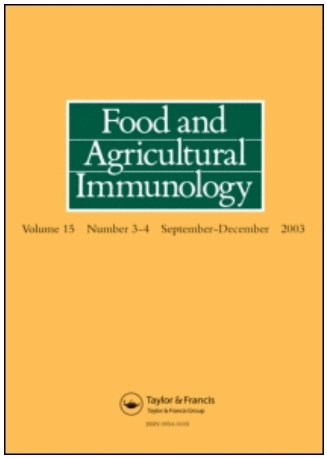

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Study of some of the mechanisms involved in the prevention against *Salmonella enteritidis* serovar Typhimurium infection by lactic acid bacteria

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The possible mechanism exerted by different lactic acid bacteria (LAB) in the protection against *Salmonella enteritidis* serovar Typhimurium (*S. typhimurium*) infection was determined. LAB was administered to BALB/c mice, and the animals were subsequently challenged with *S. typhimurium.* The inhibition of the translocation of *S. typhimurium* in the liver was correlated with a decrease in cellular apoptosis determined in slices from the small intestine of mice. The microbiocidal activity of peritoneal macrophages was increased by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, but not for the probiotic strain *L. casei* CRL 431. The levels of IFNγ and Bcl-2 positive cells in the small intestine of mice fed with the LAB were also determined by immunofluorescence. Using *in vivo* studies, we demonstrated that the biological and immune mechanisms induced by the LAB studied were different for each bacterium and were mediated by anti-*S. typhimurium* S-IgA microbiocidal activity and/or cellular apoptosis inhibition of infected immune cells.

Keywords: lactic acid bacteria; *Salmonella enteritidis* serovar Typhimurium infection; mechanisms; apoptosis

Introduction

The experimental infection of mice with *Salmonella enteritidis* serovar Typhimurium (*S. typhimurium*) provides a useful model of human fever caused by this pathogen (Raupach & Kaufmann, 2001). *Salmonella typhimurium* is a facultative intracellular pathogen that infects, replicates and persists in macrophages (Steele-Mortimer et al., 2002). After oral uptake, *S. typhimurium* reaches the small intestine where it preferentially interacts with the M cells of the Peyer's patches to reach the underlying tissue, and finds resident macrophages and dendritic cells intimately associated with the M cells (Guerrant, Steiner, Lima, & Bobak, 1999; Hersh et al., 1999). *Salmonella typhimurium* enters these professional phagocytes and resides in the resulting large membrane-bound vacuoles (Francis, Ryan, Jones, Smith, & Falkow, 1993), expressing several gene products that enhance intracellular survival by neutralising lysosomal killing mechanisms. This pathogen appears to be uncoupled from the

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main endocytic route, and after a lag period it replicates inside the host cell. The cells then die by apoptosis or lysis, releasing the bacteria to invade other cells (Finlay & Cossart, 1997). The ability of a pathogen to survive and even replicate within phagocytic cells is a potent method of evading the defense mechanisms of the host. Non-invasive and non-cytotoxic mutants are unable to induce apoptosis of macrophages, and *in vivo* infection is dramatically attenuated. Apoptosis may be an important step in the pathogenesis of *S. typhimurium* (Monack, Raupach, Hromockyj, & Falkow, 1996; van der Velden, Lindgren, Worley, & Heffron, 2000).

Live microbial supplements added to foods in order to beneficially affect the consumers are known as probiotics (Fuller 1989). A more recent definition given by the Foods and Agriculture Organization (FAO/WHO 2001) is: "Live microorganisms that when being administered in appropriate dose, they confer a benefit of health to the receiver".

Certain strains of lactic acid bacteria (LAB) are the microorganisms most commonly used as probiotics. In previous reports using mice as an experimental model, we demonstrated that the viable probiotic bacteria *Lactobacillus casei* CRL 431 administered orally for 2 days exerted a good adjuvant activity at the mucosal level. This activity provides protection against *S. typhimurium* infection and prevents its dissemination to organs, such as the liver and spleen, by producing high levels of secretory IgA specific for the pathogen (Perdigón, Alvarez, & Pesce de Ruiz Holgado, 1991; Perdigón, Alvarez, Medici, & Pesce de Ruiz Holgado, 1993; Alvarez, Gobbato, Bru, Pesce de Ruiz Holgado, & Perdigón, 1998).

