ISOFLAVONES SUPPLEMENTATION OF A PROBIOTIC FERMENTED SOY PRODUCT: EFFECTS ON QUALITY CHARACTERISTICS AND ISOFLAVONES PROFILE*

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ABSTRACT: The aim of the present study was to investigate the effect of isoflavones supplementation of a fermented soy product on its sensory acceptance, physicochemical properties and probiotic cell viable count. Additionally, we also investigated the ability of the mixed starter cultures (Enterococcus faecium CRL 183 and Lactobacillus helveticus 416) to modify the isoflavones profile of soy product during the fermentation process. Three products were analysed: soy product fermented with E. faecium CRL 183 and L. helveticus 416, isoflavone-supplemented soy product (fermented with E. faecium CRL 183 and L. helveticus 416; 50mg/100g, Isoflavin®, Galena, Brazil) and unfermented soy product. A panel of judges evaluated the acceptability of the samples on a nine point structured hedonic scale. The chemical composition namely fat, protein, ash and total carbohydrate contents, pH, enumeration of viable Lactobacillus spp. and Enterococcus spp. and quantification of isoflavones using HPLC were investigated. All determinations were conducted after 7 days storage at 10°C. The sensorial acceptance was reduced in the isoflavones-supplemented soy product, but this effect was not significant compared to the sample without isoflavones addition. Chemical composition did not differ (p<0.05) among the samples. Cell viable counts were reduced and total fermentation time was longer in the isoflavones-supplemented soy product, suggesting that the isoflavone addition could inhibit the starter cultures. However, all the products may be considered probiotic since they exhibited lactic acid bacterial populations varying from $2.3 \times 10^{9}$ up to $1.22 \times 10^{10}$ CFU/mL. Fermentation of soymilk did not change the isoflavones profile. In conclusion, it was possible to obtain a fermented soy product with a high isoflavones concentration, adequate sensory and chemical characteristics and lactic acid bacterial viability sufficiently high to characterize the product as a probiotic. The mixed starter culture was not able to convert the glycoside isoflavones into aglycone or produce equol during the fermented soy product processing.

KEYWORDS: Fermented soy product; isoflavones; probiotic; sensorial properties.

INTRODUCTION

Several studies have shown that the ingestion of soy-rich diets have health-beneficial effects including improve of lipid profile, protection against cardiovascular disease, prevention of some cancers such as breast, prostate and colon cancer and osteoporosis.1,3, 4, 25 Soybean and its derivates represent a high-quality protein source, with reduced content of saturated fat and great amount of dietary fiber and isoflavones.27 Soy Isoflavones belong to a class of compounds known as flavonoids that exhibited similarity in chemical structure and properties to estrogens.17 Isoflavones occur naturally in the soybean as β-glycosides (genistin, daidzin, glycitin), which are less estrogenic than their aglycones forms. Isoflavones glycosides are not absorbed intact across the enterocyte and require initial hydrolysis via intestinal β-glycosidase to release the aglycones forms (genistein, daidzein, glycitein) for uptake to the peripheral circulation.28 Furthermore, daidzein can be metabolized by intestinal microorganisms to equol that exhibit more estrogenic and antioxidant activities than its precursors.24 Rossi et al.20 developed a yogurt-like fermented soy product (soy yogurt), using the lactic bacterial strains Enterococcus faecium CRL 183 and Lactobacillus helveticus 416. This product had sensorial and...
technological properties similar to fermented-milk yogurt drinks and exhibited functional properties in animal tests and clinical trials.\textsuperscript{21, 22, 23}

The probiotic microorganism and soy constituents, in particular the isoflavones, are involved in the health properties of this product. However, during the processing of soy yoghurt the total isoflavones content is drastically reduced (92\% less than in the whole soybean).\textsuperscript{24} We hypothesized that supplementation of fermented soy product with isoflavones, to bring the content to approximately that found in the whole bean, would presumably reinforce its functional properties. The ability of the starter culture to convert isoflavones glycosides into aglycones and equol production during the fermented soy product manufacture has not been studied.

The aim of the present study was to evaluate the effect of isoflavones supplementation of the fermented soy product on its sensory acceptance, physicochemical properties and probiotic cell viable count. Additionally we also investigated the ability of the mixed starter cultures (\textit{E. faecium} CRL 183 and \textit{L. helveticus} 416) modify the profile of isoflavones of the soy product during the fermentation process.

