Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

# Study of the effects of spray drying in whey-starch on the probiotic capacity of *Lactobacillus rhamnosus* 64 in the gut of mice

L. Lavari[1], P. Burns[2], R. Páez[1], J. Reinheimer[2] and G. Vinderola[2]

1 INTA EEA Rafaela, Santa Fe, Argentina
2 Instituto de Lactología Industrial (INLAIN, UNL-CONICET), Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santa Fe, Argentina

**Keywords**
colitis, *Lactobacillus rhamnosus*, mice, probiotic capacity, spray drying.

**Correspondence**
Gabriel Vinderola, Instituto de Lactología Industrial (INLAIN, UNL-CONICET), Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santa Fe 3000, Argentina.
E-mail: gvinde@fiq.unl.edu.ar

2017/1062: received 6 June 2017, revised 28 July 2017 and accepted 6 August 2017

doi:10.1111/jam.13567

## Abstract

**Aims:** To evaluate the effects of spray drying of *Lactobacillus rhamnosus* 64 on its capacity to modulate the gut immune response and on the attenuation of TNBS-induced colitis in mice.
**Methods and Results:** *Lactobacillus rhamnosus* 64 was spray dried in cheese whey-starch solution and administered to mice for 3, 6 or 10 consecutive days. Peritoneal macrophage phagocytic activity, secretory IgA levels in the small intestinal fluid and TNFα, IFNγ, IL-10, IL-6 and IL-2 levels in homogenates of the small and large intestine were determined. The effects of spray drying were also evaluated in an acute model of Trinitrobenzenesulfonic acid (TNBS)-induced colitis. A shift in the regulation of immune parameters, particularly the cytokine profile, was observed for mice treated with the spray-dried culture, compared to the profile observed in animals that received the strain as fresh culture (FC). The spray-dried culture of *L. rhamnosus* 64 showed anti-inflammatory properties in murine model of TNBS-induced colitis.
**Conclusions:** The spray-drying process of *L. rhamnosus* 64 in whey-starch modified its immunomodulating capacity in healthy animals and conferred enhanced protection in an *in vivo* model of inflammation.
**Significance and Impact of the Study:** Probiotic capacity can be affected by spray drying in relation to the properties observed for the strain as an overnight FC. This fact should be taken into account when producing the culture for its application in the industry.

## Introduction

Probiotics are widely used as preventative or therapeutic agents or added into foods, and numerous human intervention studies have been performed to prove their beneficial effects in the last decade (Sanders *et al.* 2013). Probiotics can enhance the host's health and well-being since they are, at least partially, an effective prevention and control tool for certain diseases such as various types of diarrhoeal diseases, respiratory infections, chronic gastrointestinal inflammatory disorders, necrotizing enterocolitis or allergic responses (Grover *et al.* 2012). Mechanisms such as the ability to compete with pathogenic micro-organisms for adhesion sites, to antagonize pathogens or to modulate the host's immune response have been proposed to describe how these micro-organisms, acting mainly on the gastrointestinal tract, might be able to treat or prevent intestinal disorders (Syngai *et al.* 2016).

For long-term preservation of probiotic's viability and functionality, the biotechnology industry uses drying processes which involve the transition of micro-organisms from a liquid to a solid medium. Industrial manufacturing processes of dried cultures may affect microbial structures and functional properties. Factors that may affect cell viability and functionality during spray drying, such as growth media, pH of growth, growth phase at harvesting and harvesting technique,

scale of spray drying, atomization, protective agents and total solids, weere recently reviewed (Huang *et al.* 2017). Iaconelli *et al.* (2015) studied the impact of air drying, freeze drying and spray drying on *Bifidobacterium bifidum*, *Lactobacillus plantarum* and *Lactobacillus zeae*, finding that the bacteria respond differently to the three different drying processes in terms of viability and functionality (immunomodulation and hydrophobicity). They observed that adherence can be stimulated (air drying) or inhibited (spray drying) by drying processes and results of a multivariate analysis show no direct correlation between bacterial survival and functionality. In order to evaluate the impact of the drying processes, bacterial survival is usually measured soon after the dehydration process is carried out and along storage of dehydrated cultures. Nevertheless, the maintenance of cell viability after the drying process, does not always warrant the probiotic capacity. The survival of probiotic bacteria does not necessarily correlate with the preservation of their beneficial effects (Makinen *et al.* 2012). In this context, long-term preservation of both viability and probiotic capacity is a challenge for the industry as the drying processes may affect, for example, the immunomodulating capacity of probiotics (Paéz *et al.* 2013; Iaconelli *et al.* 2015). The aim of this work was to study the *in vivo* effects of spray drying of *L. rhamnosus* 64 in whey-starch on its capacity to modulate the gut immune response in the small and large intestine, and to preliminary study the effects of this dehydration technology on the attenuation of the inflammatory response in a murine model of colitis induced by TNBS.

## Materials and methods

### Bacterial strain

*Lactobacillus rhamnosus* 64 was used in this study. The strain was isolated in our laboratory from faeces of neonates (Vinderola *et al.* 2008), and showed the capacity to promote the proliferation of IgA-producing cells in the small intestine lamina propria of mice (Gregoret *et al.* 2013) and to survive spray drying (Lavari *et al.* 2014). The strain is kept in the culture collection of the INLAIN (UNL-CONICET, Santa Fe, Argentina). Overnight cultures (16 h, 37°C) were obtained in MRS (de Man, Rogosa and Sharpe) broth (Biokar, Beauvais, France), after three successive transfers from frozen (−70°C) stocks in MRS added with 18% (w/v) glycerol (Ciccarelli, Santa Fe, Argentina). Levels of viable cells were routinely checked by plating serial dilutions on MRS agar (37°C, 72 h, aerobiosis) and optical density (560 nm) reading.

### Spray drying in cheese whey-starch solution

A culture of *L. rhamnosus* 64 was obtained in a 2-l fermentor (Biostat A plus; Sartorius, Buenos Aires, Argentina), inoculated (1% v/v, achieving *c.* 7 log orders CFU per ml in the fermentor) with an overnight (37°C, aerobiosis, 18 h) culture in MRS. As culture medium in the fermentor, 9% (w/v) reconstituted cheese whey permeate (Arla Foods, Porteña, Córdoba, Argentina) supplemented with 2·5 g $l^{-1}$ yeast extract, 2·5 g $l^{-1}$ tryptone, 5 ml $l^{-1}$ Mg–Mn solution was used (Lavari *et al.* 2015). Fermentation was conducted in anaerobiosis with $CO_2$ flow (0·2 l $min^{-1}$, 200 rev $min^{-1}$ stirring) maintaining pH at 6·0 by automatic addition of sterile $Na_2CO3$ (212 g $l^{-1}$)/NaOH (160 g $l^{-1}$). Cells were harvested (4000 ***g***, 15 min, 4°C) and washed twice with PBS (pH 7·4). Cells were resuspended in (10% w/v) cheese whey (Arla Foods) plus 10% (w/v) starch (Glutal S.A., Buenos Aires, Argentina), according to a previous work (Lavari *et al.* 2015). Cell suspensions were spray dried in a laboratory scale spray dryer (Büchi mini spray dryer model B290; Flawil, Switzerland). Drying conditions were: feeding rate, 270 ml $h^{-1}$; constant inlet air temperature, 140°C; outlet temperature, 83°C; and air flux, 357 l $h^{-1}$ Survival to spray drying was assessed before and after drying by plating serial dilutions ($10^{-6}$ to $10^{-9}$) of the culture on MRS agar.

