A Novel Functional Soy-based Food Fermented by Lactic Acid Bacteria: Effect of Heat Treatment

J.G. LEBLANC, M.S. GARRO, G. SAVOY DE GIORI, AND G. FONT DE VALDEZ

ABSTRACT: The development of a novel fermented soymilk product using selected lactic acid bacteria was performed. The immunomodulatory properties of strains able to grow in soymilk were evaluated in a murine model using inbred BALB/c mice. *Lactobacillus acidophilus* CRL 43 and CRL 1064 and *Lactobacillus paracasei* CRL 75 and CRL 208 were all able to significantly increase the phagocytic index (41 ± 4) compared with the control group (16 ± 3) when given individually. These strains were then used in the elaboration of multicultured fermented soymilks that were able to stimulate an innate immune response (peritoneal macrophage activation). However, these novel fermented products sometimes caused undesired secondary effects such as microbial translocation and animal weight loss. When the same fermented products were subjected to heat treatment, they were still able to stimulate the innate immune response without causing secondary effects. These results clearly show that it is not always necessary for lactic acid bacteria to be alive to exert a beneficial physiological effect. This study could thus be used as a model in the design and future evaluation of novel fermented vegetable products.

Keywords: soymilk, lactic acid bacteria, fermentation, functional food, immune-modulation

Introduction

Soybeans have an excellent reputation for their elevated protein contents, amino acids quality, high nutritional value, and low production costs. Soy product consumption has increased because of their large beneficial properties such as being free of cholesterol, gluten, and lactose, making them suitable for lactose-intolerant consumers and vegetarians (Liu and Lin 2000). The polyunsaturated-to-saturated ratio of soymilk (SM) fatty acids is high (1.1:0.3) as is its folate concentration (Holland and others 1991). Recently, soybean and soy proteins have received much attention for their preventive effects on chronic diseases such as arteriosclerosis, cancer, osteoporosis, and menopausal disorder (Anderson and others 1999; Setchell and Cassidy 1999).

Despite these merits, many consumers find the taste of plain SM to be unappealing, and indigestible oligosaccharides such as stachyose and raffinose would limit the wide consumption of soy-based foods (Thananunkul and others 1976). Soybeans also reportedly contain phytates, which are considered antinutrients because they diminish the bioavailability of certain minerals, including magnesium, calcium, iron, and zinc (Lonnerdal 1985). For these reasons, many approaches such as fermentations are being evaluated to overcome these limitations. The taste of SM can be improved by decreasing the beany, grassy, or soy flavor by fermentation using lactic acid bacteria (LAB) (Granata and Morr 1996; Liu and Lin 2000). SM is suitable for the growth of LAB (Garro and others 1999, 2004; Chou and Hou 2000; Bezkorovainy 2001), and mixed starter cultures have been shown to produce heterogeneous metabolites that greatly improve the quality of the fermented product (Marshall and Tamine 1997; Garro and others 1998; Rossi and others 1999).

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During the past decades, there has been a renewed interest in developing functional foods using LAB as additives to enhance human and animal health (Fuller 1989). These LAB have been shown to stimulate various immune functions when administered alone (Perdigon and others 2001) and when included in traditional fermented foods such as yogurt (Faure and others 2001). To our knowledge, no published reports have used LAB in soy-based products in the goal of creating a novel functional food. SM fermented with properly selected LAB strains may become a unique functional food because it would not only improve the organoleptic characteristics but could also give the consumer beneficial properties such as stimulating their natural defense system.

The objective of this study was to formulate starter cultures using LAB strains, which could be used in functional SM-based foods. To achieve this goal, SM fermented with different LAB strains were evaluated in vivo using a murine experimental model. The effect of heat treatment on the fermented SM product was also determined.

Materials and Methods

Microorganisms and growth conditions

Streptococcus thermophilus CRL 813, *Lactobacillus acidophilus* CRL 43, *L. acidophilus*"CRL 1064, *Lactobacillus paracasei* subsp. *paracasei* CRL 75, and *L. paracasei* subsp. *paracasei* CRL 208 used in this study were obtained from the culture collection (CRL) of the Centro de Referencia para Lactobacilos (CERELA, Tucuman-Argentina). Before experimental use, cultures were propagated (2%, v/v) twice in sterile Man-Rogosa-Shape (MRS) broth (de Man and others 1960) for lactobacilli and LAPTg broth, containing per liter: 10 g glucose, 10 g yeast extract, 10 g peptone, and 1 ml Tween 80 (Raibaud and others 1961) for streptococci, and incubated at 37 °C for 16 h. All bacteria were harvested by centrifugation at 5000 × *g* for 10 min and were washed twice and resuspended in sterile saline solution (NaCl 0.15 *M*) to the same original volume. This cell suspension was used as the inoculum.

