Soymilk fermentation by *Enterococcus faecalis* VB43 leads to reduction in the immunoreactivity of allergenic proteins β-conglycinin (7S) and glycinin (11S)

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RESEARCH ARTICLE

Abstract

Food allergies represent a serious problem affecting human health and soy proteins rank among the most allergenic proteins from food origin. The proteolytic enzymes produced by lactic acid bacteria (LAB) can hydrolyse the major allergens present in soybean, reducing their immunoreactivity. Many studies have reported the ability of LAB to ferment soy-based products; while the majority of them focus on the improvement of the sensory characteristics and functionality of soy proteins, a lack of information about the role of lactic fermentation in the reduction of immunoreactivity of these proteins exists. The aim of the present study was to evaluate the capability of the proteolytic strain *Enterococcus faecalis* VB43 to hydrolyse the main allergenic proteins present in soymilk and to determine the immunoreactivity of the obtained hydrolysates. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) results of fermented soymilk demonstrated complete hydrolysis of the β-subunit from β-conglycinin and the acidic polypeptide from glycinin. Reversed phase high performance liquid chromatography (RP-HPLC) analysis of the peptides released after hydrolysis revealed the appearance of new peptides and the disappearance of non-hydrolysed proteins, indicating extensive hydrolysis of the substrate. Results from competitive enzyme-linked immunosorbent assay (ELISA) tests clearly indicated a reduction in the immunoreactivity (more than one logarithmic unit) in the fermented sample as compared to the non-fermented control. Our results suggest that the soymilk fermented by *E. faecalis* VB43 may induce lower allergic responses in sensitive individuals. The strain *E. faecalis* VB43 may be considered as an excellent candidate to efficiently reduce the immunoreactivity of soymilk proteins.

Keywords: soymilk fermentation, soy allergy, β-conglycinin, glycinin, lactic acid bacteria

1. Introduction

Soybean (*Glycine max*) is an abundant source of good quality proteins with high nutritional value and interesting functional properties (Aguirre *et al.*, 2008). This inexpensive grain from the legume family constitutes an important alternative for protein intake for vegetarians, for lactose intolerant and allergic to bovine milk individuals (Amigo-Benavent *et al.*, 2008; Frias *et al.*, 2008). Several studies suggest that soybean consumption offers health benefits such as cholesterol reduction, antioxidant activity, reduction in the risk of development of diabetes and breast tumour, etc. (Duranti, 2006; Takamatsu *et al.*, 2004; Torres *et al.*, 2006). All these facts have contributed to the increase in soybean consumption and the incorporation of its proteins in many formulations during the last decades (Aguirre *et al.*, 2014; Sloan, 2005; Song *et al.*, 2008). However, consumption of soybean or soy-based products may lead to adverse

effects mainly related to the low digestibility and allergenic potential of its proteins (Aguirre *et al.*, 2014; Bu *et al.*, 2015; FDA, 2010).

Food allergy is defined as an adverse immunological reaction in response to dietary antigens, mainly proteins (Frias *et al.*, 2008). According to the World Health Organization (WHO), food allergies represent the sixth major problem affecting human health worldwide (El-Ghaish *et al.*, 2011). About 3% of adults and 6 to 8% of children under three years of age suffer from food allergies (Frias *et al.*, 2008) although these numbers have been significantly increasing in the last years (Gray *et al.*, 2015; Hendaus *et al.*, 2016) as well as the number of implicated foods and the frequency of severe reactions (Meinlschmidt *et al.*, 2016a). In this scenario, soy proteins are considered major food allergens and soybeans belong to the group known as the 'big 8', which comprises the eight foods responsible for 90% of all diagnosed cases of immunoglobulin E (IgE)-mediated food allergies (Bu *et al.*, 2015; FDA, 2004). The incidence of soy allergy in young children under three years of age is about 1 to 6% and about 3 to 4% in adults. However, some authors suggest that the increase in soy consumption leads to higher exposure to its proteins and can result in an increase of allergy incidence (Aguirre *et al.*, 2014; Wilson *et al.*, 2005).

Even though at least 16 soybean proteins have already been described as immunoreactive to specific IgE, Gly m5 (β-conglycinin, 7S) and Gly m6 (glycinin, 11S), which represent between 70% and 80% of the total protein content, are the two main soybean allergens (Holzhauser *et al.*, 2009; Meinlschmidt *et al.*, 2016b). Both β-conglycinin and glycinin are globular proteins. While β-conglycinin is a trimeric structure composed of three subunits (α', α and β, with molecular weights of 68, 72 and 52 kDa, respectively), glycinin is made up of five subunits, each of them composed of a basic polypeptide (of approximatively 20 kDa) and an acidic polypeptide (of approximatively 40 kDa), linked by a disulfide bond. The majority of antigenic epitopes present in soy proteins are found in the α subunit of β-conglycinin and in the acidic portion of glycinin subunits (Bu *et al.*, 2015; Lagemaat *et al.*, 2007; Wilson *et al.*, 2005).