### *In vivo* trials

*Animals*

Forty-five 6-week-old male BALB/c mice (20 ± 1 g) were obtained from the random bred colony of the Centro de Medicina Comparada, Instituto de Ciencias Veterinarias del Litoral (CMC-ICiVet-Litoral, CONICET–UNL), Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe, Argentina). Animals were allowed to stand at the INLAIN animal facility for 7 days before the animal trial began. Each experimental group consisted of five mice housed together in plastic cages and kept in a controlled environment at a temperature of 21 ± 2°C with humidity at 55 ± 2%, with a 12 h light–dark cycle. Mice were maintained and treated according to the guidelines of the National Institute of Health (NIH, USA). All animals received, simultaneously and *ad libitum*, tap water and a sterile conventional balanced diet (Cooperación, Buenos Aires, Argentina) containing proteins, 230 g $kg^{-1}$; raw fibre, 60 g $kg^{-1}$; total minerals, 100 g $kg^{-1}$; Ca, 13 g $kg^{-1}$; P, 8 g $kg^{-1}$; water, 120 g $kg^{-1}$; 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).

### Effects of spray drying on the immunomodulating capacity of *Lactobacillus rhamnosus* 64

*Animals and feeding procedures*

The following groups were set: a control group, that received (by gavage) 0·2 ml $day^{-1}$ of 20% (w/v) cheese whey-starch solution for 10 consecutive days, and two probiotic-treated groups. Mice in probiotics-treated groups received daily (by gavage) 0·2 ml of a cell suspension containing $10^8$ CFU per ml of *L. rhamnosus* 64 for 3, 6 or 10 consecutive days. The strain was administered either as overnight fresh culture (FC), washed twice in PBS and resuspended in 20% (w/v) cheese whey-starch solution or as spray-dried (SD) culture in cheese whey-starch and reconstituted (to 20% w/v) in distilled water.

*Phagocytosis assay in peritoneal macrophages*

After each feeding period, animals were anaesthetized intraperitoneally (0·2 ml per mouse) with a cocktail (900 $\mu$l of ketamine (100 mg $ml^{-1}$), 900 $\mu$l of xylazine (20 mg $ml^{-1}$), 300 $\mu$l of acepromazine (10 mg $ml^{-1}$) and 7·9 ml of sterile 0·85% (w/v) NaCl solution). Animals were sacrificed by cervical dislocation. Peritoneal macrophages were harvested (in sterile conditions) by washing the peritoneal cavity with 5 ml of PBS containing 10 U $ml^{-1}$ of Heparin (Sigma Aldrich, St. Louis, MO) and 0·1% Bovine Serum Albumin (Jackson Immuno Research, West Grove, PA). The macrophage suspension was washed twice with the same buffer, and it was adjusted to a concentration of $10^6$ cells per ml using a Neubauer chamber. A heat-killed (100°C, 15 min) *Candida albicans* suspension ($10^7$ cells per ml) was opsonized with mouse autologous serum (10%) for 15 min at 37°C. Then, 0·2 ml of the opsonized yeast was added to 0·2 ml of each macrophage suspension. The mixture was incubated for 30 min at 37°C. The percentage of phagocytosis was measured as the percentage of activated macrophages (with at least one cell of yeast phagocyted) after a 100-cell count using an optical microscope (40×).

*Determination of secretory IgA in the small intestine 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 secretory-IgA (S-IgA; Sigma) and IFN-γ (BD Biosciences Pharmingen, San Diego, CA) quantification by ELISA using commercially available kits was carried out according to the procedures supplied by the manufacturers.

*Immunofluorescence test for IgA-producing cells enumeration*

The small intestine was removed for histological preparation and paraffin inclusion, according to previous reports (Vinderola *et al.* 2005). Paraffin sections (4 mm) were stained with haematoxylin–eosin followed by light microscopy examination to assess the intestinal architecture. The number of IgA-producing (IgA+) cells was determined on histological slices of samples from the ileum, not considering Peyer's patches. The immunofluorescence test was performed using α-chain-specific anti-mouse IgA fluorescein isothiocyanate (FITC) conjugate (Sigma Aldrich). Histological slices were deparaffinized and rehydrated in a series of ethanol concentrations. Deparaffinized 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. The results were expressed as the number of fluorescent, IgA+ cells per 10 fields. Positive (fluorescent cells) were counted with magnification of 400×. Data were presented as the mean value for three tissue slices for each animal for each feeding period.

*Determination of cytokines in the gut*

Small and large intestine samples (100–200 mg) were kept frozen (−70°C). Intestine samples were homogenized (Ultra Turrax T8, Ika Labortechnik, Staufen, Germany) in PBS buffer containing 1% (v/v) anti-protease cocktail (Sigma), 10 mmol $l^{-1}$ EDTA (Sigma) and 0·05% (v/v) Tween 20 (Sigma) 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 cytokine (TNFα, IFNγ, IL-10, IL-6 and IL-2) determination by ELISA using commercial kits (BD Biosciences Pharmingen), according to the procedures supplied by the manufacturer. The results were expressed as concentration of each cytokine in the intestinal fluid (pg $ml^{-1}$).

### Study of TNBS-induced acute colitis

*Animals and feeding procedures*

Thirty 6-week-old male BALB/c mice (weigh 20 ± 1 g) were allowed to stand at the INLAIN animal facility for 7 days before the animal trial began. The following groups (10 mice per group) were set: a group that received (by gavage) 0·2 ml $day^{-1}$ of 20% (w/v) cheese whey-starch solution for 10 consecutive days (control group). Mice in probiotic-treated groups received daily (by gavage) 0·2 ml of a cell suspension containing $10^8$ CFU per ml of the strain for five consecutive days. The strain was administered as an overnight FC, washed

and resuspended in 20% (w/v) cheese whey-starch, or as a culture SD in cheese whey-starch and then reconstituted (to 20% w/v) in distilled water.

