# Influence of Technological Treatments on the Functionality of *Bifidobacterium lactis* INL1, a Breast Milk-Derived Probiotic

María Florencia Zacarías, Tassia Costa Souza, Natalia Zaburlín, Denise Carmona Cara, Jorge Reinheimer, Jacques Nicoli, and Gabriel Vinderola

**Abstract:** The aim of this study is to evaluate the influence of the technological processing on the functionality of the human breast milk probiotic strain *Bifidobacterium lactis* INL1. *In vitro* antagonistic activity of *B. lactis* INL1 was detected for Gram-positive and Gram-negative pathogens. *B. lactis* INL1 was administered to mice as fresh (F), frozen (Z), spray-dried (S), or lyophilized (L) culture. Immune parameters (IgA, IL-10, and IFN-$\gamma$) were determined and histological analysis was performed to assess functionality and protection capacity against *Salmonella*. In BALB/c mice, F and S cultures induced an increase in the number of IgA-producing cells in the small intestine and IL-10 levels were increased for L culture in the large intestine. In Swiss mice, *B. lactis* INL1 increased secretory-IgA levels in the small intestine before and after *Salmonella* infection, both as F or dehydrated culture. Also, an attenuation of damage in the intestinal epithelium and less inflammatory infiltrates were observed in animals that received F and S cultures, whereas in liver only F showed some effect. The anti-inflammatory effect was confirmed in both tissues by myeloperoxidase activity and by IFN-$\gamma$ levels in the intestinal content. *B. lactis* INL1 showed inhibitory activity against pathogens and confirmed its probiotic potential in animal models. Technological processing of the probiotic strain affected its functionality.

**Keywords:** *Bifidobacterium lactis*, functionality, probiotic, *Salmonella*, technology

**Practical Application:** This work provides evidence about the influence of technology on the functionality of probiotics, which may help probiotics and functional food manufacturers to take processing into consideration when assessing the functionality of new strains.

## Introduction

In the last 20 y, the use of probiotics has proliferated considerably (Floch 2014) and a significant progress has been made in the selection and characterization of specific strains and in the demonstration of their beneficial effects. *In vitro* and *in vivo* tests in animals have been reported for selected strains of the genera *Lactobacillus* and *Bifidobacterium*, with special emphasis on the functional effects at the level of the intestinal mucosa, but the majority of them were performed using overnight active cultures of the strains. Different mouse models are widely used for immunomodulation studies in the intestine and a considerable number of them have reported that the administration of certain strains of lactobacilli and bifidobacteria increased IgA production in the intestinal lumen, the number of IgA-producing cells in the lamina propria, and favorable changes in cytokine profiles (de Moreno de LeBlanc and others 2008; Mileti and others 2009). In addition, several gastrointestinal disease animal models have been designed in order to evaluate the usefulness of some probiotic strains in their treatment and prevention, for example, the salmonellosis model (de Moreno de LeBlanc and others 2010). Probiotic bacteria are usually commercialized and added to products either as frozen or dehydrated cultures (Saarela and others 2006). Each step and variable involved in the culture production, from growth conditions, substrates, and protectants to food formulation, processing, and storage conditions, may affect probiotic properties (Jankovic and others 2010; Sanders and others 2014). However, most studies do not consider the effect of processing on the probiotic activity. The aim of this work was to further assess the functional properties of the breast milk probiotic strain *Bifidobacterium animalis* subsp. *lactis* INL1 with emphasis in studying the influence of technological processing on the functionality of the strain.

## Materials and Methods

### Microorganisms and growth conditions

*Bifidobacterium animalis* subsp. *lactis* INL1 was isolated from human breast milk (Zacarías and others 2011) and its functional and technological properties were reported (Vinderola and others 2012; Zacarías and others 2014; Burns and others 2017). The strain was kept frozen at −70 °C in MRS (de Man, Rogosa, and Sharpe) broth (Biokar, Beauvais, France) supplemented with 20% (v/v) glycerol (Ciccarelli, Buenos Aires, Argentina) at the INLAIN culture collection. Before use, the strain was cultured twice in MRS broth supplemented with 0.1% (w/v) L-cysteine hydrochloride (Biopack, Buenos Aires, Argentina) (MRS-cys) during 18 h under anaerobiosis (Anaeropack-Anaero, Mitsubishi Gas Chemical Co., Inc., Japan) at 37 °C. For the experiments of *in vitro* antagonism, the

*JFDS-2017-0411 Submitted 3/7/2017, Accepted 7/25/2017.* *Authors Zacarías, Zaburlín, Reinheimer, and Vinderola with Inst. de Lactología Industrial (INLAIN), Univ. Nacional del Litoral, Santiago del Estero 2829, 3000 Santa Fe, Argentina. Authors Souza and Nicoli are with the Dept. de Microbiologia, Inst. de Ciências Biológicas (ICB), Univ. Federal de Minas Gerais, Belo Horizonte, MG, Brazil. Author Cara is with the Dept. de Morfologia, Inst. de Ciências Biológicas (ICB), Univ. Federal de Minas Gerais, Belo Horizonte, MG, Brazil. Direct inquiries to author Vinderola (E-mail: gvinde@fiq.unl.edu.ar).*



doi: 10.1111/1750-3841.13852



Journal of Food Science A Publication of the Institute of Food Technologists

**Table 1–*In vitro* antagonistic activity of *B. lactis* INL1 against pathogenic indicators.**

| Indicator strains | Inhibition halo (mean diameter ± SD in mm) |
|---|---|
| *Bacillus cereus* ATCC 11778 | 36.87 ± 1.82 |
| *Citrobacter freundii* ATCC 8090 | 43.09 ± 1.20 |
| *Escherichia coli* ATCC 25922 | 27.16 ± 5.19 |
| *Enterobacter aerogenes* ATCC 13048 | 29.21 ± 7.57 |
| *Enterococcus faecalis* ATCC 19433 | IND |
| *Klebsiella oxytoca* ATCC 49131 | 13.88 ± 2.87 |
| *Listeria monocytogenes* ATCC 19115 | IND |
| *Micrococcus luteus* ATCC 49732 | 18.01 ± 2.19 |
| *Proteus mirabilis* ATCC 25933 | 33.09 ± 4.16 |
| *Salmonella enterica* ser. Typhi ATCC 19430 | 25.04 ± 2.15 |
| *Salmonella enterica* ser. Typhimurium ATCC 14028 | 28.37 ± 0.79 |
| *Salmonella enterica* ser. Typhimurium FUNED | IND |
| *Serratia marcescens* ATCC 8100 | 13.87 ± 6.71 |
| *Shigella sonnei* ATCC 11060 | 27.47 ± 5.26 |
| *Staphylococcus aureus* ATCC 29313 | 29.92 ± 0.66 |

IND, inhibition zone nondetected.

indicator strains listed in Table 1 were used. *Salmonella* Typhimurium FUNED, a strain of human origin isolated at the Fundação Ezequiel Dias (FUNED, Belo Horizonte, Brazil), was also used for the salmonellosis studies. These strains were maintained at −70 °C at the culture collection of the Laboratório de Ecologia e Fisiologia de Microorganismos, Inst. de Ciências Biológicas, Univ. Federal de Minas Gerais (LEFM/ICB/UFMG, Belo Horizonte, MG, Brazil), in BHI (Brain Heart Infusion) (Difco, Sparks, Nev., U.S.A.) broth containing 20% glycerol and were grown overnight (18 h, aerobic incubation, 37 °C) in BHI broth directly from the frozen stock.

