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Mortality and translocation assay to study the protective capacity of *Bifidobacterium lactis* INL1 against *Salmonella* Typhimurium infection in mice

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RESEARCH ARTICLE

Abstract

The mouse has been largely used for the study of the protective capacity of probiotics against intestinal infections caused by *Salmonella*. In this work we aimed at comparing the mortality and translocation assay for the study of the protective capacity of the human breast milk-derived strain *Bifidobacterium animalis* subsp. *lactis* INL1 on a model of gut infection by *Salmonella enterica* subsp. *enterica* serovar Typhimurium. Different doses of *S.* Typhimurium FUNED and *B. animalis* subsp. *lactis* INL 1 were administered to Balb/c mice in a mortality or a translocation assay. The survival of the control group in the mortality assay resulted to be variable along experiments, and then we preferred to use a translocation assay where the preventive administration of 10^9 cfu of bifidobacteria/mouse for 10 consecutive days significantly reduced the number of infected animals and the levels of translocation to liver and spleen, with enhanced secretory immunoglobulin A and interleukin 10 production in the small and large intestine, respectively. Ten days of *B. animalis* subsp. *lactis* strain INL1 administration to mice significantly reduced both the incidence and the severity of *Salmonella* infection in a mouse model of translocation. This work provided the first evidence that a translocation assay, compared to a mortality assay, could be more useful to study the protective capacity of probiotics against *Salmonella* infection, as more information can be obtained from mice and less suffering is conferred to animals due to the fact that the mortality assay is shorter than the latter. These facts are in line with the guidelines of animal research recently established by the National Centre for the Replacement, Refinement & Reduction of Animals in Research.

Keywords: probiotic, mouse model, *Salmonella*, infection, bifidobacteria

1. Introduction

The traditional belief that human breast milk is sterile has been changing in the last decade and it is now regarded as a continuous supply of commensal, mutualistic and/or potentially probiotic bacteria to the infant gut during breast feeding (Fernández *et al.*, 2013). In 2003, two research groups isolated lactobacilli from human breast milk (Heikkilä and Saris, 2003; Martín *et al.*, 2003) and later the presence of bifidobacteria was also reported (Gueimonde *et al.*, 2007). The presence of lactobacilli and bifidobacteria in human breast milk is of particular interest as these bacterial groups are the main ones used for the development of probiotic cultures for human consumption (Foligné *et al.*, 2013). On the other hand, correct early-life programming of the gastrointestinal tract-associated immune system, where gut microbiota plays a main role, is clue for an adequate development of the intestinal immune system. The first colonisers of the intestine, many of them derived from breast milk, play an important role in host health because they are involved in nutritional, immunologic, and physiologic functions (Nauta *et al.*, 2013). It is hypothesised that some bacteria present in the maternal gut could reach the mammary

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gland during late pregnancy and lactation through a fine selection mechanism involving gut immune cells (Fernández *et al.*, 2013). In this sense, one may preliminary think that any human breast milk isolate belonging to the genera *Lactobacillus* or *Bifidobacterium* would possess probiotic properties as they were 'naturally selected' to be present in human breast milk and then they should play, by default, a positive role in gut mucosa immune modulation. However, the guidelines released by the joint FAO/WHO working group on the evaluation of new probiotics in food (FAO/WHO, 2002) indicate that probiotic properties must be demonstrated for each specific new strain. In that context, we isolated and characterised bifidobacteria strains from human breast milk (Zacarías *et al.*, 2011). In particular, the strain *Bifidobacterium animalis* subsp. *lactis* INL1 displayed some technological (Vinderola *et al.*, 2012) and functional properties in mice (Zacarías *et al.*, 2011) that make it a potential candidate for probiotic application. Among health benefits ascribed to the consumption of probiotics, those related to enhanced gastrointestinal health are the most spread ones (Foligné *et al.*, 2013), including prevention or early resolution of different types of diarrhoea or intestinal inflammation. It is very important to note that the European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies (NDA) stated that the majority of health claims analysed by the Panel were considered as (possibly) beneficial to human health, in particular the gut health effects (Binnendijk and Rijkers, 2013). Then, following the guidelines of the FAO/WHO that require preclinical (animal) studies in suitable models and in line with the possible positive effects on gut health recognised by the EFSA to probiotics, the aim of this work was to study the protective capacity of the human breast milk-derived strain *B. animalis* subsp. *lactis* INL1 on a model of gut infection by *Salmonella enterica* subsp. *enterica* serovar Typhimurium. *S. enterica* serovar Typhi causes typhoid fever, a severe systemic disease responsible for approximately 21 million cases per year around the world. Murine models of systemic salmonellosis have been set up to understand the pathogenesis of typhoid fever, using different strains of the closely related species *S. enterica* serovar Typhimurium (Watson and Holden, 2010). The mouse model is widely used not only to study the mechanisms of the pathogenesis (Mastroeni and Sheppard, 2004), but also to study the capability of probiotic bacteria on the prevention or treatment of enteric infections caused by *Salmonella* (Castillo *et al.*, 2011; Gill *et al.*, 2001; Truusalu *et al.*, 2008). The wide array of virulence among *S. enterica* strains present in nature (Heithoff *et al.*, 2012) may explain the wide range of the infective dose reported in mice (10^5 to 10^{10} cfu/mouse) (Suitso *et al.*, 2010). As the infection was reported to depend also on the specific mice and *Salmonella* Typhimurium strains used, we determined first the optimal condition of infection before evaluating the protective effect of our strain.

