



β -Casein hydrolysate generated by the cell envelope-associated proteinase of *Lactobacillus delbrueckii* ssp. *lactis* CRL 581 protects against trinitrobenzene sulfonic acid-induced colitis in mice

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ABSTRACT

Lactobacillus delbrueckii ssp. *lactis* CRL 581, a thermophilic lactic acid bacterium used as a starter culture for the manufacture of several fermented dairy products, possesses an efficient proteolytic system that is able to release a series of potentially bioactive peptides (i.e., antihypertensive and phosphopeptides) from α - and β -caseins. Considering the potential beneficial health effects of the peptides released by *L. delbrueckii* ssp. *lactis* CRL 581 from milk proteins, the aim of this work was to analyze the anti-mutagenic and anti-inflammatory properties of the casein hydrolysates generated by the cell envelope-associated proteinase of this bacterium. The ability of α - and β -casein hydrolysates to suppress the mutagenesis of a direct-acting mutagen 4-nitroquinoline-*N*-oxide on *Salmonella typhimurium* TA 98 and TA 100 increased concomitantly with the time of casein hydrolysis. The anti-inflammatory effect of the β -casein hydrolysate was evaluated using a trinitrobenzene sulfonic acid (TNBS)-induced Crohn's disease murine model. The hydrolysate was administered to mice 10 d before the intrarectal inoculation of TNBS. The mice that received β -casein hydrolysate previously to TNBS showed decreased mortality rates, faster recovery of initial body weight loss, less microbial translocation to the liver, decreased β -glucuronidase and myeloperoxidase activities in the gut, and decreased colonic macroscopic and microscopic damage compared with the animals that did not receive this hydrolysate. In addition, β -casein hydrolysate exerted a beneficial effect on acute intestinal inflammation by increased interleukin 10 and decreased IFN- γ production in the gut. Our findings are consistent with the health-promoting attributes of the milk products fermented by *L. delbrueckii* ssp. *lactis* CRL 581 and open up new opportunities for developing novel functional foods.

Key words: bioactive peptide, lactic acid bacteria, casein hydrolysate, proteinase

INTRODUCTION

Lactobacillus delbrueckii ssp. *lactis* is a homofermentative thermophilic lactic acid bacterium (**LAB**) widely used as a starter culture for the manufacture of a variety of fermented dairy products, such as fermented sour milks and Swiss- and Italian-type cheeses (Hébert et al., 2000a). Like other LAB, *L. delbrueckii* ssp. *lactis* is auxotrophic for several AA and is, therefore, dependent on the use of exogenous nitrogen sources for optimal growth (Hebert et al., 2000b; Sadat-Mekmene et al., 2011). As milk contains only small amounts of AA and short peptides (Thomas and Mills, 1981), *L. delbrueckii* ssp. *lactis* depends on a complex proteolytic system to obtain essential AA from caseins during growth in milk. In addition to its vital role for bacterial growth in milk, this efficient proteolytic system also contributes to the development of flavor and texture of fermented products (Law and Haandrikman, 1997) and can promote human health through the release of bioactive peptides during milk fermentation (Hayes et al., 2007; Hebert et al., 2008, 2010). Antimicrobial, immunomodulatory, antithrombotic, opioid, and antioxidant activities, enhancement of mineral absorption and bioavailability, and blood pressure-lowering effects are some of the biological activities attributed to milk-derived peptides (Hayes et al., 2007; Hebert et al., 2010). Matar et al. (2001) demonstrated that immunoenhancing and antimutagenic compounds were released during milk fermentation by the proteolytic homofermentative thermophilic LAB strain *Lactobacillus helveticus* R389, whereas these bioactive compounds were not present in milk fermented by a non-proteolytic mutant. Furthermore, bioactive peptides released from milk proteins by the same proteolytic strain were able to stimulate the immune system and inhibited the growth of an immunodependent fibrosarcoma in a mouse model (LeBlanc et al., 2002). Moreover, 4 bioactive peptides derived from β -CN and α -LA from milk fermented by

Received July 15, 2011.

Accepted September 17, 2011.

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L. helveticus LH-2 upregulated cytokines and nitric oxide production by macrophages as well as stimulated phagocytic activity of these cells (Tellez et al., 2010). A peptide derived from bovine β -CN (193–209) modulates functional properties of bone marrow-derived macrophages from germfree and human flora-associated mice; this peptide could enhance antimicrobial activity of macrophages without proinflammatory effects (Sandré et al., 2001). It is well known that the nature and activity of peptides produced by milk fermentation with thermophilic LAB is strain specific (Hebert et al., 2002, 2010; Sadat-Mekmene et al., 2011). *Lactobacillus delbrueckii* ssp. *lactis* CRL 581 is able to release a series of potentially bioactive peptides (antihypertensive and phosphopeptides) from α - and β -CN (Hebert et al., 2008). Taking advantage of the powerful proteolytic system of this bacterium in the development of the organoleptic properties of fermented milk products, and considering the potential beneficial health effects of the peptides released by *L. delbrueckii* ssp. *lactis* CRL 581 from milk proteins, the aim of this work was to analyze the antimutagenic and anti-inflammatory activities of the casein hydrolysates generated by the cell envelope-associated proteinase of *L. delbrueckii* ssp. *lactis* CRL 581. The intestinal inflammation induced in mice by 2,4,6-trinitrobenzene sulfonic acid (TNBS) was selected as a model to evaluate the anti-inflammatory properties of the β -CN hydrolysate because it shares important similarities with human Crohn disease, such as transmural inflammation, lymphocyte infiltration, and Th1-dominated cytokine profiles (te Velde et al., 2006; Izcue et al., 2009). In addition, this model can be used to test alternative strategies in the preclinical phase of chronic inflammatory bowel disease (IBD; Wirtz et al., 2007). The demonstration of the antimutagenic and anti-inflammatory properties of the casein hydrolysates generated by *L. delbrueckii* ssp. *lactis* CRL 581 would enhance the health promoting attributes of this strain and open up future opportunities for developing novel functional foods.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Media

Lactobacillus delbrueckii ssp. *lactis* CRL 581 was isolated from a homemade Argentinian hard cheese and belongs to the culture collection of the Centro de Referencia para Lactobacilos (CERELA) of Argentina. This strain was grown at 40°C in a minimal defined medium (MDM) containing 5 mM CaCl₂, as described previously (Espeche Turbay et al., 2009). The MDM (pH 6.5) was prepared from concentrated individual stock solutions, which were stored at 4°C after filtration, ex-

cept for the cysteine solution that was freshly prepared. Medium and stock solutions were sterilized by filtration through a cellulose acetate membrane (0.20- μ m pore size; Sartorius AG, Göttingen, Germany). *Salmonella typhimurium* TA98 (*hisD3052 rfa Δ uvrB*) and TA100 (*hisG46 rfa Δ uvrB*) were obtained from Salmonella Genetic Stock Centre, University of Calgary, Alberta, Canada. Tests of histidine requirement, *rfa* mutation, *uvrB* mutation, and resistance factor (R-factor) were performed to confirm the genotypes of *S. typhimurium* TA98 and TA100. *Salmonella* cells were grown in nutrient broth (Difco Laboratories Inc., Detroit, MI) at 37°C overnight. The minimal glucose agar medium used for the counting of His⁺ revertants of *S. typhimurium* has been described previously (Maron and Ames, 1983).

