



Immunoreactivity, sensory and physicochemical properties of fermented soy protein isolate



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ABSTRACT

The effect of induced liquid state fermentation (*Bacillus subtilis*, *Rhizopus oryzae*, *Saccharomyces cerevisiae*, *Lactobacillus helveticus*) on the immunoreactivity, physicochemical and sensory properties of soy protein isolate (SPI) was studied. *L. helveticus* revealed the most abundant reduction in terms of immunoreactivity within soluble protein fractions, up to 100%, which could be measured by *in vitro* sandwich ELISA using mouse monoclonal anti-Glym5 antibodies (mAbs). Almost no binding was found in western blot analysis using mouse monoclonal mAbs and sera from soy sensitive individuals. Fermentation increased water- and oil-binding capacity as well as protein solubility at pH 4.0. Foaming activity was nearly doubled compared to non-fermented SPI. A decreased emulsifying capacity, foaming density, and quantity of soluble proteins at pH 7.0 were observed. Principal component analysis (PCA) confirmed decreased bitter and beany off-flavors of fermented samples compared to non-fermented SPI. Consequently, fermentation might be a promising method to produce tasty low-allergen food ingredients with good physicochemical properties.

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1. Introduction

Food allergy is an abnormal immune response to certain food components. The prevalence of food allergies is rising dramatically, and approximately 220 to 250 million people worldwide suffer from some kind of food allergy (WAO, 2013). Food allergens are typically naturally occurring proteins, with small regions, called epitopes, responsible for the immunoglobulin E (IgE)-mediated allergic response (Taylor & Hefle, 2001).

Soybean (*Glycine max* (L.) MERR.) is an important vegetable protein source for the food industry due to its considerable amount of high quality proteins and nutritional value. However, soybean is among the so-called “big 8” food allergens, which together account for over 90% of all documented food allergies in the U.S. (FDA, 2004; Taylor & Hefle, 2001). The prevalence of soy allergy is not precisely known and it is expected to escalate due to the increasing consumption of soybean products. Currently, eight allergenic proteins (Glym1–Glym8) with molecular masses ranging from 7.5 to 97 kDa have been registered by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee (www.allergen.org). However, the two storage proteins

β-conglycinin (Glym5) and glycinin (Glym6) have been related to severe reactions in European allergic populations (Amnuaycheewa & de Mejia, 2010; Holzhauser et al., 2009; Verhoeckx et al., 2015). The estimated allergy threshold is low and small amounts of soy protein, ranging from 0.0013 to 500 mg, may be enough to trigger an immune response (FDA, 2004).

So far, the only way to prevent unintended exposure to a food allergen is complete avoidance of the offending food. Recently, the need to control soy allergy by processing treatments rather than avoidance has pushed the development of new technologies, including genetic modification, thermal and non-thermal treatments (Verhoeckx et al., 2015). Currently, enzymatic hydrolysis using proteases of microbial or plant origin is the most efficient process for disrupting sequential and conformational epitopes, thereby reducing the allergenicity (Wilson, Blaschek, & de Mejia, 2005; Meinlschmidt, Schweiggert-Weisz, Brode, & Eisner, 2016; Meinlschmidt, Sussmann, Schweiggert-Weisz, & Eisner, 2016).

Fermentation is a traditional processing technology in the food industry. Besides the functions of improving the bioavailability of nutrients and reducing pathogenic bacteria in the gastrointestinal tract, a regulation of immunogenicity and possibly reduction of allergenicity by fermentation has been hypothesized (Granato, Branco, Cruz, Faria, & Shah, 2010), but has rarely been investigated. Few studies (Frias, Song, Martinez-Villaluenga, Gonzalez de Mejia, &

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Vidal-Valverde, 2008; Kobayashi, 2005; Song, Frias, Martinez-Villaluenga, Vidal-Valverde, & de Mejia, 2008; Song, Pérez, Pettigrew, Martinez-Villaluenga, & de Mejia, 2010; Yamanishi, Huang, Tsuji, Bando, & Ogawa, 1995) have confirmed the degradation of soybean allergens, particularly Glym Bd 30 K, also known as P34, during fermentation by microbial proteolytic enzymes in fermented soybean foods such as soy sauce, miso, tempeh, soybean ingredients and feed-grade soybean meals. It has also been shown that various lactic acid bacteria (LAB) are able to hydrolyze α' - and α -subunits of soybean β -conglycinin (Aguirre, Garro, & de Gioria, 2008). The studies of Frias et al. (2008) and Song et al. (2008) demonstrated that both solid- and liquid state fermentation of cracked soybean seeds, flour, or meals by various mould strains and bacteria, effectively reduced IgE-immunoreactivity by 65–99% as evaluated by an indirect ELISA with human sera. However, these research groups did not consider the effect of fermentation on the sensory and physicochemical properties of the respective soy product as fermentation can often lead to off-flavors or a loss of technofunctionality (Quinn & Beuchat, 1975; Shrestha, Dahal, & Ndungutse, 2013). As sensory and physicochemical properties are equally important parameters for the food industry, the simultaneous investigations are of considerable importance, but not examined to our knowledge up to now. Consequently, the evaluation of residual immunoreactivity of these two proteins is indispensable for the assessment of potential allergenicity of the processed food. However, reports are not available in the literature so far.

The objective of this research was to evaluate the degradation of the major soybean allergens Glym5 and Glym6 (SDS-PAGE) and the reduction of Glym5 immunoreactivity (sandwich ELISA and western blot) by induced liquid state fermentation of SPI using *Bacillus* (*B.*) *subtilis*, *Rhizopus* (*R.*) *oryzae*, *Saccharomyces* (*S.*) *cerevisiae*, and *Lactobacillus* (*L.*) *helveticus*. The sensory (smell, taste, and mouthfeeling) and physicochemical properties (protein solubility, emulsifying, foaming, oil- and water-binding capacity) have been analyzed as well.

2. Material and methods

2.1. Raw materials and chemicals

Untoasted soybeans (*G. max* (L.) MERR.) were purchased from Naturkost Ernst Weber (Munich, Germany). *L. helveticus* DSM 20075, *B. subtilis* DSM 10, and *R. oryzae* DSM 2200 were purchased from the Germany Resource Center (DSMZ, Braunschweig, Germany), while *S. cerevisiae* TMW 3.210 was obtained from the collection of the chair 'Lehrstuhl für Technische Mikrobiologie' in Weihenstephan (TMW, Freising, Germany).

All chemicals used in this study were of analytical grade and obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany) if not stated separately.