It has been demonstrated that conventional yogurt containing *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* can stop an infection caused by *S. typhimurium* (De Simone et al., 1988; Gauffin & Perdigón, 2003).

It was demonstrated by *in vitro* assays that *L. delbrueckii* subsp. *bulgaricus* and *St. thermophilus* induced an apoptosis inhibition in macrophages infected with *S. typhimurium*, and that this inhibitory effect was not due to an increase in the uptake of LAB by macrophages (Valdez, Rachid, Gobbato, & Perdigón, 2001).

Other studies in our laboratory demonstrated the immunomodulating properties of certain LAB used in the food industry (Vintiñi et al., 2000) that make them suitable as oral adjuvants, and as an alternative method in the prevention of enteropathogens.

The present research was carried out to separately analyse the effect of the three LAB-*L. delbrueckii* subsp. *bulgaricus*, *L. casei* CRL 431 (probiotic bacteria) and *St. thermophilus*-in the prevention of *S. typhimurium* infection by *in vivo* assays, and to determine the immune mechanisms involved in such preventive effects, especially the inhibitory effect of the cellular apoptosis by *in vivo* assays induced by the pathogen in the infected cells. This enhances microbiocidal activity of the macrophages or the increase in the specific anti IgA against the pathogen.

Methods

Animals

Six-week-old BALB/c mice, weighing 25–30 g, were obtained from the random-bred colony kept in our department at the Institute of Microbiology. Each experimental and control group consisted of five mice per assay. Each assay was performed in

triplicate. All animal protocols were approved by the Animal Protection Committee of the Universidad Nacional de Tucumán. All experiments comply with the current laws of Argentina.

Bacterial strain and growth conditions

The *S. typhimurium* strain from the Bacteriology Department at the Institute of Microbiology was grown as described by Perdigón et al. (1993). Briefly, bacteria were grown in Luria-Bertani (LB) agar (Gibco, USA) for 6 h at 37°C, and then washed three times by centrifugation with sterile saline solution before use.

The LAB used were *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 423, *L. casei* CRL 431 and *St. thermophilus* CRL 412, from the CERELA culture collection (San Miguel de Tucumán, Argentina). *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. casei* were cultured for 8 h at 37°C in MRS broth (Man Rogosa Sharpe; Oxoid Ltd., USA) and *St. thermophilus* in LAPTg broth for 8 h at 37°C. The microorganisms were harvested by centrifugation at $5000 \times g$ for 10 min, washed three times with a sterile saline solution, and resuspended in 10% sterile non-fat milk (NFM) to reach a concentration of 10^9 colony-forming units per milliliter (CFU/ml).

Feeding and infection procedure

Each experimental group of mice were administered daily with LAB at 10^9 CFU/ml suspended in 10% sterile NFM and administered at 1% (v/v) in drinking water. The assays were performed in four groups of animals: (a) mice given *L. delbrueckii* subsp. *bulgaricus* CRL 423 during 7 consecutive days; (b) mice given *St. thermophilus* CRL 412 for 7 consecutive days; (c) mice given *L. casei* CRL 431 for 2 consecutive days; (d) mice given only NFM 10% (control group) for each period of feeding. All animals received a conventional balanced diet (23% protein, 6% raw fiber, 10% total minerals, 1.3% Ca, 0.8% P, 12% moisture and vitamins) *ad libitum*.

The different time periods of administration were selected due to a previous result when the optimal dose to enhance gut mucosal activity was determined (Vintiñi et al., 2000).

At the end of each feeding period, the mice were challenged with 20 LD_{50} (2×10^7 CFU/ml) of *S. typhimurium* in the smooth phase using an oral catheter. This dose allowed the control mice to survive for 16 days and the morbidity was 70%. For IFNγ and Bcl-2 studies, all the LAB assayed were administered for 2, 5 or 7 consecutive days at a concentration of 10^9 cells/day per mouse using the same conditions as previously detailed.

Colonisation assays

Animals were sacrificed by cervical dislocation on days 5 and 7 after the *S. typhimurium* challenge. The liver and small intestine were removed aseptically and the intestinal fluid was collected. The small intestine was prepared for histological procedure following the Sainte-Marie technique (1962) for apoptosis determination, and the intestinal fluid was used for specific S-IgA measurement.