2. Materials and methods

Microorganisms and growth conditions

B. animalis subsp. *lactis* INL1, whose functional and technological properties were previously reported (Vinderola *et al.*, 2012; Zacarías *et al.*, 2011), was used in this study. The strain was kept frozen at -70 °C in De Man, Rogosa and Sharpe (MRS) broth (Biokar, Beauvais, France) added with 20% (v/v) glycerol (Ciccarelli, Buenos Aires, Argentina) and maintained at the INLAIN culture collection. Before use, the strain was cultured at least three times in MRS broth supplemented with 0.1% (w/v) L-cysteine hydrochloride (Biopack, Buenos Aires, Argentina) during 18 h under anaerobiosis (Anaeropack-Anaero, Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) at 37 °C. *Salmonella* Typhimurium FUNED, a strain of human origin isolated at the Fundação Ezequiel Dias (FUNED, Belo Horizonte, Minas Gerais, Brazil) was kindly provided by Dr. Jacques Nicoli (ICB, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil). *Salmonella* was kept frozen at -70 °C in Brain Heart Infusion (BHI) broth (Britania, Buenos Aires, Argentina) added with 20% (v/v) glycerol (Ciccarelli) at the INLAIN culture collection. The strain was grown overnight (aerobic incubation, 37 °C) in BHI broth directly from the frozen stock for 18 h for animal studies of infection.

Animals

BALB/c mice (male, six week-old) weighing 18-20 g were obtained from the random bred (in-bred) colony of the Centro de Experimentaciones Biológicas y Bioterio, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe, Argentina). Mice were allowed to stand at the INLAIN animal facility for a week before starting the assays and they were housed in groups of 3-5 animals/cage, depending on the experiment, and kept in a controlled environment at 21±2 °C with humidity at 55±2%, with a 12-h light/dark cycle. For assays where 7 mice were used, two cages containing 3 and 4 animals were set. After infection, mice were maintained housed in the same groups. Mice were maintained and treated according to the guidelines of the National Institute of Health (NIH, Bethesda, MD, USA). Animals received, *ad libitum*, tap water and a sterile conventional balanced diet (Cooperación, Buenos Aitres, Argentina) containing proteins, 230 g/kg; raw fibre, 60 g/kg; total minerals, 100 g/kg; Ca, 13 g/kg; P, 8 g/kg; water, 120 g/kg; and vitamins. The experiments with animals were approved by the Ethical Committee for Animal Experimentation of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe, Argentina).

Determination of the infective dose of *Salmonella*

A first set of experiments were conducted in order to implement the *in vivo* model of infection, using the strain *Salmonella* Typhimurium FUNED, before testing the protective capacity of *B. animalis* subsp. *lactis* INL1. An overnight culture (18 h) of the pathogen was washed twice with phosphate buffered saline (PBS) solution (pH 7.4). Mice (three groups of 10 animals) were challenged by intragastric intubation (0.1 ml/mouse) with a single infective dose corresponding respectively to 10^4, 10^5 or 10^6 cfu of *Salmonella* per mouse. Animals were maintained together in their cages after infection (not housed individually). The survival to infection was monitored daily until no death was observed in any group for three consecutive days. The cumulative mortality during the post-infection period was plotted against time and the results were expressed as survival (%) to infection.

Determination of the protective dose of *Bifidobacterium animalis*

A fresh overnight culture (18 h) of *B. animalis* subsp. *lactis* INL1 was prepared daily, washed twice with PBS and re-suspended in 10% (w/v) skim milk. Mice (10 animals/group) were intragastrically administered (0.1 ml/mouse), with either 10^7, 10^8 or 10^9 cfu/day of bifidobacteria for 10 consecutive days and subsequently challenged with a single infective dose (10^6 cfu/mouse) of *Salmonella* Typhimurium FUNED. Animals were maintained together in their cages after infection (not housed individually). Control animals received 0.1 ml/mouse of 10% (w/v) skim milk for 10 days before *Salmonella* challenge. Survival was monitored as previously described.