*Induction of colitis and inflammation scoring*

Animals were anaesthetized intraperitoneally as described above. TNBS (Sigma) at a dose of 100 mg $kg^{-1}$ body weight was dissolved in 0·9% NaCl/ethanol (50/50, v/v) and 50 $\mu$l was administered intrarectally at 4 cm proximal to the anus, using a 3·5F catheter. Mice were monitored daily for general appearance and behaviour and recording of body weight. On day 3 post-TNBS challenge animals were anaesthetized (as previously described), sacrificed by cervical dislocation and their colons were removed and examined to evaluate the macroscopic lesions according to the Wallace criteria (Wallace *et al.* 1989).

*Macroscopic assessment of colitis*

The study was performed according to Foligné *et al.* (2006). The colon was removed, dissected free of fat and mesentery, carefully opened and cleaned with PBS. Colonic damage and inflammation were assessed according to the Wallace criteria (Wallace *et al.* 1989). These criteria for macroscopic scoring (scores ranging from 0 to 10) have been well established in mice studies and reflect (i) the intensity of inflammation, (ii) the thickening of colonic mucosa and (iii) the extent of ulceration. The scores are: 0 = no damage, 1 = hyperaemia. No ulcers, 2 = hyperaemia and thickening of bowel wall. No ulcers, 3 = one ulcer without thickening of the bowel wall, 4 = two or more sites of ulceration or inflammation, 5 = two or more major sites of ulceration and inflammation or one site of ulceration/inflammation extending >l cm along the length of the colon, and 6–10 = if damage covered >*Z* cm along the length of the colon, the score was increased by 1 for each additional centimetre of involvement.

*Statistical analysis*

Data were analysed using the one-way ANOVA procedure of SPSS software (SPSS Inc., Chicago, IL, ver. 15.0). The differences between means were detected by Dunnet's unilateral *post hoc* test. Data were considered significantly different when $P < 0{\cdot}05$. For the colitis study, analyses were performed as comparison of experimental groups with the respective control, using the Mann–Whitney test, a nonparametric one-way analysis of variance. Differences were judged to be statistically significant when the $P < 0{\cdot}05$.

## Results

A significant reduction of half log order ($P < 0{\cdot}05$) in the level of viable cells was observed after spray drying. The count of the cell suspension before spray drying was 9·9 log CFU per ml, whereas the count after the dehydration process in the reconstituted powder was 9·4 log CFU per ml. The powder obtained presented a residual moisture of 3·8% (w/v).

The oral administration of *L. rhamnosus* 64 significantly ($P = 0{\cdot}000$) increased the phagocytic activity of peritoneal macrophages (Fig. 1) after 3 days of administration of the strain, compared with the control group (12·4 ± 0·8%), without differences between the strain administered as fresh (23·3 ± 1·8%) or as spray-dried and reconstituted culture (21·6 ± 1·7%). The activation of macrophages was observed after 3 days of administration of the strain under study, and then returned to the basal (control) value. As regards S-IgA (Fig. 2), a significant increase was observed in animals that received the FC for 6 days (121·2 ± 6·0 $\mu$g $ml^{-1}$; $P = 0{\cdot}000$) and 10 days (97·4 ± 9·9 $\mu$g $ml^{-1}$ $P = 0{\cdot}021$) and in the animals that received the spray-dried culture for 3 days ($P = 0{\cdot}01$), compared to the control (51·7 ± 9·9 $\mu$g $ml^{-1}$). Values of S-IgA between fresh and SD groups were significantly different ($P < 0{\cdot}05$) for each feeding period studied.

The histochemical study of IgA-producing cells in the lamina propria of the small intestine (Fig. 3) showed that the administration of *L. rhamnosus* 64 induced the proliferation of IgA+ cells both as fresh and as SD culture, compared to controls ($P < 0{\cdot}05$), for all the administration periods evaluated. No significant differences were observed among the three periods of administration assessed or when the strain was administered either as fresh or as a SD culture.

The levels of IFNγ, TNFα, IL-2, IL-6 and IL-10 were evaluated in homogenates of the small and large intestine by ELISA. In the small intestine, no significant differences

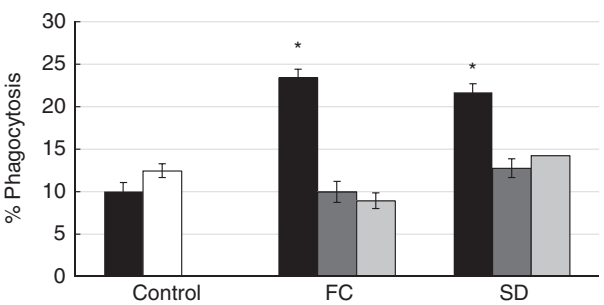


**Figure 1** Phagocytic activity of peritoneal macrophages from mice that received *Lactobacillus rhamnosus* 64 as fresh (FC) or spray-dried (SD) culture for 3 (■), 6 ( ■) or 10 (■) consecutive days, compared to control animals: untreated mice (■) and animals that received only the 20% (w/v) cheese whey-starch solution (□). Values are the mean ± SEM. *Significantly different ($P < 0{\cdot}05$) compared to control animals.

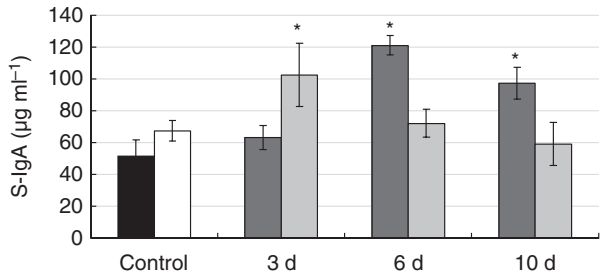


**Figure 2** Secretory IgA (S-IgA) in the small intestine content of mice that received *Lactobacillus rhamnosus* 64 as fresh (■) or as spray-dried (■) culture for 3, 6 or 10 consecutive days, compared to control animals: untreated mice (■) and animals that received only the 20% (w/v) cheese whey-starch solution (□). Values are the mean ± SEM. *Significantly different ($P < 0{\cdot}05$) compared to control animals.