The number of viable bacteria in the liver from individual animals was determined in the test and control groups. The organs were homogenised, and cell

suspensions were serially diluted and then plated on MacConkey agar plates. Culture conditions were described previously by Gauffin and Perdigón (2003). The number of CFU was counted after incubation for 48 h at 37°C. The identity of the isolated microorganisms from the white colony was verified by biochemical tests to identify *S. typhimurium*. Results were expressed as the mean of the number of CFU/g of liver.

*Determination of anti-*S. typhimurium *IgA antibodies in the intestinal fluid*

The anti-*S. typhimurium* antibodies present in the intestinal fluid of the small intestine in the test and control mice were measured by ELISA on the 5th and 7th days post-challenge. The procedure used for the collection of intestinal fluid and the ELISA test were carried out as described previously by Perdigón et al. (1991) using goat anti-mouse IgA (alpha-chain-specific) conjugated peroxidase (Sigma Chemical Co., St. Louis, MO, USA). Absorbance was measured at 493 nm. The kinetics of the enzyme-substrate reaction was extrapolated to 100 min.

Preparation of histological samples for apoptosis assay

The small intestine of 3 mice from each experimental group and from the control group were recovered on days 5 and 7 post-infection with *S. typhimurium*, and divided into three sections. The histological preparations were performed following Sainte-Marie technique for paraffin inclusion. Paraffin sections (4 μm) were cut from each tissue for apoptosis determination.

Apoptosis determination

In vivo *assays*

Apoptosis was evaluated for the presence of DNA breaks detected in the paraffin cuts of small intestine tissues using the Apoptosis Detection System kit, Fluorescein (Promega, Madison, WI, USA). The fragmented DNA of apoptotic cells was measured by incorporation of fluorescein-12-dUTP at 3′-OH ends of DNA using the enzyme Terminal deoxynucleotidyl Transferase, which forms a polymeric tail using the principle of the TUNEL assay (Gavrieli, Sherman, & Ben-Sasson, 1992). The fluorescein-12-dUTP-nick end labeled DNA was visualised directly by fluorescence microscopy. Cells were defined as apoptotic if the entire nuclear area of the cell was stained fluorescent.

Results are shown in the figures comparing the observed fluorescence for the control and test group.

Cell culture and isolation of macrophages

Microbiocidal activity

Peritoneal macrophages from the test and control mice (*ex vivo* assay) were obtained according to Valdez et al. (2001). Briefly, 10^6 cells/ml of peritoneal cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Gibco) and incubated in a Corning plate for 1 h at 37°C (5% CO_2).

After incubation, macrophages were washed with RPMI-1640 medium and infected with 10^7 CFU/ml of *S. typhimurium.* The bacteria/macrophages ratio

was 10:1. Following a 30-min incubation at 37°C, extracellular bacteria were removed by gentle washing with phosphate-buffered saline (PBS), pH: 7.4, and fresh RPMI medium containing 100 μg of gentamicin (Gm) per milliliter was added. Macrophage monolayers were incubated with RPMI supplemented with Gm for 90 min, then washed with RPMI lysed in 1% Triton X-100 for 10 min, and diluted with RPMI. Dilutions of the suspension were plated on McConkey agar plates.

The number of viable intracellular bacteria was measured after incubation for 48 h at 37°C.

Determination of IFNγ positive cells in the small intestine

This cytokine was determined in histological slices of the small intestine of mice fed for 2, 5 and 7 consecutive days with each LAB strain under study. Three mice and three samples from each experimental and control group were analysed using rabbit anti-mouse IFNγ (Peprotech Inc. Rocky Hill, NJ, USA) polyclonal antibodies and goat anti-rabbit antibody conjugated with fluorescein isothiocyanate (Jackson Immuno Research Labs. Inc.). The number of fluorescent cells was counted in 30 fields of vision at 1000 × magnification in the lamina propria of the small intestine. Results are expressed as the number of positive cells in 10 fields.