2.2. Preparation of soy protein isolate (SPI)

SPI was prepared from untoasted soybean seeds using the technique as previously described in Meinschmidt, Sussmann, et al. (2016).

2.3. Fermentation of SPI

2.3.1. Strains, media, growth conditions, and preparation of inocula

All strains were cultivated overnight under their respective optimal growth conditions (see Table 1).

For solid media, 15 g L⁻¹ agar was added to the respective broth. After cell enumeration, a calculated aliquot of the preculture was centrifuged for 10 min at 9000 × g, the pelleted cells were washed twice with Ringer solution (Merck KGaA, Darmstadt, Germany) and were resuspended in 1 ml sterile distilled water, which was used as inocula.

2.3.2. Experimental design of induced liquid state SPI fermentation

Liquid state fermentations were performed in SPI that was suspended in sterile deionized water (5% w/w, pH 6.7). Prior to inoculation, the SPI dispersion was pasteurized at 85 °C for 10 min in order to reduce the endogenous microbiota of the raw materials. After cooling down, the dispersions were supplemented with 2% (w/v) glucose (Sigma-Aldrich Inc., St. Louis, USA) to favor the growth of the inoculated strains. Two independent replicates of the SPI dispersion were inoculated with strains from precultures to obtain a concentration of either 1 × 10⁸ colony forming units (CFU) per ml of bacteria/yeast or 10% (v/v) mould.

Fermentations were carried out under strain-specific growth conditions (Table 1) for 24 and 48 h in conical flasks without agitation. Fermentation was stopped by heat treatment at 90 °C for 20 min to terminate residual proteolytic activity. All samples were neutralized (pH 7.0) with 1 M NaOH, kept at –50 °C and lyophilized (BETA 1–8, Martin Christ Gefriertrocknungsanlagen GmbH, Germany). The pH value of the samples was measured initially and after fermentation using a disinfected pH electrode (WTW, Weilheim, Germany) calibrated at pH 7.0 and 4.0. All fermentations were performed in duplicate.

2.4. Microbiological analysis

2.4.1. Determination of the total viable counts by measuring the colony forming units (CFU)

Directly after inoculation and at time intervals of 24 h and 48 h, the fermentation process was monitored by determining the total viable counts. Aliquots (100 µl) of fermented SPI were in triplicate serially diluted 1:10 (v/v) with Ringer solution (Merck, Germany) and 100 µl of certain dilutions was spread homogeneously with a Drigalski spatula in duplicate on the surface of pre-dried plate-count agar plates (Merck KGaA, Darmstadt, Germany). In addition, fermented samples were plated on strain specific agar to quantify and favor the growth of the respective strain. Plate-count agar plates were incubated aerobically at 30 °C for 48 ± 2 h, while all other plates were incubated under strain specific growth conditions (see Table 1). The colonies were counted and expressed as colony forming units per milliliter (CFU ml⁻¹).

2.4.2. Matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS)

MALDI-TOF-MS was performed to verify that the inoculated strains were dominant in the samples during fermentation. Fresh

Table 1
List of employed strains including abbreviations, and growth/culture conditions.

Strain	No.	Abbreviation	Type	Growth/culture conditions
<i>Bacillus subtilis</i>	DSM 10	<i>B. subtilis</i>	Bacteria	30 °C, aerob, nutrient
<i>Lactobacillus helveticus</i>	DSM 20075	<i>L. helveticus</i>	Lactic acid bacteria	37 °C, anaerob, MRS
<i>Rhizopus oryzae</i>	DSM 2200	<i>R. oryzae</i>	Mold	30 °C, aerob, potato dextrose
<i>Saccharomyces cerevisiae</i>	TMW 3.210	<i>S. cerevisiae</i>	Yeast	30 °C, anaerob, potato dextrose

DSM = Deutsche Sammlung von Mikroorganismen; TMW = Technische Universität Weihenstephan; MRS = DeMan, Rogosa and Sharpe

colony material (a single colony) was smeared on a polished steel MSP 96 target (Bruker Daltonik), spotted with 1 µl of a saturated α -cyano-4-hydroxy-cinnamic acid matrix solution in 50% acetonitrile-2.5% trifluoroacetic acid (Bruker Daltonik) and air dried at room temperature. The analysis ($n = 3$) of the mass spectra was performed by a Microflex LT mass spectrometer (Bruker Daltonik) using the MALDI Biotyper software package (version 3.0). For external mass calibration, a bacterial standard (Bruker Daltonics) was used.

2.5. Molecular weight distribution applying sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight distribution was determined using SDS-PAGE according to Laemmli (1970) under reducing conditions. Sample preparation and running conditions were chosen according to Meinlschmidt, Sussmann, et al. (2016) using 4–20% midi Criterion™ TGX Stain-Free™ precast gels. Protein visualization was performed by Criterion Stain-Free Gel Doc™ EZ Imager (Bio-Rad).

2.6. Sandwich enzyme-linked immunosorbent assay (ELISA)

Mouse monoclonal anti- β -conglycinin antibodies (mAbs) were generated by immunizing female BALB/c mice (Janvier Labs, Le-genest saint-Isle, France) with β -conglycinin (SIGMA, Deisenhofen, Germany). Mice with maximum antibody titers were used as donors of splenic cells, which were fused to X63.Ag8.653 myeloma cells (ACC 43, DSMZ, Braunschweig, Germany). Three positive clones (Izimab-Glym5-3, Izimab-Glym5-4 and Izimab-Glym5-5) were selected according to standard protocols (Davis, Pennington, Kubler, & Conscience, 1982) and were used in three sandwich variants to avoid detection losses due to insufficient capture efficiency. Three positive clones were selected to assemble sandwich ELISAs for determining β -conglycinin. For Sandwich 1, Izimab-Glym5-4 and Izimab-Glym5-5 were used as capture and detection antibodies, respectively, whereas Izimab-Glym5-3 (capture) and Izimab-Glym5-5 (detection) were applied in Sandwich 2. In contrast, Izimab-Glym5-5 (capture) and Izimab-Glym5-4 (detection) were chosen for Sandwich 3. Capture antibodies were immobilized onto 96-well plates (maxisorp; Nunc, Germany) at 4 °C overnight. Plates were washed three times with phosphate buffered saline, 154 mM NaCl, pH 8.0, 0.05% Tween (3xPBS-T) and blocked with Superblock blocking buffer (Life Technology GmbH, Germany). SPI and all other samples were centrifuged at 15,000 $\times$ g, 10 min and supernatants (soluble protein) were incubated for 1 h at RT in duplicate. After a further wash-step (3xPBS-T) the POD-conjugated detection-antibody was bound 1 h at RT. POD activity was determined following a third wash step (3xPBS-T) by incubating the plate with TMB-E substrate (DUNN Labortechnik, Asbach, Germany) and stopping reaction after 5 min with 0.3 M H₂SO₄. Color intensity represents a signal for the amount of captured antigen and was measured at 450 nm. Signal intensities were calculated by extrapolation to a calibration curve from standard-SPI consisting of seven known concentrations. Each sample was analyzed in duplicate.