\section*{MATERIAL AND METHODS}

\subsection*{Soymilk, Chemicals and Bacterial Strain}

Whole soybean (\textit{Foscarim} variety) was boiled at 95\(^\circ\text{C}\) for 7 minutes. The hydrated beans were drained and tritutrated. Soymilk was separated from insoluble residue by centrifugation and filtration (nylon filter). The extract was diluted (soy extract/water ratio = 1:6), homogenized and pasteurized.

Isoflavin\textsuperscript{®} (Galena, Brazil) was used for supplementation and contains at least 40.77\% of total isoflavones, which consist of 1.81\% genistin, 5.36\% genistein, 2.91\% daidzin, 29.38\% daidzein.

\textit{Enterococcus faecium} CRL183 and \textit{Lactobacillus helveticus} 416 were obtained from Reference Center for Lactobacilos (Cerela, San Miguel de Tucumán – Argentina) and Institute of Food Technology (ITAL, Campinas - SP), respectively.

\begin{table}[h]
\centering
\caption{Composition of unfermented soy product (USP), soy product (SP) and isoflavones-supplemented soy product (ISP).}
\begin{tabular}{l|c|c|c}
\hline
Ingredients/100mL & USP & SP & ISP \\
\hline
Soy milk (mL) & 82.0 & 82.0 & 82.0 \\
Soy oil (mL) & 2.6 & 2.6 & 2.6 \\
Lactose (g) & 2.0 & 2.0 & 2.0 \\
Sucrose (g) & 10.0 & 10.0 & 10.0 \\
Skin milk (g) & 3.5 & 3.5 & 3.5 \\
Gelatin (g) & 0.3 & 0.3 & 0.3 \\
Isoflavones (mg) & 0.0 & 0.0 & 50.0 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{USY= Unfermented Soy Product; SY= Soy Product; ISY= Isoflavones-Supplemented Soy Product.}

\subsection*{Fermented Soy Product Manufacture}

Fermented soy product (SP) was manufactured at UNISOJA (Development and Production Unit for Soybean Derivatives) in the Food and Nutrition Department of the Faculty of Pharmaceutical Sciences, UNESP at Araraquara (SP, Brazil), following the method described by Rossi et al.\textsuperscript{20} Soymilk, soy oil and lactose were homogenized and heated to 70\(^\circ\text{C}\). Sucrose, skin milk and gelatin were added and pasteurized (95\(^\circ\text{C}\) for 5 minutes) after prior homogenization. Each batch was cooled at 37\(^\circ\text{C}\) and the culture containing \textit{Enterococcus faecium} CRL183 and \textit{Lactobacillus helveticus} 416 (3\%) were added. Inoculated mixes were incubated at 37\(^\circ\text{C}\) until the product reached pH 4.4-4.5. Isoflavin\textsuperscript{®} was added to the soy product before the fermentation, at 50mg (total isoflavones) per 100g, to yield the isoflavones-supplemented soy product (ISP). Unfermented soy product (USP) was prepared by chemical acidification (with lactic acid) of soy product basic mixture (without bacterial culture or isoflavones). The products were storage at 10\(^\circ\text{C}\) and all determinations were conduced after 7 days of storage. Soy products composition is presented in the Table 1.

\subsection*{Acceptability Testing}

This study was performed by a panel of 50 consumers of soy products, aged between 20 and 50, recruited in the Faculty of Pharmaceutical Sciences, Araraquara. The attributes analyzed were color, aroma, flavor and overall impression. The panelists evaluated the acceptability of each one 3 samples using a nine points structured hedonic scale. The samples were coded with three-digits numbers, presented at random and individually evaluated.\textsuperscript{29}

\subsection*{Chemical Composition}

Methods for determination of chemical composition were: moisture by air-drying at 102\(^\circ\text{C}\); protein (nitrogen) by Kjeldahl method; fat by Soxhlet extraction and gravimetric determination; ash by incineration and gravimetric determination.\textsuperscript{15} Carbohydrates were calculated by formula: \% total carbohydrates= 100\% - \% (moisture+ ash + protein + fat).\textsuperscript{12}
Acidity measurement and fermentation time

The pH was monitored by a pH meter (Micronal model 320). Fermentation time was the time (minutes) required for lowering the pH values to 4.4 - 4.5.

Viable cell counts

Fermented soy products were checked microbiologically, at day 7, by counting viable cells of *E. faecium* and *L. helveticus* on two specific culture media - M17 and MRS agars, respectively.

Isoflavones profile

The extraction of isoflavones, including daidzin, genistin, daidzein, genistein and equol, was performed in accordance to Rossi et al. Isoflavones standards were purchased from Sigma Chemicals Co. (St. Louis, MO) and prepared in HPLC grade ethanol.