Preparation of Casein Hydrolysates

Lactobacillus delbrueckii ssp. *lactis* CRL 581, grown in MDM, were harvested by centrifugation (10,000 \times *g* for 10 min at 4°C) at the exponential growth phase, washed twice with 0.85% (wt/vol) saline supplemented with 10 mM CaCl₂, and resuspended in 100 mM sodium phosphate buffer (pH 7.0) to obtain a final optical density at 560 nm (OD₅₆₀) of approximately 10. This suspension was allowed to utilize the residual intracellular AA for 30 min at 40°C before casein degradation, which was carried out as described previously (Espeche Turbay et al., 2009). Briefly, washed whole cells (OD₅₆₀ = 10) were mixed with 5 mg of substrate/mL dissolved in 100 mM sodium phosphate buffer (pH 7.0) at a ratio of 1:1. As substrates, α _{S1}- and β -CN (Sigma Chemical Co., St. Louis, MO) were used. The resulting mixtures were incubated at 40°C during various time intervals (0.25, 0.5, 1, 2, and 4 h); then, the samples were centrifuged (10,000 \times *g* for 10 min at 4°C) and the supernatants were lyophilized and resuspended (1.5 mg/mL) in PBS (pH 6.8). These casein hydrolysates were used both for the antimutagenic assays and in vivo studies using an animal model of inflammation induced with TNBS. The hydrolysis of α _{S1}- and β -casein by the cell envelope-associated proteinase of *L. delbrueckii* ssp. *lactis* CRL 581 was confirmed by SDS-PAGE analysis as described previously (Hebert et al., 2008). Either Coomassie brilliant blue R-250 or silver staining (Bio-Rad Laboratories Inc., Richmond, CA) was used to visualize the proteins after SDS-PAGE.

Toxicity Test

Aliquots of 0.1 mL (1.5 mg/mL) of the α _{S1}- and β -CN hydrolysates or their vehicle (PBS, negative control) were combined with 0.1 mL of activated culture of *S. typhimurium* TA98 and TA100 (approximately 10⁸ cfu/

mL). The mixture was incubated with agitation at 37°C for 30 min. Serial dilutions were made with phosphate buffer and then 0.1 mL of the aliquot was plated onto previously prepared Petri dishes containing 25 mL of minimal glucose agar and incubated at 37°C for 48 h. After this period, the number of revertant colonies was counted and a toxicity effect on the *Salmonella* strains was confirmed if the viable count of the test sample was determined to be significantly lower than that of the negative control.

Mutagenic and Antimutagenic Tests

A direct-acting mutagen, 4-nitroquinoline-*N*-oxide (4-NQO, Sigma-Aldrich Co., St. Louis, MO), dissolved in dimethyl sulfoxide at a concentration of 0.5 µg/mL (Hsieh and Chou, 2006), was used for the mutagenic and antimutagenic tests of the casein hydrolysates using *S. typhimurium* TA98 and TA100 cells as described previously (Maron and Ames, 1983)

For the antimutagenic activity test, the pre-incubation method was followed (Maron and Ames, 1983). Briefly, 100 µL of each fraction being tested, 50 µL of mutagen solution, 100 µL of an overnight culture of *S. typhimurium* TA98 or TA100, and 0.5 mL of a 0.2 M sodium phosphate buffer (pH 7.0) were mixed and preincubated at 37°C for 30 min. Then, 3 mL of molten top agar containing histidine and biotin were added and the mixture was poured on a minimal glucose agar medium. His⁺ revertant colonies were counted after incubation at 37°C for 48 h in the dark. The antimutagenic activity was expressed as the percentage inhibition of mutagenesis as follows: inhibition (%) = 100 × [(A - B)/(A - C)], where A is the number of mutagen-induced revertants in the absence of a sample, B is the number of mutagen-induced revertants in the presence of a sample, and C is the number of spontaneous revertants in the absence of a mutagen (Rhee and Park, 2001; Saito et al., 2006). For the mutagenic assays of caseins and casein hydrolysates, 50 µL of distilled water was used instead of the mutagen solution, following the conditions described above for the antimutagenicity test. Each sample was assayed using triplicate plates and each experiment was assayed 3 times.

TNBS Induction of Acute Colitis

Intestinal inflammation was induced as described previously (de Moreno de LeBlanc et al., 2009). Briefly, mice were anesthetized by intraperitoneal injection of a mix of 100 mg/kg of BW of ketamine hydrochloride (Holliday-Scott S.A., Buenos Aires, Argentina) and 5 mg/kg of BW of xylazine hydrochloride (Rompun; Bayer, Division Sanidad Animal, Buenos Aires, Argentina).

2,4,6-Trinitrobenzene sulfonic acid (Sigma Chemical Co.) was dissolved in 0.01 M PBS, pH 7.4 containing 50% (vol/vol) ethanol to a final concentration of 20 mg/mL, and 0.1 mL (~75 mg/kg) was administered intrarectally using a catheter of 4 cm. Control group mice were inoculated with PBS containing 50% ethanol.

Animal Model of Intestinal Inflammation

Five week-old female BALB/c mice weighing 24 to 28 g, obtained from the closed random-bred colony maintained at CERELA, were used to induce the intestinal inflammation with TNBS. They were randomly assigned to 4 experimental groups: group 1 (TNBS group) mice received PBS daily for 10 consecutive days before the inoculation with TNBS to induce inflammation and then received PBS again daily for 7 d; group 2 (hydrolysate-TNBS group) mice received β-CN hydrolysate daily for 10 consecutive days before TNBS inoculation and then received PBS daily for 7 d; group 3 (control group) mice received PBS daily for 10 consecutive days and then were inoculated with the TNBS vehicle (PBS in ethanol 50% without TNBS), and finally received PBS again daily for 7 d; and group 4 (hydrolysate control group) mice received β-CN hydrolysate during the whole experiment but without TNBS inoculation. The β-CN hydrolysate (150 µg/d; LeBlanc et al., 2002) and PBS were orally administered (100 µL). Mice were group-housed (5/cage), maintained in a room with a 12-h light/dark cycle at 18 ± 2°C and had free access to water and standard rodent chow. Groups of 10 mice were used in each experiment. Body weight and animal mortality were controlled every day until the end of the experiment. Mortality rates were expressed as the percentile of deaths on the initial (total) amount of animals in each individual experimental group. All animal protocols were preapproved by the CERELA Animal Protection Committee and all experiments complied with the current laws of Argentina.

