DIVD-LS (light gray bars). P0: before digestion; P1: after buccal digestion; P2-5, P2-30, and P2-60: during gastric digestion at 5 min, 30 min, and 60 min, respectively; P3-5, P3-30, and P3-120: during intestinal digestion at 5 min, 30 min, and 120 min, respectively.

the digestive effect of pepsin, continuous mechanical shaking and dilution of the digestive slurry might also contribute to the release of soluble proteins from the soymilk matrix.^{28,37} However, DIVD-FS demonstrated a loss of soluble proteins during the gastric stage, which is probably due to the changing of its pH approaching the isoelectric point of soy protein.

Initiation of intestinal digestion further improved the soluble protein content of soymilk digesta collected from SIVD (P3-5, Fig. 4), indicating the release of soluble protein from the food matrix during this time of digestion. The subsequent intestinal digestion caused a drastic decrease ($P < 0.05$) in the soluble protein content in SIVD digesta (P3-30, P3-120, Fig. 4). This might be attributed to the generation of low molecular masses of peptides and amino acids produced by digestion hydrolysis, which did not fall within the detection range of the Bradford method.³⁸ This result was consistent with the finding of Xing *et al.*³¹

Samples collected from DIVD showed great discrepancies due to different satiety levels. DIVD-FS showed the highest soluble protein content (29.4 mg mL^{-1}), which was even higher than that of SIVD at the same period (P3-5, Fig. 4). The result indicated a fast transition of soymilk proteins from gastric digestion to intestinal digestion when a full satiety state was applied. Unlike DIVD-FS, the sample digested in a semi-satiety and limited-satiety mode showed a significantly lower soluble protein content, which was probably due to a slower gastric transition rate. Comparing the changes of soluble protein content in three DIVD samples, it can be found that the soluble protein content peaked at 5 min for sample DIVD-FS (P3-5, Fig. 4), 30 min for DIVD-SS (P3-30, Fig. 4) and 120 min for DIVD-LS (P3-120, Fig. 4). The results showed that the stage when most of the soluble proteins reached the intestine varied with the satiety degree. The lower the quantity of

soymilk digesta consumed, the later the peak would appear. In dynamic gastrointestinal digestion, the soluble protein content observed at the intestinal phase can be varied depending on the gastric transition time, but is also related to the extent of gastric and intestinal hydrolysis.

The results demonstrated that when soymilk was ingested in a full-satiety mode, most of the soluble proteins entered into the intestine rapidly at P3-5 from the stomach by the action of gastric extrusion and gastric peristalsis. This might be related to the greatest disturbance of the gastric environment when FS was applied which slowed down the efficiency of gastric digestion as indicated by the previous results on pH and particle size. The subsequent decrease of soluble protein content for FS at P3-30 and P3-120 was probably related to both the lower gastric transition rate caused by less food staying in the stomach and the progressive intestinal enzymatic hydrolysis which triggered the degradation of proteins to low molecular masses of peptides and amino acids. In contrast, improvement of the soluble protein in DIVD-LS throughout the intestinal phases suggested that the gastric conversion rate of food increased gradually even after 120 min of simulated intestinal digestion, and the soluble protein was released with the progress of intestinal digestion.

These results confirmed that samples with varied satiety levels respond differently to a series of dynamic system parameters, such as gastric/intestinal emptying, gastric/intestinal extrusion and real-time addition of enzymes, which might contribute to different gastric/intestinal digestion rates, and might further regulate the hydrolysis kinetics of soy protein in the intestinal stage.

3.4 Peptide content

The peptide contents of the samples collected before and during gastrointestinal digestion are shown in Fig. 5. The peptide contents of all the samples increased slightly during

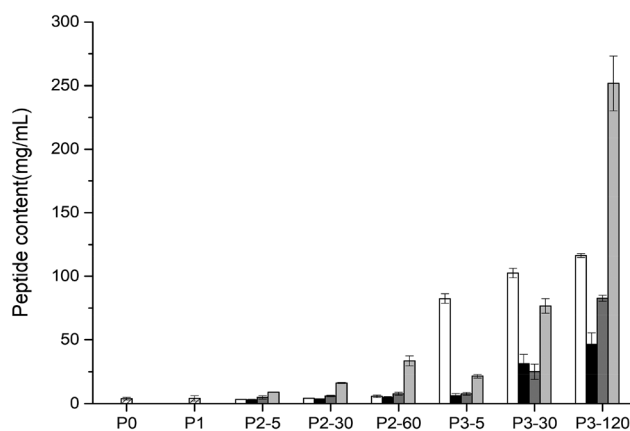


Fig. 5 Peptide content of soymilk during static and dynamic *in vitro* digestion: SIVD (white bars), DIVD-FS (black bars), DIVD-SS (dark gray bars) and DIVD-LS (light gray bars). P0: before digestion; P1: after buccal digestion; P2-5, P2-30, and P2-60: during gastric digestion at 5 min, 30 min, and 60 min, respectively; P3-5, P3-30, and P3-120: during intestinal digestion at 5 min, 30 min, and 120 min, respectively.