Determination of the protective period of administration

Three groups of mice (10 animals/group) received 10^9 cfu/day/animal of bifidobacteria, prepared daily as described above, during 3, 6 or 10 consecutive days. After those feeding periods, animals were challenged with a single infective dose (10^6 cfu/mouse) of *Salmonella* Typhimurium FUNED. Animals were maintained together in their cages after infection (not housed individually). Control animals received 0.1 ml/mouse of 10% (w/v) skim milk for 10 days before *Salmonella* challenge. No bifidobacteria were administered after *Salmonella* challenge. Survival was monitored as previously described.

Comparison of preventive vs. preventive-therapeutic administration

Twenty mice were divided into three groups named control group (C) (six animals), preventive group (P) (seven animals) and preventive-therapeutic group (T) (seven animals). Mice in groups P and T received (intragastric intubation) 10^9 cfu of *B. animalis* subsp. *lactis* INL1 for 10 consecutive days, prepared daily as described before. Animals were maintained together in their cages after infection (not housed individually). Animals in control group (C) received 10% skim milk. On the 11th day, mice received (intragastric intubation) a single infective dose (see Results for dose) of *Salmonella* Typhimurium FUNED, and continue to receive 10% skim milk (C and P groups) or *B. animalis* subsp. *lactis* INL1 (10^9 cfu/day/animal, group T). Animals were euthanised by cervical dislocation at day 5 post-challenge. Translocation of enteric bacteria was studied in internal organs. Liver and spleen were aseptically collected, weighed and homogenised (Ultra Turrax T8, Ika Labortechnik, Staufen, Germany) in sterile PBS. Serial 10-fold dilutions were made and 100 µl aliquots were plated onto MacConkey agar (Britania). Plates were incubated for 24 h (37 °C, aerobiosis). Results were expressed as a proportion of infected/non-infected animals, considering an animal infected when the translocation assay to liver was positive. Also, cell counts (Log cfu/g) in liver and spleen were carried out in all cases.

Assessment of immunoglobulin A and cytokines

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) anti-protease cocktail (P8340, Sigma, St. Louis, MO, USA). After centrifugation (2,000×*g*, 30 min, 4 °C) the supernatant fluid was collected and kept frozen at -70 °C for secretory immunoglobulin A (IgA) quantification by ELISA (Rodrigues *et al*., 2000). Portions of flushed intestines were prepared for fixation, histological preparation and paraffin inclusion, according to Vinderola *et al.* (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. The immunofluorescence test was performed using alpha-chain specific anti-mouse IgA fluorescein isothiocyanate conjugate (Sigma). Histological slices were deparaffinised and rehydrated in a series of decreasing ethanol concentrations (from absolute alcohol until 70° alcohol). Deparaffinised histological samples were treated with a dilution (1/100) of the antibody in PBS and incubated in the dark for 30 min at 37 °C. Then, samples were washed two times with PBS and examined using a fluorescent light microscope (Nikon Eclipse using a Hg lamp; Nikon, Tokyo, Japan). The results were expressed as the number of positive cells (fluorescent cells)/10 fields. Data were reported as the mean of three counts (each one in a different histological slice) for each animal. Small and large intestine samples (120-170 mg) were also obtained and kept frozen (-70 °C) for cytokine determination. Intestine samples were homogenised (Ultra Turrax T8) in PBS solution containing 1% (v/v) anti-protease cocktail (Sigma), 10 mmol/l EDTA (Sigma) and 0.05% (v/v) Tween 20 (Sigma) in a proportion of 1 ml PBS:100 mg tissue. The

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samples were then centrifuged (10 min, 10,000 rpm, 4 °C) and the supernatant was collected and kept frozen for cytokine quantification. The concentration of interleukin 10 (IL-10) and interferon-gamma (IFN-γ) was measured by ELISA using commercially available antibodies (BD Biosciences Pharmingen, San Diego, CA, USA), according to the procedures supplied by the manufacturer.

