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Soymilk fermented with *Lactobacillus rhamnosus* CRL981 ameliorates hyperglycemia, lipid profiles and increases antioxidant enzyme activities in diabetic mice

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ABSTRACT

Interest in soybeans and soy-based products has grown significantly in the last decades due to their reported nutritional and health-promoting effects. In soybean and non-fermented soy foods, isoflavones are predominantly found as glucosides, which must be hydrolyzed in order to exert their documented beneficial effects. The objective of this study was to evaluate the effect of soymilk fermented with *Lactobacillus rhamnosus* CRL981, a strain that can completely hydrolyze glucoside isoflavones due to its high beta-glucosidase activity. Using a diabetes animal model induced with streptozotocin, it was shown that the fermented soymilk was able to significantly decrease glucose levels, total cholesterol concentrations, triacylglycerols and increase antioxidant enzyme activities compared to animals that received unfermented soymilk. This study clearly shows that the adequate selection of starter cultures could be used to develop novel soy based products intended for the prevention or adjuvant treatment of diseases such as diabetes.

1. Introduction

Interest in soybeans and soy-based products has grown significantly in the last decade due to their reported nutritional and health-promoting benefits. Researchers have credited phytochemicals in soybeans, especially the polyphenolic isoflavones genistein and daidzein for some of these beneficial health effects. These isoflavones are structurally similarly to estradiol and have a high binding affinity for the primary estrogen receptor in the vascular wall (Zhang et al., 2013).

Because soy isoflavones can mimic the actions of estrogen, they are reported to play a role in the prevention cardiovascular disease (Rebholz et al., 2013), hormone dependent cancers (Yu et al., 2012), metabolic syndrome (Jungbauer & Medjakovic, 2013), and relieving postmenopausal symptoms such as osteoporosis (Chang et al., 2013).

Recently a meta-analysis reported that soy isoflavone supplementation could reduce body weight and improve glucose metabolism in non-Asian postmenopausal women (Zhang et al., 2013). Previous animal research by these same authors showed that soy isoflavones could decrease body weight and

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insulin level (Zhang, Na, Li, Zhao, & Cui, 2009). It was also shown that found that isoflavone played important roles in the regulation of glucose homeostasis in type 1 diabetic mice (Choi, Jung, Yeo, Kim, & Lee, 2008).

In soybean and non-fermented soy foods, isoflavones are predominantly found as glucosides. In order to exert a biological effect, isoflavone glucosides must be hydrolyzed. This can be accomplished partially by intestinal mucosal enzymes; however, several studies have demonstrated that lactic acid bacteria with β-glucosidase activity are able to increase the aglycone content during soymilk fermentation (Martinez-Villaluenga, Torino, Martin, Arroyo, & Garcia-Mora, 2012).

Previously it was shown that *Lactobacillus* (*L.*) *rhamnosus* CRL981 was able to proliferate in soymilk and produce a high beta-glucosidase activity achieving a complete hydrolysis of glucoside isoflavones (Marazza, Garro, & de Giori, 2009).

The objective of this study was to evaluate the biological activity of soymilk fermented with *L. rhamnosus* CRL981 using a diabetes animal model induced with streptozotocin (STZ).

2. Materials and methods

2.1. Soymilk preparation and fermentation

Fresh soymilk was prepared manually as described previously (Marazza et al., 2009). Five hundred milliliters of soymilk were inoculated (4% v/v) with *L. rhamnosus* CRL981 previously activated in soymilk. The inoculated soymilk was fermented at 37 °C for 24 h statically. Non-inoculated soymilk incubated in the same experimental conditions was used as a control. Both fermented and non-fermented milks were adjusted to pH 6.5 using sodium bicarbonate so that both the soymilks (control and fermented soymilk) have the same pH. Samples were taken at the end of this period to determine cell viability, pH, titratable acidity, organic acids, residual sugars, isoflavones and β-glucosidase activity as described previously (Marazza et al., 2009).