60 min of the gastric stage (P2-60), which might correspond to a limited hydrolysis by pepsin. This observation was consistent with the results obtained by other authors.³² Peptides in soymilk digested by SIVD increased sharply ($P < 0.05$) at the initial stage of intestinal digestion (P3-5), whereas a significant increase of peptide content in samples collected from DIVD did not occur until P3-30. This might be partially due to the different enzyme addition modes for the static and dynamic systems. For SIVD, enzyme cocktails were added immediately at the beginning of the digestion which promoted the surge in the peptide content of P3-5. In contrast, samples collected from DIVD had a delayed promotion in peptide content at P3-30 and P3-120 regardless of the satiety degree. At the end of intestinal digestion, DIVD-LS digesta possessed the highest amount of peptides (251.7 mg mL^{-1}), followed by SIVD (116.4 mg mL^{-1}), DIVD-SS (82.8 mg mL^{-1}) and then DIVD-FS (46.4 mg mL^{-1}) in the descending order. The results indicated that different satiety levels affected the release of peptides from the soymilk matrix. It is interesting that DIVD-LS showed the highest peptide content compared to the other two DIVD samples. This probably corresponded to multiple factors such as suitable pH conditions for the functionality of gastric enzymes, higher enzyme/substrate ratios, and lower gastric digestion rates, indicated by the previous results on pH and soluble protein content.

3.5 Electrophoresis

The hydrolysis patterns of soymilk were detected by SDS-PAGE and are shown in Fig. 6. At P0 and P1, the bands indicating

the main components of soy protein were observed as follows: α' ($\sim 75 \text{ kDa}$), α ($\sim 68 \text{ kDa}$), and β ($\sim 50 \text{ kDa}$) subunits of 7S globulin and A_3 ($\sim 39 \text{ kDa}$), acidic ($\sim 35 \text{ kDa}$), and basic ($\sim 20 \text{ kDa}$) subunits of glycinin (11S protein) (P0 and P1, Fig. 6A–D), although their concentration in P1 was lower due to the dilution of simulated saliva. The bands obtained were similar between soymilk digested by SIVD and DIVD-FS during the gastric digestion stage, and both 7S and 11S protein bands showed a decrease in intensity along with gastric digestion but were still visible at the end of gastric digestion (P2, Fig. 6A and B). This decrease in the band intensity could be attributed to the degradation by enzymes and dilution of simulated gastric fluid. These results were different from those obtained by Xing *et al.*, in which the 7S globulin subunit disappeared within 1 min.³¹ This is mainly due to the differences in the food matrix and gastrointestinal model applied, but is similar to the results presented in Nguyen *et al.*³⁹ At the first 30 min of gastric digestion, the intensity of the globulin bands in DIVD-FS appeared to be stronger than those in SIVD. At the end of gastric digestion (P2-60, Fig. 6A and B), the bands of the six major soy protein subunits were visible in SIVD and DIVD-FS samples, however, SIVD showed additional intact bands at 23–39 kDa and smeared bands at the molecular weight ranging between 15 and 20 kDa. In addition, the residue band intensity of the 11S basic subunit in SIVD (66.52%) was significantly higher than that in DIVD-FS (47.44%). This might be due to the different enzyme addition modes between the two systems, which caused the change of the hydrolysis kinetics of soy protein. Except the 11S basic