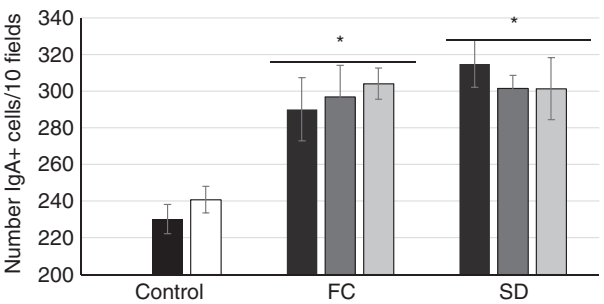


**Figure 3** Effects of the oral administration of *Lactobacillus rhamnosus* 64 as fresh (FC) or spray-dried (SD) culture for 3 (■), 6 (■) or 10 (■) consecutive days on the number of IgA-producing cells in the lamina propia of the small intestine, compared to control animals: untreated mice (■) and animals that received only the 20% (w/v) cheese whey-starch solution (□). Values are the mean ± SEM. *Significantly different ($P < 0{\cdot}05$) compared to control animals.

were observed for IL-2, IL-6 and IL-10 for any of the periods of administration evaluated (3, 6 and 10 days) when the strain was administered as fresh or SD culture, compared to control mice. No significant differences were observed between the control group and the group that received only 20% (w/v) cheese whey-starch solution for 10 days. A significant increase in IFN$\gamma$ was observed, compared to the control group ($88{\cdot}5 \pm 25{\cdot}4$ pg g$^{-1}$), when mice received the strain for 10 days (Fig. 4) as fresh ($262{\cdot}5 \pm 43{\cdot}2$ pg g$^{-1}$, $P = 0{\cdot}058$) or spray-dried cultures ($292{\cdot}8 \pm 52{\cdot}7$ pg g$^{-1}$, $P = 0{\cdot}012$ respectively), without differences between them. The concentration of TNF$\alpha$ in homogenates of the small intestine of mice that received the FC for 10 consecutive days was lower ($1675 \pm 364$ pg g$^{-1}$, $P = 0{\cdot}006$) than in untreated animals ($3477 \pm 277$ pg g$^{-1}$). For the SD culture this fact was observed for 3 days of administration ($1938 \pm 422$ pg g$^{-1}$, $P = 0{\cdot}019$ respectively) (Fig. 4). Additionally, a significant difference ($P < 0{\cdot}05$) was observed for this parameter in animals that received fresh or SD cells for 3 days.

In the large intestine, no changes were observed for the expression of IL-2 and IL-6 after the administration of *L. rhamnosus* 64, either as fresh or SD culture (data not shown). An increase in IL-10 expression was observed for the FC for all feeding periods ($P = 0{\cdot}043$, $0{\cdot}017$, $0{\cdot}031$ for 3, 6 or 10 days respectively), but only after 3 days ($P = 0{\cdot}049$) of the administration of the SD culture (Fig. 5). Additionally, a significant difference ($P < 0{\cdot}05$) was observed for IL-10 in mice that received fresh or SD cells for 10 days. In relation to the expression of the pro-inflammatory cytokines IFN$\gamma$ and TNF-$\alpha$, a significant decrease ($P < 0{\cdot}05$), compared to control values, was observed for both cytokines when the strain was administered as FC or spray-dried culture for all administration periods, without differences between treatments and periods of administration (Fig. 6).

The effect of oral administration of *L. rhamnosus* 64 in a model of acute colitis induced by TNBS was evaluated. The macroscopic evaluation of the tissues showed that a thickening of the large intestine wall occurred in the control group, as in the group that received *L. rhamnosus* 64 as a FC. The colonic tissue showed larger areas of ulceration and necrosis with respect to the group of animals

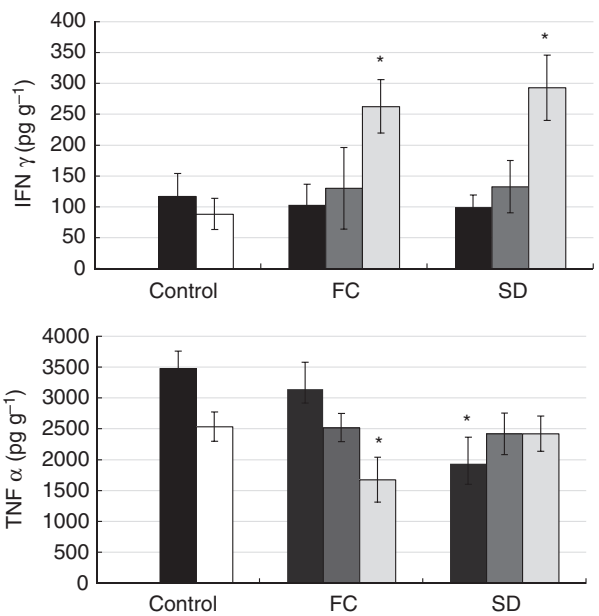


**Figure 4** Effects of the oral administration of *Lactobacillus rhamnosus* 64 as fresh (FC) or spray-dried (SD) culture for 3 (■), 6 (■) or 10 (■) consecutive days on the production of IFN$\gamma$ (above) or TNF$\alpha$ (below) in homogenates of the small intestine, compared to control animals: untreated mice (■) and animals that received only the 20% (w/v) cheese whey-starch solution (□). Values are the mean ± SEM. *Significantly different ($P < 0{\cdot}05$) compared to control animals.

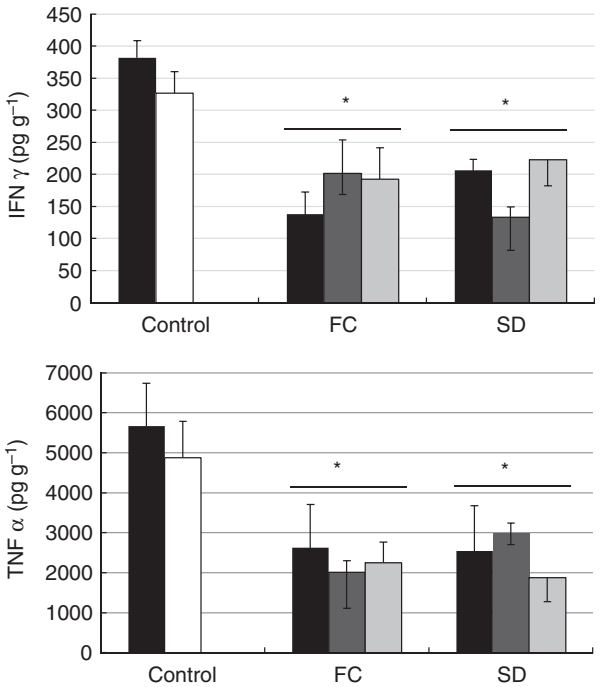


**Figure 5** Effects of the oral administration of *Lactobacillus rhamnosus* 64 as fresh (FC) or spray-dried (SD) culture for 3 (■), 6 (■) or 10 (■) consecutive days on the production of IL-10 in homogenates of the large intestine (above) and small intestine (below) compared to control animals: untreated mice (■) and animals that received only the 20% (w/v) cheese whey-starch solution (□). Values are the mean ± SEM. *Significantly different ($P < 0{\cdot}05$) compared to control animals.