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Preparation of fermented soymilk

The substrate used was a commercial SM, which had the following composition: 3.0% proteins, 4.5% lipids, 3.5% sugars, 0.5% ash, and 88.5% water. The sugar fraction contained 2.8% sucrose, 2.0% glucose, and 0.7% stachyose. Before being used, SM was sterilized at 115 °C for 20 min and cooled to 37 °C. Final pH was 6.8 to 7.0. The substrate was inoculated separately with each cell suspension at 1% (v/v). All inoculated samples were incubated statically at 37 °C for 4 h, and the fermentation was stopped by rapidly cooling the fermented milk in an iced bath and then chilled in water bath before being used. One half of the fermented milk was used directly in the animal trials, and the other half was heat-treated before animals feeding.

Heat treatment

The fermented SM was placed in a water bath at 80 °C for 40 min with shaking every 5 min and immediately cooled to 4 °C. The heat treatment was repeated 3 times, and its efficiency was determined using the plate dilution method. Serial dilutions of each sample were plated in mass using MRS and LAPTg agar for the enumeration of lactobacilli and streptococci, respectively, and plates were incubated at 37 °C for 48 h. Colonies were counted and results were expressed as colony-forming unit per milliliter (CFU/mL).

Animal trials

BALB/c mice weighing 20 to 25 g from the inbred closed colony maintained at CERELA were divided into 14 groups, each 1 consisting of at least 5 animals (10 for controls). Mice were fed a solid conventional diet (Rodent Chow: 32% protein, 5% fat, 2% fiber, and 60% nitrogen-free extract) and water ad libitum and were maintained in a room with a 12-h light/dark cycle at 18 ± 2 °C. At the end of each feeding period, mice were sacrificed by cervical dislocation.

To study the effect of each LAB strain on the immune response, 5 experimental groups and a control group were used. Overnight cultures of previously selected LAB (see "Microorganisms and growth conditions" section) were washed twice with sterile saline solution and resuspended in SM at a concentration of 3.0×10^8 CFU/mL. This cell suspension was diluted in the drinking water (1:30, v/v) to a final dose of 1.0×10^7 CFU/mouse/d and administered ad libitum during 7 consecutive days. The drinking water was changed daily.

In a second study, the drinking water was replaced by fermented SM given ad libitum (maximum daily dose of 5 mL per mouse) during 7 d. In this study, 2 large groups of mice were used, consisting of 4 subgroups (3 experimental groups and a control) that were fed either the fermented SM or the heat-treated fermented SM.

Peritoneal macrophage activation

Peritoneal macrophage activation was evaluated using the technique described by Valdez and others (1997). Briefly, 5 mL saline solution containing 0.1% albumin and 10 U/mL heparin was injected into the peritoneal cavity. The peritoneal liquid was collected and macrophages were concentrated by centrifugation (1000 × *g* for 4 min). Heat-treated, opsonized yeast were added to the macrophages and incubated at 37 °C for 15 min. Macrophages were observed immediately using a light microscope at 400×. The phagocytosis index (%) was defined as the number of macrophages that have ingested yeast/total number of macrophages.

Microbial translocation

Microbial translocation was determined following the protocol of Rodriguez and others (2001). The spleen and liver were aseptically removed and homogenized in 5.0 mL sterile 0.1% peptone solution. Serial dilutions of the homogenate were plated in triplicate in the following media: MRS for enumeration of lactobacilli, McConkey for analysis of enterobacteria, and blood-supplemented brain-heart infusion (BHI) for enumeration of anaerobic and aerobic bacteria. Bacterial growth was evaluated after incubation of the plates at 37 °C for 48 to 72 h.

Statistical analysis

Unless otherwise indicated, all results in this article are the means of 3 independent trials ± standard deviation, and significant differences between experimental groups were determined using the Paired Student *t* test with a 95% confidence interval.

Results and Discussion

Our 1st objective was to evaluate the capacity of selected LAB strains to enhance the innate immune response. *L. acidophilus* strains (CRL 43 and CRL 1064) and the *L. paracasei* strains (CRL 75 and CRL 208) were able to significantly increase the phagocytic index compared with the control group (Figure 1). Indeed, macrophages activation was almost 2-fold higher (39 ± 2, 41 ± 3, 41 ± 2, and 40 ± 2 for CRL 43, CRL 1064, CRL 75, and CRL 208, respectively) than the control group (16 ± 3). In contrast, the *S. thermophilus* group showed no significant difference compared with the control group (19 ± 2).