### *In vitro* antagonism assay

The antagonistic capacity of *B. lactis* INL1 was studied by the double agar layer diffusion assay according to Nardi and others (1999). Briefly, a 5 μL sample of an overnight culture of the bifidobacteria strain was spot-inoculated on the surface of MRS-cys agar and incubated for 48 h at 37 °C in an anaerobic chamber (Forma Scientific Company, Marietta, Ohio, U.S.A.) containing an atmosphere of 85% $N_2$, 10% $H_2$, and 5% $CO_2$. The cells of the colony were inactivated by exposure to chloroform for 30 min and the residual chloroform was allowed to evaporate. Cell inactivation was confirmed by culturing a colony on MRS broth. For the determination of the antagonistic activity, the plate was overlaid with 3.5 mL of BHI soft agar (0.75% w/v) that had been inoculated with $10^7$ colony forming units (CFUs) per mL of the indicator strain and incubated for 24 h at 37 °C in aerobiosis. The antagonistic activity was determined as the presence of an inhibition zone around the spot. When observed, the diameter of the inhibitory zone was determined with a digital pachymeter (Digimatic Calipar, Mitutoyo, Japan).

### Effect of technological treatments on *B. lactis* INL1 functionality in BALB/c mice

**Animals.** Forty BALB/c mice (male, 6-wk-old) weighing 19.8 ± 0.4 g were obtained from the random bred colony of the Centro de Medicina Comparada del Inst. de Ciencias Veterinarias del Litoral (CMC-ICiVet-Litoral, CONICET–UNL), Facultad de Ciencias Veterinarias, Univ. Nacional del Litoral (Esperanza, Santa Fe, Argentina). Animals were maintained for a week at the INLAIN animal facility before starting the experiments. Mice were housed in plastic cages ($n = 5$) and kept in a controlled environment at 21 ± 2 °C with 55 ± 2% of humidity and with a 12-h light/dark cycle. Mice were maintained and treated according to the guidelines of the Natl. Inst. of Health (NIH, U.S.A.) and experiments were approved by the Ethical Committee for Animal Experimentation of the Facultad de Ciencias Veterinarias, Univ. Nacional del Litoral (Esperanza, Santa Fe, Argentina).

**Bacterial cultures and feeding procedure.** A fresh (F), frozen (Z), spray-dried (S), or lyophilized (L) culture of *B. lactis* INL1 was used to feed the animals. The cell suspension for F bifidobacteria was prepared daily from an overnight culture (18 h) that was washed twice with PBS (phosphate buffered saline) buffer and resuspended in 20% (w/v) skim milk. To prepare Z, S, and L cultures, fresh cultures of *B. lactis* INL1 (produced in batch culture at pH free (not controlled), 18 h, 37 °C, anaerobiosis) grown on MRS-cys broth were centrifuged (2750 × *g*, 10 min, 5 °C), washed twice with PBS, and resuspended (10× concentration) in 20% (w/v) skim milk. Afterward, cell suspensions were aliquoted. For the Z group, the culture was frozen at −70 °C, and for the S group, the culture was dehydrated by spray drying (Büchi mini spray dryer model B-290, Flawil, Switzerland) using an inlet temperature of 160 °C, an outlet temperature of 83 °C, and a feed rate of 0.0075 L/min. For the L group, the culture was lyophilized (freeze-dried) for 24 h in a single chamber freeze drier (beta 2 to 16, Christ, Osterode, Germany) under a chamber pressure of 37 Pa, a primary drying temperature of −15 °C and a secondary drying temperature of +25 °C. Animals were divided in 5 groups (8 animals per group) and were housed according to a randomized block design (RBD), with 1 animal of each group of treatment (F, Z, S, or L) per cage. Animals received by gavage (0.1 mL) approximately $10^8$ CFU of *B. lactis* INL1 as F, Z, S, or L culture for 10 consecutive days. Control animals (C) received 0.1 mL per mouse of 20% (w/v) reconstituted skim milk. All animals received, simultaneously and *ad libitum*, tap water and a sterile conventional balanced diet (Cooperación, Buenos Aires, Argentina).

**Assessment of immunoglobulin A (IgA) and IL-10.** After the feeding period, animals were weighed, anesthetized intraperitoneally with a cocktail of ketamine/xylazine/acepromazine, and sacrificed by cervical dislocation. Intestinal contents were recovered by washing the small intestine from the duodenum to the distal ileum with 5 mL of PBS containing 20 μL of protease inhibitors (P8340, Sigma-Aldrich, St. Louis, Mo., U.S.A.). After centrifugation (2000 × *g*, 30 min, 4 °C), the supernatant was carefully collected and stored at −70 °C for secretory IgA (S-IgA) quantification by Enzyme-Linked ImmunoSorbent Assay (ELISA) (Rodrigues and others 2000). Portions of flushed intestines were prepared for fixation, histological preparation, and paraffin inclusion, according to Vinderola and others (2005). The number of IgA-producing (IgA+) cells was determined on histological slices of samples from the ileum near Peyer's patches and from the large intestine as described by Zacarías and others (2014) and results were expressed as the number of positive cells (fluorescent cells, IgA+ cells) per 10 fields. Small and large intestine samples (100 to 200 mg) were also obtained and kept frozen (−70 °C) for IL-10 determination. Intestine samples were homogenized (Ultra Turrax T8, Ika Labortechnik, Staufen, Germany) in PBS buffer containing 1% (v/v) antiprotease cocktail (Sigma-Aldrich), 10 mM EDTA (Sigma-Aldrich), and 0.05% (v/v) Tween 20 (Sigma-Aldrich) in a proportion of 1 mL PBS per 100 mg tissue. The samples were then centrifuged (9500 × *g*, 10 min, 4 °C) and the supernatant was collected and kept frozen for IL-10 quantification by ELISA using commercially available

antibodies (BD Biosciences Pharmingen, San Diego, Calif., U.S.A.), according to the procedures supplied by the manufacturer.

### Influence of technological treatments on the functionality of *B. lactis* INL1 and on its protective capacity against *Salmonella* infection in Swiss mice

**Animals, feeding, and infection procedures.** In a 1st assay, 24 conventional mice (6–wk-old; weight 18 to 20 g) derived from a NIH Swiss germ-free colony were obtained from the LEFM/ICB/UFMG (Belo Horizonte, Brazil) colony and were divided in 4 groups: control (C), fresh culture (F), spray-dried (S), and lyophilized (L). Experiments were carried out in microisolators (Uno Roestvaststaal, BV, Zevenaar, The Netherlands). For all the animals, water and commercial autoclavable diet (Nuvital, Curitiba, PR, Brazil) were sterilized by steam and administered *ad libitum*. Mice were maintained in a ventilated animal caging system (Alesco Ltda., Campinas, SP, Brazil) with controlled lighting (12 h light, 12 h dark), humidity (60% to 70%), and temperature (22 ± 1 °C). All experimental procedures were carried out according to the standards set forth by the Brazilian College for Animal Experimentation. The study was approved by the Ethics Committee in Animal Experimentation of the UFMG. All animals from treated groups received (by gavage) $10^8$ CFU/d of *B. lactis* INL1 as F, S, or L culture for 10 consecutive days and subsequently were challenged with a single infective dose ($10^6$ CFU/mouse) of *S.* Typhimurium FUNED. Animals were maintained together in their cages after infection (not housed individually). Control animals (C) received 0.1 mL/mouse of 20% (w/v) skim milk for 10 d before *Salmonella* challenge. Cultures of *B. lactis* INL1 were prepared as described before. Animals were anesthetized and euthanized by cervical dislocation 10 d postinfection (d.p.i.). Liver and intestines were aseptically collected and divided to be homogenized and used for the determination of myeloperoxidase (MPO) activity or histological and morphometric analysis. A 2nd experiment was conducted to assess the immune response before and after the infection. Fifty-two female Swiss mice, also from LEFM/ICB/UFMG colony, were used. Animals were divided in 4 groups (C, F, S, and L) and were treated under the same conditions as described before. All treated animals received (gavage) $10^8$ CFU/d of *B. lactis* INL1 as F, S, or L culture, while C animals received 20% (w/v) skim milk for 10 consecutive days. On the 11th day, 4 mice from each group were sacrificed as previously described for the measurement of immune parameters (S-IgA and IFN-$\gamma$ concentrations) before *Salmonella* challenge. Remaining animals were challenged with a single infective dose ($10^6$ CFU/mouse) of *S.* Typhimurium FUNED and were sacrificed at 4 and 8 d.p.i. In all cases (challenged and unchallenged animals), small intestine contents were recovered for S-IgA and IFN-$\gamma$ quantification.