Nowadays, the only method to completely avoid soybean-related allergic reactions is the dietary exclusion, which is difficult and has nutritional disadvantages (Meinlschmidt *et al.*, 2016a). Therefore, some technological strategies have been proposed to reduce soy proteins immunoreactivity and they are mainly focused on the modification of the antigenic epitopes, leading to a loss in their binding capability to specific IgE. In this attempt heating, glycation, enzymatic hydrolysis, genetic modifications and microbial fermentation procedures have been proposed (Bu *et al.*, 2015; Gomaa and Boye, 2013; Kasera *et al.*, 2015; Lagemaat *et al.*, 2007; Wang *et al.*, 2014; Wilson *et al.*, 2005).

The proteolytic system of lactic acid bacteria (LAB) is very important for their growth in complex matrices especially those rich in proteins and poor in free amino acids (Hebert *et al.*, 2008). This system involves the action of proteinases, peptidases and transport enzymes, which are responsible for the hydrolysis of proteins and the availability of free amino acids, essential for bacterial growth (Bu *et al.*, 2010; Lozo *et al.*, 2011). These microorganisms are traditionally used for the production of many fermented products, both from vegetal and animal origins, and the proteolysis is considered one of the most important biochemical process that occur during lactic fermentation, since it contributes to improve the sensory characteristics of the fermented products (Pescuma *et al.*, 2010; Savijoki *et al.*, 2006).

Several studies in literature describe the hydrolysis of soy proteins by fermentation with LAB cultures, resulting in improvement of their technological and sensory characteristics, nutritional value and digestibility, in addition to release of bioactive peptides with beneficial health effects (Aguirre *et al.*, 2008, 2014; Meinlschmidt *et al.*, 2016a,b; Pescuma *et al.*, 2013; Rodriguez de Olmos *et al.*, 2015). However, few studies on the reduction of the immunoreactivity of soy main allergens by LAB have been carried out (Frias *et al.*, 2008; Meinlschmidt *et al.*, 2016c; Song *et al.*, 2008, 2010). In addition, the success of this approach depends on the LAB strain and the fermentation conditions used (Hebert *et al.*, 2000; Wilson *et al.*, 2005). Therefore, the study of new LAB strains able to hydrolyse soybean main allergens, leading to a reduction in soy protein immunoreactivity is of great interest and could help in the development of new hypoallergenic soy-based products. The aim of this study was to evaluate the capability of the proteolytic LAB strain *Enterococcus faecalis* VB43 to hydrolyse the main allergenic proteins present in soymilk and to evaluate the immunoreactivity of the obtained hydrolysates. The choice of this strain was based on its strong proteolytic activity against bovine milk proteins, observed in previous experiments performed in our laboratory (unpublished data). Therefore, the study of *E. faecalis* VB43 proteolytic activity against soy main allergens may contribute to increase the knowledge about the potential application of this strain in the reduction of food allergies.

2. Material and methods

Bacterial strain

The proteolytic strain *E. faecalis* VB43, previously isolated from Brazilian artisanal hard cheese and belonging to the collection culture from the Food Microbiology laboratory from the Faculty of Pharmaceutical Sciences of the University of Sao Paulo (Sao Paulo, Brazil), was used for soymilk fermentation. The culture was kept at -80 °C in M17 broth (Oxoid, Basingstoke, UK) containing 20%

(w/v) of glycerol (Sigma-Aldrich, Munich, Germany) as cryoprotector.

Inoculum preparation and soy milk fermentation

To prepare cell suspensions for inoculation of soymilk samples, *E. faecalis* VB43 was activated twice in M17 broth at 37 °C for 24 h. Cells were harvested by centrifugation (10,000×*g* for 5 m at 4 °C) and washed twice in sterile 0.85% (w/v) saline solution to remove carryover nutrients from the media. Then, cells were resuspended in sterile 0.85% (w/v) saline solution to the original volume.

To stimulate proteinase production, the obtained cell suspension was surface-planted (200 µl) on milk-citrate agar (MCA) composed of UHT skim milk (44%, v/v), Na-citrate (0.8%, w/v), yeast extract (0.1%, w/v), glucose (0.5%, w/v) and agar (1.5%, w/v), all components from Sigma-Aldrich (Fira *et al.*, 2001). After incubation at 37 °C for 24 h, cells were collected from the surface of the plates by adding 1 ml of sterile saline solution (0.8% NaCl, w/v), supplemented with 10 mM of $CaCl_2$, and scraping with the help of a Drigalski loop. The cell suspension was washed twice in the same diluent (10,000×*g* for 5 m at 4 °C) and resuspended to 1 ml in Na-phosphate buffer (100 mM, pH 7.0); this last suspension was used to inoculate soymilk samples.