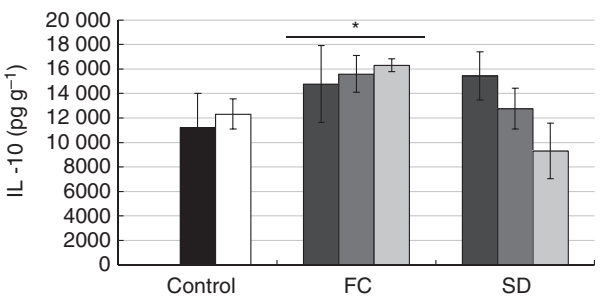


**Figure 6** Effects of the oral administration of *Lactobacillus rhamnosus* 64 as fresh (FC) or spray-dried (SD) culture for 3 (■), 6 (■) or 10 (■) consecutive days on the production of IFNγ (above) or TNFα (below) in homogenates of the large intestine, compared to control animals: untreated mice (■) and animals that received only the 20% (w/v) cheese whey-starch solution (□). Values are the mean ± SEM. *Significantly different ($P < 0{\cdot}05$) compared to control animals.

that received *L. rhamnosus* 64 as a SD culture. The animals treated with the SD culture presented a lower degree of inflammation compared to the FC group ($P = 0{\cdot}0018$) and compared to the control group ($P = 0{\cdot}043$) (Fig. 7). Colon shortening was significantly greater in the control group than in the group that received the SD culture ($P = 0{\cdot}044$) (Fig. 7). A significant diminution in the expression of IL-2 and TNFα were observed when *L. rhamnosus* 64 was administered as fresh ($P = 0{\cdot}001$, $0{\cdot}029$ for IL-2 and TNFα respectively) or SD culture ($P = 0{\cdot}014$, $0{\cdot}039$, for IL-2 and TNFα respectively), whereas an increase ($P = 0{\cdot}025$) in IL-10 expression was observed only when the strain was administered as SD culture (Fig. 8).

## Discussion

Technological factors such as sugar ingredients' concentration in the culture medium and moment of cell harvesting during biomass production, the protectants and technology used for culture preservation and the food matrix where a probiotic is included may modify its probiotic capacity without direct correlation with changes in the level of viable cells (Vinderola *et al.* 2011; Lavari *et al.* 2015). In this work, we aimed at studying the impact of spray drying of *L. rhamnosus* 64 on its immunomodulating capacity in the gut, a strain with potential technological properties for application in food as a probiotic (Lavari *et al.* 2014, 2015).

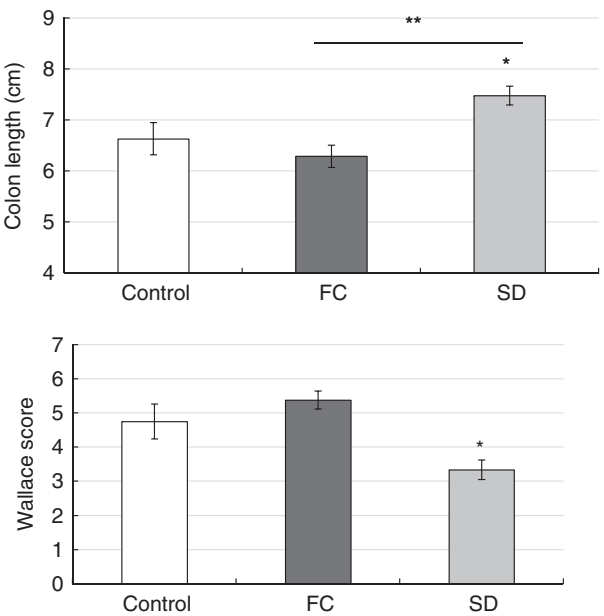


**Figure 7** Colon length and Wallace inflammation scores after 5 days of TNBS challenge in mice that received *Lactobacillus rhamnosus* 64 as fresh (FC) or spray-dried (SD) culture for five consecutive days, compared to control animals. Values are the mean ± SEM. *Significantly different ($P < 0{\cdot}05$) compared to control animals. **Significantly different ($P < 0{\cdot}05$).

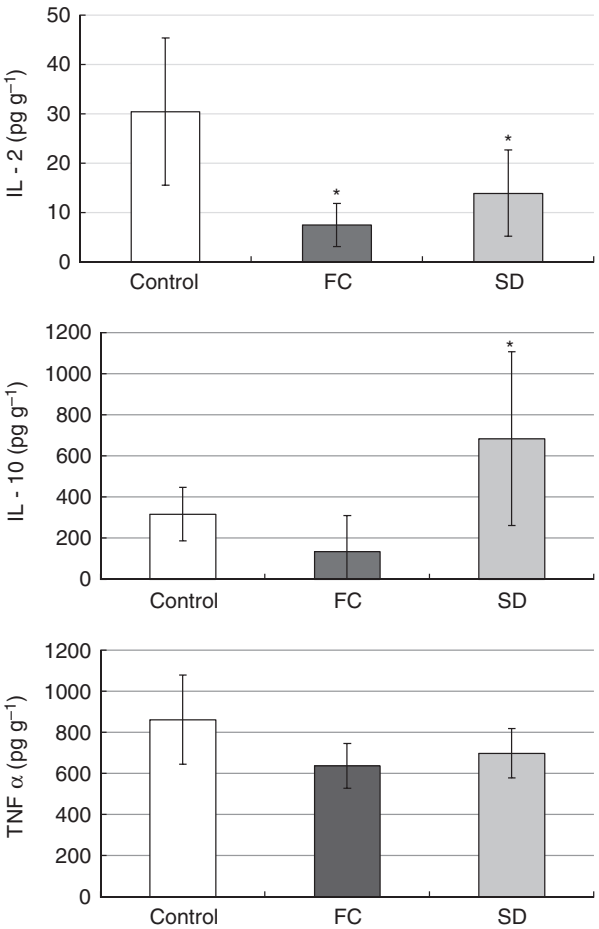


**Figure 8** Concentration of cytokines in homogenates of the large intestine of mice after 5 days of TNBS challenge in mice that received *Lactobacillus rhamnosus* 64 as fresh (FC) or spray-dried (SD) culture for five consecutive days, compared to control animals. Values are the mean ± SEM. *Significantly different ($P < 0{\cdot}05$) compared to control animals.

The increase in the phagocytic activity of peritoneal macrophages has been considered an indicator of immune modulation at distant sites following the intake of probiotic micro-organisms or functional foods in animal models (Vinderola *et al.* 2005) and in healthy humans (Vanderhoof *et al.* 1999), whereas the elimination of fermented foods in the diet of healthy volunteers for 2 weeks decreased the phagocytic activity of leucocytes (Yan and Polk 2004; Olivares *et al.* 2006). In this work, an enhanced phagocytic activity of murine peritoneal macrophages was observed after 3 days of administration of the strain under study. It is interesting to note that there was a fast activation (after 3 days of administration) but short duration (by day 6 the phagocytic activity returned to control values) of the systemic immune response induced, when compared to the local (gut mucosa), and more prolonged response observed for IgA-producing cells. This type of behaviour for the pahogytic activity of peritoneal macrophages was observed in a previous study (Vinderola *et al.* 2005). In the present work and in the work referenced, the activation of macrophages occurred in the short term (after 3 days of the beginning of the administration of the strain under study) and returned to the state of homoeostasis or equilibrium, also quickly.