Measurement of Bcl-2 positive cells in the small intestine

The Bcl-2 positive cells were determined in histological sections of the small intestine of the test mice treated for 2, 5 and 7 days with each LAB under study. After removal of the paraffin and rehydration in a graded ethanol series, paraffin sections (4 μm) were incubated with a 1% blocking solution of BSA-Hank for 30 min. Hamster anti-mouse Bcl-2 monoclonal antibodies (diluted in saponin–HBSS) were applied to the sections for 75 min at room temperature. The sections were then treated with rabbit anti-hamster fluorescein isothiocyanate (Jackson Immuno Research Labs Inc.) for 45 min at room temperature, and washed in saponin–HBSS. The number of fluorescent cells was counted in the lamina propria of the small intestine in 30 fields at 1000 × magnification. Results are expressed as the number of positive cells in 10 fields of vision.

Statistical analysis

Data were expressed as the mean (M) of three independent experiments ± standard errors of the mean (SEM). The Student's *t*-test was used to calculate the statistical significance of the results compared with the control.

Results

Colonisation assays

On analysing the preventive effect of the oral administration of the LAB assayed against *S. typhimurium* infection, we noticed that the probiotic bacteria at the feeding periods assayed were effective since colonisation in the livers of the test groups was lower (about 50%) than in the control mice for both 5 and 7 days post-challenge. Figure 1 shows these results.

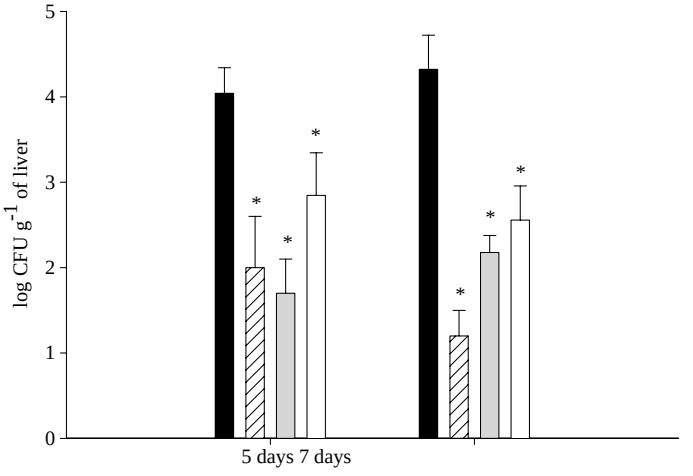

Figure 1. Effect of LAB administration on *Salmonella enteritidis* serovar Typhimurium colonisation. The CFU were determined in MacConkey agar using liver samples from different mice groups: control (black bars), mice fed for 2 days with *L. casei* CRL 431 (diagonal lines bars), mice fed for 7 days with *L. delbrueckii* subsp. *bulgaricus* (gray bars) and with *St. thermophilus* (white bars). All the groups were challenged with *S. typhimurium* and the samples were obtained 5 and 7 days post-challenge. *Significant differences between mice fed with different lactobacilli and the control group ($p < 0.05$).

*Determination of anti-*S. typhimurium *specific IgA*

In the determination of the specific anti-*Salmonella* S-IgA present in the intestinal fluid, we observed that *L. casei* CRL 431 induced an increase in the levels of IgA anti-*Salmonella* in comparison with the NFM controls 5 and 7 days post-challenge; this increase was more important on day 7 after the challenge (0.853 and 0.504 for *L. casei* and control groups, respectively). *Lactobacillus delbrueckii* subsp. *bulgaricus* only showed the highest absorbance values for 5 days after the challenge with respect to the control group, while for *St. thermophilus* the values were slightly enhanced on days 5 and 7 after the challenge compared with the control. These results are expressed in Figure 2.

Apoptosis in vivo *studies*

On analysing the inhibition of apoptosis of the immune cells, this study found that *S. typhimurium* induced an increase in the number of apoptotic cells in Peyer's patches and in the lamina propria of the small intestine in the control group (Figure 3a,b). The animals fed with *L. delbrueckii* subsp. *bulgaricus* and challenged with *S. typhimurium* showed a great inhibition of the cellular apoptosis (Figure 3c,d), while for *St. thermophilus* and *L. casei*, we found a similar number of apoptotic cells, the inhibitory effect was not marked in relation to the control group (Figure 3e).