Isoflavones quantification was carried out on a high performance liquid chromatography (HPLC - Shimadzu®), in a system provided with auto sampler (SIL-10AF), diode array ultraviolet (UV) visible detector (SPD-10MA), quaternary pump, vacuum degasser, and Hypersil ODS C18 (250mm x 4.6mm) reverse-phase column (Supelco®). All reagents used in isoflavones extraction and HPLC analyses were filtered through a 0.22 μ or 0.45 μ membrane (PTFE – Millipore).

HPLC isocratic elution was used to isolate the isoflavones for detection and was composed of acetic acid-water 60% (2:98 v/v - solvent A) and methanol-acetonitrile 40% (80:20 v/v – solvent B), set a flow rate of 1.1mL/min, during 40 minutes. The diode array UV-visible detector was set at dual wavelengths of 262nm to measure and fermentation time of soy products.

The identification of isoflavones was confirmed by HPLC retention time and ultraviolet spectral analysis.

Statistical Analysis

Quantitative results were reported as mean ± SEM. The data were tested by analysis of variance (ANOVA) and the means were compared across groups by the Tukey test, significance being declared when p ≤ 0.05. All analyses were carried out with the BIOSTAT statistical package.

RESULTS AND DISCUSSION

The concentration and profile of isoflavones in soy-based products vary with the processing conditions. In an earlier study, it was found that during the stages of processing of the fermented soy product the total isoflavones content fell from 66.6mg/100g in whole soybean to 5.2mg/100g in the final product. These changes are due the heat treatment applied to the bean, from losses to the cooking water and to the insoluble residue (okara) and from the diluting effect. The supplementation of the fermented soy product with isoflavones, proposed in this study, was carried out in order to restore part of the losses due to processing, without exceeding the naturally occurring concentrations in the soybean.

Sensory Analysis

The sensorial acceptance was reduced in the isoflavones-supplemented soy product, but this effect was not significant (p>0.05) compared to the control sample (without added isoflavones) (Figure 1).

Despite no significant reduction in the acceptance, judges reported the presence of bitter and astringent residual more intense in isoflavones-supplemented product. Matsuura et al. showed that the isoflavones aglycones - genistin and daidzein - confer the unpleasant aftertaste to the aqueous extract of soybeans. In this study, the isoflavones commercial mixed used for supplementation was mainly composed by aglycone forms, favoring the intensification of residual flavors. In this way, use of higher concentrations of this isoflavones supplement could produce unpleasant bitter taste and impair the product acceptance.

On the other hand, unfermented soy product showed scores of texture / body, flavor and aroma lower than of fermented products (p<0.05). Unfermented soy product was prepared identically to the fermented product, excluding only the lactic cultures and, consequently, the stage of fermentation, which contributes to the development of aroma and body of the products (Figure 1).

Chemical Composition Acidity Measurement and Fermentation Time

Table 2 shows the chemical composition, acidity measurement and fermentation time of soy products.

Unfermented soy product, fermented soy product and isoflavones-supplemented soy product had similar chemical composition. However, the composition of the products differed from previously described by Rossi et al., with higher levels of protein, ash and moisture and lower concentrations of lipid and carbohydrate. The raw materials used, mainly soybean, may be cited as responsible for these variations.

The addition of isoflavones to the product resulted in an increase of 40 minutes in the time required for lowering the pH values to 4.4 - 4.5, but the fermentation time did not exceed that found in previous studies. In this study pH of fermented soy products ranged from 4.45 to 4.55, indicating that the mixed starter culture is able to producing enough acids even after isoflavones addition.
Viable Cell Count

Viable cell count were reduced and total fermentation time was longer in the isoflavones-supplemented soy product, suggesting that the supplementation could inhibit the probiotic bacteria (Table 3). However, all the products exhibited count of acid lactic bacteria between 2.3 x 10⁹ and 1.22 x 10¹⁰ CFU/mL, which is an enough condition for a product to be considered probiotic.⁵,⁶

Isoflavones Profile

The approximate retention times of glycoside and aglycone isomers of isoflavones standards and that found in soy product supplemented with isoflavones are shown in Figure 2.

Fermented soy product and unfermented soy product contained a total of 8.04 and 8.03 mg isoflavones per 100 ml, respectively. Isoflavones-supplemented soy product exhibited a 6.4-fold increase in the total isoflavones content (51.26mg/100mL) (Table 4).