2.2. Animal model

Four-week-old male BALB/c mice weighing 16–20 g were obtained from the inbred closed colony maintained at CERELA (Centro de Referencia para Lactobacilos, San Miguel de Tucumán, Argentina). In order to induce diabetes, Balb/c mice received a single intraperitoneal injection (50 mg/kg animal live weight) of streptozotocin (STZ, Sigma, Argentina) dissolved in 0.1 mM citrate buffer (pH 4.5). Some of the STZ induced animals (STZ group) then received unfermented soymilk (STZ + SM group) or soymilk fermented with *L. rhamnosus* CRL981 (STZ + SM981). Each soymilk was given to each mouse individually (5 ml each day) in drinking tubes as a replacement of their drinking water starting 2 days after STZ injection during 4 weeks. A non STZ treated group of animals was also included (Control group). All groups contained 15 mice of equal average weight.

All animals received a balanced diet (23% proteins, 6% raw fiber, 10% total minerals, 1.3% Ca, 0.8% P, 12% vitamin mixture, Balanced/Autoclaved Rodent Diet, Batistela, Buenos Aires, Argentina) *ad libitum* and were maintained in a room with a 12-h light/dark cycle at 18 ± 2 °C.

At different times, animals from each group were anaesthetized intraperitoneally using a mixture of ketamine hydrochloride (Holliday-Scott S.A., Buenos Aires, Argentina; 100 μg/g body weight), and xylazine hydrochloride (Rompun, BAYER, Division Sanidad Animal, Buenos Aires, Argentina; 5 μg/g body weight).

Blood samples were taken by cardiac puncture and serum was obtained after incubating the blood at room temperature (21 °C) during 30 min followed by centrifugation (3000*g* during 15 min at 4 °C). Glucose (Glu), glycosylated haemoglobin (HbA1c), total cholesterol (Ch), high and low lipoproteins (HDL y LDL, respectively), triacylglycerols (TG), urea, creatine, glutamic-pyruvic transaminase (GPT) and glutamic-oxaloacetic transaminase (GOT) activities were determined using commercial kits (Wiener Lab, Argentina).

Microbial translocation to liver and spleen was determined following previously described protocols (del Carmen et al., 2011). Briefly, the liver was aseptically removed, weighed and homogenized in 5.0 ml sterile 0.1% (w/v) peptone solution. Serial dilutions of the homogenate were plated in triplicate in the following media: MRS, BHI and, MacConkey (Britania Laboratories, Buenos Aires, Argentina). Bacterial growth was evaluated after incubation at 37 °C for 48–72 h.

Catalase (CAT) and superoxide dismutase (SOD) activities were determined in liver samples as previously described (LeBlanc et al., 2011). Protein concentration was determined by use of a protein assay according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard.

All animal protocols were preapproved by the Animal Protection Committee of CERELA and all experiments complied with the current laws of Argentina.

2.3. Statistical analysis

Statistical analysis were performed with the software package Minitab 14 (Minitab, State College, PA, USA) using ANOVA GLM followed by a Tukey's posthoc test, and $P \leqslant 0.05$ was considered significant. Unless otherwise indicated, all values ($n = 9$) were the means of three independent trials ± standard deviation (SD). No significant differences were observed between individual replicates.

3. Results and discussion

3.1. Animal growth

A diabetic mouse model was chosen since it was found that isoflavone aglycones play important roles in the regulation of glucose homeostasis in type 1 diabetic mice (Choi et al., 2008). In the present study Balb/c mice were induced with an intraperitoneal injection of streptozotocin (STZ), an antibiotic that acts selectively on pancreatic beta-cells that are responsible for insulin secretion (Lee, 2006). Mice were either given i) a fermented soymilk product (STZ + SM981 group) containing a total of 45.4 ± 0.4 mg isoflavone aglycones/l (13.5 ± 0.2 mg daidzein/l and 31.9 ± 0.2 mg genistein/l) and no residual isoflavone glycosides or ii) unfermented soymilk (STZ + SM group) that contained a total of 11.7 ± 0.4 mg iso-

flavone aglycones/l (3.3 ± 0.2 mg daidzein/l and 8.4 ± 0.2 mg genistein/l) and 24.9 ± 0.3 mg isoflavone glycosides/l (4.8 ± 0.1 mg daidzin/l and 20.1 ± 0.2 mg genistin/l).