Secretory IgA has many functions, such as inhibiting pathogens from inhabiting the intestine and impairing the attack of toxins to mucosal surfaces, but it can also keep the balance of the intestinal microbiota and increase the immune function (Baldassano *et al.* 2013). As a functional trait, the capacity of enhancing mucosal IgA in the gut after oral administration of bacteria is a desirable attribute for probiotic candidates (Galdeano and Perdigón 2004), and it has been linked to enhanced protection against *Salmonella* infection in the gut (Vinderola *et al.* 2007; Steinberg *et al.* 2014). Frece *et al.* (2005) demostrated that a 10-day feeding period is adequate to expect an increase of IgA in the gut of mice when candidate probiotics are orally administered in a murine model. In this work, although both forms of administration (fresh or spray-dried culture) achieved an increase in the levels of S-IgA, the dynamics were different and dependent on the technological treatment, that is, the fact that the culture was administered as a fresh (increase in S-IgA for 6 and 10 days of administration) or spray-dried and reconstituted culture (increase in S-IgA 3 days of administration). Different profiles in S-IgA were observed between fresh and SD cultures but not in the number of IgA-producing cells in the lamina propria. This change in the kinetics of IgA production was previously observed for probiotic micro-organisms (Paéz *et al.* 2013) and for soluble antigens (Burns *et al.* 2010) when administered as fresh or SD ingredients, however, the mechanisms remain unknown. These results highlight the need to conduct functional studies with the strain under the technological format in which it is intended to be used, since probiotic capacity might be modified by technological treatments (Vinderola *et al.* 2011), especially dehydration processes (Iaconelli *et al.* 2015), compared to that observed for FCs obtained under laboratory conditions.

Although promotion of IgA at intestinal level is a relevant parameter to establish the functional capacity of a potential probiotic culture, a standardized protocol for this purpose has not yet been harmonized by the scientific community. Furthermore, it has not been suggested yet whether the functional capacity of a probiotic candidate may be better predicted by measuring S-IgA by ELISA in the luminal contents or by determining IgA-producing cells by immunohistochemistry at the small

intestine lamina propria. While ELISA test measures the levels of (dimeric) S-IgA soluble in the intestinal contents, immunohistochemical analysis quantifies plasma cells with IgA molecules as membrane receptors, responsible for IgA secretion into the intestinal lumen (Macpherson *et al.* 2012). Even though proliferation of this cell population in the mucosal lamina propria and dimeric IgA excretion into the intestinal space (lumen) are sequential phenomena, there are no studies of IgA promotion where both methodologies (ELISA and immunohistochemistry) were compared in order to determine their correlation. During this work, an increased number of IgA-producing cells in the small intestine was noticed, both for fresh and SD cultures and for the three administration periods evaluated. However, this resulted in enhanced S-IgA in the luminal content of the small intestine on day 6 and 10 of the administration of the FC, and on day 3 of administration of the SD culture. There is no clear mechanism for the lack of direct correlation between both ways of quantifying this phenomenon. However, we may hypothesize that the difference may be related to the fact that half-life for IgA+ plasma cells in the lamina propria is 5 days (Cerutti and Rescigno 2008), whereas S-IgA in the small intestine is subjected to luminal degradation and dragging and elimination through faeces caused by peristaltic movements. Anyway, and for the aim of our study, the measure of S-IgA allowed to see a difference in probiotic capacity due to the spray drying that was not noticed by quantifying IgA-producing cells by immunohistochemistry.

Cytokines are low molecular weight peptides that regulate the function of the immune cells that produce them and also act on other cell populations. These molecules are the agents responsible for intercellular communication to induce the activation of specific membrane receptors, cell proliferation and differentiation, chemotaxis, growth and modulation of immunoglobulin secretion. Beyond the activation of peritoneal macrophages and the enhancement of IgA levels, the administration of a probiotic candidate may exert effects on cytokines expression. However, there is no direct correlation between the administration of a probiotic and an expected or desired profile of cytokines expression when several cytokines are assessed, except in some particular cases where, for instance, the administration of a strain with anti-inflammatory properties is expected to enhance IL-10 levels (Foligné *et al.* 2007) or when the administration of a strain with capacity to protect from *Salmonella* infection is expected to raise the levels of IFNγ (Castillo *et al.* 2011). One of the main mechanisms of protection of probiotics in case of intestinal inflammatory pathologies is the induction of the synthesis of regulatory cytokines, such as IL-10 (Mileti *et al.* 2009). This cytokine is one of the most frequently analysed ones when studying the immunomodulatory profile of a probiotic strain. Although it is often considered anti-inflammatory (de Kivit *et al.* 2012), it is actually a regulatory cytokine that can be produced by different immune cells and lead, not only to a humoral or cellular immune response but also to the development of oral tolerance (Johansson *et al.* 2012; Rescigno 2013). Increased expression of IL-10 is positively related to the potential of controlling or moderating inflammatory bowel processes *in vivo* such as intestinal inflammation or various types of diarrhoea (Foligné *et al.* 2007; Paul *et al.* 2012). As observed in this work, the increase in IL-10 was also reported by Castillo *et al.* (2011) after feeding mice for 7 days with *Lactobacillus casei* CRL 431. Active treatment with probiotics induces changes in the immune response of individuals with inflammatory bowel disease, characterized by a decrease in the number of monocytes and dendritic cells producing IFN and increased secretion of IL-10 and TGFβ (Pathmakanthan *et al.* 2004). A review of results from human trials using meta-analysis concluded that probiotic treatment may be effective for patients with inflammatory disorders such as ulcerative colitis during the remission period (Sang *et al.* 2010).

In order to evaluate the impact of the dehydration processes on the characteristics of probiotic cultures, bacterial survival has been the classic parameter for determining the resistance to the process after rehydration. However, studies on the impact of the drying processes on the viability of micro-organisms often do not take into account aspects related to the probiotic capacity, beyond cell viability. In fact, the survival of probiotic bacteria to the dehydration process does not necessarily correlate with the preservation of its beneficial effects (or probiotic capacity). Previous studies have shown that the same probiotic strain, produced and/or preserved under different storage conditions, may present different susceptibility to the adverse conditions of the gut, different ability to adhere to the intestinal epithelium or different immunostimulatory capacity, although viability is maintained (Saarela *et al.* 2009; Vinderola *et al.* 2012). The results obtained in this part of the work are in line with those published by several authors who evaluated the probiotic capacity *in vitro* or *in vivo*, where the effect of the dehydration process modified the functionality of the cultures in some of the measured parameters (Makinen *et al.* 2012; Paéz *et al.* 2013; Iaconelli *et al.* 2015). Therefore, performing studies of probiotic capacity in the technological format or in the food matrix used as a vehicle, will allow a more appropriate estimation of the probiotic capacity of the strain with respect to the observations that can be made using cultures obtained under laboratory conditions. The mechanisms by which spray drying

altered the cytokine-inducing capacity of the strain, remain unknown.