**Histological and morphometric analysis.** The organs (liver and ileum) were removed from mice after opening of the abdominal cavity and washed with PBS. The organs were transferred to Bouin solution with 2% glacial acetic acid for prefixation during 10 min, and then fixed by immersion in formaldehyde (4% v/v) for 24 h. The samples were processed routinely for paraffin embedding and submitted to microtome to obtain histological slides of 3 to 5 $\mu$m thick. The slides were stained with hematoxylin and eosin (HE), coded and scored in a blinded manner by optical microscopy (BX51 microscope, Olympus, Tokyo, Japan). Ileum histological analysis was based on a semiquantitative scoring system in which 4 features were evaluated: (a) extent of destruction of normal mucosal architecture; (b) presence and degree of cellular infiltration; (c) presence of ulceration; and (d) of oedema. These features were considered as indicators of inflammation and were graded from 0 to 3 being 0 equal to normal and 1, 2, and 3, mild, moderate, and extensive damage, respectively. The scores for each feature were summed with a maximum possible score of 12. For morphometric examination of the liver, images (10×) were obtained from the HE slices and inflammatory foci were considered as damage index. The infiltrated areas were measured using ImageJ software (NIH, Bethesda, Md., U.S.A.) and the parameter $I$ = infiltrated area ($\mu$m)/total area ($\mu$m) was calculated (3 slices analyzed for each animal).

**Determination of MPO activity.** The extent of neutrophil or polymorphonuclear (PMN) neutrophils accumulation in ileum and liver was measured by determining MPO activity according to Vieira and others (2012). Briefly, portions of ileum or liver were homogenized (100 mg of tissue per 1.9 mL of buffer) in pH 4.7 buffer (0.1 M NaCl, 0.02 M $Na_3PO_4$, 0.015 M EDTA) and centrifuged (12000 × *g*, 4 °C, 10 min). Precipitates were suspended in HETAB 0.5% (Sigma-Aldrich) in phosphate buffer (pH 5.4), and after homogenization and freezing/thawing sessions, suspensions were centrifuged and supernatants were recovered. For MPO quantification, 25 $\mu$L of supernatant were added to 25 $\mu$L of 3,3′,5,5′-tetramethylbenzidine (TMB) in dimethylsulfoxide (DMSO) and incubated at 37 °C for 5 min. After that, 100 $\mu$L of $H_2O_2$ was added and incubated. Reaction was stopped by adding $H_2SO_4$ and read at 450 nm. Results were expressed in arbitrary units (based on absorbance) by 100 mg of tissue.

**Determination of S-IgA and IFN-$\gamma$ in intestinal fluid.** Small intestine contents were recovered by gently pressing the small intestine from the duodenum to the distal ileum, weighed, and diluted 1:4 in PBS supplemented with 1% (v/v) antiprotease cocktail (P8340, Sigma). After centrifugation (2000 × *g*, 30 min, 4 °C), the supernatant was collected and kept frozen at −70 °C for S-IgA (Sigma) and IFN-$\gamma$ (BD Biosciences Pharmingen) quantification by ELISA using commercially available kits according to the procedures supplied by the manufacturers.

### Statistical analysis

Results were expressed as mean ± SEM (standard error of the mean). For IgA-producing cells, S-IgA, and cytokines, the difference between groups was evaluated using one-way analysis of variance (ANOVA) followed by multiple-comparison compared with control-group Dunnett's method. Data were analyzed using SPSS 15.0 software (SPSS Inc., Chicago, Ill., U.S.A.). Histological scores were analyzed by a nonparametrical test (Mann–Whitney), while MPO values were analyzed using a one-way ANOVA and Tukey's comparison test. Both results were analyzed using GraphPad Prism software version 5.00 for Windows (GraphPad Software, San Diego, Calif., U.S.A.). In all cases, means were considered statistically different when $P < 0.05$.

## Results and Discussion

### *In vitro* antagonism assay

The ability of beneficial bacteria to inhibit the growth of pathogenic microorganisms in the gut is considered one of the mechanisms of the probiotic action. Bifidobacteria are found in the gastrointestinal tract (GIT) in a reverse correlation to undesirable bacteria such as clostridia and *Escherichia coli*, as observed from clinical feeding studies which would suggest that bifidobacterial metabolic activities have a role in preventing the overgrowth of undesirable microorganisms (Russell and others 2011). Several mechanisms have been suggested for the inhibitory action of

bifidobacteria, including a decrease of the local pH via the production of organic acids, the inhibitory action of undissociated organic acid molecules, the competition for nutrients or adhesion sites, the stimulation of the host's immunity, and the production of specific antibacterial substances (Cheikhyoussef and others 2008). The results of the *in vitro* antagonistic activity of *B. lactis* INL1 are shown in Table 1. From 15 pathogenic strains evaluated, *B. lactis* INL1 showed the capacity of inhibiting the growth of 12 of them, including both Gram-positive and Gram-negative pathogens. Although *in vitro*, *B. lactis* INL1 inhibitory results are encouraging, particularly for the potential treatment or prevention of some infections caused by such diversity of pathogens. It is also interesting to note that *B. lactis* INL1 had a differential behavior for 2 strains of the same species and serotype, *S.* Typhimurium, being able to inhibit the development of the collection strain (ATCC 14028) but not of the strain isolated in the Ezequiel Dias Foundation (FUNED, Fundação Ezequiel Dias, Brazil), as it was also observed by Souza and others (2013) for 2 other bifidobacteria strains of human origin, *B. breve* $110^{1A}$ and *B. bifidum*$^{2A}$. This fact once again supports the concept that effects are strain-dependent and that when it comes to the functional characterization of a probiotic microorganism, results cannot be extended to other strains. The results of inhibitory activity displayed by *B. lactis* INL1 could be used as a 1st step to select other *in vivo* pathology models and further study the potential protective role of the probiotic strain.