Determination of the microbiocidal activity of peritoneal macrophages

The microbiocidal activity of peritoneal macrophages was analysed by *ex vivo* studies in the test and control mice. We found that *L. delbrueckii* subsp. *bulgaricus*

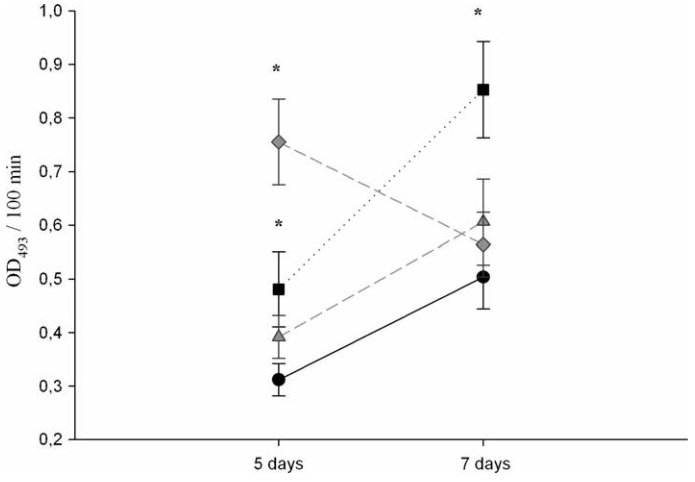

Figure 2. Determination of specific IgA anti-*Salmonella enteritidis s*erovar Typhimurium. The specific IgA anti-*S. typhimurium* were measured by ELISA test using the intestinal fluid from different mice groups: control (black circles and line), mice fed during 2 days with *L. casei* (black square and dotted line), mice fed for 7 days with *L. delbrueckii* subsp. *bulgaricus* (gray diamonds and broken line) and with *St. thermophilus* (gray triangles and broken line). Specific IgA was determined in samples obtained 5 and 7 days post-challenge with *S. typhimurium.*

and *St. thermophilus* were more effective in inducing this activity than *L. casei*, where no increase in microbiocidal activity was found with respect to the control values. Figure 4 shows these results.

Effect of LAB on Bcl-2 and IFNγ positive cells

The activation of immune cells induced by LAB by means of Bcl-2 and IFNγ positive cells was analysed. It was observed that the increase in Bcl-2 positive cells was LAB dose-dependent. The expression of the Bcl-2 protein was significantly increased in mice fed with *L. casei* for 2, 5 and 7 days and *L. delbrueckii* subsp. *bulgaricus* for 2 and 7 days. *Streptococcus thermophilus* did not increase the number of Bcl-2 positive cells compared with the control (Table 1).

The number of IFNγ positive cells was different for each LAB assayed. *L. delbrueckii* subsp. *bulgaricus* increased these cells for all periods assayed, and showed a significant increase after 7 days of administration, while *St. thermophilus* induced a significant increase in all periods of administration with respect to the controls. *Lactobacillus casei* increased the IFNγ positive cells at all periods of administration in comparison with the NFM control, although these values were lower than those obtained for *St. thermophilus.* These results are shown in Table 1.

Discussion

Several virulent mechanisms are involved in the pathogenesis of *S. typhimurium* infection. The host is protected against bacterial pathogens by physical and immunological barriers, where the gut microflora plays an important role. The