As shown in Fig. 1, STZ administration significantly affected animal growth. All animals started at the same weight (average of 17.3 ± 0.9 g) and control animals (Control group) showed a constant growth rate reaching values of 26.8 ± 0.5 g at the end of the experiment (day 28), whereas the animals that were induced with STZ (STZ group) showed a significantly lower growth rate, with a final average live weight of 22.2 ± 0.9 g. The decrease in animal growth was expected since it is known that STZ can provoke DNA damage and protein loss, causing hypoinsulinemia that prevents the use of carbohydrates as energy sources, reason for which it was used as the test model for diabetes in hundreds of published animal experiments. The animals that received soymilk fermented with *L. rhamnosus* CRL981 after induction with STZ (STZ + SM981 group) showed the highest growth rates (final live weight of 29.1 ± 1.2 g after 28 days) of all the assayed groups. This significant increase compared to the unfermented soymilk group could be explained by the elevated isoflavone aglycones concentrations in the fermented product (Marazza et al., 2009). STZ induced animals that received unfermented soymilk (STZ + SM group) also showed a significant increase in animal growth (final weights of 25.9 ± 1.0 g) compared to those of the STZ group, probably due the presence of beneficial compounds (micronutrients and macronutrients such as vitamins and proteins) present in soymilk. No significant differences in food or water intake were observed between all the groups analyzed (data not shown).

3.2. *Blood glucose concentrations*

Diabetes can originate from the destruction of pancreatic β cells that in turn generate a decrease in insulin levels (Ramkumar et al., 2004). As a result, there is an increase in plasmatic glucose levels (hyperglucemia) that is characteristic of diabetes. In this study, STZ- induced diabetic mice showed a significant increase (103.4%) of glucose levels (Fig. 2a) compared to the Control group animals. The administration of soymilk fermented with *L. rhamnosus* CRL981 to STZ-induced diabetic mice (STZ + SM981) was able to significantly reduce the glucose levels by 40.2% compared to those from the STZ group and did not show any significant differences compared to the control group, demonstrating that the consumption of soymilk fermented with *L. rhamnosus* CRL981 was able to prevent the onset of induced diabetes. The animals that received unfermented soymilk (STZ + Soy group) also showed a significant decrease in serum glucose concentrations compared to the STZ animals (169.4 ± 1.4 *vs.* 216.8 ± 7.8 mg/dl) but this value did not reach those of the Control group animals or those that received the fermented soymilk (106.6 ± 5.2 and 129.6 ± 2.1 mg/dl, respectively). It was previously shown that the administration of soy and genistein to STZ-induced diabetic rats was able to decrease glucose levels by 20.5% (Lee, 2006). These results suggest that the consumption of soy by itself is beneficial for preventing or treating diabetes, but that a more pronounced effect is observed when soymilk is fermented with β-glucosidase producing lactic acid bacteria liberating the isoflavones aglycones (biologically active forms). The hypoglycemic effect of the isoflavones observed in this study could be related to an increased secretion of insulin as shown by the administration of soy products and

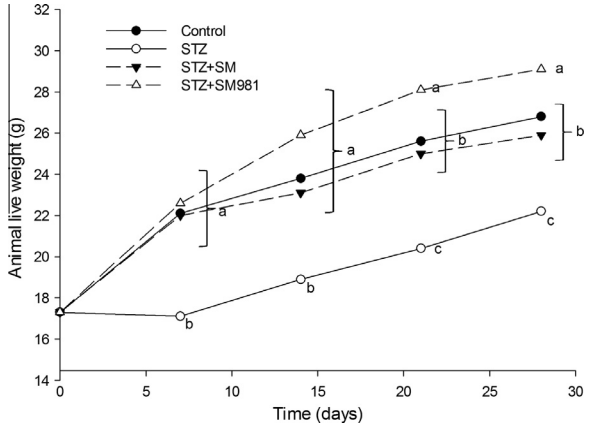

Fig. 1 – Animal live weight. Data is expressed as the means (n = 15) and standard deviations (below 10% were omitted for clarity.[a,b,c] Means with different letters indicate that a significant difference ($p \leqslant 0.05$) exists at that time point.