For fermentation assays, 200 µl of UHT soymilk (Soja Nature Bio; Bjorg, Saint Genis Laval, France) were inoculated (5%, v/v) with *E. faecalis* VB43 cell suspension, obtained from MCA, following incubation at 37 °C for 24 h (test samples). Non-fermented control samples were prepared by adding 5% (v/v) of sterile Na-phosphate buffer (100 mM, pH 7.0) to UHT soymilk and incubating under the same conditions as test samples.

Hydrolysis of soymilk proteins

Proteins and peptides resulting from the assayed soymilk fermentation were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). SDS-PAGE was carried out in a vertical slab electrophoresis cell (Biorad Mini Protean 3 System, Hercules, CA, USA). Samples were diluted (1:9) in a solubilisation buffer composed of 50 mM Tris-HCl (Euromedex, Souffelweyersheim, France), pH 6.8; SDS (4%, w/v); glycerol (20%, w/v); 2-mercaptoethanol (3%, v/v) and bromophenol blue (0.07%, w/v) (all from Sigma-Aldrich) and heated at 60 °C for 5 min. Heated samples were centrifuged (10,000×*g* for 5 m at 25 °C), and the supernatants were further heated at 100 °C for 3 min. After the last heating, the samples were loaded onto 12% (w/v) polyacrylamide gels and the proteins were separated by electrophoresis in the following conditions: 10 mA on the stacking gel (7.8% acrylamide, w/v) and 20 mA on the separation gel (12% acrylamide, w/v). Migration buffer was composed of 50 mM Tris, 0.384 M glycine (Euromedex) and 0.1% (w/v) SDS. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R250 overnight, destained during approximately 3 h with a solution composed of 30% (v/v) ethanol and 5% (v/v) acetic acid (both from Carlo Erba Reagents, Val de Reuil, France) and scanned with an Image Scanner III (GE Healthcare, Chicago, IL, USA) for visualisation of the bands.

Hydrolysis of soymilk proteins by the *E. faecalis* VB43 was observed by comparing the obtained SDS-PAGE band profile with that of the non-fermented control. A reduction in the intensity of the colour of at least one of the bands, representing soymilk proteins, was considered as a positive result for hydrolysis.

Peptide profile generated by hydrolysis of soymilk proteins

The peptide profile generated by hydrolysis of soymilk proteins by *E. faecalis* VB43 was assessed by reversed phase high performance liquid chromatography (RP-HPLC), according to El-Ghaish *et al.* (2010). UHT soymilk incubated in the absence of the proteolytic strain was used as control.

Before injection in the column, the samples were mixed (1:1, v/v) with 9 M urea (Acros Organics, Geel, Belgium) and centrifuged (12,000×*g* for 5 m at 4 °C) to remove the cells. The supernatant was injected (20 µl) in a C_{18} symmetry® column 3.5 µm (4.6×100 mm, Waters, Milford, MA, USA) and the analysis was carried out in a HPLC System Alliance (Waters), in the following conditions: flow rate, 0.2 ml/min; equilibrium buffer (solvent A), 98.0% water, 2.0% acetonitrile, 0.05% trifluoroacetic acid (TFA) (v/v/v); elution buffer (solvent B), 80% acetonitrile, 20% water and 0.04% TFA (v/v/v). All reagents were from Carlo Erba Reagents, Val de Reuil, France. Elution was performed with a linear gradient of solvent B, ranging from 0 up to 100%. The detection was performed at 220 nm, using a photodiode array detector (model 996, Waters) and the data were analysed by Empower software (Waters). The peptide profile obtained for the fermented sample was compared with that obtained for the control (non-fermented soymilk).

Screening for immunoglobulin E specificity of sera from soy allergic patients

A series of 14 sera from patients allergic to soy proteins (children between 3 to 5 years of age), presenting values of specific IgE between 3.4 and 64.9 ng/ml, were obtained from the Laboratory of Immuno-Allergology of Academic Hospital (Angers-Nantes, France) and checked for their specific reactivity to β-conglycinin (7S) and glycinin (11S) by direct enzyme-linked immunosorbent assay (direct ELISA) according to Ahmadova *et al.* (2013). The use of human

sera was approved by the internal Ethical Committee of the hospital. The purified 7S and 11S fractions were obtained at laboratory scale, following the method described by Guéguen *et al.* (1984). For coating the plates, a mixed solution of 7S and 11S, containing 5 μg/ ml of each purified protein, was prepared by diluting the proteins in 1 M phosphate buffered saline (PBS) (0.136 M NaCl, 2.68 mM KCl, 1.76 mM KH_2PO_4 and 10.14 mM Na_2HPO_4), pH 7.4 (mix 7S/11S).