Statistical analysis

Data (% of survival) of the mortality assays were evaluated by Fisher's exact test. Data of colonisation in liver and spleen, secretory IgA in small intestine fluid and IL-10 and IFN-γ in large intestine homogenates were analysed using the one-way ANOVA procedure of SPSS software version 15.0 (SPSS Inc., Chicago, IL, USA). The differences between means were detected by Dunnet's unilateral post hoc test. Data were considered significantly different when $P<0.05$. Principal component analysis (PCA) was used to get an overview of the data and it was computed for variables measured in both the small and large intestine (number of IgA-producing cells/10 fields and the concentration of IL-10 and IFN-γ in tissue homogenates) and it was performed using R software (version 2.12.2) (R Development Core Team, 2011).

3. Results

Determination of the infective dose of *Salmonella*

Six week-old male mice challenged with a single dose of 10^4, 10^5 or 10^6 cfu/mouse of *Salmonella* Typhimurium showed a survival of 90, 70 and 50%, respectively (Figure 1). No significant differences in the cumulative survival (P=0.244) were observed between the groups that received 10^4 and 10^5 cfu/mouse of *Salmonella* Typhimurium, nor differences were detected neither between animals that received 10^5 and 10^6 cfu/mouse of *Salmonella* Typhimurium (P=0.454). However, significant differences (P=0.049) were observed between groups that received 10^4 and 10^6 cfu/mouse of the pathogen. The dose that induced a survival of 50% was chosen for further studies (10^6 cfu/mouse of *Salmonella* Typhimurium).

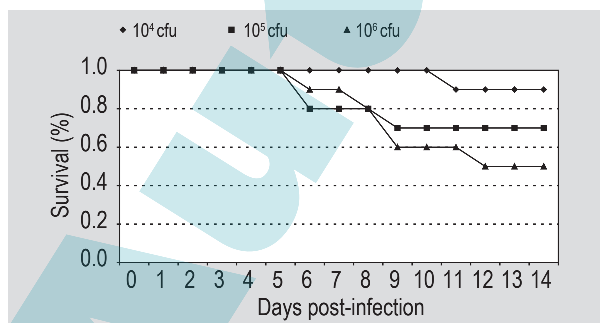

Figure 1. Survival of BALB/c mice to an infection with a single infective dose (10 animals/group) of *Salmonella* Typhimurium FUNED.

Determination of the protective dose of *Bifidobacterium animalis*

In order to define the optimal dose of bifidobacteria, mice received 10^7, 10^8 or 10^9 cfu/day of the strain for 10 consecutive days. Mice were then challenged with 10^6 cfu/mouse of *Salmonella* Typhimurium FUNED (Figure 2). The survival of the control group was 40%, whereas no significant differences (P=0.905) were observed among groups that received bifidobacteria, although the group that received 10^9 cfu of bifidobacteria presented 50% of survival. Considering previous studies where mice received for 10 days, 10^9 cfu of bifidobacteria per animal (Zacarías *et al.*, 2011), we performed a third assay, in which mice received the same amount of bifidobacteria daily for 3, 6 or 10 consecutive days and were challenged with 10^6 cfu/mouse of *Salmonella*. No control animals survived to infection (Figure 3), whereas the group that received bifidobacteria for 10 consecutive days presented a significant (P=0.01) higher survival.

Preventive vs. preventive-therapeutic administration of *Bifidobacterium animalis*

B. animalis subsp. *lactis* INL1 was administered for 10 consecutive days, either only before challenge (preventive group) or for 10 consecutive days before and after *Salmonella* challenge (preventive-therapeutic group). Total counts were performed in liver and spleen at day 5 post infection (Figure 4). Infected animals were considered those in which the translocation assay was positive in liver (presence of enterobacteria in agar plates). The proportion of infected/total animals was 5/6 for the control group, 2/7 for the preventive group and 4/7 for the preventive-therapeutic group. The statistical analysis

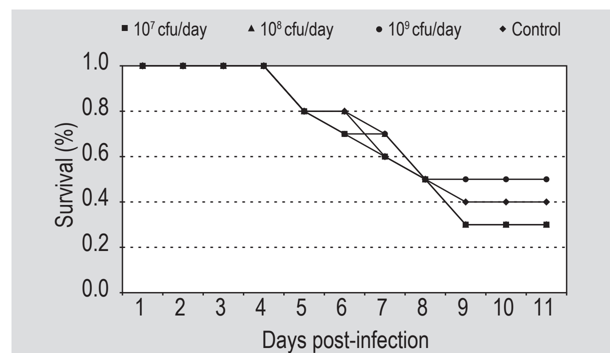

Figure 2. Survival of BALB/c mice fed for 10 consecutive days with *Bifidobacterium animalis* subsp. *lactis* INL1 (10 animals/group) and challenged with a single infective dose (10^6 cfu) of *Salmonella* Typhimurium FUNED. Control animals received no bifidobacteria, but were challenged with *Salmonella*.