High-level activation of peritoneal macrophages could be attributed to enhanced innate immune response or caused by bacterial translocation resulting from the ingestion of the LAB. Microbial translocation was tested in mice fed with the strains that showed a significant increase in peritoneal macrophages activation. Administration of LAB strains in a murine model showed different effects on bacterial translocation. *L. paracasei* strains (CRL 208 and CRL 75) do not cause bacterial translocation in the liver and spleen (Figure 2a and 2b, respectively). However, in these visceral organs, bacterial count increased significantly (10^2 to 10^3 CFU/g organ) in the *L. acidophilus* groups (strains CRL 1064 and CRL 43) compared with the control group. These results could explain the increased values of phagocytic index because peritoneal macrophages are activated when the liver and spleen are infected (Perdigon and others 2001).

From the results obtained, 3 mixtures were prepared with the

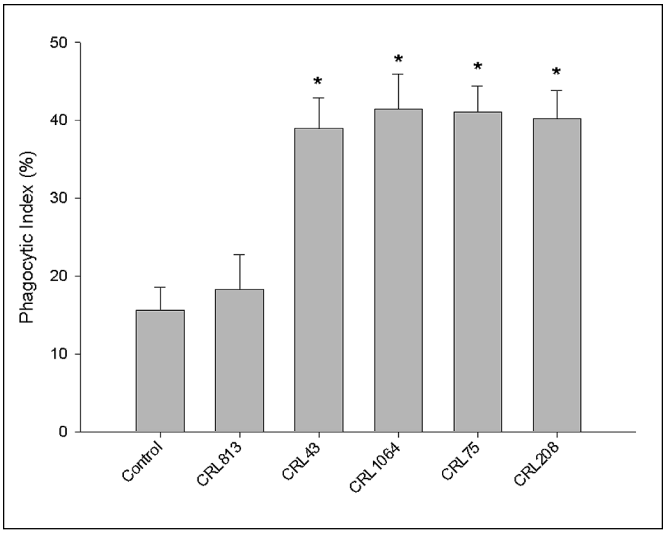

Figure 1—Peritoneal macrophage activation (phagocytosis index) of mice fed individual strains of lactic acid bacteria (LAB) during 7 d. *$P < 0.05$.

LAB strains: Mixture "A" contained *L. paracasei* CRL 75 plus *L. acidophilus* CRL 43 (ratio 1:1); mixture "B" contained *L. paracasei* CRL 75 plus *S. thermophilus* CRL 813 (ratio1:1); and mixture "C" contained *L. paracasei* CRL 75 plus *L. paracasei* CRL 208 (ratio 1:1). *L. acidophilus* CRL 43 and *S. thermophilus* CRL 813 were not able to be grown together so this mixture was not used in further assays.

Figure 3 shows the peritoneal macrophage activation of mice fed with the different fermented SM. All 3 soy products were able to significantly increase the phagocytic index compared with the noninoculated control (16 ± 3). Soymilk fermented with starter A showed the highest index (46 ± 6) followed by B and C (40 ± 5 and 39 ± 4, respectively). Because the LAB strains present in these fermented products can cause potential adverse effects such as microbial translocation in visceral organs, analyses were simultaneously performed using the heat-treated product after the fermentation period (4 h). No viable bacteria were detected in the heat-treated product (data not shown). When mice were fed heat-treated fermented SM, the control value (19 ± 1) did not differ significantly from untreated SM. Interestingly, heat-treated fermented SM were still able to significantly increase phagocytic index, although to a lower extent than the untreated fermented SM. Heat-treated SM fermented with starter culture B showed the highest levels of phagocytosis (31 ± 2) followed by C and A (27 ± 3 and 25 ± 3, respectively). This unintended result is remarkable because the heat-treated fermented SM is still able to induce an innate immune response even though the bacterial strains are not viable. Furthermore, no microbial translocation was observed after the administration of the heat-treated fermented product (data not shown), indicating that this functional food does not possess the same risks observed using live bacterial strains. Although this is the 1st report of an immunomodulating effect after heat-treating fermented soymilk, mechanisms by which LAB can exert biological effects when not alive are numerous (Matar and others 2003). Soluble compounds produced by LAB during milk fermentation can be used to prevent gastrointestinal disorders and cancer (Biffi and others 1997); the supernatant of fermented milk cultured with *L. casei* and *L. acidophilus* increased the immune response independent of the presence of lactobacilli (Perdigon and others 1986); and peptidic fractions cause by the action of LAB during milk fermentation can produce antitumor and immunomodulating responses (LeBlanc and others 2002).