Direct ELISA was carried out using 96-well micro-titration, flat bottom, white plates (Nunc, Thermo Scientific, Courtaboeuf, France). Plates were coated for 3 h at 30 °C with 100 μl of 7S/11S mix per well. The negative control wells were coated with 1 M PBS. After coating, plates were washed three times with PBS buffer containing 0.1% (v/v) Tween 20 (PBS/T) and saturated with 250 μl of PBS/T buffer added of 1% (v/v) polyvinyl alcohol (PBS/T/PVA) per well. After incubation for 1 h at 37 °C, the plates were washed again (three times with PBS/T) and saturated wells were added of 100 μl of each serum (diluted 20 times in PBS/T/PVA buffer). The wells coated with PBS (negative controls) were added of 100 μl of PBS/T/PVA without any sera. The plates were incubated overnight at 4 °C and washed three times with PBS/T buffer. The secondary antibody (Ab_2-polyclonal antihuman IgE, alkaline-phosphatase-conjugated, produced in goat; Sigma-Aldrich), diluted 1,000 times with PBS/T/PVA, was added to the wells (100 μl per well) and the plates were further incubated at 37 °C for 2 h and washed three times with PBS/T. Development step was carried out by adding 150 μl (per well) of 4-methylumbelliferyl phosphate (4-MUP, Sigma-Aldrich), diluted 1:4 (v/v) in 1M Tris-HCl pH 9.8, and incubating for 90 m at room temperature in the dark. Fluorescence was measured using a Synergy HT micro plate reader (BioTek Instruments, Winooski, VT, USA) and Gen 5, version 2.05 software (BioTek Instruments), with a 360 nm excitation filter and a 460 nm emission filter.

The sera presenting high specificity to the 7S/11S mix were selected to constitute the pool to be used for the competitive ELISA tests to evaluate the antigenicity of fermented soymilk; the sera showing no specificity to the 7S/11S mix were selected to be used as negative controls. Before use, all pools of sera were diluted in a 1:19 (v/v) ratio with PBS/T/PVA buffer.

Competitive ELISA

The IgE binding ability of the hydrolysed proteins in fermented soymilk was analysed by competitive ELISA, as described by Pescuma *et al.* (2011). Before addition to the ELISA plates, the fermented soymilk samples were solubilised (1:9, v/v) in PBS/T/PVA buffer (pH 8.2, adjusted with 1M NaOH). Non-fermented soymilk was used as control.

Competitive ELISA was carried out using 96-well micro-titration, flat bottom white plates. Coating and saturation steps were carried out as described in the previous section. Whilst the saturation step was taking place, dilutions of fermented and control samples (from 1000 to 0.02 μg/ml of antigen, prepared in PBS/T/PVA) were separately mixed (1:1, v/v) with the pool of 7S/11S specific sera (20 times diluted in PBS/T/PVA buffer) and incubated at 37 °C for 1 h. After the incubation, these mixtures were transferred (100 μl per well) to the plates coated with 7S/11S mix. Plates were incubated overnight at 4 °C, washed three times with PBS/T and added of 100 μl of Ab_2 (prepared as described in the previous section). The plates were further incubated at 37 °C for 2 h and washed three times with PBS/T. Development and fluorescence measurement steps were carried out as described in the previous section.

To calculate the IgE inhibition percentages by the antigens in the fermented soymilk, specific sera mixed (1:1, v/v) with PBS buffer without any antigen [fluorescence value of 100% free IgE (i.e. 0% inhibition)] and samples of non-specific IgE sera mixed with PBS buffer [fluorescence value of 0% free IgE (i.e. 100% inhibition)] were included in the ELISA plates. The inhibition of IgE binding was calculated as described by Taheri-Kafrani *et al.* (2009), using the following equation:

$$[(Fluo_0 - Fluo_x) / (Fluo_0 - Fluo_m)] \times 100$$

Where $Fluo_0$ was the fluorescence obtained for the samples without antigen (specific pool mixed with PBS), which corresponded to 0% of inhibition; $Fluo_x$ was the fluorescence obtained for hydrolysates and control samples (with different concentrations of antigen); and $Fluo_m$ was the fluorescence obtained for samples without specific IgE (non-specific pool mixed with PBS), which corresponded to minimal fluorescence detected.