## Effect of technological treatments on the functionality of *B. lactis* INL1 in BALB/c mice

A significant increase in the number of IgA-producing cells was observed in the small intestinal lamina propria of mice that received bifidobacteria as fresh ($P = 0.004$) or spray-dried culture ($P = 0.019$), whereas no differences were observed for animals that received the strain as frozen or lyophilized cultures (Figure 1). No effects were observed in the large intestine for any of the groups under study, even if a slight tendency to enhanced IgA-producing cells was observed in animals that received the spray-dried culture (data not shown). In a previous assay (Zacarías and others 2011), *B. lactis* INL1 was administered to BALB/c mice as fresh culture for 3, 6, and 10 d and a significant increase in the number of IgA-producing cells in the lamina propria of the small and large intestine was observed. The differences in the results for the administration of F culture could indicate the variability sometimes observed in *in vivo* trials, due to either intrinsic or external uncontrolled factors, as for example, differences in microbiota composition and physiology between batches of mice obtained even from the same vendor and maintained under the same environmental conditions (Hoy and others 2015; Rausch and others 2016). Total S-IgA was also determined in the small intestine fluid and no differences were observed among groups. The main function of S-IgA is to protect mucosal surfaces against pathogenic and nonpathogenic microorganisms (Macpherson and others 2001) and an increase on its levels is a desirable probiotic feature. However, in this assay no significant differences were detected between control and treated groups for this parameter, although IgA-producing cells proliferated in the lamina propria of the small intestine from F and S groups. The important role of IgA in the intestinal homeostasis and immune protection has been known for decades and it is becoming increasingly clear that the intestinal mucosa utilizes multiple follicular and extrafollicular sites as well as multiple T cell-dependent and T cell-independent pathways to generate protective IgA responses (Cerutti and Rescigno 2008). Although the generation of the intestinal IgA-producing plasma cells is studied extensively, the molecular mechanisms regulating the IgA maintenance and memory responses are still poorly understood (Hu and others 2011). In the small intestine of mice, the average half-life of lamina propria plasma cells is estimated to be 5 d but a maximum life span in the order of 6 to 8 wk has been observed (Mesin and others 2011). Considering this life span, the lack of correlation in our study between the proliferation of $IgA^+$ cells in the lamina propria of small intestine and the content of S-IgA in the intestinal lumen after 10 d of probiotic intervention, could be due to regulatory mechanisms in the production or secretory path of the antibody. IL-10 is one of the most frequently assessed cytokines when the immune profile of a potential probiotic strain is under study. Although it is often considered as an anti-inflammatory cytokine (de Kivit and others 2014), its main role is regulatory since it can be produced by different cellular subsets, including Treg cells, and it can led to the development of oral tolerance (Johansson and others 2012; Rescigno 2013). In this work, IL-10 levels were analyzed and a significant higher concentration ($P = 0.015$) was observed in the large intestine of animals that received the strain as a lyophilized culture (Figure 2), compared to control animals. As for the influence of technological treatments, previous studies showed differences in resistance to the GIT conditions, adherence, and immunomodulatory properties for the same strain produced or preserved under different conditions (Saarela and others 2009; Vinderola and others 2012). Recently, Cinque and others (2016) found that production conditions affected some *in vitro* immunomodulatory properties of the probiotic commercial product VSL#3, stressing the importance of reconfirming

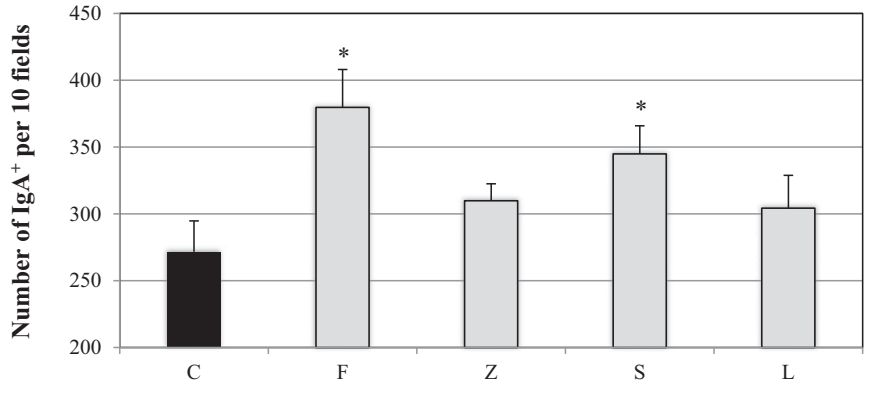


**Figure 1–Effect of the oral administration (ca. $10^8$ CFU/d/mouse) of *B. lactis* INL1 as fresh (F), frozen (Z), spray-dried (S), or lyophilized (L) culture for 10 consecutive days on the number of IgA-producing cells (IgA+ per 10 fields) in the lamina propria of the small intestine of BALB/c mice, compared to control (C) animals. *significantly different ($P < 0.05$) from control.**

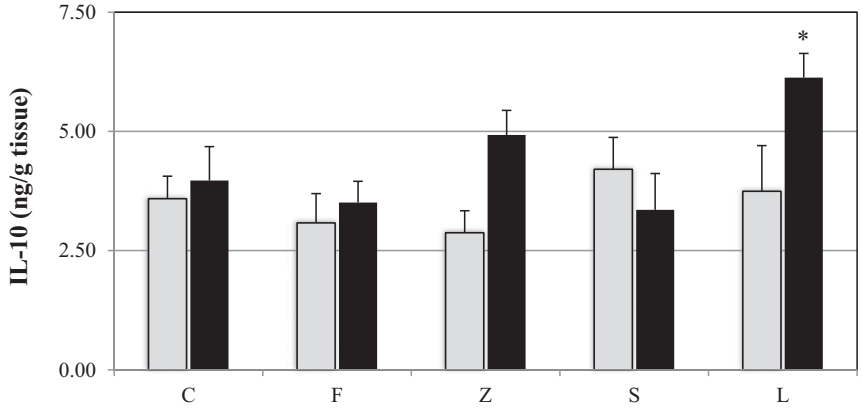


Figure 2–Effect of the oral administration (ca. $10^8$ CFU/d/mouse) of *B. lactis* INL1 as fresh (F), frozen (Z), spray-dried (S), or lyophilized (L) culture for 10 consecutive days on the concentration of IL-10 in small (▒) and large intestine (■) homogenates of BALB/c mice, compared to control (C) mice. *significantly different ($P < 0.05$) from control.

safety and/or efficacy for any probiotic product after changing the manufacturing process. In this work, an enhanced proliferation of IgA+ cells was observed when fresh and spray-dried cultures were administered. Frozen and lyophilized cultures did not show this effect; however, an increase or tendency to increase the IL-10 production was observed for these groups. It is interesting to note that both cultures share a common step: freezing. Sousa and others (2015) described different resistances to simulated gastric digestion for probiotic strains kept frozen, both free or microencapsulated, compared to fresh cultures. This could indicate that freezing may affect functionality somehow without modifying viability. For example, it has been postulated that osmotic shock or formation of intracellular ice may affect some properties of probiotics. As for freeze drying, whereas no significant differences in the adhesion capability to Caco-2 cells or in the resistance to simulated gastrointestinal transit of *B. animalis* BF052 were detected among lyophilized and nonlyophilized cells (Charnchai and others 2016), du Toit and others (2013) found that freeze drying of probiotics, included *B. lactis* Bb12, had an effect on adhesion potential, showing an overall significant decrease in the ability of the lyophilized probiotic to exclude, displace, or outcompete certain pathogens. IL-10 induction was only evident in animals treated with the lyophilized culture in the large intestine. Even if strains that suppress the production of pro-inflammatory cytokines and/or enhance the production of regulatory cytokines (IL-10 and TGF-$\beta$) and Th3 cells are preferred, there is a large variation in the cytokine response induced by different strains or species of probiotics (Saxelin and others 2005). Due to the great number of cells of the innate and adaptive immunity residing next to a complex commensal microbiota, the GIT uses complex regulatory mechanisms to maintain homeostasis of the immune system (Peterson and Artis 2014). Hart and others (2004) observed an increase in the IL-10 levels that inhibited Th1 cells proliferation when the commercial probiotic consortium VSL#3 was administered and this effect was mainly due to the *Bifidobacterium* strains present in the product. Furthermore, Jeon and others (2012) observed also an increase in this cytokine in the colon when a probiotic strain of *B. breve* was administered. However, not all commercial strains have shown an induction in the secretion of IL-10 or at least results have not been conclusive, as is the case for the most studied probiotic strains worldwide, *L. rhamnosus* GG and *B. lactis* Bb12 (Roller and others 2004; Kekkonen and others 2008). So, even if an increase in luminal S-IgA levels or induction of IL-10 is desired, these results are not discouraging regarding *B. lactis* INL1 as a probiotic candidate.