2.7. Western blot

Monoclonal antibodies specific for β -conglycinin were used for detecting β -conglycinin by western blot. SPI and fermented samples were separated by SDS-PAGE using precast gradient (4–15%) tris-glycine gels (BioRad, München, Germany) according to standard protocol (Laemmli, 1970). After electrophoresis, proteins were blotted onto nitrocellulose membranes using semi-dry blotter Perfect blue (Peglab, VWR, Erlangen, Germany) according to manufacturer's instructions using Towbin buffer (25 mM Tris,

192 mM glycine with 10% MetOH, 0.02% SDS) as transfer buffer. Membranes were blocked with 5% skim milk powder in PBS-T (phosphate-buffered saline, 154 mM NaCl, 0.05% Tween 20, pH 8.0) and incubated overnight with mAbs at 4 °C. After washing in PBS-T, membranes were incubated with detection antibody Horse-radish-peroxidase (HRP)-conjugated goat anti-mouse IgG (Fc Fragment specific) (Dianova, Hamburg, Germany) for 1 h at room temperature.

Serum samples of allergy patients were collected in the Dermatology, Venerology and Allergy Clinic, University of Leipzig (Leipzig, Germany ethics proposal 428-12-17122012). Three serum samples out of 50 different patients suffering from soy allergy, showed *in vitro* Glym5-reactivity (CAP class 2, f432, measured with Phadia 100, Phadia, Thermo Scientific, Freiburg Germany). Two of these serum samples were used to verify results, obtained with mouse monoclonal anti- β -conglycinin Abs in western blot. Conditions for electrophoresis and blotting were the same as used for analysis with mAbs. Human serum was diluted twofold and detection of bound Abs was carried out with mouse-anti-human-IgE (ϵ -chain specific)-HRP (Biozol, Eching, Germany). HRP activity was visualized by using chemiluminescence substrate supersignal west femto (Thermo Fisher, Pierce, Bonn, Germany) with subsequent exposure of CL-XPosure films.

2.8. Chemical composition and physicochemical properties

2.8.1. Chemical composition

The protein and dry matter content was determined as described by AOAC methods using a protein calculation factor of $N \times 6.25$ (AOAC, 2005a, 2005b).

2.8.2. Physicochemical properties

2.8.2.1. Protein solubility. Protein solubility of the samples was determined at pH 4.0 and 7.0 following the method of Morr et al. (1985).

2.8.2.2. Emulsifying capacity. The emulsifying capacity (EC) was determined in duplicate as suggested by Wang and Johnson (2001).

2.8.2.3. Foaming properties (activity, density, and stability). Foaming activity was determined according to Phillips, Haque, and Kinsella (1987), whereas the foaming density and stability were analyzed as described elsewhere Meinlschmidt, Sussmann, et al. (2016).

2.8.2.4. Water- and oil-binding capacity. Water-binding capacity (WBC) was analyzed according to the AACC 56-20 official method (AACC, 2000). Oil-binding capacity (OBC) was determined as described by Ludwig, Ludwig, and Pingel (1989).

2.9. Sensory evaluation

2.9.1. Quantitative descriptive analysis (QDA)

Sensory profiling of the samples was performed using descriptive sensory analysis as described in ISO 6564:1985. The sensory panel consists of ten panellists (7 female, 3 male), selected and trained according to guidelines in ISO 8586-1:1993. A broad list of attributes characteristic for the samples in terms of smell (*fermented, beany, yeasty*), taste (*fermented, bitter, beany*), and mouth-feeling (*mouthcoating, astringent*) have been developed by the panel (1 month, 2-times per week). The attributes were rated on a 10-cm continuous scale (0 = no perception; 10 = strong perception).

2.9.2. Samples preparation and sensory evaluation

For sensory evaluation, samples were mixed with tap water to a protein concentration of 2.5% (w/w) and adjusted to pH 7.0 with

1 M NaOH. The panel was presented with five samples (10 ml) per session (performed in two sessions, 1 h each), which were served to the panel in randomized order at room temperature in small plastic cups, coded by arbitrary numbers (three-digits). Water and plain crackers were provided for palate cleansing in between. The panel was instructed to evaluate the sensory attributes for smell, taste, and mouthfeeling as mentioned in Section 2.9.1 using the 10-cm scale. Each panellist did a monadic evaluation of the samples at individual speed. Sensory evaluation was performed in duplicate.

2.10. Statistical analysis

Statistical analysis was performed using SPSS (SPSS v 20.0 SPSS Inc. Chicago, IL, USA). All chemical data were analyzed using one-way analysis of variance (ANOVA) and means were generated and adjusted with the Bonferroni post hoc test. Statistically significant differences were considered at $p < 0.05$.

QDA data were also analyzed using the one-way ANOVA, followed by Tukey's honest significance difference (HSD) test. In order to find similarities and differences between the samples and relations between the attributes, the results of the descriptive sensory analysis were processed by means of multivariate statistical PCA using the R-based PanelCheck software (Nofima, Ås, Norway; version 1.4.0.).

3. Results and discussion

For the experiments, a SPI, containing a dry matter content of 94.4% and a protein content of 94.6% was prepared (see Section 2.2).

All fermentations were monitored for 24 and 48 h, but all samples fermented for 48 h – except *L. helveticus* – were not suitable as food ingredients as the sensory properties were negatively affected. Therefore, fermentations with all strains were performed for 24 h, while fermentation with *L. helveticus* was additionally carried out for 48 h due to the pleasant taste.