Animal growth is a general indicator of health. Poor growth and weight loss are often signs of sickness, disease, or malnutrition. Figure 4 shows the weight differences of the animals during the period of the trial (7 d). The control group that received unfermented SM showed a normal weekly weight gain (1.5 ± 1 g). Heat treatment did not significantly affect this value (1.5 ± 1 g). When fed the fermented SM, mixtures A and C had similar animal weight gains (2 ± 1 g and 1 ± 0.5 g, respectively), whereas mixture B showed a significant decrease in animal weight (–1 ± 0.5 g). When mice received heat-treated fermented SM, all mixtures showed similar weight gain (1.5 ± 1, 1 ± 0.5, and 1.5 ± 0.5 for A, B, and C, respectively).

This result, along with the activation of peritoneal macrophages shows that the heat-treated product is able to exert a biological effect on the host. Although most studies dealing with LAB strains have shown that they exert their activities when given in a live form, it is well known that LAB possess different mechanisms by which they can enhance an immune response (Perdigon and others 2001). These uncommonly studied mechanisms allow strains to exert a biological effect when they are administered in a nonviable form. A heat treatment reduced but did not eliminate the immunomod-

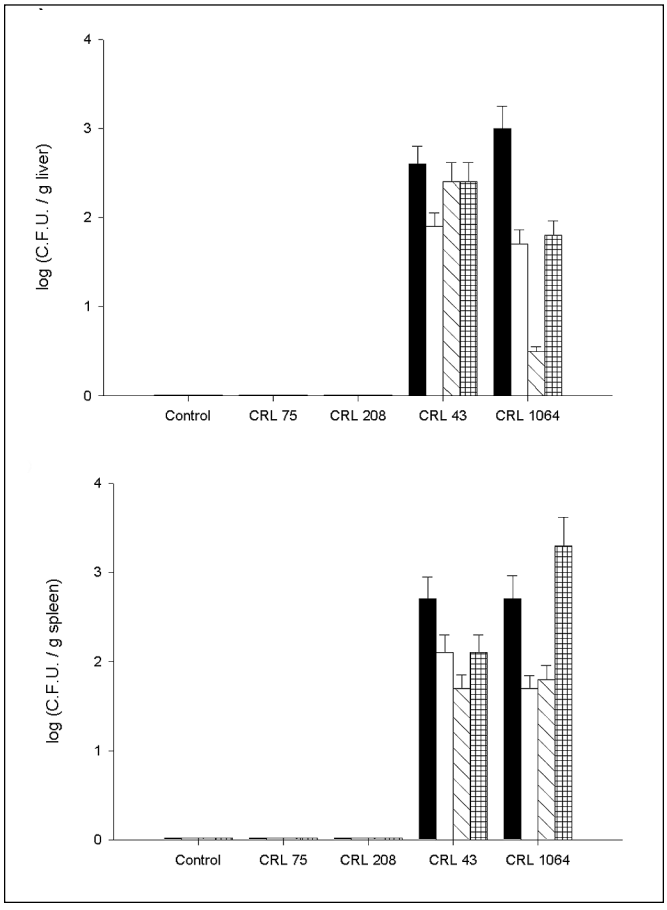

Figure 2—Microbial translocation in liver (a) and spleen (b) of mice fed lactic acid bacteria (LAB) during 7 d with individual strains capable of increasing the peritoneal macrophage activity. Results are expressed as colony-forming units per gram (CFU/g) organ of lactobacilli (*black boxes*), enterobacteria (*empty boxes*), anaerobic microorganisms (*diagonally stripped boxes*) and aerobic microorganisms (*checkered boxes*).