## Effect of technological treatments on the functionality of *B. lactis* INL1 in Swiss mice

**Histological evaluation of ileum and liver.** Histological sections of the small intestine and liver were analyzed and the severity of tissue damage was assessed by a cumulative clinical score ranging from 0 (absence of inflammatory markers) to 12 (highest magnitude of inflammatory damage). Intestinal infections due to *Salmonella* species in mice manifest through ileal lesions, which can be extended to jejunum and cecum. These lesions are characterized by the presence of ulcers associated with diffuse infiltrations of macrophages, neutrophils, and lymphocytes in the adjacent mucosa (Greaves 2012). Figure 3(A) shows the total scores resulting from histopathological analysis of ileum for control animals (C) or mice that received *B. lactis* INL1 as F, S, or L culture. The total score was the result of the sum of 4 parameters analyzed: impaired intestinal architecture, the presence of inflammatory infiltrates, oedema, and/or ulcers. Signs of inflammation were evident in all animals belonging to the infection control group (total score per animal $> 0$ in all cases), and although not significant, a trend in attenuation of ileal inflammatory damage ($P = 0.082$) was observed when *B. lactis* INL1 was administered as a fresh culture. When each parameter was considered individually, the decrease in intestinal epithelial alterations observed for groups receiving the bifidobacteria as fresh or spray-dried culture when compared to the control group ($P = 0.035$ and $P = 0.026$, respectively, Figure 3b) shows to be more particularly responsible for this trends. A lower presence of inflammatory infiltrates was also observed for these 2 groups although not statistically significant ($P = 0.101$ and $P = 0.171$, respectively, Figure 3C). On the other hand, liver histopathology of the probiotic-treated groups was similar to the control group and a great variability was observed between individuals of the same group. However, based on the morphometric examination, an attenuation from a moderate degree of infiltration observed for the control group culture ($I = 0.03$) to a mild degree of infiltration for the group receiving *B. lactis* INL1 as fresh culture ($I = 0.008$) could be considered (Figure 4).

**Determination of tissue MPO activity.** Neutrophil recruitment in intestine, liver, and spleen is one of the most notable features of salmonellosis and is one of the 1st mechanisms of virulence of the pathogen. To corroborate the histopathological observations described above, MPO activity was determined in the same tissues and a significant reduction was observed in the enzyme activity in the ileum from all the groups receiving *B. lactis* INL1 (Figure 5A), while this effect was observed in the liver only after the administration of bifidobacteria as fresh culture

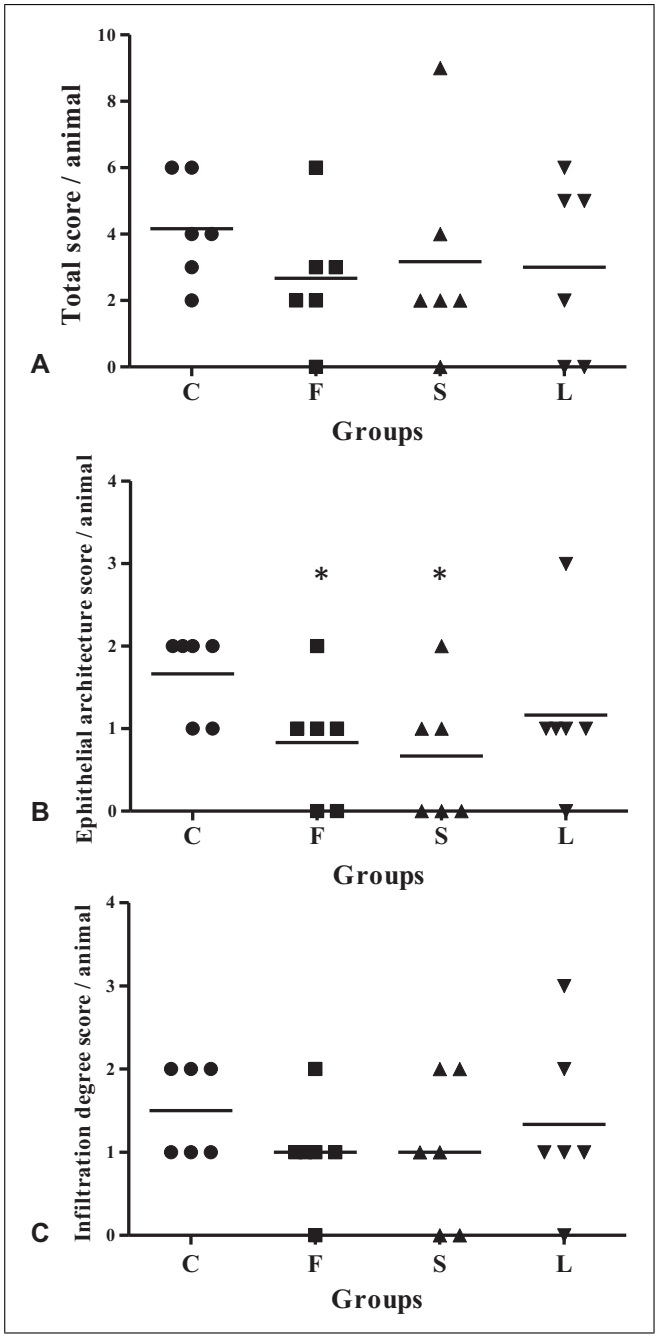


Figure 3–Microscopic histological damage scores for ileum of *Salmonella*-challenged Swiss mice at day 10 post infection: (A) Total damage score; (B) damage to epithelial architecture score; and (C) infiltration degree score. Before infection animals received (oral administration) ca. $10^8$ CFU/d/mouse of *B. lactis* INL1 as fresh (F), spray-dried (S), or lyophilized (L) culture or skim milk (control animals, C) for 10 consecutive days. *significantly different ($P < 0.05$) from control. Horizontal lines indicate the mean value.

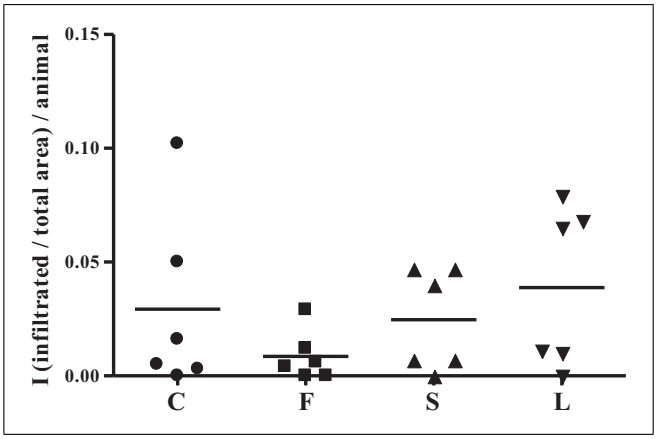


Figure 4–Semiquantitative determination of inflammation in liver by morphometric measurement of infiltrated areas of *Salmonella*-challenged Swiss mice at day 10 postinfection. Parameter *I* was defined as the ratio infiltrated area/total area of HE stained liver sections. Before infection animals received (oral administration) ca. $10^8$ CFU/d/mouse of *B. lactis* INL1 as fresh (F), spray-dried (S), or lyophilized (L) culture or skim milk (control animals, C) for 10 consecutive days. *significantly different ($P < 0.05$) from control. Horizontal lines indicate the mean value.