3.1. pH value and microbial growth during fermentation

Fermentation with *L. helveticus* showed a progressive reduction in pH value after 24 and 48 h with an initial pH value of 6.7 lowering to pH 4.5 and 4.2, respectively. This sharp decrease ($\Delta\text{pH} = 2.2\text{--}2.5$) is characteristic for LAB strains. During fermentation, *L. helveticus* grew rapidly, converting the supplemented glucose into organic acids, mainly lactic-acid, thereby lowering the pH and inhibiting the growth of other competing microorganisms (Doblado, Frias, Munoz, & Vidal-Valverde, 2003). SPI was inoculated with *L. helveticus* at a cell density of 1×10^8 CFU ml⁻¹, and in the course of fermentation a growth of up to 7.5×10^9 CFU ml⁻¹ after 48 h was detected. After 24 h, *B. subtilis*, *S. cerevisiae*, and *R. oryzae* lowered the pH value to pH 5.1, 5.9, and 6.5, respectively. These strains produce only minor organic acids from fermentable sugars (hexoses) and, therefore, the lowering of the pH was rather minor compared to *L. helveticus*. This phenomenon was also observed by Song et al. (2008). An increase in viable cells was detected for both *B. subtilis* and *S. cerevisiae*, rising from initially 1×10^8 CFU ml⁻¹ up to 5×10^8 CFU ml⁻¹ or 7.5×10^8 CFU ml⁻¹, respectively. Following fermentation with *R. oryzae*, the visual colonies displayed the wooly texture on the surface on the plates, thereby showing the growth of this mould and the absence of other bacteria on plate-count agar.

MALDI-TOF-MS analyses reported a reliable identification of the inoculated strains with log(score) values of ≥ 2.3 (highly probable species identification) and confirmed the absence of competitive

strains and the ability of the inoculated strains to dominate fermentation.

3.2. Molecular weight distribution and residual immunoreactivity

Fig. 1A shows the electrophoretic patterns of all samples. The electrophoretic profile of non-fermented SPI (lane 2) presents high intensity bands of β -conglycinin subunits α' (~72 kDa), α (~68 kDa), β (~53 kDa), and glycinin, which is mainly composed of the acidic subunit "A" (29–33 kDa) and the basic subunit ("B", 18–22 kDa) (Amnuaycheewa & de Mejia, 2010). The protein identification by means of LC-MS/MS was described previously Meinschmidt, Schweiggert-Weisz, et al. (2016).

Among the fermented samples, electrophoretic patterns of samples fermented with *B. subtilis* (Fig. 1A, lane 3) and *L. helveticus* (Fig. 1A, lanes 6 and 7) showed the greatest reduction in protein band intensity corresponding to β -conglycinin subunits. In contrast, both acidic and basic subunits of glycinin were less affected. This might be due to the complex structure and disulfide bridges of glycinin, which impedes enzymatic hydrolysis of proteins. Aguirre et al. (2008) reported that β -conglycinin subunits were the preferred substrate for different LAB strains. Various different proteolytic enzymes have been identified in LAB (Savijoki, Ingmer, & Varmanen, 2006). It is well known that exploitation of milk proteins in the course of fermentation is initiated by cell-envelope proteinases (CEP), a subtilisin-like serine protease, that degrades proteins into oligopeptides, shorter peptides and amino acids (Christensen, Dudley, Pederson, & Steele, 1999). In addition, a number of distinct intercellular peptidases, including endo-, amino-, tri- and dipeptidases are involved, which might hydrolyze soy proteins (Christensen et al., 1999). The degradation of major soybean allergens following fermentation with *B. subtilis* was also reported by Yamanishi et al. (1995) and Frias et al. (2008). *S. cerevisiae* (Fig. 1A, lane 5) and *R. oryzae* (Fig. 1A, lane 4) were less effective in the degradation of soy proteins as the electrophoretic pattern did not changed compared to non-fermented SPI.

The residual immunoreactivity of the soluble proteins of all samples is presented in duplicate (1st and 2nd experiment) in Fig. 1B (sandwich ELISA) and Fig. 2 (western blot). Each sandwich ELISA (Sandwich 1–3) in Fig. 1B is composed of two different antibodies as described in the Material and Method section (see Section 2.6), recognizing different epitopes of β -conglycinin. Therefore, the result of ELISA analysis represents the concerted immunoreactivity of both epitopes. The immunoreactivity was calculated in relation to non-fermented SPI, which was set to 100%. Reduced immunoreactivity is measured if either of both epitopes failed in binding. Hence separate tests for each antibody were performed in western blot, and both capture and detection Abs revealed reduced immunoreactivity in the fermented samples (Fig. 2A and B). These results verified the ELISA-data (Fig. 1B). All fermented samples showed a reduction in immunoreactivity in the first experiment of fermentation in western blot using any antibody applied previously in ELISA regardless of the strain. However, differences among both experiments (1st and 2nd experiment) and the strains can be found.

For all strains assayed – except for *S. cerevisiae* and *R. oryzae* – SDS–PAGE results greatly coincide with the ELISA and western blot results. Although SDS–PAGE analysis of the sample prepared with *S. cerevisiae* and *R. oryzae* suggests that this strain did not alter the allergic potential of SPI, ELISA and western blot analyses disprove this assumption. A reason could be that both strains changed the conformational epitopes on the surface of the soy proteins, which did not lead to changes in electrophoretic patterns (Fig. 1A, lane 4 and 5), but lead to great changes in IgE-binding ability to human serum.