A curve of IgE inhibition was built using the average fluorescence values obtained from three separate experiments; the Origin Pro version 8.5.1 (Origin Labs, Northampton, MA, USA) was applied. The data were fitted into a sigmoidal curve, using the Logistic5 model and the protein concentration needed for 50% inhibition (IC_{50}) was calculated for each sample, using the values extrapolated from the curve.

3. Results and discussion

Hydrolysis of soymilk proteins and peptide profile of hydrolysates

Fermentation of soymilk by *E. faecalis* VB43 resulted in the hydrolysis of all three subunits of β-conglycinin (α', α and β), and both basic and acidic polypeptides from glycinin, as observed in the SDS-PAGE gel of fermented and non-fermented (control) samples (Figure 1). However,

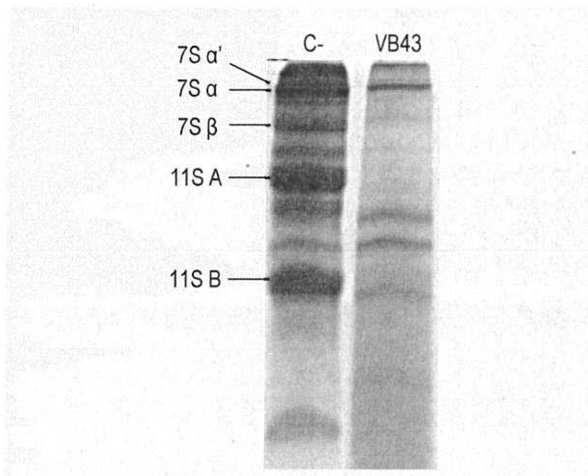

Figure 1. SDS-PAGE showing the hydrolysis profile of soymilk proteins, after fermentation by *Enterococcus faecalis* VB43 (at 37 °C for 24 h). C-, negative control (soymilk incubated in the absence of the proteolytic strain); VB43, test sample (soymilk fermented by *E. faecalis* VB43); 7S α', 7S α and 7S β, α', α and β subunits of β-conglycinin, respectively; 11S A and 11S B, acidic and basic polypeptides of glycinin, respectively.

the degree of hydrolysis of the different protein fractions varied. Regarding β-conglycinin, the β subunit was the most degraded fraction by the proteolytic enzymes of *E. faecalis* VB43. The total hydrolysis of this protein fraction was observed after 24 h by the disappearance of the 7S subunit band. On the other hand, both the α' and α subunits were somehow resistant as only a partial hydrolysis was achieved at the end of the fermentation period. Concerning the glycinin fractions, the disappearance of the band corresponding to the acidic polypeptide was observed indicating the total hydrolysis of the 11S polypeptide fraction. In contrast, a partial hydrolysis of the basic polypeptide fraction by the strain *E. faecalis* VB43 was detected. It has been claimed that the majority of the antigenic epitopes in soy proteins are found in the α subunit of β-conglycinin and in the acidic portion of glycinin subunits (Lagemaat *et al.*, 2007; Wilson *et al.*, 2005).

Fermentation of soymilk by *E. faecalis* VB34 resulted in partial hydrolysis of the α subunit of β-conglycinin and complete hydrolysis of the glycinin acidic portion suggesting that a relevant reduction in the allergenicity of these proteins may occur. The suitability of the application of LAB fermentation to hydrolyse allergenic soy proteins has been demonstrated by other authors (Aguirre *et al.*, 2008, 2014; Frias *et al.*, 2008; Meinlschmidt *et al.*, 2016c). Aguirre *et al.* (2008) studied the ability of different *Lactobacillus* spp. strains to hydrolyse soy proteins and found that β-conglycinin was the preferred fraction to hydrolysis while the basic polypeptide of glycinin was the least degraded one. The authors suggested that the chemical and physical structures of the basic polypeptide from glycinin hampered the access of LAB enzymes to the cleavage sites, making this fraction more resistant to hydrolysis. In opposite to what was observed in the present work, β subunit was the least affected fraction by lactic fermentation while higher degrees of hydrolysis were observed against α' and α subunits indicating that the hydrolysis pattern was strain-dependent. As occurred in our study, Aguirre *et al.* (2014) observed that LAB strains were more effective degrading the acidic polypeptide from glycinin than its basic portion concluding that the higher hydrophobicity and compactness of the basic glycinin polypeptide are responsible for the resistance to hydrolysis.