Sampling Procedures and Colon Histology

Samples were obtained from each group on d 0 (basal sample just before TNBS inoculation) and 3 and 7 d after TNBS inoculation. Three mice of each group were killed at each sampling period, and the experiments were repeated 3 times. Large intestines and ceca were removed, visually inspected for macroscopic evaluation, and prepared for histological evaluation using standard methods (Sainte-Marie, 1962). Serial paraffin sections of 4 µm from the large intestine tissues were made and stained with hematoxylin-eosin for light microscopy examination. For the colon histology, the microscope slides were blind examined by 2 researchers and the

extent of damage and colonic inflammation was scored according to the criteria defined previously by Ameho et al. (1997) as follows: colitis grade 0: histological findings identical to normal mice; colitis grade 1: mild mucosal or submucosal inflammatory infiltrate (admixture of neutrophils) and edema, punctate mucosal erosions often associated with capillary proliferation, and muscularis mucosae intact; colitis grade 2: grade 1 changes involving 50% of the specimen; colitis grade 3: prominent inflammatory infiltrate and edema (neutrophils usually predominating) frequently with deeper areas of ulceration extending through the muscularis mucosae into the submucosa and rare inflammatory cells invading the muscularis propriae but without muscle necrosis; colitis grade 4: grade 3 changes involving 50% of the specimen; colitis grade 5: extensive ulceration with coagulative necrosis bordered inferiorly by numerous neutrophils and lesser numbers of mononuclear cells and necrosis that extends deeply into the muscularis propria; and colitis grade 6: grade 5 changes involving 50% of the specimen.

Bacterial Translocation

The liver was aseptically removed, weighed, and homogenized in 5.0 mL of sterile 0.1% (wt/vol) peptone solution. Serial dilutions of the homogenate were plated in triplicate in the following media: de Man, Rogosa, and Sharpe (MRS) agar; MacConkey agar; and brain-heart infusion agar to detect a wide range of microorganisms. Bacterial growth was analyzed after incubation of the plates at 37°C for 48 to 72 h.

β -Glucuronidase Activity Assay

The cecum content (0.2 g) was suspended in 300 μ L of potassium phosphate buffer (0.1 M, pH 7.0), centrifuged at $9,000 \times g$ for 15 min at 4°C and the supernatant was used to determine the β -glucuronidase activity as described by Goldin et al. (1980). The reaction mixture (200 μ L), consisting of 0.02 M potassium phosphate buffer, 0.1 mM EDTA, 1 mM phenolphthalein-glucuronic acid (Sigma Chemical Co.), and 20 μ L of intestinal sample, was incubated at 37°C for 15 min. The reaction was stopped by addition of 1 mL of 0.2 M glycine buffer (pH 10.4) containing 0.2 M NaCl. Absorbance was read at 540 nm using a VERSA Max Microplate Reader (Molecular Devices LLC, Sunnyvale, CA). The amount of released phenolphthalein was calculated from a standard curve. One unit of enzyme was defined as the amount of enzyme necessary to release 1 μ g of phenolphthalein at 37°C in 1 min. Specific activity was expressed as units of enzyme per milligram of protein. The protein concentration was determined by using a

protein assay according to the manufacturer's instructions (Bio-Rad Laboratories Inc.).

Myeloperoxidase Activity Assay

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was determined by an *o*-dianisidine- H_2O_2 method (Bradley et al., 1982). Briefly, samples of large intestine were weighed and homogenized in 0.5% hexadecyl-trimethylammonium bromide (HTAB; Sigma Chemical Co.) in 50 mM potassium phosphate buffer (pH 6.0) before sonication for 10 s in an ice bath. The homogenates were freeze-thawed 3 times repeating the sonication and then centrifuged at $20,000 \times g$ for 15 min at 4°C; the resulting pellet and supernatant were analyzed. The MPO activity level was determined spectrophotometrically: 0.1 mL of the sample was mixed with 2.9 mL of 50 mM potassium phosphate buffer (pH 6.0) containing 0.167 mg of *o*-dianisidine hydrochloride/mL and 0.0005% (vol/vol) H_2O_2 . Absorbance was measured at 460 nm (Cary 50; Varian Inc., Middelburg, the Netherlands). One unit of MPO was defined as the amount of enzyme degrading 1 μ mol of H_2O_2 per minute at 20°C and was expressed in units per gram of wet tissue. Units of MPO activity per minute were calculated from a standard curve using purified peroxidase enzyme (Sigma Chemical Co.). Specific activity was expressed as units of enzyme per milligram of protein. The protein concentration was determined by using a protein assay according to the manufacturer's instructions (Bio-Rad Laboratories Inc.).

Determination of Cytokines in Intestinal Fluids

Intestinal fluids were collected from the large intestines of mice, previous to the histological fixation, with 1 mL of PBS and immediately centrifuged at $5,000 \times g$ for 15 min at 4°C. The supernatants were recovered and stored at -20°C until cytokine determinations. BD OptEIA mouse cytokine ELISA kits from BD Biosciences Pharmingen (San Diego, CA) were used to determine the concentration of the cytokines (IFN γ and IL-10). The results were expressed as concentration of each cytokine in the intestinal fluid (pg/mL).

Statistical Analysis

Statistical analyses were performed with the software package Minitab 14 (Minitab Inc., State College PA) using ANOVA general linear models, followed by a Tukey post-hoc test, and $P < 0.05$ was considered significant. Unless otherwise indicated, all values ($n = 10$) were the means of 3 independent trials \pm standard

deviation. No significant differences were observed between individual replicates.

RESULTS

Mutagenic and Antimutagenic Activity of Casein Hydrolysates

Toxicity test results showed that the survival of *S. typhimurium* TA98 and TA100 in the presence of either casein or casein hydrolysates was close to that of the controls (data not shown), indicating that these fractions were not toxic toward the tested *Salmonella* strains. On the other hand, the number of revertants in the presence of either casein hydrolysates or non-hydrolyzed α - and β -casein for *S. typhimurium* TA98 and TA100 were close to that observed for the negative control (spontaneous revertants; Table 1). These data indicated that up to 150 μ g of caseins and their hydrolysates were neither toxic nor mutagenic to *S. typhimurium* TA98 and TA100.

The antimutagenic activity of the α - and β -CN hydrolysates, generated by *L. delbrueckii* ssp. *lactis* CRL 581 after 0.25, 0.5, 2, and 4 h of incubation, against 4-NQO on *S. typhimurium* TA98 and TA100, is shown in Table 2. A slight antimutagenic effect against 4-NQO was observed after 15 min of casein hydrolysis: 4% inhibition for the α -CN hydrolysate on *S. typhimurium* TA98 and 3 and 12% inhibition for the β -CN hydrolysate on TA98 and TA100 strains, respectively. The ability of α - and β -CN hydrolysates to suppress the mutagenesis of 4-NQO increased concomitantly with the time of hydrolysis until 4 h of incubation (Table 2). Previously, we observed that the relative amounts of the cleavage products derived from α - and β -CN changed significantly during the course of incubation (from 0 to 4 h), as several degradation bands could be visualized (Hebert et al., 2008). These proteins were completely digested after 4 h of degradation, corresponding with the highest activity against the mutagenesis induced by 4-NQO (Table 2).

Effect of β -CN Hydrolysate in Mouse TNBS-Induced Colitis: Analysis of Mortality, BW, and Intestinal Histology

All animals that received a TNBS inoculation showed a significant decrease in BW after 3 d of inoculation compared with the control animals (Figure 1). However, the administration of β -CN hydrolysate previous to TNBS attenuated the deleterious weight changes compared with those of the TNBS group at d 7 post-TNBS inoculation (Figure 1). Moreover, animals that received β -CN hydrolysate before TNBS inoculation showed a lower mortality rate ($22 \pm 2\%$) compared with those from the TNBS group ($35 \pm 5\%$).