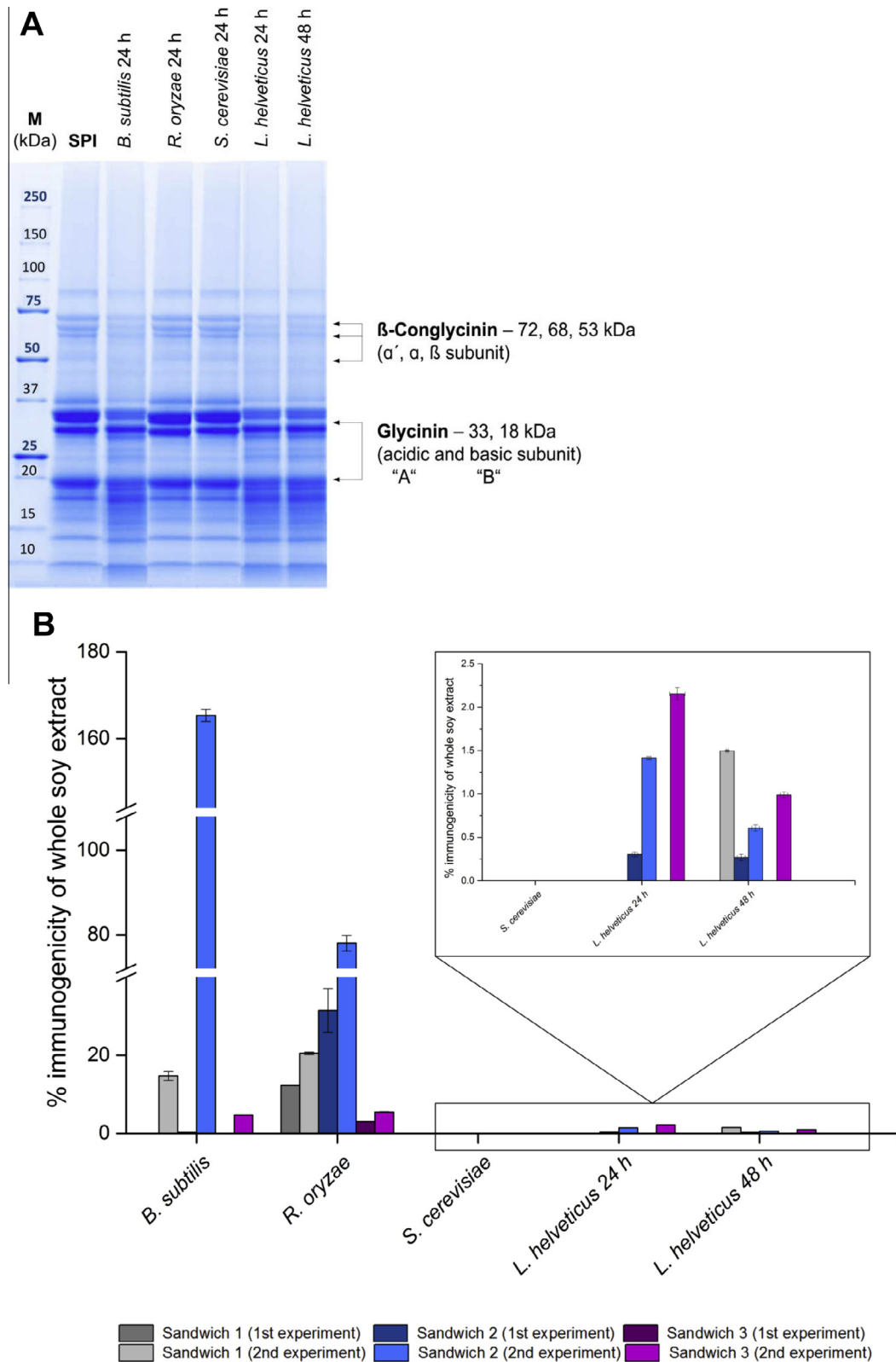


Fig. 1. SDS–PAGE patterns (A) and sandwich ELISA (B) using mouse monoclonal anti-β-conglycinin (Glym5) Abs (Izimag-Glym5-3, Izimag-Glym5-4, Izimag-Glym5-5). M – molecular weight standard indicated in kilo Dalton (kDa); SPI – soy protein isolate; electrophoresis was carried out with 4–20% polyacrylamide gradient gels. α', α- and β-subunits of β-conglycinin; "A" and "B": acidic and basic subunit of glycine. Results of three different sandwich ELISAs are shown. Each ELISA was performed in duplicate (1st and 2nd experiment).

The greatest reduction of up to nearly 100% in immunoreactivity to mAbs against β-conglycinin was achieved by fermentation with bacteria (*B. subtilis* and *L. helveticus*) and yeast (*S. cerevisiae*). Moulds like *R. oryzae* grow at a slower rate compared to bacteria,

generating lower β-conglycinin alteration, thereby giving a higher immune response with anti-β-conglycinin mAbs (Fig. 1B and 2). Among the strains assayed, almost no binding to soy sensitive serum (Fig. 2D and E) was found for samples fermented with

