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Potentiation of the humoral immune response elicited by a commercial vaccine against bovine respiratory disease by *Enterococcus faecalis* CECT7121

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Received: 5 July 2017 / Accepted: 20 December 2017
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RESEARCH ARTICLE

Abstract

Vaccination against pathogens involved in bovine respiratory disease (BRD) is a useful tool to reduce the risk of this disease however, it has been observed that the commercially available vaccines only partially prevent the infections caused by *Pasteurella multocida* and *Mannheimia haemolytica*. Therefore, it is recommended to search for new adjuvant strategies to minimise the economic impact of this respiratory syndrome. A possibility to improve the conventional vaccine response is to modulate the immune system with probiotics, since there is accumulating evidence that certain immunomodulatory strains administered around the time of vaccination can potentiate the immune response. Considering veterinary vaccines are frequently tested in murine models, we have developed an immunisation schedule in BALB/c mice that allows us to study the immune response elicited by BRD vaccine. In order to evaluate a potential strategy to enhance vaccine efficacy, the adjuvant effect of *Enterococcus faecalis* CECT7121 on the murine specific humoral immune response elicited by a commercial vaccine against BRD was studied. Results indicate that the intragastric administration of *E. faecalis* CECT7121 was able to induce an increase in the specific antibody titres against the bacterial components of the BRD vaccines (*P. multocida* and *M. haemolytica*). The quality of the humoral immune response, in terms of antibody avidity, was also improved. Regarding the cellular immune response, although the BRD vaccination induced a low specific secretion of cytokines in the spleen cell culture supernatants, *E. faecalis* CECT7121-treated mice showed higher interferon-γ production than immunised control mice. Our results allowed us to conclude that the administration of *E. faecalis* CECT7121 could be employed as an adjuvant strategy to potentiate humoral immune responses.

Keywords: *Enterococcus faecalis* CECT7121, BRD vaccine, antibody, IgG avidity test, ELISA

1. Introduction

The microbiota is essential to human and animal health, as it contributes to food digestion and the development and optimal functioning of the immune system (Lebeer *et al.*, 2010). In the last decades, interest in the beneficial functions of the microbiota has resulted in the search for and selection of specific species with putative health-promoting capacities for the treatment of conditions in which the microbiota – or its optimal functioning – is disturbed. These microorganisms, well-known as probiotics, are live microorganisms that, upon their administration, are able to influence the composition and activity of the intestinal microbiota, to improve the nonspecific intestinal barrier and to reinforce or modulate mucosal and systemic immune responses (Maldonado Galdeano *et al.*, 2007). The immune modulation mediated by probiotics has been demonstrated by the proliferation of leukocytes, antibody production, increasing phagocyte activity and changes in cytokine expression (Barberi *et

ISSN 1876-2883 print, ISSN 1876-2891 online, DOI 10.3920/BM2017.0081

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al., 2015; Frei *et al.*, 2015). Thus, probiotics have been employed to control infections and intestinal inflammatory diseases, to avoid and treat allergy processes, to prevent several types of cancers and to stimulate the immune system (Chiang and Pan, 2012; Hardy *et al.*, 2013; Sarowska *et al.*, 2013). The mechanisms by which probiotic microorganisms exert these beneficial effects are varied and are generally strain-dependent (Khang and Im, 2015). Most studies that assess the role of probiotics in the immune response have been conducted with probiotic strains selected from the *Lactobacillus* and *Bifidobacterium* genera (Barberi *et al.*, 2015; Lebeer *et al.*, 2010). However, studies with other probiotics that belong to the lactic acid bacteria include *Enterococcus faecalis* strains, have also been demonstrated to confer beneficial effects to the host (Lebeer *et al.*, 2010; Nami *et al.*, 2015).