Mice treated with TNBS showed severe macroscopic and microscopic damage, which included a loss of crypts, necrosis, and focal influx of inflammatory cells in the mucosa and submucosa (Figure 2 and Table 3). Premature death resulted mainly from an excessive inflammatory reaction, as assessed by postmortem autopsy and, considering the histology and other parameters studied in the mice killed during the study, this group should have had a greater mortality rate.

The mice that received exclusively the vehicle as well as the hydrolysate control group had similar macroscopic and microscopic appearance of their colons compared with the normal mice (Figure 2) and maintained their BW during the whole experiment (Figure 1). A small number of these animals had mucosal or submucosal infiltrates in focalized regions of the colon.

The animals that received 10 d of β -CN hydrolysate before the induction of inflammation showed, in the samples taken at d 3 and 7 post-TNBS inoculation, significantly lower damage scores compared with the TNBS group (Table 3 and Figure 2).

Microbial Translocation to the Liver

Mice from the TNBS group increased significantly the number of cfu/g in the 3 growth media assayed [MacConkey; brain-heart infusion; and de Man, Rogosa,

Table 1. Mutagenic effect induced by α - and β -CN and their hydrolysates against *Salmonella typhimurium* TA98 and TA100

Sample	Revertants (cfu/plate) ¹	
	<i>S. typhimurium</i> TA98	<i>S. typhimurium</i> TA100
Control (distilled water) ²	280 \pm 25	120 \pm 12
α -CN (150 μ g)	256 \pm 18	123 \pm 11
α -CN hydrolyzed (150 μ g) ³	253 \pm 19	115 \pm 13
β -CN (150 μ g)	265 \pm 23	132 \pm 12
β -CN hydrolyzed (150 μ g) ³	268 \pm 25	111 \pm 9

¹Results are presented as means \pm standard deviations from at least 3 separate experiments.

²Spontaneous revertants.

³ α - and β -CN were hydrolyzed by *Lactobacillus delbrueckii* ssp. *lactis* CRL 581 for 4 h at 40°C.

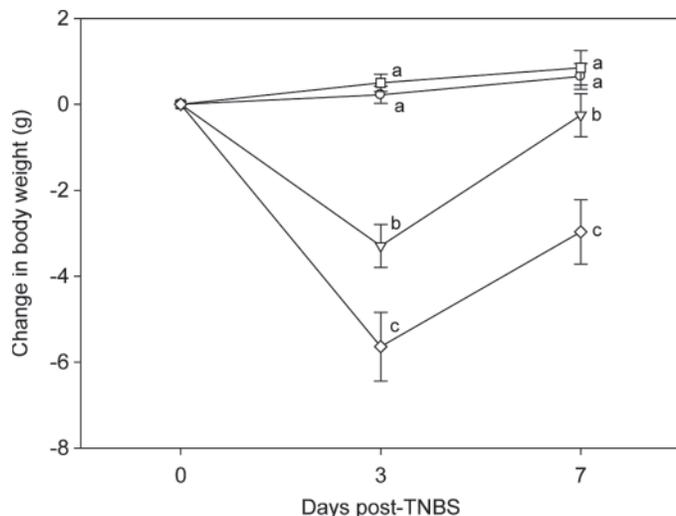


Figure 1. Changes in the BW of animals from the control group (○), hydrolysate control group (□), hydrolysate-trinitrobenzene sulfonic acid (TNBS) group (▽), and TNBS group (◇). Each point represents the average \pm standard deviation of changes in the BW data from 30 mice (10 mice from each separate trial). Means at each time point without a common letter (a–c) differ significantly ($P < 0.05$).

and Sharpe (MRS)] compared with the mice inoculated with only alcohol (Figure 3). However, animals that received β -CN hydrolysate for 10 d before TNBS inoculation had significantly decreased microbial growth in the liver compared with the TNBS group in both samples (3 and 7 d post-TNBS inoculation, Figure 3).

Enzymatic Activities in the Large Intestine

β -Glucuronidase activity increased significantly 3 d after TNBS inoculation in both groups of mice that re-

ceived this inflammatory agent compared with control groups, with this activity being significantly lower in the animals from the hydrolysate-TNBS group compared with the TNBS group. Seven days post-TNBS inoculation, β -glucuronidase activity decreased significantly in the hydrolysate-TNBS and TNBS groups compared with their values obtained in the previous sample (3 d), but only the mice from the group administered β -CN hydrolysate before TNBS had these values decreased to the basal level (Figure 4).

The MPO activity, an inflammatory marker, significantly increased at 3 and 7 d post-TNBS inoculation in the TNBS group mice compared with the basal data (Figure 5). The administration of β -CN hydrolysate prior to TNBS inoculation decreased MPO activity compared with the TNBS group, with the values reached at 7 d post-TNBS being similar to those obtained in the control groups (Figure 5).

Cytokine Profiles

The concentration of IFN- γ in the large intestine fluid of mice from the TNBS group was significantly higher in samples taken at d 3 and 7 post-TNBS inoculation compared with the control groups (Figure 6A). The animals that received the β -CN hydrolysate prior to TNBS injection showed decreased levels of this cytokine compared with the TNBS group at d 3 and 7 post-TNBS; with the levels of this cytokine being similar to those in the control groups (Figure 6A).

The analysis of IL-10 levels in the large intestine fluids showed that the administration of the hydrolysate without another stimulus (hydrolysate control group) maintained increased levels of this cytokine compared

Table 2. Antimutagenic activity of α - and β -CN hydrolysates against 4-nitroquinoline-*N*-oxide (4-NQO) on *Salmonella typhimurium* TA98 and TA100

Sample ¹	Inhibition ratio ² (%)	
	<i>S. typhimurium</i> TA98	<i>S. typhimurium</i> TA100
α -CN (control)	ND ³	ND
α -CN hydrolyzed (0.25 h)	4 \pm 0.6	ND
α -CN hydrolyzed (0.5 h)	51 \pm 4.5	42 \pm 3.3
α -CN hydrolyzed (2 h)	59 \pm 5.1	56 \pm 4.2
α -CN hydrolyzed (4 h)	66 \pm 5.4	71 \pm 6.5
β -CN (control)	ND	ND
β -CN hydrolyzed (0.25 h)	3 \pm 0.5	12 \pm 0.9
β -CN hydrolyzed (0.5 h)	11 \pm 0.9	46 \pm 4.3
β -CN hydrolyzed (2 h)	58 \pm 5.1	53 \pm 3.9
β -CN hydrolyzed (4 h)	71 \pm 5.9	82 \pm 4.3

¹The samples consisted of 150 μ g of each fraction. The caseins were degraded by *Lactobacillus delbrueckii* ssp. *lactis* CRL 581 for 0 (control), 0.25, 0.5, 2, and 4 h (these times are indicated in parentheses).

²Inhibition ratio (%) = $100 \times [(A - B)/(A - C)]$, where A is the number of mutagen-induced revertants in the absence of a sample, B is the number of mutagen-induced revertants in the presence of a sample, and C is the number of spontaneous revertants in the absence of a mutagen. Results are presented as means \pm standard deviations from at least 3 separate experiments.

³Not detected in the assay conditions.