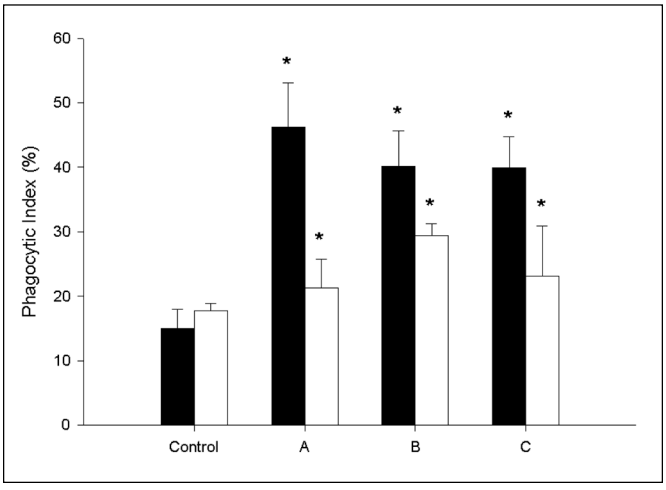

Figure 3—Peritoneal macrophage activation (phagocytosis index) of mice fed fermented soymilk (SM) (*black boxes*) or heat-treated fermented SM (*empty boxes*) during 7 d. *$P < 0.05$.

ulatory effects of candidate probiotic strains (Pessi and others 1999), and the cell-free extract of a LAB-fermented product was able to activate peritoneal macrophages (Hatcher and Lamprech 1993).

The selected LAB strains used as single or mixed starter cultures for SM fermentation showed high technological performance in SM (unpublished data). The decreases in pH value correlated well with the increases in titratable acidity (data not shown). Several strains produced lactic acid at a rate and final concentration suitable for fermented SM products. *S. thermophilus* CRL 813 reached a pH of < 4.60 after 4 h of incubation at 37 °C. Under the same conditions, only *L. acidophilus* CRL 43 acidified SM below pH 4.90. *S. thermophilus* CRL 813 and *L. acidophilus* CRL 1064 were able to produce aroma compounds (diacetyl and acetoin), whereas *L. acidophilus* CRL 43 did not. It is well established that *L. paracasei* strains are producers of the aroma compounds. Moreover, these strains enhanced viscosity of the SM food by producing exopolysaccharides. All the strains tested were able to coagulate soy proteins after 4 h of incubation, time after which fermentation was stopped (see "Materials and Methods" section).

Conclusions

This study shows the use of LAB in the development of novel soy-based products. In addition to the immune system stimulation preventing a wide range of diseases, this product with improved technological and organoleptic characteristics would contribute to satisfy human nutritional requirements.

The results of this study show that the fermented SM products were able to stimulate an innate immune response (peritoneal macrophage activation), even after receiving a heat treatment. The application of heat treatment similar to the one in this study could be used to eliminate risks of incorporating novel strains that could potentially cause dangerous secondary effects (such as microbial translocation and/or animal weight loss). In our study, the bacterial translocation observed after the ingestion of *L. acidophilus* was abolished when the product fermented with this LAB was heat-treated. Also, the weight loss observed after the ingestion of some mixtures was not seen when the same mixture was heat-treated before administration.

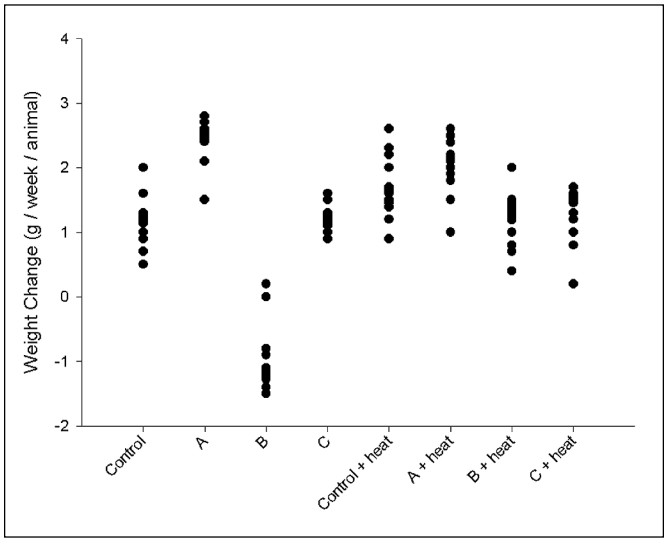

Figure 4—Weekly live animal weight variation of mice fed fermented soymilk (SM) and heat-treated fermented SM. Each point represents 1 animal.

Heat treatment could also be used to increase the shelf life and facilitate the storage of products derived from microbial fermentation of soy-based products. From the obtained results, mixture B made from the fermentation of SM by *L. paracasei* CRL 75 and *S. thermophilus* CRL 813 could be used in the formulation of a novel soy-based food because these LAB were able to grow in this complex medium and gave it immunomodulatory properties.

To our knowledge, this study represents the 1st report of a fermented soy-based product that is able to stimulate an immune response when the starter microorganisms have been heat-killed. This study could thus be used as a model in the design and future evaluation of novel fermented vegetable products.

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