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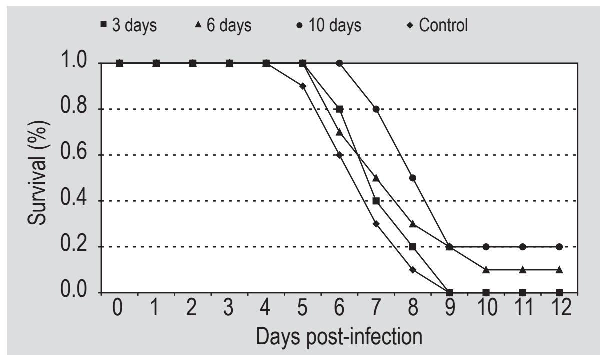

Figure 3. Survival of BALB/c mice fed for 3, 6 or 10 consecutive days with 10^9 cfu/day of *Bifidobacterium animalis* subsp. *lactis* INL1 (10 animals/group) and challenged with a single infective dose (10^6 cfu) of *Salmonella* Typhimurium FUNED. Control animals received no bifidobacteria, but were challenged with *Salmonella*.

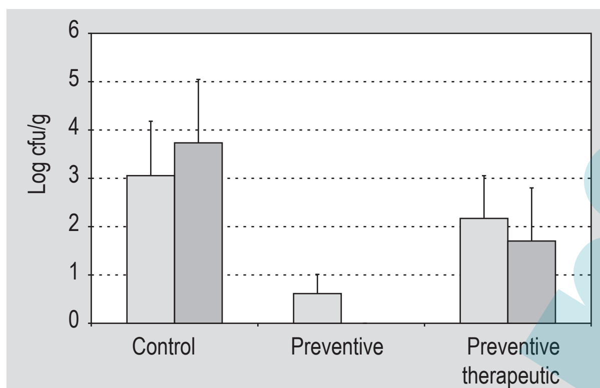

Figure 4. Translocation assay: total counts in liver (light grey columns) and spleen (dark grey columns) of mice by day 5 after *Salmonella* infection. Animals received 10^9 cfu/day of *Bifidobacterium animalis* subsp. *lactis* INL1 in 10% skim milk (Preventive and Preventive-therapeutic groups, 7 animals/ group) or 10% skim milk alone (Control group, 6 animals) for 10 consecutive days and were challenged with a single infective dose (10^6 cfu) of *Salmonella* Typhimurium FUNED. In the Preventive-therapeutic group, animals continued receiving the bifidobacteria during the course of the infection after *Salmonella* challenge. [a,b] Significantly different compared to the corresponding control (*P*=0.024 for counts in liver and *P*=0.001 for counts in spleen, respectively).

determined that proportions were significantly different among them (*P*=0.04). The unilateral Dunnet test applied determined that the proportion of non-infected animals in the preventive group was significantly higher than in the control group (*P*=0.05). However, no differences in the proportions were observed between the preventive-therapeutic and the control groups (*P*=0.24). Unexpectedly, the administration of bifidobacteria after *Salmonella* challenge did not contribute to enhanced protection against translocation.

The severity of the infection was evaluated considering the level of liver and spleen colonisation in infected animals (Figure 4). Infected animals in the control group presented an average colonisation between 3 and 4 log orders in liver and spleen, whereas for the preventive-therapeutic group infection was about 2 log orders in both organs. On the contrary, for the preventive group, no spleen colonisation was observed (*P*=0.001) whereas liver colonisation showed significant lower values (*P*=0.024) of less than 1 log order by day 5 post-challenge. Total secretory IgA was quantified in the small intestine fluid whereas IFNγ and IL-10 were assessed in large intestine tissue homogenates (Table 1). Both S-IgA and IL-10 contents were significantly enhanced (*P*=0.064 and 0.01, respectively) in the small and large intestine, respectively, of animals of the preventive group.

The number of IgA-producing cells/10 fields in the small and large intestine lamina propria and the concentration of IL-10 and IFNγ in homogenates of the small and large intestine of mice at day 5 post *Salmonella* infection were considered for a PCA to obtain a structural representation of the variability of the responses and to determine how the responses distributed for the different groups (control, preventive, preventive-therapeutic) (Figure 5) with the aim of providing additional tools to visualise the fundamental differences among treatments.