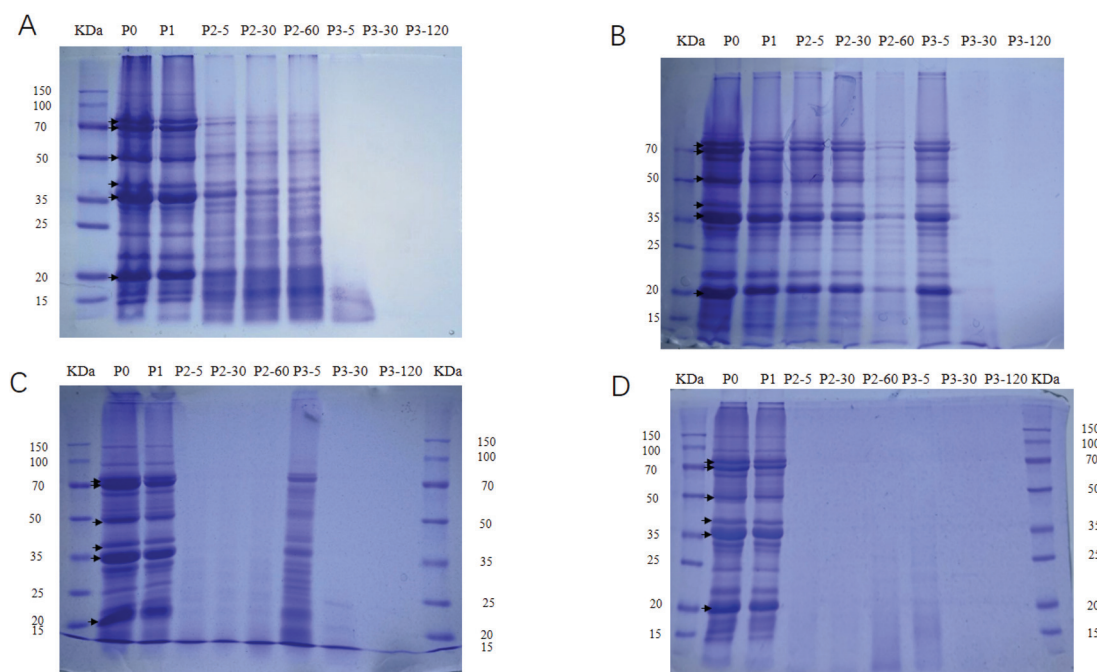


Fig. 6 SDS-PAGE analysis of *in vitro* digested soymilk by SIVD (A), DIVD-FS (B), DIVD-SS (C) and DIVD-LS (D). P0: before digestion; P1: after buccal digestion; P2-5, P2-30, and P2-60: during gastric digestion at 5 min, 30 min, and 60 min, respectively; P3-5, P3-30, and P3-120: during intestinal digestion at 5 min, 30 min, and 120 min, respectively. The arrows in each picture represent α' , α , and β subunits of β -conglycinin and A_3 , acidic, and basic subunits of glycinin from top to bottom.

subunit, no significant difference was found in the major globulins which showed similar residue band intensities at 35%–45% between the two groups.

However, in terms of DIVD-SS and DIVD-LS, the major globulins were hydrolyzed rapidly within 5 min after the initiation of gastric digestion allowing visibility of only a smear of bands corresponding to peptides less than 35 kDa (P2, Fig. 6C and D). The absence of intact bands might be due to a high degree of hydrolysis in the corresponding digesta.

Subsequent intestinal digestion led to the disappearance of all visible globulins in SIVD (P3, Fig. 6A) except polypeptides that have molecular masses less than 20 kDa at P3-5 (Fig. 6A). This indicated a rapid degradation of a large number of protein macromolecules. However, intact bands of all major globulins were observed in DIVD-FS and DIVD-SS at P3-5, although DIVD-SS demonstrated less intensity than that obtained from DIVD-FS. Unlike the gastric and intestinal digestions were carried out independently in SIVD, DIVD was a successive loading system in which samples passed through the artificial stomach and intestine consecutively by rolling and extrusion of the gastrointestinal tract. The result showed that part of the major globulins might “escape” from the gastric digestion and enter the intestinal tract intact in a full-satiety and semi-satiety mode. However, this was not the case when limited-satiety mode was applied. All samples, regardless of the digestive mode, showed rapid degradation of all visible protein bands in the subsequent intestinal digestion (P3-30, P3-120) indicating intensive hydrolysis which was in accordance with the significant increase in the peptide content in the intestinal phase (Fig. 5).

3.6 Antigenic properties

The uptake of food digesta through the intestinal epithelium might further induce a series of immune reactions including allergenicity. Soymilk digesta at P3-5 and P3-120 by both SIVD and DIVD were subjected to analysis to determine the protein

antigenicity and the results are shown in Fig. 7. The results suggested that most samples (SIVD, DIVD-SS, DIVD-LS) at P3-5 possessed decreased antigenicity after gastric digestion, which indicated a significant proteolytic destruction of epitopes. However, sample P3-5 collected from the full-satiety mode (DIVD-FS) showed comparable antigenicity to the value obtained at P0 which was significantly higher than the other three samples. This result was not surprising based on the previous SDS-PAGE pattern which showed the presence of all major globulin bands at P3-5 for DIVD-FS corresponding to the major soy allergens. It seems that epitopes were hardly disrupted for soymilk digesta at P3-5 in a full-satiety serving mode, which may consequently increase the potential risk of allergenicity. Semi-satiety (DIVD-SS) and limited-satiety (DIVD-LS) modes demonstrated antigenicity significantly lower ($P < 0.05$) than that of DIVD-FS. The result implied that the satiety levels impact greatly on the antigenicity of soy protein especially at the initial stage of intestinal digestion.