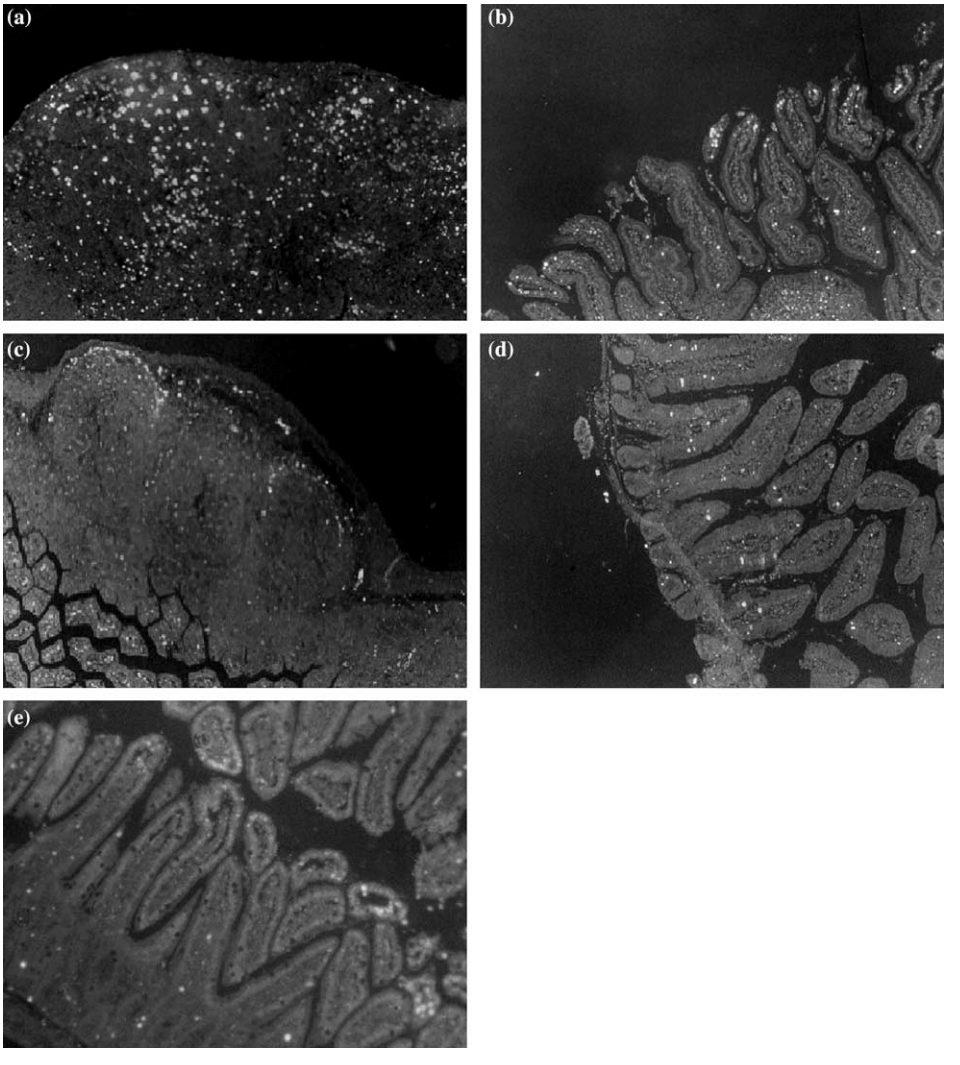

Figure 3. Apoptosis determination in small intestine tissues. The apoptotic cells were determined by TUNEL test in the small intestine: Peyer's patch of mouse 7 days after challenge with *S. typhimurium* (control group). Magnification 400 ×. Villi of small intestine of mouse 7 days after challenge with *S. typhimurium* (control group). Magnification 400 ×. Peyer's patch of mouse fed for 7 days with *L. delbrueckii* subsp. *bulgaricus* and challenged with *S. typhimurium* (7 days after challenge). Magnification 400 ×. Villi of small intestine of mouse fed for 7 days with *L. delbrueckii* subsp. *bulgaricus* and challenged with *S. typhimurium* (7 days after challenge). Magnification 400 ×. Villi of mouse fed 7 days with *St. thermophilus* and challenged with *S. typhimurium* (7 days after challenge). Magnification 400 ×.

main function of the normal intestinal microflora is to prevent colonisation by pathogenic bacteria through a mechanism called 'barrier effect' (Ducluzeau, 1989). Thus, the microflora naturally prevents the attachment of enteropathogenic bacteria to the epithelial cells. Mucus, produced by goblet cells, and lysozymes from Paneth cells are other mechanisms involved in the non-specific immune response of the host