The first two principal components explained 55% of the total variability of the data. The coefficients are shown in Table 2. The first component showed a parallel effect or a parallel movement of variables: if one of the responses increases in a mouse, the same happened to the other responses in the same animal. The first component also grouped the individual responses of the control and the preventive group at the right of the chart, whereas the individual responses of the preventive-therapeutic group were located to the left of the chart. The individual responses of the preventive treatment were visible scattered in the two-dimensional space. In contrast, the samples of

Table 1. Concentration of total secretory IgA (S-IgA) in the small intestine content and IFN-γ and IL-10 in large intestine homogenates at day 5 post-*Salmonella* infection.

	Concentration (mean ± standard error of the mean)		
	Control	Preventive	Preventive therapy
S-IgA (µg/g)	275.5±28.2[a]	371.4±20.6[b]	268.6±24.1[a]
IFN-γ (pg/g)	622.9±62.4[a]	732.6±142.1[a]	239.6±81.4[b]
IL-10 (pg/g)	3,613.5±538.9[a]	7,191.7±1,070.5[b]	4,088.1±1148.6[a]

[a,b] Values in rows with different superscripts are significantly different (*P*=0.0064, 0.0128 and 0.0122 for S-IgA, IFNγ and IL-10, respectively).

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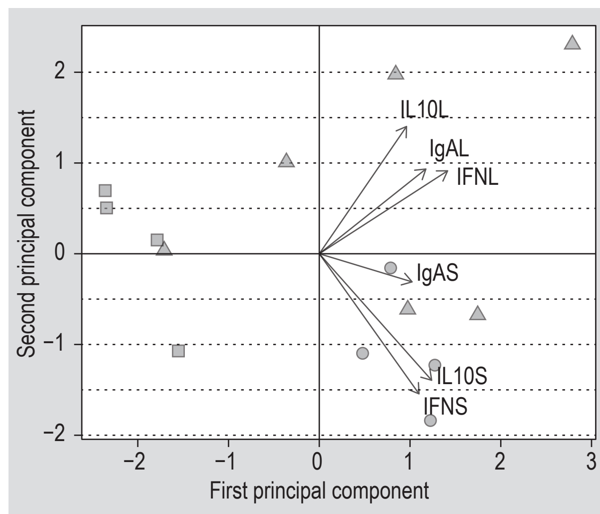

Figure 5. Principal components analysis for the variables IgA-producing cells/10 fields in the small (IgAS) and large (IgAL) intestine lamina propria (immunohistochemistry) and IL-10 and INF-γ in tissue homogenates of the small (IL10S, IFNS) and large (IL10L, IFNL) intestine (ELISA), at day 5 post-*Salmonella* infection.

the control and preventive-therapeutic group showed a much stronger clustering; suggesting that infected animals (control and preventive-therapeutic groups) apparently acquired a rather uniform infection. The second component separated the response of the preventive group (upper side of the chart) from the response of the control group (lower side of the chart), whereas the response of the preventive-therapeutic group remained distributed in the middle of the chart.

4. Discussion

A wide array of virulence among *Salmonella* Typhimurium strains was reported (Suitso *et al.*, 2010). Therefore, we set up a first experiment to define the optimal infectious dose of *Salmonella* Typhimurium FUNED, the strain available in this study. The survival was dose-dependent (Figure 1). The dependence of survival with the infective dose was reported by Wijburg *et al.* (2006), who found that 10^4, 10^5 or 10^6 cfu of *Salmonella* Typhimurium allowed a survival of 80, 0 and 0%, respectively, in C57BL/6 mice. The dependence of survival with age was also previously informed by Ren *et al.* (2009) in C57BL/6 mice, where old mice (22-24 months of age) were more sensitive to *Salmonella* infection than adult mice (4-6 months of age). Using seven-eight week old Balb/c male mice but a different *Salmonella* Typhimurium strain, Zoumpopoulou *et al.* (2008) found a survival of 30% in mice infected with $5{\times}10^4$ cfu of *Salmonella*. Truusalu *et al.* (2008) reported a 91% survival in four to six week-old Swiss NIH line mice challenged with $5{\times}10^4$ cfu/mouse. Working with the same *Salmonella* strain that we used in this study, Silva *et al.* (2004) and Martins *et al.* (2005) reported a 0 and 20% survival, respectively, in conventionalized Swiss

Table 2. Coefficients of the principal components analysis of the responses of IgA-producing cells in the small (IgAS) and large (IgAL) intestine lamina propria (determined by immunohistochemistry) and IL-10 and INF-γ in homogenates of the small (IL10S, IFNS) and large (IL10L, IFNL) intestine (determined by ELISA).