In *in vivo* models using healthy animals, different probiotic strains have demonstrated the ability to promote immune defences by enhancing parameters of both natural and acquired immunity (Díaz-Ropero *et al.* 2006). These observations could explain the protective role of these strains in models of pathologies, for example, in reducing inflammation in models of inflammatory bowel disease in mice (Peran *et al.* 2007). A preliminary study was conducted in order to explore the potential of *L. rhamnosus* 64 for the prevention of inflammation in a model of acute colitis. Regarding the weight of the animals after the administration of TNBS, no significant differences were observed between the groups evaluated (Fig. 7), coinciding with that published by Preising *et al.* (2010). According to Philippe *et al.* (2011), animals treated with *B. bifidum* S17 showed a trend towards partial inhibition of TNBS-induced weight loss, although this effect was not significant, probably because in the acute colitis model the period between administration and the sacrifice is short. From a histopathological point of view, the strain conferred partial protection against TNBS-induced colitis, only when it was administered as a SD culture. This can be explained by the reduction in the expression of pro-inflammatory cytokines (IL-2 and TNFα) and an enhancement of IL-10 production, observed only when mice received SD *L. rhamnosus* 64. The downregulation of the expression of pro-inflammatory cytokines and the enhancement of regulating factors was also observed for a spray-dried strain of bifidobacteria in the same model of acute colitis (Burns *et al.* 2017). The difference in effectiveness between fresh and SD culture might be due may be to the extra charge of nonviable cells present in the SD culture, as result of the, rather small but significant ($P < 0{\cdot}05$) loss in cell viability (*c.* 0·5 log orders) during spray drying, as animals received the strain on a basis of viable cells per millilitre. It was reported that nonviable cells can also exert health effects in the gut (Ouwehand and Salminen 1998).

We conclude that *L. rhamnosus* 64 has the capacity to promote intestinal and distant site defences, mediated by IgA and peritoneal macrophages respectively. The application of the technology of spray drying to the strain, in order to develop a dehydrated culture, significantly modified its effects on the immune response in all parameters measured in the small and large intestine, but not in distant sites, where the response was of short duration. The strain under study in this work displayed capacity to confer enhanced protection in a model of acute colitis in mice as SD culture. That property is mediated by the downregulation of pro-inflammatory cytokines and upregulation of IL-10. Further studies are needed in order to unveil the mechanisms by which the technological treatment applied to the strain (spray drying) induced changes in the dynamics of the immunomodulating capacity of the strain in healthy animals and in an *in vivo* model of inflammation.

## Conflict of Interest

The authors declare no conflicts of interest.

## References

Baldassano, S., Amato, A., Cappello, F., Rappa, F. and Mule, F. (2013) Glucagon-like peptide-2 and mouse intestinal adaptation to a high-fat diet. *J Endocrinol* **217**, 11–20.

Burns, P., Molinari, F., Beccaria, A., Páez, R., Meinardi, C., Reinheimer, J. and Vinderola, G. (2010) Suitability of buttermilk for fermentation with *Lactobacillus helveticus* and production of a functional peptide-enriched powder by spray-drying. *J Appl Microbiol* **109**, 1370–1378.

Burns, P., Alard, J., Hrdỳ, J., Boutillier, D., Páez, R., Reinheimer, J., Pot, B., Vinderola, G. *et al.* (2017) Spray-drying process preserves the protective capacity of a breast milk-derived *Bifidobacterium lactis* strain on acute and chronic colitis in mice. *Sci Rep* **7**, 43211.

Castillo, N.A., Perdigón, G. and de LeBlanc, A. (2011) Oral administration of a probiotic lactobacillus modulates cytokine production and TLR expression improving the immune response against *Salmonella enterica* serovar *Typhimurium* infection in mice. *BMC Microbiol* **11**, 177.

Cerutti, A. and Rescigno, M. (2008) The biology of intestinal immunoglobulin a responses. *Immunity* **28**, 740–750.

de Kivit, S., Saeland, E., Kraneveld, A.D., van de Kant, H.J., Schouten, B., van Esch, B.C., Knol, J., Sprikkelman, A.B. *et al.* (2012) Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. *Allergy* **67**, 343–352.

Díaz-Ropero, M.P., Martín, R., Sierra, S., Lara-Villoslada, F., Rodríguez, J.M. and Xaus, J. (2006) Two Lactobacillus strains, isolated from breast milk, differently modulate the immune response. *J Appl Microbiol* **102**, 337–343.

Foligné, B., Nutten, S., Steidler, L., Dennin, V., Goudercourt, D., Mercenier, A. and Pot, B. (2006) Recommendations for improved use of the murine TNBS-induced colitis model in evaluating anti-inflammatory properties of lactic acid bacteria: technical and microbiological aspects. *Dig Dis Sci* **51**, 390–400.

Foligné, B., Nutten, S., Grangette, C., Dennin, V., Goudercourt, D., Poiret, S., Dewulf, J., Brassart, D. *et al.* (2007) Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. *World J Gastroenterol* **13**, 236–243.

Frece, J., Kos, B., Beganovic, J., Vukovic, S. and Suskovic, J.S. (2005) *In vivo* testing of functional properties of three

selected probiotic strains. *World J Microbiol Biotechnol* **21**, 1401–1408.

Galdeano, C.M. and Perdigón, G. (2004) Role of viability of probiotic strains in their persistence in the gut and in mucosal immune stimulation. *J Appl Microbiol* **97**, 673–681.

Gregoret, V., Perezlindo, M.J., Vinderola, G., Reinheimer, J. and Binetti, A.A. (2013) A comprehensive approach to determine the probiotic potential of human-derived *Lactobacillus* for industrial use. *Food Microbiol* **34**, 19–28.

Grover, S., Rashmi, H.M., Srivastava, A.K. and Batish, V.K. (2012) Probiotics for human health – new innovations and emerging trends. *Gut Pathogens* **4**, 15.

Huang, S., Vignolles, M.L., Chen, X.D., le Loir, Y., Jan, G., Schuck, P. and Jeantet, R. (2017) Spray drying of probiotics and other food-grade bacteria: a review. *Trends Food Sci Technol* **63**, 1–17.

Iaconelli, C., Lemetais, G., Kechaou, N., Chain, F. and Bermudez-Humaran, L.G. (2015) Drying process strongly affects probiotics viability and functionalities. *J Biotechnol* **214**, 17–26.