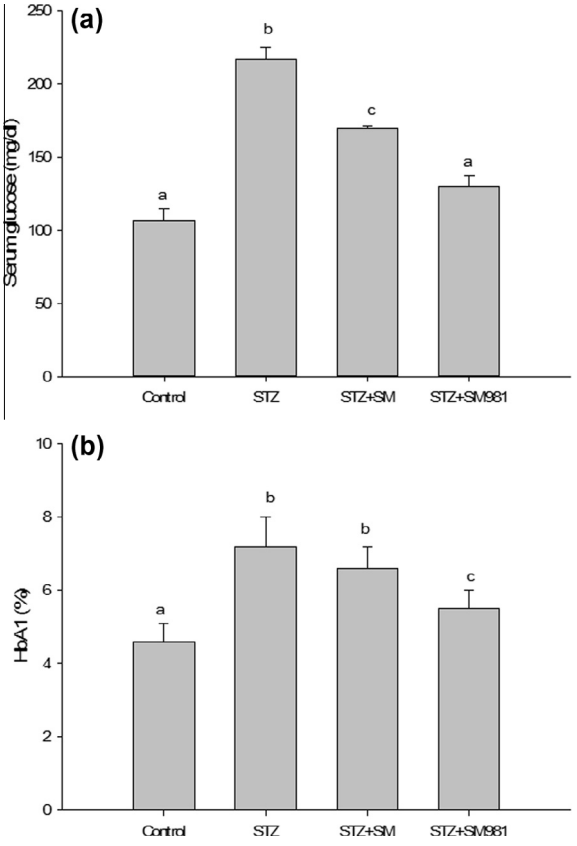

Fig. 2 – Glucose metabolism. Serum glucose concentrations (a) and HbA1 percentages (b) of mice from different experimental groups expressed as the means (n = 15) ± standard deviation.[a,b,c] Means with different letters indicate that a significant difference ($p \leqslant 0.05$) exists.

in turn increase peripheral metabolism of glucose (Lee, 2006). Previous studies have shown that the administration of soy beans to normo-glycemic mice significantly increased the mRNA that encodes for insulin, indicating that soy can stimulate the production of this hormone by pancreatic β cells (Fushiki & Iwai, 1989).

Glycosylated haemoglobin (HbA1c) arises from the non-enzymatic attachment of glucose to haemoglobin. They are formed and accumulate in the red cell in proportion to the blood glucose level. Their concentration reflects the long-term average glucose level and is thus useful as an indicator of diabetic control. Moreover, their concentration also has been associated with cardiovascular disease, nephropathy, and retinopathy and others suggesting that the HbA1c monitoring in patients with type 1 diabetic patients may improve outcomes (Larsen, Horder, & Mogensen, 1990). In this study it was shown that STZ induced mice showed an increase of 56.5% (Fig. 2b) in HbA1c levels compared to Control animals. The STZ induced animals that received soymilk fermented with *L. rhamnosus* CRL981 produced a significant decrease (23.6%) compared to STZ mice. This decrease was not observed in STZ mice supplemented with unfermented soymilk suggesting that the low isoflavone aglycone concentration in soymilk or the presence of isoflavone glycones is not sufficient to exert a biological effect. These results suggest that the beneficial effect of soy isoflavones on glucose levels was significant throughout the study and the fermented product could thus be useful in preventing diabetes and associated diseases.