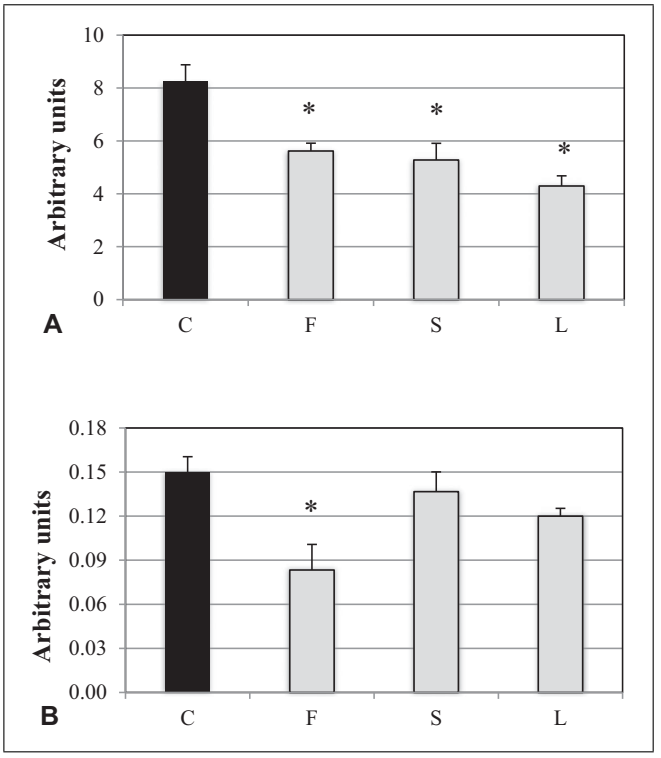


Figure 5–Activity of neutrophil MPO in ileum (A) and liver (B) of Swiss mice challenged with *Salmonella* Typhimurium at day 10 postinfection. Before infection animals received (oral administration) ca. $10^8$ CFU/d/mouse of *B. lactis* INL1 as fresh (F), spray-dried (S), or lyophilized (L) culture or skim milk (control animals, C) for 10 consecutive days. *significantly different ($P < 0.05$) from control.

(Figure 5B). Results from the histopathological analysis showed a decrease of epithelial damage caused by the pathogen in the ileum for F and S cultures, and a lower, although not statistically significant, presence of inflammatory infiltrates, whereas liver analysis showed an attenuation from a moderate to a mild degree of infiltration when control and S and L cultures were compared to F culture. The MPO results confirmed the observations of the histopathological analysis, reinforcing the hypothesis of a modulation of the inflammatory response mediated by *B. lactis* INL1, and this was influenced by the dehydration process. It could also be observed that in this case, the technological processes applied to the strain affected their ability to induce anti-inflammatory mechanisms in extra-intestinal sites, since only the fresh culture managed to "extend" to the liver the effect observed at ileum level.

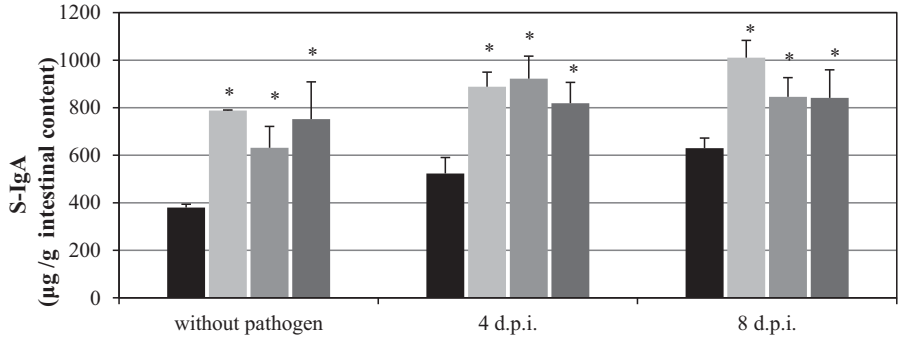


Figure 6–Concentration of secretory IgA (S-IgA) in intestinal content of Swiss mice that received ca. $10^8$ CFU/d/mouse of *B. lactis* INL1 as fresh (□), spray-dried (▒), or lyophilized (■) culture for 10 consecutive days, prior to *Salmonella* infection (without pathogen) or at 4 and 8 d postinfection (4 d.p.i. and 8 d.p.i., respectively) compared to their corresponding control (■). *significantly different ($P < 0.05$) from the corresponding control.

**S-IgA and IFN-$\gamma$ levels in intestinal content.** Nonspecific binding of S-IgA to pathogenic bacteria is functionally important for the defense of the intestinal epithelium. In a study of knockout mice lacking S-IgA an increase in the invasion of *S.* Typhimurium to epithelial cells was observed, while in another trial it was observed that the presence of S-IgA limited translocation of aerobic bacteria from the intestinal lumen to the mesenteric lymph nodes (Suzuki and Nakajima 2014). In order to increase the knowledge about the potential protective effect of *B. lactis* INL1 in a salmonellosis model in Swiss mice and to evaluate the influence of technological treatments on its functionality, the induction of S-IgA and IFN-$\gamma$ production in the intestinal lumen was studied. When bifidobacteria were administered for 10 d as fresh or dehydrated (lyophilized or spray dried) cultures, a significant increase in the levels of S-IgA in the intestinal lumen was observed compared to the control group. This behavior was also observed after 4 and 8 d.p.i. and levels were also increased compared to their respective controls for all groups receiving the bacterial strain, irrespective of the technological treatments applied (Figure 6). The induced secretion of IgA was generally more pronounced for fresh than dehydrated cultures, compared to control (except for 4 d.p.i.). Furthermore, when the levels of S-IgA for each group (F, S, and L) were separately compared applying a *post hoc* Dunnett test, it was observed that for the F groups, preinfection levels were not significantly different to those for 4 d.p.i., but increased at 8 d.p.i. For the S groups, this raise in S-IgA level was observed at 4 d.p.i. and then returned to preinfection levels at 8 d.p.i. Finally, for L groups, no significant changes were observed in antibody levels at 4 and 8 d.p.i. compared to their preinfection values. That is, *B. lactis* INL1 was able to induce the secretion of S-IgA when administered as both fresh or dehydrated culture, prior and post-*Salmonella* infection, but the magnitude and behavior of this increased humoral immune response over time was affected by the technological treatment applied. However, *B. lactis* INL1 was not able to induce IgA secretion into the intestinal lumen of BALB/c mice that received F, S, or L cultures, although an increase of IgA-producing cells in the small intestine lamina propria for F or S treated groups was observed. These facts together reinforce the importance of both the animal model and the experimental design, as well as of the effect of technological processes on the functionality of any strain under study for a potential probiotic application.

IFN-$\gamma$ is one of the most potent and pleiotrophic cytokines (Ellis and Beaman 2004). When the presence of IFN-$\gamma$ in the intestinal content was analyzed, it was observed that the administration of *B. lactis* INL1 for 10 d did not alter luminal baseline levels. However, once the animals were challenged with *S.* Typhimurium FUNED, a significant increase in the levels of this cytokine at 4 d.p.i. for all groups that received *B. lactis* INL1 was observed. In the case of the dehydrated (S and L) groups, the effect was still observed by day 8 d.p.i. (Figure 7). The role of IFN-$\gamma$ in the immune response against *S.* Typhimurium is ambiguous and complex, because its participation is essential in the early postinfection period to activate macrophages and to trigger a physiological inflammatory response (Lapaque and others 2009). However, if these levels are not adequately regulated (by IL-12 and IL-23), they may eventually lead to an acute pathological inflammation and tissue damage (Awoniyi and others 2012). It is also considered that its participation indicates the interface between the innate and the adaptive immune responses (Kaiser and others 2012) so that an increase in levels of this cytokine, especially several days after initiation of infection, could indicate the activation of adaptive immune mechanisms by T cells. As for the relationship between IFN-$\gamma$ and neutrophils, it is interesting to note that IFN- $\gamma$ treatment of PMNs has been shown to downregulate IL-8 and some