Parameter	Principal component	
	1	2
IgAS	0.36	0.11
IgAL	0.41	-0.33
IL10S	0.44	0.49
IL10L	0.34	-0.49
IFNS	0.39	0.54
IFNL	0.50	-0.32

NIH control mice that received 10^5 cfu or 10^4 cfu/mouse of the enteropathogen. In another study using, again, the same *Salmonella* strain of this study, a 40% survival was reported in conventionalised Swiss NIH control mice for an infective dose of 10^4 cfu/mouse (Martins *et al.*, 2009). Finally, De Moreno de Leblanc *et al.* (2010), using five to six week-old BALB/c mice and a locally-isolated *Salmonella* Typhimurium strain, reported 30% survival by day 10 post-infection in mice challenged with 10^7 cfu of the pathogen. When considering all these reports together, it seems that the dose of *Salmonella* able to induce a mild infection in mice is highly conditioned by factors related to the enteropathogen available (strain and dose) and to the animals used (strain, sex, age). Then, it seems that for each study about the effects of probiotics against enteric infections, the infective dose of *Salmonella* must be adjusted first. We decided to use the highest dose (10^6 cfu of *Salmonella)* that led to 50% mortality to evaluate the protective effect of *B. animalis* subsp. *lactis* INL1. For assays 2 and 3, the 10-day feeding period with bifidobacteria was chosen according to a previous study (Zacarías *et al.*, 2011) in which it was shown to be effective for the proliferation of IgA-producing cells in the small intestine mucosa. In the third assay, a significant enhanced survival was observed. Taken into consideration the three independent mortality assays performed, it is worth to note that the same single infective dose of *Salmonella* (10^6 cfu/mouse) induced different survival to infection in control animals (50, 40 or 0%, in mortality assay 1, 2 and 3, respectively) and survival reached a steady state by day 12 (assay 1) or by day 9 (assays 2 and 3). The reasons of this variability remains unknown, as the strain and animals were managed exactly under the same conditions in the three assays, but it might be related to the fact that the process of *Salmonella* infection in mice is considered to be dynamic and heterogeneous and dependent on multiple variables that underlie the complex processes that take place in the host (Watson and Holden,

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2010). Additionally, we cannot ruled out certain variability due to possible *Salmonella* cross-contamination due to coprophagy in mice housed together after infection. This certain variability in the survival rate of the control group (animals that received only *Salmonella*) was also observed in germ-free and conventional mice in this mouse model of infection (Lima-Filho *et al.*, 2004; Maia *et al.*, 2001; Martins *et al.*, 2005, 2007, 2009, 2010; Silva *et al.*, 1999, 2004).

IgA is the main immunoglobulin in the gut surfaces where its main function is to exert the immune exclusion of pathogenic bacteria or viruses by intimate cooperation with the innate non-specific defence mechanisms (Brandtzaeg *et al.*, 1987). As a functional trait, the capacity of enhancing mucosal IgA in the gut after oral administration is a desirable attribute for probiotic bacteria (Galdeano *et al.*, 2009) and in previous works this feature was linked to the protection against *Salmonella* infection (Vinderola *et al.*, 2007). If we consider previous results (Zacarías *et al.*, 2011) and current data, it might be assumed that a feeding period of 10 days with 10^9 cfu/day/mouse of bifidobacteria might be effective in enhancing gut IgA and lessening mortality due to *Salmonella* infection. Taking into account that mice dead occurs as a result of large bacterial loads in the liver and spleen (Watson and Holden, 2010), we decided to switch from a mortality assay to a translocation trial, where the level of infection of (normally sterile) liver and spleen is studied after *Salmonella* challenge (Bao *et al.*, 2000; Vinderola *et al.*, 2007). As in experiments of mortality, survival reached a steady state (no mouse death) between days 9 and 12, we decided to perform the translocation assay at day 5 post-infection. The translocation assay showed that preventive, but not preventive-therapeutic administration of bifidobacteria for 10 consecutive days diminished both the incidence and the severity of *Salmonella* infection (Figure 4). It is worth to note that the administration of bifidobacteria after *Salmonella* challenge did not contribute to enhanced protection against translocation. In this sense, increased translocation followed prebiotics administration was reported in mice (Petersen *et al.*, 2009) and in rats (Bovee-Oudenhoven *et al.*, 2003; Ten Bruggencate *et al.*, 2003) and it was hypothesised that increased intestinal acidification might be related to translocation. Then, we might speculate that metabolic activity of bifidobacteria along intestinal transit might have induced a similar effect taking into consideration previous reports.