3.3. *Cholesterol and triacylglycerol concentrations*

In STZ induced mice, it has been shown that total cholesterol (Ch) levels and plasmatic triacylglycerols (TG) increase and that the supplementation with genistein was able to reduce these levels (Lee, 2006) as did aglyconated isoflavones (Kawakami, Tsurugasaki, Nakamura, & Osada, 2005). However, other studies have shown that soy isoflavones did not modify TG concentrations in obese diabetic rats (Ali, Velasquez, Hansen, Mohamed, & Bhathena, 2004) or in STZ induced rats that received different concentrations of isoflavones (240, 480 or 1920 mg/100 g diet) in their diets (Hsu, Chiu, & Yeh, 2003). In the present study, it was shown that STZ induced mice had 1.3 times more cholesterol, 2.1 times more low-density lipoprotein cholesterol (LDL) and 1.5 times more TG than mice from the Control group (Fig. 3). The administration of soymilk fermented with *L. rhamnosus* CRL981 in STZ mice was able to reduce Ch concentrations and TG to values that were similar to the control group and showed a 20% decrease of LDL compared to STZ mice. Similar results were obtained from STZ induced animals that were supplemented with unfermented soymilk, showing that soymilk by itself had a significant effect on lowering lipids and cholesterol regardless of if this product was fermented or not. No differences in high-density lipoprotein cholesterol (HDL) levels were observed between the experimental groups.

3.4. *Liver and kidney functions*

Hyperglycemia can generate, over a long period of time, damage to small and large blood vessels that can affect the functionality of certain organs such as liver and spleen. In previous studies, it was show that STZ induced rats showed an increase in glutamic-pyruvic transaminase (GPT) and glutamic-oxaloacetic transaminase (GOT) activities compared to the control group (liver function indicators) and an increase in urea concentration in urine (kidney function indicator), and that genistein supplementation suppressed these increases (Lee, 2006). In the present study no significant changes in GPT, GOT or urea were observed between all of the animal groups (data not shown). The use of mice in this study instead of rats might explain the differences observed with previous studies (Lee, 2006).

3.5. *Antioxidant activity*

It was previously shown by our group that soymilk fermented with *L. rhamnosus* CRL981 showed increased antioxidant activities *in vitro* compared to unfermented soymilk due to the presence of isoflavone aglycones (Marazza, Nazareno, de Giori, & Garro, 2012). The evaluation of the fermented product using an animal model is essential in order to evaluate its biological potential from an antioxidant point of view. The cytotoxic action of STZ in diabetic animal models is associated with the generation of reactive oxygen species (ROS) that cause oxidative damage culminating in β-cell destruction through the induction of apoptosis and suppression of insulin biosynthesis (Saxena, Srivastava, Kale, & Baquer, 1993). In addition, it has been shown in STZ mice that catalase (CAT) and superoxide dismutase (SOD) activities are abnormally decreased, favoring the accumulation of ROS such as hydrogen peroxide and superoxide radicals (Lee, 2006). In the present study, the STZ induced mice (STZ group) showed a significant decrease in hepatic CAT and SOD activities compared to those of the Control group (50.5% and 55.4%, respectively, Table 1). When STZ induced mice received soymilk fermented with *L. rhamnosus* CRL981 (STZ + SM981 group), there was a significant increase in CAT and SOD activities compared to STZ induced mice (STZ group), reaching activities that were similar to the Control group. STZ induced mice that received unfermented soymilk (STZ + SM) showed a significant increase in CAT (but not SOD) compared to STZ mice but remained lower than in the Control group. It was previously shown that an increase in CAT and SOD activities was observed in rats induced with STZ that received a diet supplemented with genistein (Lee, 2006). The concentration of isoflavones seem to be an important factor since the low dose administration of genistein (0.1 g/kg) in mice during 2 weeks did not significantly affect antioxidant enzymes in liver (Breinholt, Lauridsen, & Dragsted, 1999). In the present study, the increase in CAT and SOD could be due to the biologically active soy isoflavones present in the fermented soymilk (approximately 8 and 3 g genistein and daizein/kg live weight) due to microbial bioconversion since unfermented soymilk did not show the same level of increase (Marazza et al., 2009). These results are similar to other reported (Hamden, Jaouadi, Carreau, Aouidet, & Elfeki, 2011) where it has been shown that soy isoflavones can increase the antioxidant system of diabetic mice through a mechanism that is similar to human estrogen.