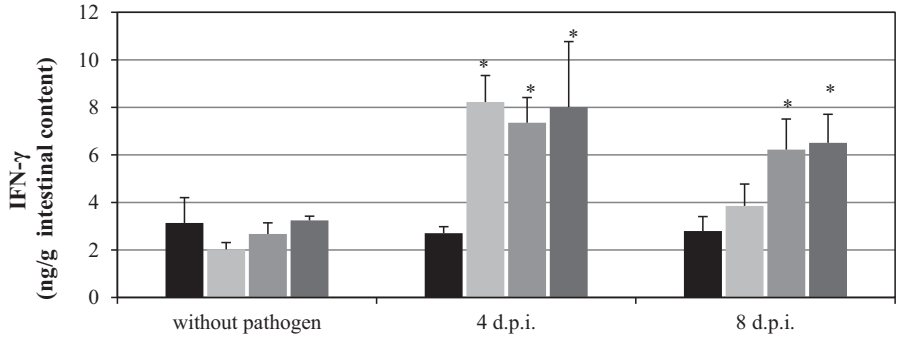


Figure 7–Concentration of secretory IFN-$\gamma$ in intestinal content of Swiss mice that received ca. $10^8$ CFU/d/mouse of *B. lactis* INL1 as fresh (□), spray-dried (▒), or lyophilized (■) culture for 10 consecutive days, prior to *Salmonella* infection (without pathogen) or at 4 and 8 d postinfection (4 d.p.i. and 8 d.p.i., respectively) compared to their respective controls (■). *significantly different ($P < 0.05$) from the corresponding control.

chemokines that recruit neutrophils, and upregulate chemokines that are chemoattractants for components of the acquired immune response, specifically T cells. This could indicate that IFN-$\gamma$ may act as a signal to halt PMN recruitment and infiltration (Ellis and Beaman 2004; Marchi and others 2014). In this work, the administration of *B. lactis* INL1 prior to *Salmonella* infection led to an attenuation in the inflammatory response to the pathogen both in ileum and liver, at 10 d.p.i. This was evidenced by both histological and MPO analysis. Also, when IFN-$\gamma$ levels were studied, an increase in this cytokine concentration was observed, especially at 4 d.p.i., associated with a stimulation of IgA-mediated response. Although technological treatments, as spray drying or freeze drying, affected the functional effect of the probiotic strain, in both cases a protective function was evident, but in lesser extent than for fresh culture. Based on these results, modifications at process level could be made in order to try to save the functional features of the strain, particularly for freeze drying, paying special attention in the freezing step. When considered together, results suggest that the administration of *B. lactis* INL1 either as fresh or dehydrated culture, could trigger a protective response against *Salmonella* infection through an induction of nonspecific humoral response and by regulating the inflammatory process.

## Conclusion

*B. lactis* INL1 showed the ability to inhibit a wide range of pathogens in an *in vitro* test as well as probiotic functional properties in 2 different murine models. However, technological processing of the probiotic strain affected its functionality. Whereas in BALB/c mice only an induction in the number of IgA-producing cells in small intestine was evident for fresh and spray-dried cultures, in Swiss mice the administration of *B. lactis* INL1 induced a significant increase in the levels of total S-IgA in the intestinal content of animals treated with both fresh and dehydrated cultures. Different responses at immunological and histological levels were observed between fresh and dehydrated cultures in the response to an infection with *S.* Typhimurium. The results indicate the possible dependence of functionality when using different mouse models, and more importantly, the importance of studying probiotic strains as close as possible to the technological format in which it is intended to be used.

## Acknowledgments

María Florencia Zacarías is a postdoctoral fellow of CONICET (Argentina) and Tassia Costa Souza was a doctoral fellow of CNPq (Brazil). This work has been supported by the following projects: Argentina-MINCyT and Brazil-CAPES: BR/09/14 "Evaluación del potencial tecnológico y probiótico de bifidobacterias aisladas de leche materna para el desarrollo de alimentos funcionales"; "Desarrollo de cultivos probióticos nacionales a partir de cepas autóctonas de lactobacilos y bifidobacterias", Proyecto CAI+D 2011, 501 201101 00136 LI, 2013–2015; "Cultivos microbianos autóctonos para la producción de alimentos funcionales para humanos y animales utilizando secado spray", Proyecto PICT-2013-0260, ANPCyT N° 214/14. Authors thank Dr. Roxana Páez from INTA EEA Rafaela (Santa Fe, Argentina) for spray drying of the strain and Dr. Flaviano Martins for assistance in cytokine determination.

## Author Contributions

J. Reinheimer, J. Nicoli, and G. Vinderola designed the study and revised the manuscript. M.F. Zacarías performed the trials. M.F. Zacarías and G. Vinderola drafted the manuscript. N. Zaburlín and T. Souza assisted on sample processing in *in vivo* trials and D. Cara assisted on histopathological analysis.