Table 3. Mean histological damage score of colon sections of mice that were untreated (hydrolysate control group), administered with alcohol (control group), trinitrobenzene sulfonic acid (TNBS) alone (TNBS group), or with β -CN hydrolysate before the TNBS inoculation (hydrolysate-TNBS group)

Group	Days post-TNBS ¹	
	3	7
Hydrolysate control	0.30 \pm 0.20 ^a	0.30 \pm 0.20 ^a
Control	1.33 \pm 0.58 ^b	0.50 \pm 0.35 ^a
TNBS	4.67 \pm 0.58 ^c	3.50 \pm 0.50 ^c
Hydrolysate-TNBS	2.17 \pm 0.75 ^b	1.77 \pm 0.55 ^b

^{a-c}Means within a column with different superscripts differ ($P < 0.05$).

¹Each value represents the mean \pm standard deviation.

with the control group in both samples studied (Figure 6B). The animals from the TNBS group maintained the concentration of IL-10 similar to the hydrolysate

control group but significantly higher than the control group. It is important to note the significant increase observed in the mice that received β -CN hydrolysate before TNBS in the first samples taken 3 d post-TNBS inoculation compared with the other test and control groups.

DISCUSSION

Bioactive peptides derived from casein are of particular interest in food science and nutrition because they have been shown to play physiological roles, including opioid-like features, as well as immunostimulating and anti-hypertensive activities, and the ability to enhance calcium absorption (Silva and Malcata, 2005; Hebert et al., 2010). Bioactive peptides are hidden in a latent state within the primary sequences of casein and whey pro-

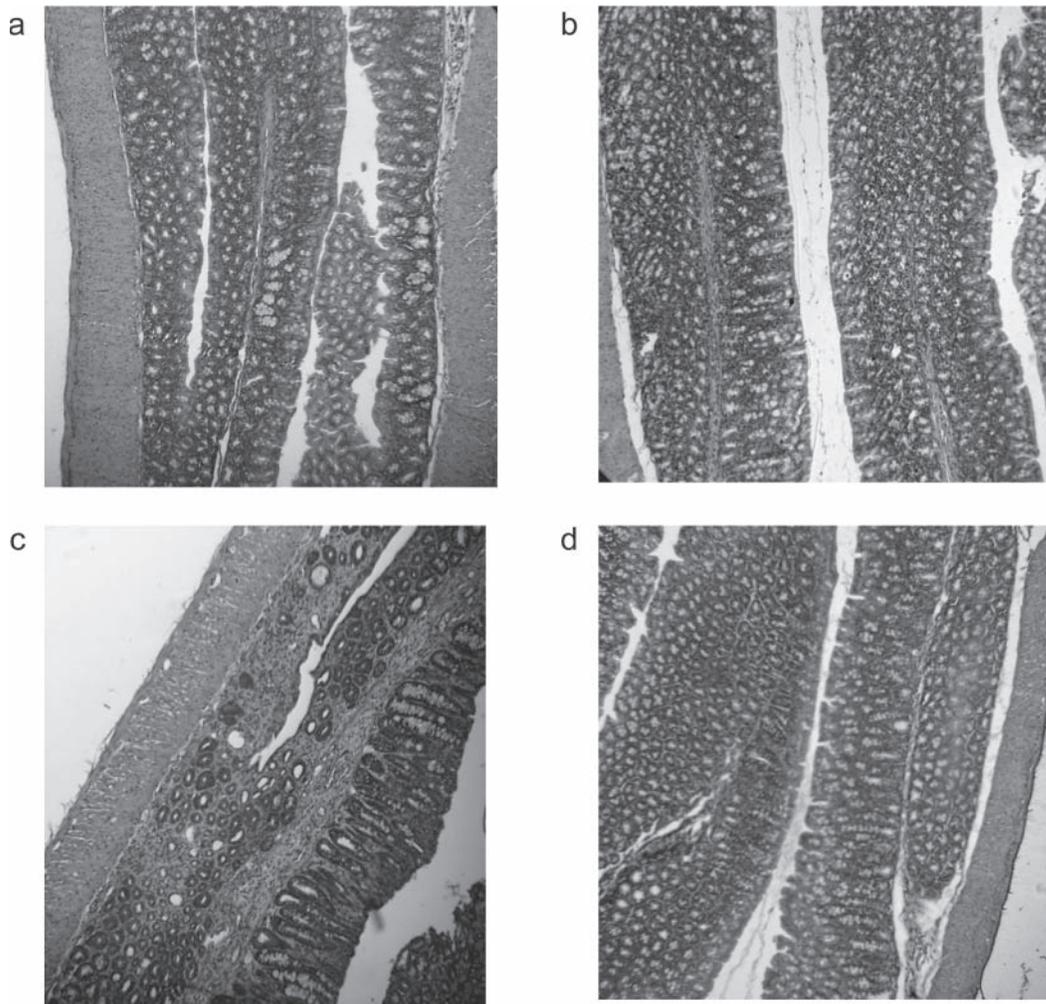


Figure 2. Representative hematoxylin-eosin-stained paraffin-wax-embedded sections of the colons of mice from the control group (a), hydrolysate control group (b), trinitrobenzene sulfonic acid (TNBS) group (c), and hydrolysate-TNBS group (d) at 3 d post-TNBS inoculation as observed at 100 \times magnification.

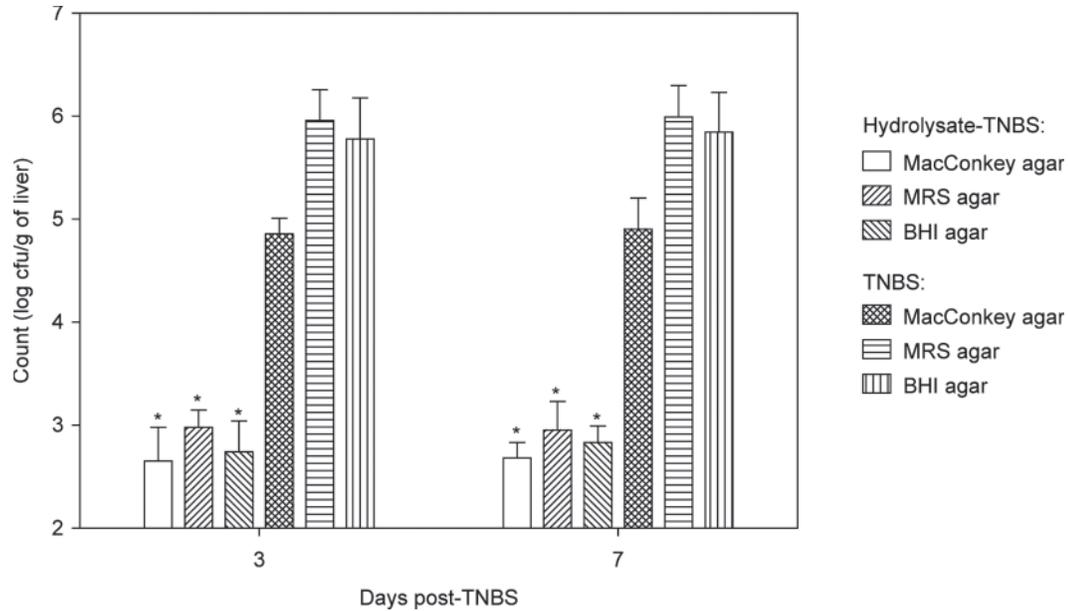


Figure 3. Microbial growth of liver samples from the hydrolysate-trinitrobenzene sulfonic acid (TNBS) and TNBS groups in MacConkey agar; de Man, Rogosa, and Sharpe (MRS) agar; and brain-heart infusion (BHI) agar. The control group was not represented because no growth was observed in the scales presented in this figure. Asterisks indicate that the value is different from the value for the TNBS group ($P < 0.05$). Error bars indicate the standard deviation.