The daily consumption of 200g of isoflavones-supplemented soy product would provide 102.52mg of total isoflavones, corresponding to 1.43mg/kg body weight for a 70-kg adult. According to the literature this isoflavones concentration seem sufficient to exert health effects.⁵,⁶,¹⁰,¹⁸,¹⁹

It has been proposed that intestinal microorganisms play a key role in the metabolism and bioavailability of isoflavones, as they hydrolyze the glycoside components via β-glicosidase, releasing the more bioavailable aglycone forms.²⁶

Table 2 – Chemical composition of unfermented soy product (USP), soy product (SP) and isoflavones-supplemented soy product (ISP).

<table>
<thead>
<tr>
<th>Composition</th>
<th>USP</th>
<th>SP</th>
<th>ISP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (%)</td>
<td>3.85 ± 0.04ᵃ</td>
<td>3.90 ± 0.00ᵃ</td>
<td>3.85 ± 0.00ᵃ</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>2.32 ± 0.02ᵃ</td>
<td>2.30 ± 0.08ᵃ</td>
<td>2.26 ± 0.09ᵃ</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>9.93 ± 0.16ᵃ</td>
<td>9.70 ± 0.15ᵃ</td>
<td>10.06 ± 0.11ᵃ</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>0.90 ± 0.00ᵃ</td>
<td>0.90 ± 0.07ᵃ</td>
<td>0.90 ± 0.00ᵃ</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>83.00 ± 0.16ᵃ</td>
<td>83.20 ± 0.00ᵃ</td>
<td>82.93 ± 0.17ᵃ</td>
</tr>
<tr>
<td>Final pH</td>
<td>4.6</td>
<td>4.45</td>
<td>4.55</td>
</tr>
<tr>
<td>Fermentation Time (minutes)</td>
<td>-</td>
<td>240</td>
<td>280</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM (n=3). Statistical comparison of groups: means with identical letters in the same line do not differ significantly (p≤0.05).

FIGURE 1 – Sensory evaluation of unfermented soy product (USP), soy product (SP) and isoflavones-supplemented soy product (ISP).

Values represent mean (n=50). Statistical comparison of groups: means with * differ significantly (p≤0.05).
Table 3 – Viable cell count of soy product and isoflavones-supplemented soy product.

<table>
<thead>
<tr>
<th>Bath</th>
<th>Lactobacillus sp. (CFU/mL)</th>
<th>Enterococcus (CFU/mL)</th>
<th>Lactobacillus sp. (CFU/mL)</th>
<th>Enterococcus sp. (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$1.22 \times 10^{10}$</td>
<td>$1.01 \times 10^{10}$</td>
<td>$2.80 \times 10^9$</td>
<td>$3.05 \times 10^9$</td>
</tr>
<tr>
<td>2</td>
<td>$1.21 \times 10^{10}$</td>
<td>$1.01 \times 10^{10}$</td>
<td>$3.10 \times 10^9$</td>
<td>$3.20 \times 10^9$</td>
</tr>
<tr>
<td>3</td>
<td>$1.15 \times 10^{10}$</td>
<td>$1.22 \times 10^{10}$</td>
<td>$2.30 \times 10^9$</td>
<td>$2.65 \times 10^9$</td>
</tr>
</tbody>
</table>

FIGURE 2 – Chromatograms for standards (A) and isoflavones-supplemented soy product (B).

1=daidzin, 2=genistin, 3=daidzein, 4=equol, 5=genistein. ------ 280nm; _____ 260nm.
Table 4 – Isoflavones profile (mg/100g) of unfermented soy product (USP), soy product (SP) and isoflavones-supplemented soy product (ISP).

<table>
<thead>
<tr>
<th></th>
<th>Daidzin</th>
<th>Genistin</th>
<th>Daidzein</th>
<th>Genistein</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>USP</td>
<td>2.00 ± 0.03b</td>
<td>5.76 ± 0.05b</td>
<td>0.26 ± 0.01b</td>
<td>-</td>
<td>8.03 ± 0.07b</td>
</tr>
<tr>
<td>SP</td>
<td>2.09 ± 0.04b</td>
<td>5.69 ± 0.04b</td>
<td>0.26 ± 0.01b</td>
<td>-</td>
<td>8.04 ± 0.01b</td>
</tr>
<tr>
<td>ISP</td>
<td>4.68 ± 0.27a</td>
<td>6.72 ± 0.02a</td>
<td>3.26 ± 0.94a</td>
<td>7.25 ± 0.22</td>
<td>51.26 ± 1.12a</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM (n=3). Statistical comparison of groups: means with identical letters in the same column do not differ significantly (p<0.05).