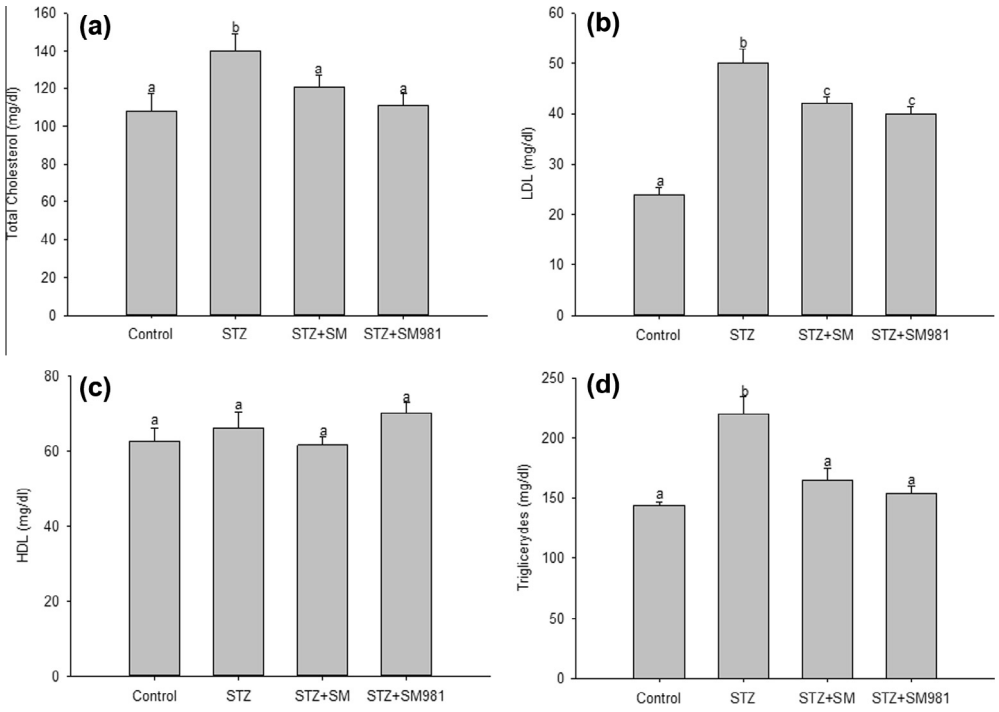

Fig. 3 – Lipid metabolism. (a) Total colesterol (b), LDL, (c), HDL, and (d) triacylglycerols of mice from different experimental groups expressed as the means ($n = 15$) ± standard deviation. [a,b,c] Means with different letters indicate that a significant difference ($p \leqslant 0.05$) exists.

Table 1 – Antioxidant enzyme activities in liver of mice from different experimental groups expressed as the means (n = 15) ± standard deviation. [a,b,c] Means with different letters within an enzyme type indicates that a significant difference ($p \leqslant 0.05$) exists.

	Control	STZ	STZ + SM	STZ + SM981
CAT[1]	0.55 ± 0.05^{a}	0.27 ± 0.04^{b}	0.48 ± 0.02^{c}	0.57 ± 0.04^{ac}
SOD[2]	50.5 ± 4.4^{a}	22.5 ± 2.7^{b}	26.3 ± 3.1^{b}	40.1 ± 5.9^{c}

[1] Catalase.
[2] Superoxide dismutase.

3.6. *Safety evaluation of fermented soymilk*

No microbial translocation was observed in all the animal groups, including those that received soymilk fermented with *L. rhamnosus* CRL 981 (data not shown). These results indicate that the fermented soybean product do not cause alterations in the intestinal mucosa, and may be considered as an indicator of the biological safety of the product and the LAB used in its preparation (LeBlanc et al., 2010).

4. Conclusions

The results of this study clearly show that soymilk fermented with *L. rhamnosus* CRL981 was able to improve the health status of diabetic animals as shown by increases in animal growth, decreases in blood glucose and HbA1, decreased cholesterol and triacylglycerol concentrations, and an increase in antioxidant activities in the liver. These beneficial effects would be principally due to the capacity of this strain to increase the aglyconated isoflavones in soymilk by its high β-glycosidase activity converting them into the biologically active forms since unfermented soymilk did not show similar levels of improvements. This study could be the basis to elaborate novel fermented foods using food grade microorganisms to convert soy isoflavones to their biologically active forms and be useful for the prevention or complementary treatment of diseases such as diabetes.

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