teins, requiring enzymatic proteolysis for their release. Thus, peptides with physiological effects are largely found in fermented milks and cheeses. The proteolytic system of *L. delbrueckii* ssp. *lactis* CRL 581, a starter

culture in the manufacture of fermented milks and cheeses, contributes to the release of bioactive peptides or their precursors. The present study demonstrated that the peptides released from α - and β -CN exhib-

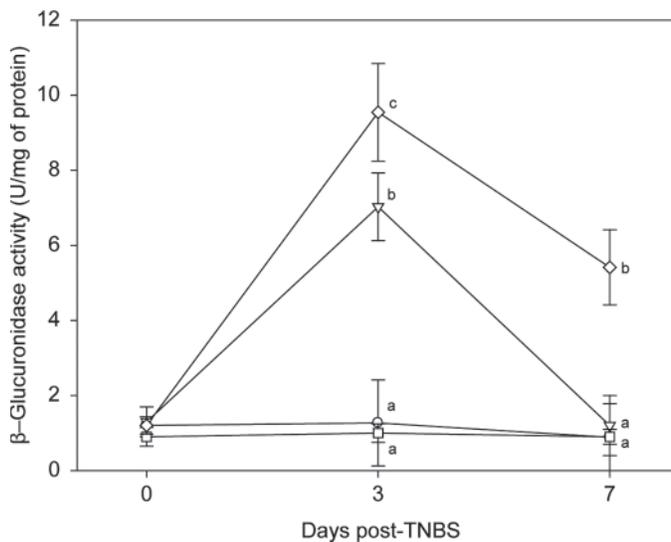


Figure 4. β -Glucuronidase activity in the large intestine content of the control group (○), hydrolysate control group (□), hydrolysate-trinitrobenzene sulfonic acid (TNBS) group (◇), and TNBS group (▽) mice. Each point represents the average \pm standard deviation of β -glucuronidase activity data from 15 mice (5 mice from each separate trial). Means at each time point without a common letter (a-c) differ significantly ($P < 0.05$).

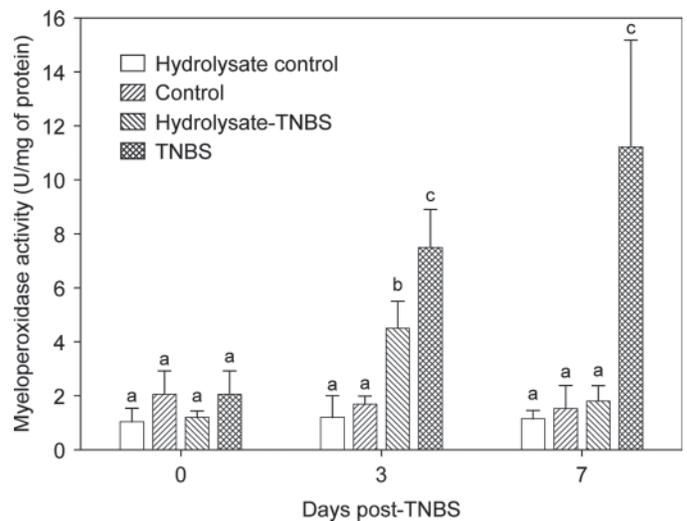


Figure 5. Myeloperoxidase activity in the large intestine content of the hydrolysate control group, control group, hydrolysate-trinitrobenzene sulfonic acid (TNBS) group, and TNBS group mice. Each point represents the average \pm standard deviation of myeloperoxidase activity data from 15 mice (5 mice from each separate trial). Means for each time point without a common letter (a-c) differ significantly ($P < 0.05$).

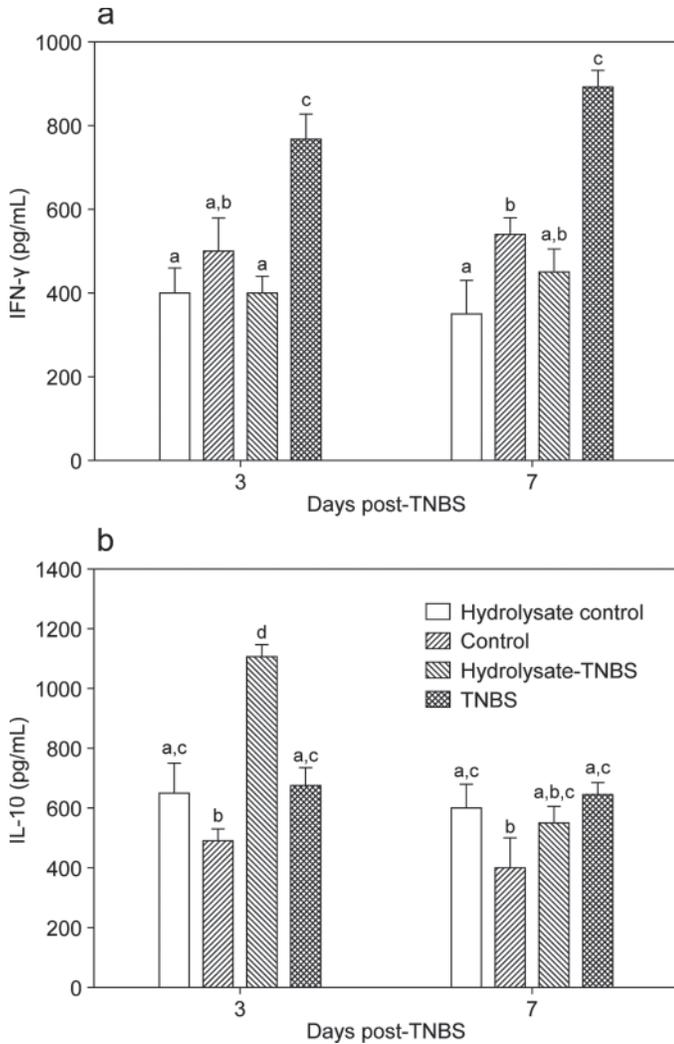


Figure 6. Interferon γ (a) and IL-10 (b) concentrations in the contents of the large intestines of the hydrolysate control group, control group, hydrolysate-trinitrobenzene sulfonic acid (TNBS) group, and TNBS group mice. Means for each time point without a common letter (a–d) differ significantly ($P < 0.05$). Error bars indicate the standard deviation.

ited antimutagenic properties in vitro. Evidence that β -CN hydrolysate generated from *L. delbrueckii* ssp. *lactis* CRL 581 have anti-inflammatory properties has emerged from the experimental murine TNBS-induced colitis model (te Velde et al., 2006; Izcue et al., 2009). The exact causes of IBD are still unknown; however, it is believed that chronic inflammation is a result of aberrant immune response and deregulated intestinal microbiota in genetically predisposed hosts (Strober et al., 2007). The association of chronic inflammation with several malignant diseases has also been extensively reported. This relationship could be mediated by cytokines or by reactive oxygen species generated

by inflammatory phagocytes that can cause injury to target cells, contributing to cancer development.