Johansson, M.A., Saghafian-Hedengren, S., Haileselassie, Y., Roos, S., Troye-Blomberg, M., Nilsson, C. and 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**, e49315.

Lavari, L., Páez, R., Cuatrin, A., Reinheimer, J. and Vinderola, G. (2014) Use of cheese whey for biomass production and spray drying of probiotic lactobacilli. *J Dairy Res* **81**, 267–274.

Lavari, L., Ianniello, R., Paez, R., Zotta, T., Cuatrin, A., Reinheimer, J., Parente, E. and Vinderola, G. (2015) Growth of *Lactobacillus rhamnosus* 64 in whey permeate and study of the effect of mild stresses on survival to spray drying. *Food Sci Technol* **63**, 322–330.

Macpherson, A.J., Geuking, M.B., Slack Siegfried, E., Hapfelmeier, K. and McCoy, D. (2012) The habitat, double life, citizenship, and forgetfulness of IgA. *Immunol Rev* **245**, 132–146.

Makinen, K., Berger, B., Bel-Rhlid, R. and Ananta, E. (2012) Science and technology forthe mastership of probiotic applications in food products. *J Biotechnol* **162**, 356–365.

Mileti, E., Matteoli, G., Iliev, I.D. and 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**, e7056.

Olivares, M., Díaz-Ropero, M.P. and Gómez, N. (2006) The consumption of two probiotic strains, *Lactobacillus coryniformis* CECT5711 and *Lactobacillus gasseri* CECT5714, boost the immune system of healthy humans. *Int Microbiol* **9**, 47–52.

Ouwehand, A.C. and Salminen, S.J. (1998) The health effects of cultured milk products with viable and non-viable bacteria. *Int Dairy J* **8**, 749–758.

Paéz, R., Lavari, L., Audero, G., Cuatrin, A., Zaritzky, N., Reinheimer, J. and Vinderola, G. (2013) Study of the effects of spray-drying on the functionality of probiotic lactobacilli. *Int J Dairy Technol* **66**, 155–161.

Pathmakanthan, S., Li, C.K., Cowie, J. and Hawkey, C.J. (2004) *Lactobacillus plantarum* 299: beneficial *in vitro* immunomodulation in cells extracted from inflamed human colon. *J Gastroenterol Hepatol* **19**, 166–173.

Paul, G., Khare, V. and Gasche, C. (2012) Inflamed gut mucosa: downstream of interleukin-10. *Eur J Clin Invest* **42**, 95–109.

Peran, L., Sierra, S. and Comalada, M. (2007) A comparative study of the preventative effects exerted by two probiotics, *Lactobacillus reuteri and Lactobacillus fermentum,* in the trinitrobenzenesulfonic acid model of rat colitis. *Br J Nutr* **97**, 96–103.

Philippe, D., Heupel, E., Blum-Sperisen, S. and Riedel, C.U. (2011) Treatment with *Bifidobacterium bifidum* 17 partially protects mice from Th1-driven inflammation in a chemically induced model of colitis. *Int J Food Microbiol* **149**, 45–49.

Preising, J., Philippe, D., Gleinser, M., Wei, H., Blum, S., Eikmanns, B.J., Niess, J.H. and Riedel, C.U. (2010) Selection of bifidobacteria based on adhesion and anti-inflammatory capacity *in vitro* for amelioration of murine colitis. *Appl Environ Microbiol* **76**, 3048–3051.

Rescigno, M. (2013) Mucosal immunology and bacterial handling in the intestine. *Best Pract Res Clin Gastroenterol* **27**, 17–24.

Saarela, M.H., Alakomi, H.L., Puhakka, A. and Matto, 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**, 1204–1212.

Sanders, M.E., Guarner, F., Guerrant, R., Holt, P.R., Quigley, E.M., Sartor, R.B., Sherman, P.M. and Mayer, E.A. (2013) An update on the use and investigation of probiotics in health and disease. *Gut* **62**, 787–796.

Sang, L.X., Chang, B., Zhang, W.L., Wu, X.M., Li, X.H. and Jiang, M. (2010) Remission induction and maintenance effect of probiotics on ulcerative colitis: a meta-analysis. *World J Gastroenterol* **16**, 1908–1915.

Steinberg, R.S., Silva, L.C., Souza, T.C., Lima, M.T., de Oliveira, N.L.G., Vieira, L.Q., Arantes, R.M.E., Miyoshi, A. *et al.* (2014) Safety and protective effectiveness of two strains of *Lactobacillus* with probiotic features in an experimental model of salmonellosis. *Int J Environ Res Public Health* **11**, 8755–8776.

Syngai, G.G., Gopi, R., Bharali, R., Dey, S., Lakshmanan, G.M. and Ahmed, G. (2016) Probiotics - the versatile functional food ingredients. *J Food Sci Technol* **53**, 921–933.

Vanderhoof, J.A., Whitney, D.B., Antonson, D.L., Hanner, T.L., Lupo, J.V. and Young, R.J. (1999) *Lactobacillus* GG in the prevention of antibiotic-associated diarrhea in children. *J Pediatr* **135**, 564–568.

Vinderola, C.G., Duarte, J., Thangavel, D., Perdigon, G., Farnworth, E. and Matar, C. (2005) Immune response in

distant sites by orally administered kefir. *Eur J Inflamm* **3**, 15–24.

Vinderola, G., Matar, C., Palacios, J. and Perdigón, G. (2007) Mucosal immunomodulation by the non-bacterial fraction of milk fermented by *Lactobacillus helveticus R389*. *Int J Food Microbiol* **115**, 180–186.

Vinderola, G., Capellini, B., Villarreal, F., Suárez, V., Quiberoni, A. and Reinheimer, J. (2008) Usefulness of a set of simple *in vitro* tests for the screening and identification of probiotic candidate strains for dairy use. *LWT- Food Sci Technol* **41**, 1678–1688.

Vinderola, G., Binetti, A., Burns, P. and Reinheimer, J. (2011) Cell viability and functionality of probiotic bacteria in dairy products. *Front Microbiol* **2**, 70.

Vinderola, G., Zacarías, M.F., Bockelmann, W., Neve, H., Reinheimer, J. and Heller, K.J. (2012) Preservation of functionality of *Bifidobacterium animalis* subsp. *lactis* INL1 after incorporation of freeze-dried cells into different food matrices. *Food Microbiol* **30**, 274–280.

Wallace, J.L., Braquet, P., Ibbotson, G.C., MacNaughton, W.K. and Cirino, G. (1989) Assessment of the role of platelet-activating factor in an animal model of inflammatory bowel disease. *J Lipid Mediat* **1**, 13–23.

Yan, F. and Polk, D.B. (2004) Commensal bacteria in the gut: learning who our friends are. *Curr Opin Gastroenterol* **6**, 565–571.