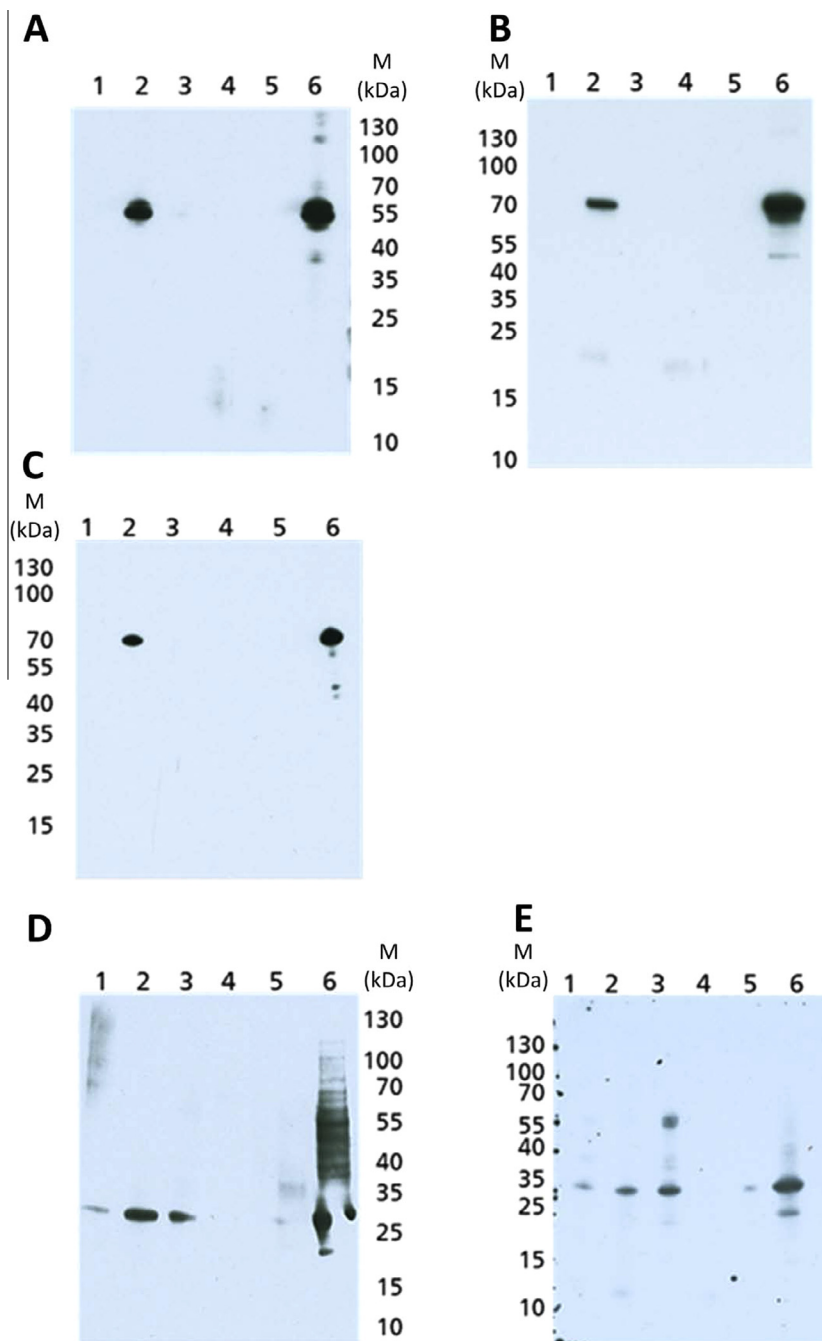


Fig. 2. Western blot analysis of all samples applying three different mouse monoclonal antibodies (Izimag-Glym5-3, Izimag-Glym5-4, Izimag-Glym5-5) used in the ELISA experiments above as coating-Abs (A–C) and two sera from soy sensitive individuals (D and E). Representative samples containing equal protein contents (determined by Bradford assay) of SPI, fermented by microorganisms or non-fermented ((1) *B. subtilis* 24 h, (2) *S. cerevisiae* 24 h, (3) *R. oryzae* 24 h, (4) *L. helveticus* 48 h, (5) *L. helveticus* 24 h and (6) non-fermented SPI), were blotted onto nitrocellulose membranes. M – molecular weight standard indicated in kilo Dalton (kDa). Electrophoresis was carried out with 4–15% polyacrylamide gradient gels. Membranes were incubated with mouse monoclonal anti- β -conglycinin antibodies (A–C) and serum samples from soy sensitive individuals (D and E).

L. helveticus, presumably as a result of a combined effect of proteolysis and acid-induced protein denaturation. The enormous reduction in immunoreactivity due to fermentation of soybean meals and flour following fermentation with a LAB (*Lactobacillus plantarum*) and yeast (*S. cerevisiae*) was also observed by Song et al. (2008) and Frias et al. (2008). They attributed the reduction in immunoreactivity to decreased protein solubility due to fermentation. Reasons for decreased protein solubility (see Section 3.3.2, protein solubility) at neutral pH could be due to the fact that microorganisms and enzymes involved in the fermentation process

can easily hydrolyze soluble proteins, modifying epitopes with lower affinity to IgE-Abs. Further, new peptides were formed, and the conditions used to extract original proteins were not adequately extracting the new peptides. In addition, during fermentation, proteases and peptidases produced by LAB might cleave proteins into peptides and amino acids; hence, the structure of the epitopes might be altered or even destroyed (El-Ghaish et al., 2011).

In the present paper only β -conglycinin was monitored with specific Abs in the different fermented SPIs. Other allergenic components of soy e.g. glycinin (Glym6), Kunitz inhibitor (GlymTI) or

profilin (Glym3) could contribute to the allergenicity as well. However β -conglycinin (Glym5) and glycinin (Glym6) are the most abundant storage proteins of soybeans and sensitization against Glym5 and Glym6 is highly indicative for severe allergic reactions (Holzhauser et al., 2009). We could show by SDS–PAGE analysis that proteins with molecular weights exactly matching those of the subunits of Glym5 and Glym6 disappear by fermentation using *B. subtilis* and *L. helveticus* in soluble extracts. Additionally, two human serum samples from soy sensitive individuals show an overall decrease in immunoreactivity in the fermented protein

extracts and not solely in the immunoreactive bands corresponding to β -conglycinin.

3.3. Effects of fermentation using *L. helveticus* on the sensory profile and physicochemical properties of SPI

According to the results given, *L. helveticus* was most effective in the reduction in immunoreactivity and almost no binding to monoclonal mouse Abs and human serum in western blot could be found. Therefore, samples fermented with *L. helveticus* for 24 and