Hydrolysis of soymilk proteins was confirmed as revealed by the RP-HPLC assay; peaks representing intact proteins in control samples were absent in the fermented soymilk (Figure 2). Besides, the peptide profile changed after fermentation of soymilk by *E. faecalis* VB 43, as new peaks of lower molecular weight appeared suggesting the release of medium-sized and less hydrophobic peptides. These results indicate that the proteolytic strain *E. faecalis* VB 43 was able to hydrolyse the main proteins present in soymilk. Other authors also observed a change in the peptide profile of soy-based products after the action of proteolytic enzymes from different LAB strains (Aguirre *et al.*, 2008, 2014; Pescuma *et al.*, 2013). According to these authors, the release of small and more hydrophilic peptides may be responsible for the desirable flavours in fermented soy products, in addition to modifications of antigenic epitopes, leading to a lower antigenic response.

Effect of fermentation by proteolytic strain on the immunoreactivity of soymilk

From the 14 sera screened for the presence of specific IgE for β-conglycinin and glycinin soy proteins, evaluated by the direct ELISA test, 12 presented fluorescence values higher than the negative control and were considered as positive for specific IgE (Figure 3). However, the amount of specific IgE in each serum varied and the most immunoreactive ones were the S1, S6, S9, S10 and S13 samples, which were chosen to compose the pool of specific sera used in the competitive ELISA test. On the other hand, the sera S5 and S7 presented lower fluorescence values than the negative control, indicating that even though these sera came from allergic patients, the amount of specific IgE present on them was too low to be detected. Therefore, these two sera were considered as negative for the presence of specific IgE for the 7S/11S mix and used in the competitive ELISA assay to represent the 0% free IgE fluorescence values (wells added of non-specific sera) in the calculation of inhibition percentages for fermented and non-fermented soymilk samples. It is important to mention that since sera S5 and S7 were used in competitive ELISA, its immunoreactivity with fermented and non-fermented soymilk was also evaluated, but once more no reaction was observed.

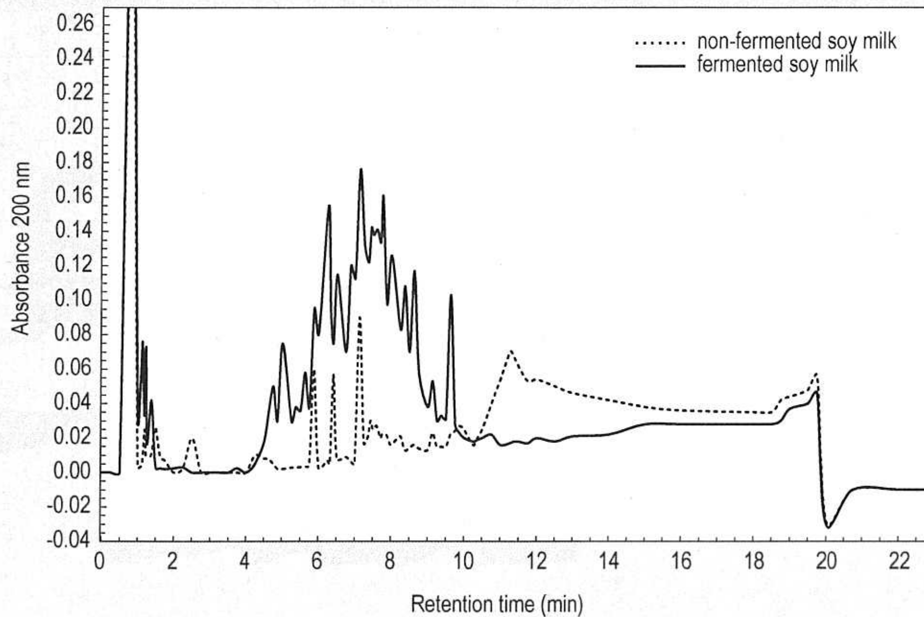

Figure 2. Comparison of the reverse phase-HPLC profiles of soymilk samples, both fermented by *Enterococcus faecalis* VB43 (at 37 °C for 24 h) and non-fermented (control).

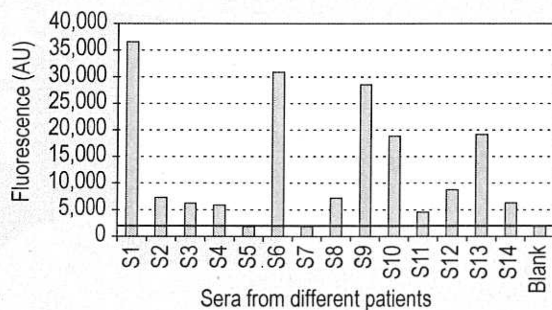

Figure 3. Screening for IgE specificity of the sera from soybean-allergic patients to β-conglycinin and glycinin protein fractions, performed by direct ELISA.