## References

Awoniyi M, Miller SI, Wilson CB, Hajjar AM, Smith KD. 2012. Homeostatic regulation of Salmonella-induced mucosal inflammation and injury by IL-23. PLoS ONE 7(5):e37311.
Burns P, Alard J, Hrdy J, Boutillier D, Páez R, Reinheimer J, Pot B, Vinderola G, Grangette C. 2017. Spray-drying effect on the protective capacity of the breast milk-derived *Bifidobacterium lactis* INL1 on acute and chronic colitis in mice. Sci Rep 7:43211.
Cerutti A, Rescigno M. 2008 The biology of intestinal immunoglobulin: a responses. Immunity 28(6):740–50.
Charnchai P, Jantama SS, Prasitpuriprecha C, Kanchanatawee S, Jantama K. 2016. Effects of the food manufacturing chain on the viability and functionality of *Bifidobacterium animalis* through simulated gastrointestinal conditions. PLoS One 11(6):e0157958.
Cheikhyoussef A, Pogori N, Chen W, Zhang H. 2008 Antimicrobial proteinaceous compounds obtained from bifidobacteria: from production to their application. Int J Food Microbiol 125(3):215–22.
Cinque B, La Torre C, Lombardi F, Palumbo P, Van der Rest M, Cifone MG. 2016. Production conditions affect the *in vitro* anti-tumoral effects of a high concentration multi-strain probiotic preparation. PLoS ONE 11(9):e0163216.
de Kivit S, Tobin MC, Forsyth CB, Keshavarzian A, Landay AL. 2014. Regulation of intestinal immune responses through TLR activation: implications for pro- and prebiotics. Front Immunol 5:60.
de Moreno de LeBlanc A, Chaves S, Carmuega E, Weill R, Antóine J, Perdigón G. 2008. Effect of long-term continuous consumption of fermented milk containing probiotic bacteria on mucosal immunity and the activity of peritoneal macrophages. Immunobiol 213(2): 97–108.
de Moreno de LeBlanc A, Castillo NA, Perdigón G. 2010. Anti-infective mechanisms induced by a probiotic *Lactobacillus* strain against *Salmonella enterica* serovar Typhimurium infection. Int J Food Microbiol 138(3):223–31.
du Toit E, Vesterlund S, Gueimonde M, Salminen S. 2013. Assessment of the effect of stress-tolerance acquisition on some basic characteristics of specific probiotics. Int J Food Microbiol 165(1):51–6.
Ellis TN, Beaman BL. 2004. Interferon-gamma activation of polymorphonuclear neutrophil function. Immunology 112(1):2–12.
Floch MH. 2014. Recommendations for probiotic use in humans: a 2014 update. Pharmaceuticals 7(10):999–1007.
Greaves P. 2012. Digestive system. In Histopathology of preclinical toxicity studies: interpretation and relevance in drug safety evaluation. 4th ed. Amsterdam: Academic Press. p 325–422.
Hart AL, Lammers K, Brigidi P, Vitali B, Rizzello F, Gionchetti P, Campieri M, Kamm MA, Knight SC, Stagg AJ. 2004. Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut 53(11):1602–9.
Hoy YE, Bik EM, Lawley TD, Holmes SP, Monack DM, Theriot JA, Relman DA. 2015. Variation in taxonomic composition of the fecal microbiota in an inbred mouse strain across individuals and time. PLoS One 10(11):e0142825.
Hu S, Yang K, Yang J, Li M, Xiong N. 2011. Critical roles of chemokine receptor CCR10 in regulating memory IgA responses in intestines. Proc Natl Acad Sci U S A 108(45): E1035–44.
Jankovic I, Sybesma W, Phothirath P, Ananta E, Mercenier A. 2010. Application of probiotics in food products-challenges and new approaches. Curr Opin Biotechnol 21(2): 175–81.
Jeon SG, Kayama H, Ueda Y, Takahashi T, Asahara T, Tsuji H, Tsuji NM, Kiyono H, Ma JS, Kusu T, Okumura R, Hara H, Yoshida H, Yamamoto M, Nomoto K, Takeda K. 2012. Probiotic *Bifidobacterium breve* induces IL-10-producing Tr1 cells in the colon. PLoS Pathog 8(5):e1002714.
Johansson MA, Saghafian-Hedengren S, Haileselassie Y, Roos S, Troye-Blomberg M, Nilsson C, Sverremark-Ekström E. 2012. Early-life gut bacteria associate with IL-4-, IL-10- and IFN-$\gamma$ production at two years of age. PLoS One 7(11), e49315.
Kaiser P, Diard M, Stecher B, Hardt WD. 2012. The streptomycin mouse model for *Salmonella* diarrhea: functional analysis of the microbiota, the pathogen's virulence factors, and the host's mucosal immune response. Immunol Rev 245(1):56–83.
Kekkonen RA, Lummela N, Karjalainen H, Latvala S, Tynkkynen S, Jarvenpaa S, Kautiainen H, Julkunen I, Vapaatalo H, Korpela R. 2008. Probiotic intervention has strain-specific anti-inflammatory effects in healthy adults. World J Gastroenterol 14(13):2029–36.
Lapaque N, Walzer T, Méresse S, Vivier E, Trowsdale J. 2009. Interactions between human NK cells and macrophages in response to *Salmonella* infection. J Immunol 182(7):4339–48.
Macpherson AJ, Hunziker L, McCoy K, Lamarre A. 2001. IgA responses in the intestinal mucosa against pathogenic and non-pathogenic microorganisms. Microbes Infect 3:1021–35.
Marchi LF, Sesti-Costa R, Ignacchiti MD, Chedraoui-Silva S, Mantovani B. 2014. *In vitro* activation of mouse neutrophils by recombinant human interferon-gamma: increased phagocytosis and release of reactive oxygen species and pro-inflammatory cytokines. Int Immunopharmacol 18(2):228–35.
Mesin L, Di Niro R, Thompson KM, Lundin KE, Sollid LM. 2011. Long-lived plasma cells from human small intestine biopsies secrete immunoglobulins for many weeks in vitro. J. Immunol 187:2867–74.
Mileti E, Matteoli G, Iliev ID, Rescigno M. 2009. Comparison of the immunomodulatory properties of three probiotic strains of *Lactobacilli* using complex culture systems: prediction for *in vivo* efficacy. PLoS One 4(9):e7056.
Nardi RD, Santos ARM, Carvalho MAR, Farias LM, Benchetrit LC, Nicoli JR. 1999. Antagonism against anaerobic and facultative bacteria through a diffusible inhibitory compound produced by a *Lactobacillus* sp. isolated from the rat fecal microbiota. Anaerobe 5:409–11.
Peterson LW, Artis D. 2014. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nat Rev Immunol 14(3):141–53.
Rausch P, Basic M, Batra A, Bischoff SC, Blaut M, Clavel T, Gläsner J, Gopalakrishnan S, Grassl GA, Günther C, Haller D, Hirose M, Ibrahim S, Loh G, Mattner J, Nagel S, Pabst O, Schmidt F, Siegmund B, Strowig T, Volynets V, Wirtz S, Zeissig S, Zeissig Y, Bleich A, Baines JF. 2016. Analysis of factors contributing to variation in the C57BL/6J fecal microbiota across German animal facilities. Int J Med Microbiol 306(5):343–55.

Rescigno M. 2013. Mucosal immunology and bacterial handling in the intestine. Best Pract Res Clin Gastroenterol 27(1):17–24.
Rodrigues AC, Cara DC, Fretez SH, Cunha FQ, Vieira EC, Nicoli JR, Vieira LQ. 2000. *Saccharomyces boulardii* stimulates sIgA production and the phagocytic system of gnotobiotic mice. J Appl Microbiol 89(3):404–14.
Roller M, Rechkemmer G, Watzl B. 2004. Prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* modulates intestinal immune functions in rats. J Nutr 134(1):153–6.
Russell DA, Ross RP, Fitzgerald GF, Stanton C. 2011. Metabolic activities and probiotic potential of bifidobacteria. Int J Food Microbiol 149(1):88–105.
Saarela M, Virkajarvi I, Alakomi HL, Sigvart-Mattila P, Mättö J. 2006. Stability and functionality of freeze-dried probiotic *Bifidobacterium* cells during storage in juice and milk. Int Dairy J 16:1477–82.
Saarela MH, Alakomi HL, Puhakka A, Mättö J. 2009. Effect of the fermentation pH on the storage stability of *Lactobacillus rhamnosus* preparations and suitability of *in vitro* analyses of cell physiological functions to predict it. J Appl Microbiol 106:1204–12.
Sanders ME, Klaenhammer TR, Ouwehand AC, Pot B, Johansen E, Heimbach JT, Marco ML, Tennilä J, Ross RP, Franz C, Pagé N, Pridmore RD, Leyer G, Salminen S, Charbonneau D, Call E, Lenoir-Wijnkoop I. 2014. Effects of genetic, processing, or product formulation changes on efficacy and safety of probiotics. Ann N Y Acad Sci 1309:1–18.
Saxelin M, Tynkkynen S, Mattila-Sandholm T, de Vos WM. 2005. Probiotic and other functional microbes: from markets to mechanisms. Curr Opin Biotechnol 16(2):204–11.
Sousa S, Gomes AM, Pintado MM, Silva JP, Costa P, Amaral MH, Duarte AC, Rodrigues D, Rocha-Santos TAP, Freitas AC. 2015. Characterization of freezing effect upon stability of probiotic loaded, calcium-alginate microparticles. Food Bioprod Process 93: 90–7.
Souza TC, Silva AM, Drews JRP, Gomes DA, Vinderola G, Nicoli J. 2013. *In vitro* evaluation of *Bifidobacterium* strains of human origin for probiotics functional foods. Benef Microbes 4:179–86.
Suzuki K, Nakajima A. 2014. New aspects of IgA synthesis in the gut. Int Immunol 26(9):489–94.
Vieira EL, Leonel AJ, Sad AP, Beltrão NR, Costa TF, Ferreira TM, Gomes-Santos AC, Faria AM, Peluzio MC, Cara DC, Alvarez-Leite JI. 2012. Oral administration of sodium butyrate attenuates inflammation and mucosal lesion in experimental acute ulcerative colitis. J Nutr Biochem 23(5):430–6.
Vinderola CG, Duarte J, Thangavel D, Perdigón G, Farnworth E, Matar C. 2005. Immunomodulating capacity of kefir. J Dairy Res 72:195–202.
Vinderola G, Zacarías MF, Bockelmann W, Neve H, Reinheimer J, Heller KJ. 2012. Preservation of functionality of *Bifidobacterium animalis* subsp. *lactis* INL1 after incorporation of freeze-dried cells into different food matrices. Food Microbiol 30(1):274–80.
Zacarías MF, Binetti A, Laco M, Reinheimer J, Vinderola G. 2011. Preliminary technological and probiotic characterization of bifidobacteria isolated from breast milk for use in dairy products. Int Dairy J 21:548–55.
Zacarías MF, Reinheimer J, Forzani L, Grangette C, Vinderola G. 2014. Mortality and translocation assay to study the protective capacity of *Bifidobacterium lactis* INL1 against *Salmonella* Typhimurium infection in mice. Benef Microbes 5(4):427–36.