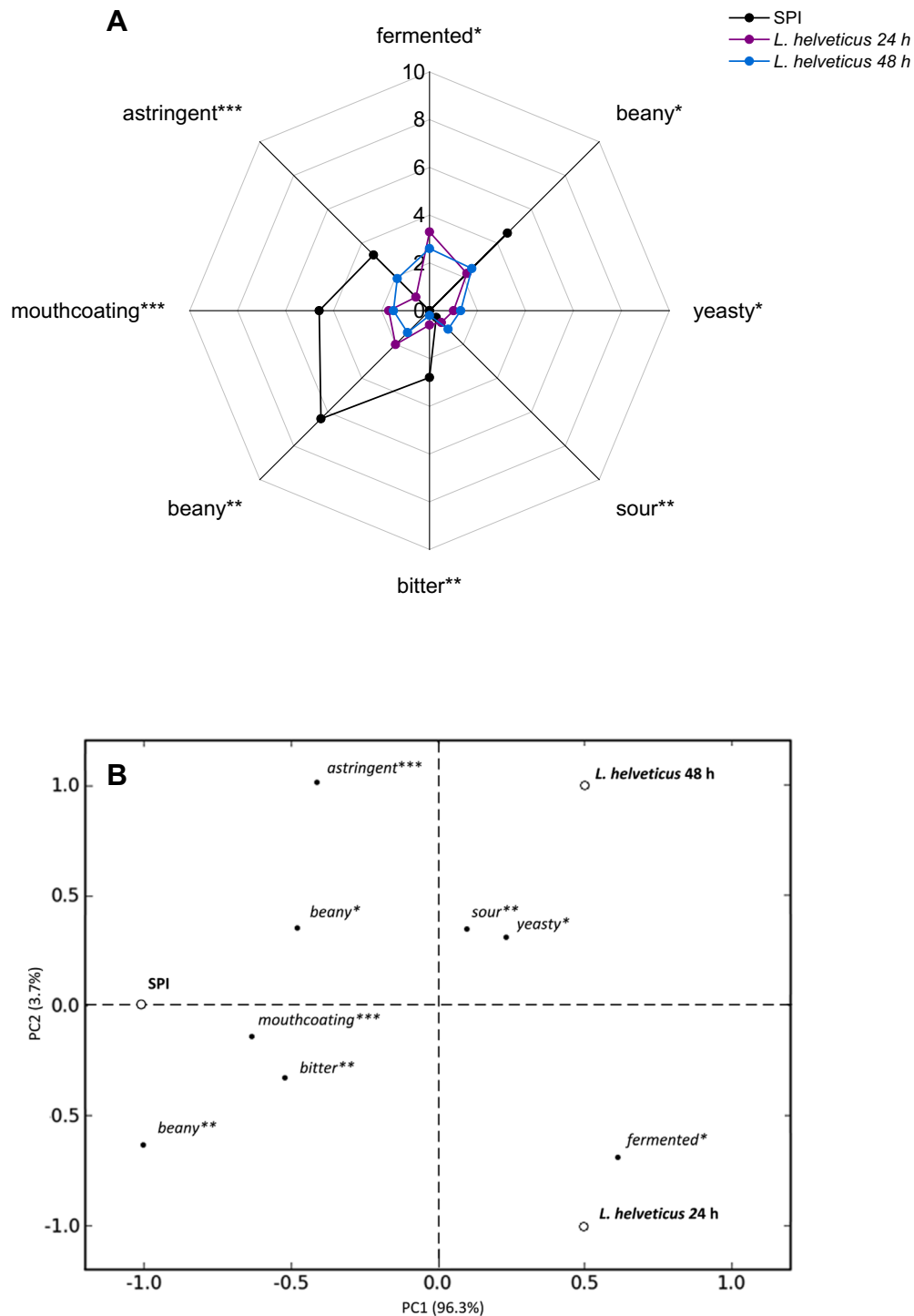


Fig. 3. Taste profile (A) and bi-plot (B) showing non-fermented SPI and SPI fermented with *L. helveticus* for 24 and 48 h in relation to descriptive attributes based on the descriptive sensory data. Each value is expressed as mean scored on the 10-cm continuous scale from 0 (no perception) to 10 (strong perception) by a 10-member panel. *smell; **taste; ***mouthfeeling.

48 h were analyzed in more detail, including the physicochemical and sensory properties.

3.3.1. Sensory profile

3.3.1.1. Quantitative descriptive analysis (QDA). The results of QDA are shown in Fig. 3A. Non-fermented SPI evaluation resulted in the following smell-scaling: *fermented* (0.0), *beany* (4.6), *yeasty* (0.0); taste-scaling: *sour* (0.4), *bitter* (2.8) and *beany* (6.4); mouthfeeling-scaling: *mouthcoating* (4.6) and *astringent* (3.3). During fermentation, the samples exhibited a *fermented* (2.6–3.3) and *yeasty* (1–1.3) smell as well as a slight *sour* (0.7–1.1) taste. In general, panellists agreed that all negative attributes associated with soybeans like *beany*, *bitter*, *mouthcoating* and *astringent* were significantly ($p < 0.05$) lower following fermentation compared to non-fermented SPI. Soy is often rejected due to a *beany* off-flavor, which results from lipid oxidation and lipoxygenase catalyzed oxidation of soybean oil to volatile compounds such as isopentanol, *n*-hexanal and hexanol (Shogren, Mohamed, & Carriere, 2003). Pinthong, Macrae, and Rothwell (1980) observed that fermented soy milk contained less *n*-hexanal, which was converted into *n*-hexanoic acid, thereby reducing the *beany* off-flavor. The *fermented* and *sour* taste might be attributed to the production of lactic acid, which results in a tangy lactic acid taste characteristic for fermented soy products. During fermentation, bitterness was significantly ($p < 0.05$) reduced from 2.8 initially to 0.6 and 0.2 after 24 and 48 h, respectively. Bitterness, which is believed to result from the accumulation of hydrophobic peptides, i.e. peptides rich in proline and leucine, is a serious quality concern facing the food industry. Specific enzymatic debittering strategies have focused on the application of proline specific exo- and endopeptidases given the contribution of proline residues to peptide/hydrolysate bitterness. Other exopeptidase activities, including carboxypeptidases, have been detected in a number of lactic acid bacteria, but their role has mainly been described with respect to the dairy processes (Savijoki et al., 2006). *L. helveticus* is an important industrial starter culture that is known to express endopeptidases with post-proline specificity such as PepO2 and PepO3, which sufficiently degrade *bitter* peptides in cheese during ripening, which might explain the reduced bitterness of the fermented samples (Soeryapranata, Powers, & Unlu, 2007).

3.3.1.2. Principal component analysis (PCA). PCA was employed on QDA data to identify the most important sensory attributes that have an impact on sample characteristics. The results are shown in Fig. 3B (bi-plot) and Table 2 (scaled scores and loadings).

The extracted and uncorrelated principal components (PCs) PC1 and PC2 accounted for 96.3% and 3.7% (100% in total) of the variation across the samples, respectively. The bi-plot (Fig. 3B) reveals great differences between non-fermented SPI and fermented samples. The PC1 dimension (factor loadings > 0.550) was mainly described by the attributes *fermented* smell (0.613), *beany* taste (−1.000), and *mouthcoating* mouthfeeling (−0.632). In contrast, the PC2 dimension is mainly explained by the attributes *fermented* smell (−0.693), *beany* taste (−0.630), and *astringent* mouthfeeling (1.000). According to the scaled loadings and location (scaled scores) in Table 2, PC1 mainly explains a contrast between fermented samples and non-fermented SPI (*beany*), while PC2 presents a contrast between *fermented* and *astringent*.

Non-fermented SPI loaded on the negative side of PC1 and is explained by the attributes *beany* (smell and taste), *bitter*, and *mouthcoating* (Fig. 3B). In contrast, and in great accordance with the QDA results, the fermented samples were less explained by these attributes, but *fermented*, *sour* and *yeasty* profiles became dominant. The attribute *fermented* was loaded on the sample following fermentation for 24 h, while attributes such as *sour* and *yeasty* became dominant after 48 h of fermentation. This might

Table 2

Principal component (PC) scaled scores and scaled loadings for SPI and SPI samples fermented with *L. helveticus* for 24 and 48 h obtained by principal component analysis (PCA).

Samples	Scaled scores		Scaled loadings		
	PC1	PC2	Attributes	PC1	PC2
SPI	−1.000	0.005	<i>Fermented</i> *	0.613	−0.693
<i>L. helveticus</i> 24 h	0.496	−1.000	<i>Beany</i> *	−0.475	0.350
<i>L. helveticus</i> 48 h	−1.000	0.995	<i>Yeasty</i> *	0.234	0.307
			<i>Sour</i> **	0.099	0.338
			<i>Bitter</i> **	−0.514	−0.340
			<i>Beany</i> **	−1.000	−0.630
			<i>Mouthcoating</i> ***	−0.632	−0.145
			<i>Astringent</i> ***	−0.411	1.000

Loadings with an absolute letter greater 0.550 are shown in bold type.

* Smell.

** Taste.

*** Mouthfeeling.

presumably be due to further production of lactic acid and aroma compounds characteristic for fermented products.

3.3.2. Physicochemical properties

The results of the physicochemical properties (protein solubility, emulsifying, water- and oil-binding capacity, foaming activity, density, and stability) are summarized in Table 3.