The results of the competitive ELISA on the immunoreactivity of the β-conglycinin and glycinin protein fractions with specific IgE for both soymilk samples (fermented, non-fermented) were fitted into a sigmoidal curve applying a Logistic5 model. The fitting of data resulted in R-square values of 0.99, 0.98 for non-fermented and fermented samples, respectively, indicating that the model was well adjusted. Binding of specific IgE to immunoreactive antigens was significantly weaker in fermented than in non-fermented soymilk (Figure 4) indicating that the peptides released by the proteolytic enzymes of *E. faecalis* VB43 were less recognised by specific IgE than non-hydrolysed proteins present in the control. The calculated IC_{50} values for non-fermented and fermented soymilk were 0.19 and 3.40 μg/ml, respectively, which represents a reduction of 94.4% in the immunoreactivity of hydrolysates. This more than one log reduction in immunoreactivity indicate that the fermentation of soymilk by *E. faecalis* VB43 resulted in a less antigenic product. However, the clinical relevance of this 94.4% immunoreactivity reduction will depend on the sensitivity threshold of patients and can only be determined by human challenge studies. The inhibition curves shown in Figure 4 also indicate that the increase in protein concentration was followed by a progressive increase in the IgE binding inhibition for all tested samples. It is worth noting that although the peptides released after hydrolysis were less immunoreactive than non-hydrolysed proteins and that a complete degradation of some fractions of β-conglycinin and glycinin occurred (as revealed by SDS-PAGE), a recognition by specific IgE was still detected, probably due to the presence of a few conserved antigenic epitopes.

In a proteolytic process occurring during fermentation, a change in the structure of the allergens may be detected interfering with the antigen-antibody complex and consequently reducing the IgE binding to specific epitopes from immunoreactive proteins. Song *et al.* (2008) achieved 100% of immunoreactivity reduction in a fermented soy protein concentrate ingredient and Frias *et al.* (2008) also found high levels of antigenicity reduction (from 96 to 99%) after liquid-state fermentation of soy flour by *L. plantarum* CECT 748T. These authors comment that these levels of immunoreactivity reduction may be important for some allergic individuals and that the clinical significance of