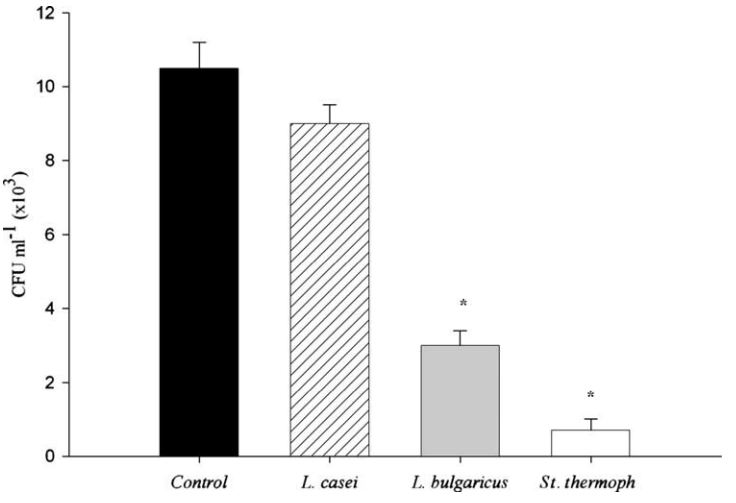

Figure 4. Microbiocidal activity of peritoneal macrophages. The macrophages monolayers of control and tests groups were infected with 10^7 CFU/ml of *Salmonella enteritidis* serovar Typhimurium. After incubation, the macrophages were lysed and the number of viable intracellular bacteria was determined. The samples were plated in MacConkey agar. The results are expressed as CFU/ml ($\times 10^3$) of samples from different experimental mice groups: control (black bars), mice fed for 2 days with *L. casei* (diagonal lines bars), mice fed for 7 days with *L. delbrueckii* subsp. *bulgaricus* (gray bars) and with *St. thermophilus* (white bars). *Significant differences between mice fed with different lactobacilli and the unfed control ($p < 0.05$).

at the intestinal level (Brandtzaeg, 1995). There are reports about the effect of some LAB used as probiotic bacteria in the prevention of enterobacteria (Gill, 2000).

When two or more strains share the same property, in this case the prevention of colonisation, the mechanisms by which they are able to exert such activity could be different. Thus, the aim of the present study was to analyse some biological mechanisms such as microbiocidal activity of macrophages and immune mechanisms involved in the prevention of *S. typhimurium* infection. When the preventive capacity of the LAB assayed against the pathogen was analysed, it was demonstrated that the

Table 1. Effect of LAB administration on the number of IFNγ and Bcl-2 positive cells.

	Bcl-2			IFNγ		
	2 days	5 days	7 days	2 days	5 days	7 days
L. casei	85 ± 10*	120 ± 26*	113 ± 23*	124 ± 15*	116 ± 18*	85 ± 19*
L. delbrueckii subsp. *bulgaricus*	87 ± 16*	50 ± 10	75 ± 15*	59 ± 22*	72 ± 18*	209 ± 34*
St. thermophilus	63 ± 20	39 ± 11	40 ± 11	200 ± 12*	156 ± 24*	115 ± 5*
Control		39 ± 6			18 ± 2	

The values are expressed as mean ± SD of the number of positive cells (fluorescent cells) counted in 10 fields of vision at 1000 × magnification (cells/10 fields).
*Significant differences between mice fed with different lactobacilli and the unfed control ($p < 0.05$).
There is only one value for each cytokine control because it did not modify in the three samples taken.

LAB were able to prevent the infection against the enteropathogen (Figure 1). The different mechanisms by which these LABs exerted the preventive capacity against *S. typhimurium* were determined. Some of the mechanisms involved in protection against *S. typhimurium* are: specific secretory IgA, cellular apoptosis inhibition and an increase in the microbiocidal activity of the macrophages.

It has been extensively demonstrated that IgA is involved in the defense against pathogens (Lamm, Nedrud, Kaetzel, & Mazanec, 1996). IgA secreted into the intestinal lumen can neutralise the pathogen by preventing its adhesion to the epithelial cells of the intestine or to the M cells of the Peyer's patches. This immunoglobulin can inhibit the internalisation of the *S. typhimurium* in the gut and prevent it spreading to deep tissues and to the liver or spleen. IgA can also neutralise the toxins produced by enterobacteria. Thus, for prevention against infection, the stimulation of the intestinal immune system should be induced by specific oral vaccination or by the use of an oral polyclonal immunoadjuvant. To date, no oral vaccine has been developed to prevent diarrhea caused by viruses or bacteria. Much effort has been made in the selection of the appropriate viral vector or attenuated pathogen strains that will not induce side effects, such as the recovery of virulence factors (Gupta & Siber, 1995).