In recent years, our research studies have focused on the immunomodulatory activity of *E. faecalis* CECT7121. This non-pathogenic bacterium implants and remains in the murine intestinal mucosa for at least 18 days, stimulating local mucosal immunity without inducing deleterious inflammation (Castro *et al.*, 2007, 2016). The immunomodulatory capacity of *E. faecalis* CECT7121 has been demonstrated in different biological models in mice: an infection with *Salmonella*, a challenge with lymphoma LBC cells and an ovalbumin-induced allergic response (Castro *et al.*, 2007, 2010, 2012). Recently, we have demonstrated that the immune stimulation triggered by *E. faecalis* CECT7121 involves the activation of the innate response and the consequent development of Th1-related, cellular adaptive responses (Molina *et al.*, 2015). Regarding the humoral immune response, it is speculated that the ingestion of this bacterial strain could also modulate antibody production. In this regard, the European Food Safety Agency (EFSA) states that vaccination protocols may be employed in order to evaluate the potential role of probiotic strains in improving the humoral immune response against antigen challenges (EFSA, 2011). In relation to vaccination studies, we have previously shown that the administration of *E. faecalis* CECT7121 can induce a stronger cellular anti-tetanic and anti-diphtheric response in BALB/c mice immunised with a diphtheria-tetanus-*Bordetella pertussis* vaccine (Castro *et al.*, 2008).

The use of vaccines in the control of infectious diseases has been a major contributor to health. Bovine respiratory disease (BRD) is a general term for the respiratory disease syndrome. The BRD aetiology is multi-factorial; it is a variety of physical and physiological stressors acting in combination to predispose the animal to pneumonia. BRD can be caused by several pathogens individually or as co-infections. It is well-known that the key to preventing respiratory disease is to reduce stress and to vaccinate against the viruses and bacteria that cause disease. Vaccination against pathogens involved in BRD is a useful tool to reduce the risk of this disease; however, commercially available vaccines only partially prevent infections caused by *Pasteurella multocida* and *Mannheimia haemolytica* (Bednarek *et al.*, 2012; Fulton, 2009; Rice *et al.*, 2008). Therefore, it is now widely recommended to seek new adjuvant strategies to minimise the economic impact of this respiratory syndrome. One possible way to improve the conventional vaccine response is to modulate the immune system with probiotics (Bandeira Roosa *et al.*, 2012; Dhama *et al.*, 2015; Kang and Im, 2015).

Taking the above into account, the aim of this work was to study the effects of *E. faecalis* CECT7121 on the specific humoral immune response elicited by a commercial vaccine against BRD in order to evaluate a potential strategy to enhance vaccine efficacy. The study compared naïve BALB/c mice with mice intragastrically pre-treated with *E. faecalis* CECT7121 before the subcutaneous immunisation with the BRD vaccine. Our results allowed us to conclude that the administration of *E. faecalis* CECT7121 could be used as an adjuvant strategy to potentiate humoral immune responses elicited by vaccination.

2. Material and methods

Animals

Mice were housed and handled in accordance with the 'Guide for the Care and Use of Laboratory Animals' (National Research Council, 2011). The animal studies were approved by the Ethics Committee at Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (permit number 0044155-2015). All efforts were made to minimise the suffering of laboratory animals. Technical staff was responsible for monitoring the physical condition of the animals twice a day. Mice were sacrificed by cervical dislocation.

Female BALB/c mice were obtained from the Animal Facility at the Facultad de Ciencias Veterinarias (Universidad de Buenos Aires, Argentina) and were kept at the Animal Facility of the Instituto de Estudios de la Inmunidad Humoral 'Prof. Dr. Ricardo A. Margni' (CONICET-UBA, Argentina). Mice were 6- to 8-weeks old and weighed roughly 20-25 g each at the beginning of the experiment. For the experiments, animals were divided and housed in groups to 4/cage, with controlled air temperature (20-22 °C) and humidity, and with 12 h light/dark cycles. Animals were allowed access to food and water *ad libitum*.

Bacterial suspensions

E. faecalis CECT7121 was grown in triptone soy broth (Biokar Diagnostics, Allonne, France) at 37 °C for 18 h corresponding to stationary phase of growth. After incubation, the culture was harvested by centrifugation at

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4,000 rpm for 15 min (4 °C) and washed three times with sterile phosphate buffered saline (PBS).