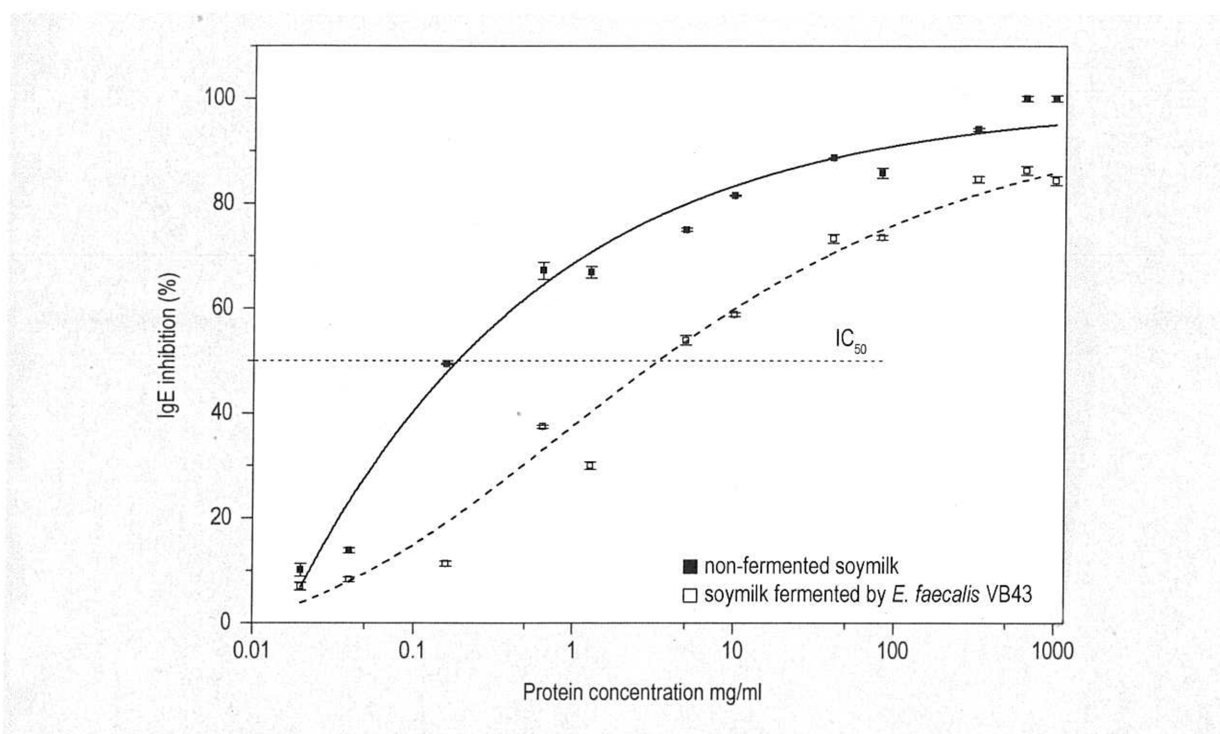

Figure 4. Comparison of the ability of specific IgE, present in sera of soy-allergic patients, to bind the antigens present in soymilk fermented by *Enterococcus faecalis* VB43 (at 37 °C for 24 h) and non-fermented soymilk, analysed by competitive ELISA. IC_{50} = protein concentration needed for 50% inhibition of IgE. Data points represent mean ± standard deviation of three independent experiments.

these numbers will depend on their sensitivity to soy proteins, as well as on the inclusion rate of the soy protein in the finished product consumed. Meinlschmidt *et al.* (2016c) reported 100% of immunoreactivity reduction after liquid-state fermentation of β-conglycinin by *Lactobacillus helveticus* DSM 20075 and suggested that a combined effect of proteolysis and acid-induced protein denaturation were responsible for the modifications in the conformational epitopes present in this protein, leading to changes on IgE binding capability. Even though *in vitro* tests of immunoreactivity cannot be used as an absolute diagnostic parameter for allergy, the results obtained by ELISA tests performed with sera from patients with declared soy allergy are relevant and can be used as a tool for the assessment of the risk of a product to trigger allergic reactions, since it can demonstrate a clear dose-response effect of immunoreactive antigens (Frias *et al.*, 2008). The results presented in this study clearly demonstrate that fermentation of UHT soymilk by *E. faecalis* VB43 provokes extensive hydrolysis of soy main allergens (β-conglycinin and glycinin), leading to a reduction in the immunoreactivity of the fermented product. According to Song *et al.* (2008), depending on the proteolysis degree and hydrolysis conditions, microbial fermentation could potentially decrease or even eliminate soy-related allergenic reactions. Therefore, soy-based products fermented by *E. faecalis* VB43 could present a potential for the development of less immunoreactive products.

As limitations of the this study the authors present the fact that all experiments were performed *in vitro* and cannot be used to predict allergenic reactions *in vivo*. Therefore, clinical tests would be necessary before making any conclusions about the application of the studied strain in the manufacture of hypoallergenic products. Besides, only two antigens from soy were investigated. Even though Gly m5 and Gly m6 are the most abundant proteins in soybean and considered as the main allergens from this food, soybean contains other allergenic components that may not be affected by fermentation with *E. faecalis* VB43 and contribute to maintain the immunoreactivity of the final product. Finally, before proposing the elaboration of a novel hypoallergenic soy-based product fermented by *E. faecalis* VB43, other aspects should also be taken into account, such as the physicochemical parameters of the final product and its sensory acceptation. Another important point to be discussed is the safety of the proteolytic LAB strain used. The application of enterococci in foods may be a controversial issue, since some species have already been associated to nosocomial infections. Therefore, checking the safety of *Enterococcus* spp. is of utmost importance before considering its application in foods. Previous

experiments, performed in our laboratory (unpublished data), confirmed that *E. faecalis* VB43 lacks the majority of virulence genes frequently associated with this species (*asa*1, *agg*, *efa*Afs, *hyl*, *esp*, *cyl*L_L and *cyl*L_S). This strain was positive only for *ace* (adhesin of collagen protein) and *gel*E (gelatinase), both genes frequently found in *Enterococcus* spp. strains isolated from dairy products and presenting interesting potential for application in foods (Ahmadova *et al.*, 2011; Martín-Platero *et al.*, 2009). Although many virulence genes have already been found in *Enterococcus* spp isolated from food, up to now, implication of these strains in cases of infections have never been reported. Furthermore, *Enterococcus* spp. is part of the autochthonous microbiota of many fermented food products and are frequently studied for their important role in the development of sensory characteristics, production of bacteriocins and reduction of immunoreactivity (Chajecka-Wierzchowska *et al.*, 2016).

4. Conclusions

Our results indicate that *E. faecalis* VB43 presents strong proteolytic activity against both β-conglycinin and glycinin from soy. Fermentation of soymilk resulted in complete hydrolysis of the 7S β subunit and the acidic polypeptide from 11S; in addition, a partial hydrolysis of the other fractions of these two proteins were noticed reducing their ability to bind to the specific IgE present in sera of soy-allergic patients. The strain *E. faecalis* VB43 may be an interesting candidate to be used in the manufacture of hypoallergenic soy-based products.

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