Subsequently, at the end of digestion (P3-120), the antigenicity of all digested samples was significantly lower ($P < 0.05$) than that of soymilk as expected. The hypoallergenic characteristic of hydrolyzed proteins is attributed to the disruption of allergenic epitopes during hydrolysis.⁴⁰ Compared with soymilk, the antigenicity of the samples digested by SIVD, DIVD-FS, DIVD-SS and DIVD-LS was decreased by 89%, 94%, 91% and 87%, respectively. The samples digested by DIVD-LS decreased the least and showed antigenicity which was significantly higher ($P < 0.05$) than DIVD-SS and DIVD-FS. This sequence was consistent with the peptide content at the same time point (P3-120). A previous article has found that the increase of degree of hydrolysis might result in the exposure of epitopes within the protein or form new epitopes.⁴¹ However, whether the increase of antigenicity of DIVD-LS at P3-120 is due to this reason remains to be further studied.

4. Conclusion

In this study, soymilk consumed at three satiety degrees was compared in terms of soy protein digestive behavior and antigenicity by using a dynamic *in vitro* gastrointestinal simulated digestive system (DIVD), and a static *in vitro* gastrointestinal simulated digestive system (SIVD) was used as a reference. Results showed that the digestive pattern and antigenicity of soy protein varied with satiety degrees. DIVD-LS with minimum food intake had the highest protein digestibility, whereas DIVD-FS had a lower protein digestibility in the gastric and early intestinal digestion stages, as revealed by the results of peptide content and electrophoresis. The low digestibility further led to the persistence of major allergens in the early intestinal digestion for DIVD-FS. This was in accordance with the observation of the shorter gastric transit time of DIVD-FS for which the peak of soluble protein content appeared the earliest at P3-5, whereas DIVD-LS appeared at the latest at P3-120. The study suggested that different satiety levels exerted different dilution effects on simulated digestion

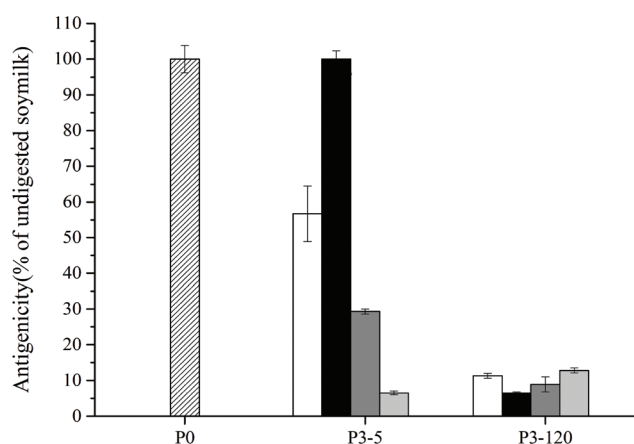


Fig. 7 The antigenicity of soymilk (P0) and soymilk digesta by SIVD (white bars), DIVD-FS (black bars), DIVD-SS (dark gray bars), and DIVD-LS (light gray bars) at 5 min and 120 min of intestinal digestion (P3-5 and P3-120, respectively).

juice, resulting in the differences in pH and enzyme concentration, and thus further affected digestive behaviour in terms of digestibility, gastric transit time and antigenicity of soymilk protein. The static *in vitro* digestion protocol as a reference showed a good reference to different satiety degrees of DIVD, although a descriptive result was obtained at the early stage of intestinal digestion. The current study showed that a full satiety consumption mode of soymilk is accompanied by lower digestibility and higher antigenicity risk which is not suggestive of a healthy diet mode.

Abbreviation

SIVD	Standardized static <i>in vitro</i> digestion
DIVD	DIVRSD-II dynamic <i>in vitro</i> digestion
DIVD-FS	Dynamic <i>in vitro</i> digestion full satiety
DIVD-SS	Dynamic <i>in vitro</i> digestion semi satiety
DIVD-LS	Dynamic <i>in vitro</i> digestion limited satiety
GIS	Gastrointestinal digestion simulation
OPA	O-Phthaldialdehyde
TCA	Trichloroacetic acid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSF	Simulated salivary fluid
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid

Conflicts of interest

There are no conflicts to declare.

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