P. multocida serotype A and serotype D and *M. haemolytica* were grown in brain heart infusion (Biokar Diagnostics) for 18 h at 37 °C and continuous shaking (150 rpm). Bacterial suspensions were then washed with sterile PBS between steps (8,000 rpm, 10 min, 4 °C), and subjected to heating (80 °C, 60 min). Heat-killed microorganisms (*P. multocida* (PM) and *M. haemolytica* (MH)) were used to stimulate cell cultures and for ELISA plates coating. Biochemical tests were routinely performed for identification. Complete inactivation of the bacteria prior to adding to cell culture was controlled employing sheep blood and chromogenic agar plates (Britania, Buenos Aires, Argentina).

Bovine respiratory disease commercial vaccine

The Biopoligen® HS vaccine (Biogénesis Bagó, Buenos Aires, Argentina) was employed in this study. The vaccine is formulated with inactivated bovine herpesvirus types 1 and 5; bovine viral diarrhea virus types 1 and 2; parainfluenza 3 virus; formaldehyde-inactivated *M. haemolytica*; *P. multocida; Histophilus somni* and aluminium hydroxide gel as adjuvant.

Enterococcus faecalis CECT7121 intragastric administration and immunisation

E. faecalis CECT7121 suspensions were prepared using standard growth curves, which were constructed by plotting the OD620 values against agar plate counts of freshly prepared, serially diluted cultures and adjusted to approximately 3.0×10^8 cfu/ml in sterile PBS (Castro *et al.*, 2007). A schematic representation of the *E. faecalis* CECT7121 and BRD inoculations is shown in Figure 1. Briefly, live *E. faecalis* CECT7121 suspensions were administered by intragastric (i.g.) route (0.2 ml/mice/day) on days -3, -2, -1 and 14, 15, 16 of the BRD immunisation schedule. Mice were immunised with the commercial vaccine on days 0, 15 and 27 by subcutaneous injection (0.25 ml) on the mouse´s back. Animals were randomly divided into two experimental groups (n=4): Vac group-mice inoculated i.g. with PBS and vaccinated with BRD vaccine; Ef/Vac group-mice inoculated i.g. with *E. faecalis* CECT7121 and vaccinated. Non-immunised control groups (treated or not with the probiotic) were also added to the study. Blood samples were obtained on days 0, 10, 27 and day 38 when mice were sacrificed. Serum samples were separated and stored at -20 °C until assayed for antibody levels.

Levels of specific antibodies

Each animal was periodically bled by the maxillary vein. Specific antibody levels (anti-PM and anti-MH immunoglobulin (Ig)G, IgG1 and IgG2a) were measured by an in house indirect ELISA. Briefly, 100 µl heat-killed bacterial suspensions (10 µg/ml in PBS) were employed as coating antigen. After blocking with 10% (v/v) foetal calf serum (FCS; Natocor, Buenos Aires, Argentina) in PBS, serum samples were incubated for 2 h at 37 °C. Bound antibodies were detected using an horseradish peroxidase (HRP)-conjugated anti-mouse IgG serum (Cappel, Cochranville, PA, USA), HRP-conjugated anti-mouse IgG1 or IgG2a sera (Bethyl Laboratories Inc., Montgomery, TX, USA). To determine the titre of each serum sample, two-fold serial dilutions were performed. All determinations were performed in duplicate. Absorbance values were obtained after spectrophotometric reading (450-570 nm) in an ELISA plate reader (Multiskan EX; Thermo Scientific, Waltham, MA, USA). Antibody titres were calculated as the EC50 (half maximal) value obtained by a four-parameter non-linear regression curve in a log reciprocal-dilution response curve.