In the present work, the administration of β -CN hydrolysate generated by *L. delbrueckii* ssp. *lactis* CRL 581 showed a beneficial effect in the prevention of the inflammatory process. Mice that received the hydrolysate showed decreased mortality rates, BW loss, and microbial translocation to the liver compared with the TNBS group. These effects were not observed by using non-hydrolyzed β -CN instead of hydrolysate β -CN, demonstrating the preventive effect of this hydrolysate, which was administered 10 d previous to TNBS inoculation. It was also observed that this group of mice showed decreased colonic macroscopic and microscopic damage caused by the inflammatory agent (Table 3; Figure 2).

It is known that the development of spontaneous or chemically induced colitis is influenced by the intestinal microbiota. This is supported by the reduction or absence of intestinal inflammation in TNBS- or dextran sulfate sodium-induced models using antibiotic-treated and germ-free animals (Rath et al., 1999; Verdù et al., 2000; Chandran et al., 2003). The intestinal microbiota can produce toxic substances, such as endotoxins by gram-negative bacteria, and harmful enzymes, such as the procarcinogenic β -glucuronidase (Goldin et al., 1980). Trinitrobenzene sulfonic acid inoculation induced an increase in β -glucuronidase activity in the large intestine of the mice, which may promote the development of colitis. On the other hand, the administration of β -CN hydrolysate prior to TNBS inoculation decreased the intestinal β -glucuronidase activity values compared with the TNBS group, reaching the basal enzyme levels at d 7 post-TNBS inoculation.

Myeloperoxidase is an enzyme produced mainly by polymorphonuclear leukocytes and is a source of free radicals that contribute to oxidative damage and pathogenesis of IBD (Krawisz et al., 1984). After 3 d of TNBS inoculation, MPO activity increased significantly in the TNBS group compared with the control animals (Figure 5). This increase was significantly decreased by the preventative administration of β -CN hydrolysate, reaching a basal level in the sample taken 7 d post-TNBS inoculation. On the contrary, mice that received non-hydrolyzed β -CN before TNBS inoculation showed similar levels of MPO activity to the TNBS group (data not shown). Because MPO activity is a biochemical marker of neutrophil infiltration, this observation suggests that β -CN hydrolysate administered prior to the inflammatory drug exerted a protective effect on IBD by decreasing polymorphonuclear infiltration in the colonic tissue. The absence of increases in MPO activity in the large intestines from the hydrolysate control

group showed that the administration of these peptides by itself was safe and did not induce inflammatory cells infiltration.

Finally, considering that these differences in the infiltrating cells between the hydrolysate-TNBS and TNBS groups could be related to disequilibrium in the cytokine profile resulted from Th1-mediated inflammation (Strober et al., 2004), we preliminarily investigated the regulation on the release of 2 cytokines to the large intestinal fluid by the β -CN hydrolysate. Interferon γ was analyzed as a pro-inflammatory cytokine characteristic of the Th-1 immune response, which is increased in the TNBS-induced IBD model. The results showed that β -CN hydrolysate significantly decreased the release of IFN- γ after TNBS inoculation (Figure 6A) compared to the TNBS group, which maintained the release of this cytokine significantly increased in the intestinal fluid at both 3 and 7 d after TNBS induction. Interleukin 10 was another cytokine analyzed because it is the most important anti-inflammatory cytokine at the intestinal level. In our model, it was observed that both groups inoculated with TNBS had significantly increased IL-10 release to the intestinal fluid in the samples obtained 3 d after TNBS administration, compared with the control group. This is an expected immune response against the inflammatory agent, as was previously observed using a TNBS-induced inflammation model and a colon cancer model where the carcinogen induced an important inflammatory response in the intestinal tissue (de Moreno de LeBlanc et al., 2007; Chaves et al., 2011). The significant increases obtained for IL-10 in the samples taken 3 d after TNBS inoculation in the group that received the β -CN hydrolysate are related to the decrease observed in IFN- γ levels and the anti-inflammatory effect of this hydrolysate. At d 7 post-TNBS induction, the IL-10 levels were similar in the hydrolysate-TNBS and TNBS groups (Figure 6). This decrease in IL-10 concentration in the mice that received β -CN hydrolysate is in accordance with the inflammation decrease, showing the importance of the early release of this anti-inflammatory cytokine to prevent the inflammatory damage caused by the drug. It is also noteworthy that the administration of β -CN hydrolysate in healthy mice (hydrolysate control group) maintained an anti-inflammatory balance between both cytokines analyzed, which could be related to their protective effect against IBD.

Our experimental results showed the antimutagenic effect of α - and β -CN hydrolysates generated by *L. delbrueckii* ssp. *lactis* CRL 581 and the correlation of this in vitro result with the anti-inflammatory effect of the β -CN hydrolysate in a TNBS-induced colitis mouse model. The administration of the hydrolysate to the mice decreased the macroscopic and microscopic

damage observed in the inflammation model through beneficial changes in the intestinal microbiota enzyme activity and by modulating the balance of pro- and anti-inflammatory cytokine production. These anti-inflammatory effects were not observed by using non-hydrolyzed β -CN instead of β -CN hydrolysate (data not shown), demonstrating the beneficial health effects of the peptides released by *L. delbrueckii* ssp. *lactis* CRL 581 from β -CN. Bioactive peptides generated by milk protein proteolysis can either produce local effects in the gastrointestinal tract or be absorbed through the intestine to enter intact into the blood circulation and exert systemic effects (Hebert et al., 2010). The results obtained in this work indirectly support the concept that some beneficial actions of the milk-derived peptides are the result of local action.

In this paper, we scientifically demonstrated that casein hydrolysates, generated by milk fermentation with *L. delbrueckii* ssp. *lactis* CRL 581, contribute with a variety of bioactive compounds to a positive effect on human health, specifically antimutagenic and anti-inflammatory properties. The powerful proteolytic system of this LAB opens up future opportunities to develop novel functional food with potential health-promoting properties.

ACKNOWLEDGMENTS

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, San Miguel de Tucumán, Argentina), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, Buenos Aires, Argentina), and Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT, Tucumán, Argentina).

REFERENCES

- Ameho, C. K., A. A. Adjei, E. K. Harrison, K. Takeshita, T. Morioka, Y. Arakaki, E. Ito, I. Suzuki, A. D. Kulkarni, A. Kawajiri, and S. Yamamoto. 1997. Prophylactic effect of dietary glutamine supplementation on interleukin 8 and tumour necrosis factor α production in trinitrobenzene sulphonic acid induced colitis. *Gut* 41:487–493.
- Bradley, P. P., R. D. Christensen, and G. Rothstein. 1982. Cellular and extracellular myeloperoxidase in pyogenic inflammation. *Blood* 60:618–622.
- Chandran, P., S. Satthaporn, A. Robins, and O. Eremin. 2003. Inflammatory bowel disease: Dysfunction of GALT and gut bacterial flora (I). *Surgeon* 1:63–75.
- Chaves, S., G. Perdigon, and A. de Moreno de LeBlanc. 2011. Yoghurt consumption regulates the immune cells implicated in acute intestinal inflammation and prevents the recurrence of the inflammatory process in a mouse model. *J. Food Prot.* 74:801–811.
- de Moreno de LeBlanc, A., S. Chaves, and G. Perdigon. 2009. Effect of yoghurt on the cytokine profile using a murine model of intestinal inflammation. *Eur. J. Inflamm.* 7:97–109.
- de Moreno de LeBlanc, A., C. Matar, and G. Perdigon. 2007. The application of probiotics in cancer. *Br. J. Nutr.* 98:S105–S110.