3.3.2.1. Protein solubility. Non-fermented SPI had a protein solubility of 5.0% and 44.0% at pH 4.0 and 7.0, respectively. Data presented in Table 3 indicated that fermentation drastically lowered protein solubility at pH 7.0, while the solubility at acidic conditions was increased. Protein solubility at pH 7.0 was nearly halved by fermentation compared to non-fermented SPI. *L. helveticus* produced a large amount of acid, which might have resulted in irreversibly coagulation of proteins, and thereby decreasing protein solubility. Further, enzyme/microorganisms inactivation (90 °C, 20 min) might have promoted aggregation and cross-linking of partially hydrolyzed soy proteins. In contrast, protein solubility at pH 4.0 was almost doubled after fermentation. Sadowska, Fornal, Vidal-Valverde, and Frias (1999) and Elkhailifa, Schiffler, and Bernhardt (2005) also reported the enhancing effect of fermentation on protein solubility of lentils and sorghum flour in acidic pH ranges, whereas it was decreased at a neutral pH value.

3.3.2.2. Emulsifying capacity. Non-fermented SPI exhibited the highest EC of 660 ml g^{−1}, while fermentation resulted in a significantly ($p < 0.05$) decreased EC (475–483 ml g^{−1}). As mentioned before, the exposure of buried hydrophobic regions and high-temperature enzyme/microorganism inactivation promoted aggregation and cross-linking of partially hydrolyzed proteins. Soluble proteins are surface active and are known to promote oil-in-water emulsions. At pH 7.0, the solubility of the fermented samples was reduced, thus the ability to form an emulsion was negatively affected. This was in confirmation with the observations reported by Quinn and Beuchat (1975), who also found a reduced EC in defatted peanut flour following fermentation with *R. microsporus* var. *oligosporus*.

3.3.2.3. Water- and oil-binding capacity. Fermented SPI samples exhibited an increased WBC up to 3.5 ml g^{−1} (48 h), while non-fermented SPI had a WBC of about 2.6 ml g^{−1}. An enhanced WBC might be due to the decrease in solubility, which means that the higher content of insoluble proteins were able to bind more water. Non-fermented SPI did not show any OBC, whereas fermentation increased OBC to 2.9 ml g^{−1}. This might be due to a higher content of hydrophobic or lipophilic groups, which enables oil retention

Table 3

Physicochemical properties in terms of protein solubility, emulsifying capacity, water- and oil-binding capacity, and foaming properties of SPI and the SPI samples fermented with *L. helveticus* for 24 and 48 h.

	Protein solubility		Emulsifying capacity (mL g ⁻¹)	Water-binding capacity (mL g ⁻¹)	Oil-binding capacity (mL g ⁻¹)	Foaming activity (%)	Foaming stability (%)	Foaming density (g L ⁻¹)
	pH 4.0 (%)	pH 7.0 (%)						
SPI	5.0 ± 1.1 ^a	44.0 ± 0.6 ^a	660 ± 5 ^a	2.6 ± 0.1 ^a	0.0 ± 0.0 ^a	552 ± 5 ^a	90 ± 0 ^a	215 ± 5 ^a
<i>L. helveticus</i> 24 h	11.3 ± 0.8 ^b	18.2 ± 0.4 ^b	483 ± 3 ^b	3.4 ± 0.0 ^b	2.7 ± 0.1 ^b	996 ± 42 ^b	90 ± 0 ^a	114 ± 0 ^b
<i>L. helveticus</i> 48 h	12.4 ± 2.8 ^b	16.5 ± 0.0 ^c	475 ± 10 ^b	3.5 ± 0.1 ^b	2.9 ± 0.1 ^b	1085 ± 13 ^c	90 ± 0 ^a	91 ± 2 ^c

Results are expressed as means ± standard deviation. Means followed with different superscript letters (a–c) within each column are significantly different ($p < 0.05$) following one-way ANOVA (Bonferroni).

(Dixon & Larry, 1981). Ahmed, Ramaswamy, and Alli (2006) and Elkhailifa et al. (2005) also found an enhanced OBC and WBC after fermentation of peanut flour and sorghum meal and flour with fungal and LAB strains.

3.3.2.4. Foaming activity, density and stability. Non-fermented SPI had a foaming activity of 552%, which was nearly doubled (996–1085%) after fermentation. Due to the partial hydrolysis of soy proteins, more flexible and low-molecular weight peptides were formed. Thereby, peptides could transfer to the air–water interface more rapidly and the protein–protein interaction improved the strength of the viscoelastic cohesive film, dropping the surface tension (Tsumura et al., 2005). As might be expected, the foaming density of the fermented samples significantly ($p < 0.05$) decreased compared to non-fermented SPI. The foaming density was reduced from initially 215 g L⁻¹ to 114 and 91 g L⁻¹ after fermentation for 24 and 48 h, respectively. However, a foaming stability of about 90% could be found for all samples.

4. Conclusions

In conclusion, fermentation of SPI significantly reduced the immunoreactivity of soluble soy proteins tested in this study as demonstrated by sandwich ELISA and western blot analyses. The highest performance among the strains assayed was obtained by fermentation with *L. helveticus*. Furthermore, *L. helveticus* exhibits great sensory properties with a significantly ($p < 0.05$) decreased bitter taste and good physicochemical properties in terms of increased foaming activity and maintained foaming stability. *L. helveticus* is “generally recognized as safe” (GRAS) LAB, which can be applied as a “natural” food preservative and starter culture for the production of hypoallergenic food ingredients that combine pleasant taste and good physicochemical properties. In addition, *L. helveticus* is known to possess many common probiotic properties such as prevention of gastrointestinal infections, making the fermented food products healthier (Taverniti & Guglielmetti, 2012).

Although Glym5 immunoreactivity was almost completely inhibited, other allergenic components of soy e.g. glycinin (Glym6), Kunitz Trypsin Inhibitor (GlymTI) or proflin (Glym3) could contribute to the allergenicity as well. Therefore, the immunoreactivity of these protein fractions has to be analyzed by further ELISAs and western blots. The level of immunoreactivity reduction in this study might be important for a lot of allergic individuals depending upon their sensitivity threshold; however, the clinical relevance of these findings still needs to be confirmed by, for example, controlled human challenge studies. However, it is worth note that aggregation and loss of protein solubility, rather than the epitope destruction, may be responsible for the observed decrease in immunoreactivity of the tested soy epitope. Future studies on immunoreactivity of variety of soluble and insoluble protein fractions are necessary to understand mechanism of inactivation of soy allergenic proteins (epitopes) by fermentation.

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