Our results showed that *L. casei* was the only LAB able to induce a significant increase in the specific S-IgA (Figure 2). With *St. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* the values obtained were slightly higher than those in the control group; however, these increases were significant lower than those found with *L. casei*. This fact could indicate that the preventive effect against *S. typhimurium* exerted by *L. bulgaricus* and *St. thermophilus* could be mediated by mechanisms other than specific IgA, which is in agreement with a previous paper when by *in vitro* assay, we demonstrated that *L. delbrueckii* subsp. *bulgaricus* and *St. thermophilus* were able to inhibit apoptosis of the peritoneal macrophages infected with *S. typhimurium* (Valdez et al., 2001).

Since apoptosis is a well-demonstrated process by which *Salmonella* can disseminate to the deep tissues (Monack, Bouley, & Falkow, 2004), we determined by *in vivo* studies from the small intestine if the LAB assayed were able to inhibit the cellular apoptosis induced by the pathogen in the immune cells of the innate immunity associated to the gut. We demonstrated the remarkable effect *L. delbrueckii* subsp. *bulgaricus*. These finding show that some LAB can exert a preventive effect against *Salmonella* infection through apoptosis.

The inhibition of the cellular apoptosis observed led us to consider why this process occurred, considering that in previous studies the LAB assayed were more involved in the immunostimulation (Vintiñi et al. 2000; Perdigón, Maldonado Galdeano, Valdez, & Medici, 2002) than in the opposite process of apoptosis. Thus, we analysed the microbiocidal activity induced by LAB and two markers: the anti-apoptotic protein Bcl-2 and the cytokine IFNγ.

Bcl-2 is an anti-apoptotic member of the Bcl-2 family. The Bcl-2 gene is responsible for cellular proliferation. In our study, with the exception of *St. thermophilus*, Bcl-2 positive cells were increased. We suggest that the decrease in the number of apoptotic cells observed, especially in mice fed with *L. delbrueckii* subsp. *bulgaricus*, could be explained by the important increase in the number of anti-apoptotic protein Bcl-2 positive cells found in the small intestine. *Streptococcus thermophilus* did not induce an increase in the number of Bcl-2 positive cells with respect to the untreated control; and

this LAB did not exert an important effect on apoptosis inhibition. *Lactobacillus casei* induced an increase in Bcl-2 positive cells, although its effect in the apoptosis inhibition was similar to *St. thermophilus*, perhaps Bcl-2 increases to favor the immune cell activation.

IFNγ is a cytokine that can stimulate oxidant radical production by activating macrophages and increasing the expression of class I and II histocompatibility antigens. The increase in the number of IFNγ positive cells observed in our study (Table 1) show the cellular activation caused by LAB in agreement with the Bcl-2+ cells.

The increase of IFNγ positive cells induced by *L. delbrueckii* subsp. *bulgaricus* is consistent with the increase in the microbiocidal capacity of the peritoneal macrophages after oral administration of these LAB. Although induced *L. casei* increases the number of positive cells for this cytokine, they are insufficient to induce an increase in the microbiocidal activity of the peritoneal macrophages. This *Lactobacillus* also causes increases in other regulatory cytokines (IL-4 and IL-10) that could modulate the IFNγ effect, as was demonstrate in a previous paper (Perdigón et al., 2002). The increase in the Bcl-2+ cells may enhance the microbiocidal activity of the macrophages from the Peyer's patches, and it would explain the inhibition in the cellular apoptosis observed.

Conclusion

To conclude, we demonstrated that the LAB assayed can induce different mechanisms in the prevention of an infection with *S. typhimurium.* The preventive effect of *L. casei* would be mediated mainly by an increase in the specific S-IgA that neutralises the pathogen and prevents pathogen internalisation in the gut. *L. delbrueckii* subsp. *bulgaricus* was effective in the prevention of *S. typhimurium* mainly through of the inhibition of apoptosis mechanisms that avoid *Salmonella* dissemination. *Lactobacillus delbrueckii* subsp. *bulgaricus* may also induce an increase in the microbiocidal activity of the macrophages from the Peyer's patches or from the lamina propria of the gut, as observed in peritoneal macrophages. *Streptococcus thermophilus* exerts a partially protective effect by apoptosis inhibition and by the important increase in the microbiocidal activity, the increase in this activity could be mediated by the high levels of IFNγ determined in our experiments.

In spite of the results obtained in the present study, other mechanisms, such as competitive inhibition induced by LAB, could be involved in the prevention of enteropathogen bacteria infection.

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