- Espeche Turbay, M. B., G. Savoy de Giori, and E. M. Hebert. 2009. Release of the cell-envelope-associated proteinase of *Lactobacillus delbrueckii* subspecies *lactis* CRL 581 is dependent upon pH and temperature. *J. Agric. Food Chem.* 57:8607–8611.
- Goldin, B. R., L. Swenson, J. Dwyer, M. Sexton, and S. L. Gorbach. 1980. Effect of diet and *Lactobacillus acidophilus* supplements on human fecal bacterial enzymes. *J. Natl. Cancer Inst.* 64:255–261.
- Hayes, M., R. P. Ross, G. F. Fitzgerald, and C. Stanton. 2007. Putting microbes to work: Dairy fermentation, cell factories and bioactive peptides. Part I: Overview. *Biotechnol. J.* 2:426–434.
- Hebert, E. M., G. Mamone, G. Picariello, R. R. Raya, G. Savoy, P. Ferranti, and F. Addeo. 2008. Characterization of the pattern of α_{s1} - and β -casein breakdown and release of a bioactive peptide by a cell envelope proteinase from *Lactobacillus delbrueckii* subsp. *lactis* CRL 581. *Appl. Environ. Microbiol.* 74:3682–3689.
- Hébert, E. M., R. Raya, and G. Savoy de Giori. 2000a. Use of SDS-PAGE of cell-wall proteins for rapid differentiation of *Lactobacillus delbrueckii* ssp. *lactis* and *Lactobacillus helveticus*. *Biotechnol. Lett.* 22:1003–1006.
- Hebert, E. M., R. R. Raya, and G. S. De Giori. 2000b. Nutritional requirements and nitrogen-dependent regulation of proteinase activity of *Lactobacillus helveticus* CRL 1062. *Appl. Environ. Microbiol.* 66:5316–5321.
- Hébert, E. M., R. R. Raya, and G. S. de Giori. 2002. Modulation of the cell-surface proteinase activity of thermophilic lactobacilli by the peptide supply. *Curr. Microbiol.* 45:385–389.
- Hebert, E. M., L. Saavedra, and P. Ferranti. 2010. Bioactive peptides derived from casein and whey proteins. Pages 233–249 in *Biotechnology of Lactic Acid Bacteria: Novel Applications*. F. Mozzi, R. Raya, and G. Vignolo, ed. Wiley-Blackwell, Ames, IA.
- Hsieh, M. L., and C. C. Chou. 2006. Mutagenicity and antimutagenic effect of soymilk fermented with lactic acid bacteria and bifidobacteria. *Int. J. Food Microbiol.* 111:43–47.
- Izcue, A., J. L. Coombes, and F. Powrie. 2009. Regulatory lymphocytes and intestinal inflammation. *Annu. Rev. Immunol.* 27:313–338.
- Krawisz, J. E., P. Sharon, and W. F. Stenson. 1984. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models. *Gastroenterology* 87:1344–1350.
- Law, J., and A. Haandrikman. 1997. Proteolytic enzymes of lactic acid bacteria. *Int. Dairy J.* 7:1–11.
- LeBlanc, J. G., C. Matar, J. C. Valdéz, J. LeBlanc, and G. Perdigon. 2002. Immunomodulating effects of peptidic fractions issued from milk fermented with *Lactobacillus helveticus*. *J. Dairy Sci.* 85:2733–2742.
- Maron, D. M., and B. N. Ames. 1983. Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.* 113:173–215.
- Matar, C., J. C. Valdez, M. Medina, M. Rachid, and G. Perdigon. 2001. Immunomodulating effects of milks fermented by *Lactobacillus helveticus* and its non-proteolytic variant. *J. Dairy Res.* 68:601–609.
- Rath, H. C., K. H. Wilson, and R. B. Sartor. 1999. Differential induction of colitis and gastritis in HLA-B27 transgenic rats selectively colonized with *Bacteroides vulgatus* or *Escherichia coli*. *Infect. Immun.* 67:2969–2974.
- Rhee, C. H., and H. D. Park. 2001. Three glycoproteins with anti-mutagenic activity identified in *Lactobacillus plantarum* KLAB21. *Appl. Environ. Microbiol.* 67:3445–3449.
- Sadat-Mekmene, L., M. Genay, D. Atlan, S. Lortal, and V. Gagnaire. 2011. Original features of cell-envelope proteinases of *Lactobacillus helveticus*. A review. *Int. J. Food Microbiol.* 146:1–13.
- Sainte-Marie, G. 1962. A paraffin embedding technique for studies employing immunofluorescence. *J. Histochem. Cytochem.* 10:250–256.
- Saito, J., Y. Sakai, and H. Nagase. 2006. In vitro anti-mutagenic effect of magnolol against direct and indirect mutagens. *Mutat. Res.* 609:68–73.
- Sandré, C., A. Gleizes, F. Forestier, R. Gorges-Kergot, S. Chilmonczyk, J. Leonil, M.-C. Moreau, and C. Labarre. 2001. A peptide derived from bovine β -casein modulates functional properties of bone marrow-derived macrophages from germfree and human flora-associated mice. *J. Nutr.* 131:2936–2942.
- Silva, S. V., and F. X. Malcata. 2005. Caseins as source of bioactive peptides. *Int. Dairy J.* 15:1–15.
- Strober, W., I. Fuss, M. Boirivant, and A. Kitani. 2004. Insights into the mechanism of oral tolerance derived from the study of models of mucosal inflammation. *Ann. N. Y. Acad. Sci.* 1029:115–131.
- Strober, W., I. Fuss, and P. Mannon. 2007. The fundamental basis of inflammatory bowel disease. *J. Clin. Invest.* 117:514–521.
- te Velde, A. A., M. I. Verstege, and D. W. Hommes. 2006. Critical appraisal of the current practice in murine TNBS-induced colitis. *Inflamm. Bowel Dis.* 12:995–999.
- Tellez, A., M. Corredig, L. Y. Brovko, and M. W. Griffiths. 2010. Characterization of immune-active peptides obtained from milk fermented by *Lactobacillus helveticus*. *J. Dairy Res.* 77:129–136.
- Thomas, T. D., and O. E. Mills. 1981. Proteolytic enzymes of starter bacteria. *Neth. Milk Dairy J.* 35:255–273.
- Verdù, E. F., P. Bercik, B. Cukrowska, M. A. Farre-Castany, H. Bouzourene, E. Saraga, A. L. Blum, I. Corthésy-Theulaz, H. Tlaskalova-Hogenova, and P. Michetti. 2000. Oral administration of antigens from intestinal flora anaerobic bacteria reduces the severity of experimental acute colitis in BALB/c mice. *Clin. Exp. Immunol.* 120:46–50.
- Wirtz, S., C. Neufert, B. Weigmann, and M. F. Neurath. 2007. Chemically induced mouse models of intestinal inflammation. *Nat. Protoc.* 2:541–546.