Although we used a isogenic strain of mice (inbred clones of genetically identical individual), it is remarkable that, in the translocation assay, not all challenged animals, in the control group, got infected by *Salmonella*. If we consider that mouse death occurs when large amounts of the pathogen colonises liver and spleen (Watson and Holden, 2010), then we might expect that mice that survives in the mortality assays, are indeed those which are not infected by *Salmonella* in a translocation assay when assessed at day 5 post-infection. In our work, 1/6 and 5/7 of the animals in the control and preventive group, respectively, were not infected by *Salmonella* in the translocation assay, then we might expect a survival of 17% and 70% in a mortality assay. Then, the use of a translocation assay instead of a mortality one would shorten experiments, diminishing then suffering in lethally infected laboratory animals. Additionally, more information might be obtained using a translocation assay (infected/non infected ratio and organs and tissues to determine levels of colonisation and immune parameters) compared to a mortality assay (only percentage of animals that survived to infection). Shortening manipulation periods and maximising information derived from animals are facts that are in line with the ARRIVE guidelines for animals research of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (Kikenny *et al.*, 2010), however further research is needed in order to propose the alternative use of a translocation assay over a mortality assay for the study of the protective capacity of probiotics against enteric infections.

Some immune parameters were determined in the gut of animals in the translocation assay as well. Enhanced S-IgA and IL-10 was observed in the small and large intestine of animals in the preventive group (Table 1). In previous works, enhanced mucosal S-IgA was linked to enhanced survival to *Salmonella* infection (Vinderola *et al.*, 2007). Although it is known that enhanced S-IgA can protect the intestinal epithelium from enteric pathogens and toxins, surprisingly little is known about the molecular mechanisms by which this is achieved (Mantis and Forbes, 2010). One possible mechanism is the inhibition of the enteropathogen motility and entry into epithelial cells, that was reported as a previously unrecognised capacity of S-IgA to 'disarm' microbial pathogens on mucosal surfaces and prevent colonisation and invasion of the intestinal epithelium (Forbes *et al.*, 2008). Another mechanism that contributed to enhanced protection was mediated by enhanced IL-10 in the large intestine, which is known for its regulatory and anti-inflammatory effects (Paul *et al.*, 2012).

Finally, PCA of data was used in order to get an overview of the data distribution. The individual responses of the animals of the preventive treatment were observed scattered in the chart whereas the responses of the control and preventive-therapeutic group resulted much more clustered. This kind of grouping of responses in infected animals was previously reported by Gerritsen *et al.* (2011) when correlating probiotic therapy and acute pancreatitis-associated microbiota in rats. Putaala *et al.* (2010) also demonstrated a higher clustering of individual responses in Caco-2 cells challenged with *Escherichia coli* O157:H7 when compared with the individual responses of Caco-2 cells challenged with probiotics. Individual responses that cluster together forming a relatively small group, suggest that the response is consistent and homogeneous (Putaala

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et al., 2010). The second component also indicated that the movement of variables in the small intestines went to the opposite direction compared to the movement in the large intestine, this could be related to the fact that mucosal immune responses are compartmentalised (Macpherson *et al.*, 2012). This occurred for the three variables measured (IgA-producing cells, IL-10 and IFN-γ). The length of the arrows and the position of the final points are similar for the variables inside each group, indicating that those variables were highly correlated and behaved in a similar way in each mouse. A biological interpretation of data movement along the principal components chart would suggest that the second component was efficient in differentiating the control from the preventive group, whereas the preventive-therapeutic group remained between these two groups. Clustering or scattering of individual immune responses in infected or non-infected animals, respectively, may be a useful tool provided by PCA to further characterise the mitigation of *Salmonella* infection by probiotics. Further research involving the study of other immune parameters is still needed in this regard.

Modulation of host defence responses and protection against infectious diseases are among the most distinctive benefits of bifidobacteria. We selected a *B. animalis* subsp. *lactis* strain (INL1) as a potential candidate for probiotic application. The aim of the present study was to determine, using a mouse model, the capacity of this bifidobacteria strain to achieve protection against *S.* Typhimurium infection. The results obtained demonstrated that 10 days of *B. animalis* subsp. *lactis* strain INL1 administration reduced both the incidence and the severity of *Salmonella* infection (regarded as the proportion of infected animals and as the level of liver and spleen colonisation, respectively). Several mechanisms and cells are involved in this protective effect against *Salmonella*, notably the induction of secretary IgA antibodies responses. The positive effect was also associated with an enhancement of total s-IgA in the small intestine as well as with an increase in the level of intestinal IL-10, indicating an associated anti-inflammatory effect. There are still some issues tough that deserve further study, which could make more suitable a translocation assay compared to a mortality assay for the study of the functional properties of probiotics.

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