Some microorganisms possess β-glycosidase activity, which may hydrolyze isoflavones glycoside to aglycone in fermented soymilk. Tsangalis et al. reported that β-glycoside forms comprised approximately 92% of the total isoflavones in unfermented soymilk. Authors also found that fermentation with B. pseudolongum, B. longum-a, and B. animalis caused a significant increase in isoflavones aglycone levels, via the β-glycosidase catalyzed hydrolysis of isoflavones glycoside conjugates.

In the present study, isoflavones profile of fermented soy product and unfermented soy product were similar, with predominance of glycoside forms (96.6% and 96.8%, respectively) (Table 4). Genistin contributed to the greatest concentration of glycoside isoflavones in both of the products. These results indicate that the microorganisms used in soymilk fermentation (L. helveticus 416 and E. faecium CRL 183) have no activity of β-glycosidase, required to isoflavones biotransformation. The aglycone forms were predominant (77.8%) in the isoflavones-supplemented soy product and daidzein was the highest of individual isomer with 32.62mg/100g. The high concentration of aglycone form in this product may be explained by the composition of the commercial mixed used in isoflavones supplementation (isoflavin® - 95.6% isoflavones aglycons).

Equol is a daidzein metabolite, exclusively formed by intestinal microbiota. Several studies have indicated that clinical effectiveness of isoflavones is a function of the individual ability to produce equol. Transformation of daidzein to equol in soy foods has been proposed as an alternative to enhance the beneficial effects of isoflavones. Tsangalis et al. showed that Bifidobacterium pseudolongum, Bifidobacterium longum-a, and Bifidobacterium animalis promoted hydrolysis of isoflavones malonyl-, acetyl- and β-glucosides to aglycones, and transformed daidzein to equol in soymilk. The daidzein metabolite was formed predominantly during the exponential growth of these strains.

In this study, equol was not detected in soy product, supplemented or not with isoflavones, showing that L. helveticus 416 and E. faecium CRL 183 are not able to metabolize daidzein into equol during the fermentation of soymilk.

The specific bacterial species involved in equol production in vivo are not completely known. Decroos et al. isolated a mixed fecal culture - Enterococcus faecium EPI1, Lactobacillus mucosae EPI2, Finegoldia magna EPI3 and Veillonella sp. - capable to stimulate equol production in a fecal culture of non-equol producing. Modulation of intestinal microbiota also have been proposed to stimulate the equol production. Previous studies showed that regular ingestion of soy product fermented with L. helveticus 416 and E. faecium CRL 183 modify the intestinal microbiota of rats. This characteristic of the fermented soy product could promote the stimulation of in vivo equol production, but this effect was not verified yet.

CONCLUSION

In conclusion, it was possible to obtain an isoflavones-supplemented soy product with adequate sensory and chemical characteristics and the viability of lactic acid bacteria was enough to characterize the product as a probiotic. The mixed starter culture of Enterococcus faecium CRL183 and Lactobacillus helveticus 416 was not able to convert the glycoside isoflavones into aglycone or produce equol during the soy product processing.

as análises foram conduzidas após sete dias de estocagem das amostras a 10°C. A aceitação do produto fermentado suplementado com isoflavonas foi reduzida, porém esse efeito não foi significativo em relação à amostra não suplementada. As amostras apresentaram composição química semelhante. O número de células viáveis foi reduzido e o tempo total de fermentação foi superior para o produto suplementado com isoflavonas, sugerindo que a adição de isoflavonas pode inibir as culturas iniciadoras. Entretanto, todos os produtos fermentados analisados podem ser considerados probióticos, pois apresentaram populações de bactérias lácticas variando de 2,3 x 10⁹ a 1,22 x 10¹⁰ UFC/mL. A fermentação do extrato aquoso de soja não alterou o perfil de isoflavonas dos produtos. Em conclusão, o presente trabalho possibilitou a obtenção de um produto fermentado de soja contendo concentração elevada de isoflavonas, características sensoriais e químicas adequadas e número de bactérias lácticas viáveis suficiente para caracterizar o produto como probiótico. A cultura iniciadora mista não foi capaz de converter as isoflavonas glicosídicas em agliconas ou de produzir equol durante o processamento dos produtos.

PALAVRAS-CHAVE: Produto fermentado de soja; isoflavonas; probiótico; propriedades sensoriais.

REFERENCES