Avidity test

A similar indirect ELISA was performed employing a chaotropic agent (urea; Mallinckrodt plc., Dublin, Ireland) for dissociation of the immune complexes (Ibison *et al.*, 2012; Özkul *et al.*, 2008; Pour Abolghasem *et al.*, 2011; Zakeri *et al.*, 2011). Different urea concentrations (8, 6 and 2 M) and serum dilutions (1/1000 for 6 and 8 M urea and 1/5,000 for 2 M urea) were employed. To evaluate antibody avidity, the ELISA described above was modified to include an antibody disassociation step prior to addition of the HRP-conjugated secondary antibody. Briefly, 1/1000 and 1/5,000 diluted sera (only sera from day 38) were

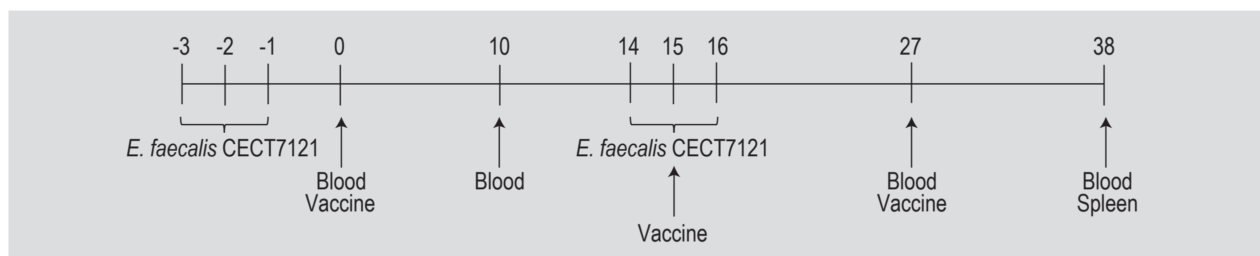

Figure 1. Immunisation schedule of mice with bovine respiratory disease vaccine and intragastric administration of *Enterococcus faecalis* CECT7121.

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incubated on the previously coated plates (2 h, 37 °C). After incubation, the content of the wells was discarded and half of the wells were treated with 100 µl urea for 10 min at 37 °C, whereas the remaining half was treated with PBS to measure the total antibody binding. The reaction was continued as above. The optical density (OD) obtained from these wells treated with urea was considered as the remaining OD (OD_{UREA}) and was compared to the OD of the same serum sample but without the addition of the dissociating agent (OD_{TOTAL}). The relative avidity index (RAI) was defined as the proportion of antibodies binding after treatment with urea for each dilution (Relative Avidity index [OD_{UREA}/OD_{TOTAL}]×100), and the avidity index (AI) was calculated as [(OD_{UREA} /OD_{TOTAL})×OD_{TOTAL}]×100). Results were presented as mean RAI ± SEM (standard error of the mean) and AI ± SEM of murine sera from animals of the same experimental group.

Specific cellular immune response

On day 38, individual spleen cell suspensions of each immunised or control mouse were prepared in culture medium RPMI 1640 (Gibco, New York, NY, USA) containing 10% (v/v) FCS, 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 0.25 µg/ml amphotericin B (Gibco), 300 µg/ml L-glutamine (MP Biomedicals Inc., Solon, OH, USA) and 220 µg/ml pyruvate (J.T. Baker, Phillipsburg, NJ, USA). One hundred microliters of splenocytes (4.0×10^6 cells/ml) were cultured with the same volume of RPMI alone (basal) or with heat-killed PM and MH (4.0×10^6 cfu/ml, ratio bacteria:splenocyte 1:1) in quadruplicates at 37 °C and 5 % CO_2. Concanavalin A (10 µg/ml, Vector Labs, Burlingame, CA, USA) was used as positive control. After 72 h, culture supernatants were collected and stored at -80 °C until assayed for cytokine levels.

Determination of cytokine levels

Cytokine concentrations were tested individually in spleen culture supernatants employing BD OptEIA ELISA kits (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. Results were expressed as mean concentration ± SEM of animals from the same experimental group.

Statistical analysis

All values were presented as means with their standard errors. When variables had a normal distribution (D'Agostino-Pearson omnibus normality test) and showed homoscedasticity (F-test or Bartlett's test to compare variances), parametric tests were employed: unpaired Student's t-test or one-way ANOVA plus Tukey's multiple comparison *post-hoc* tests. When samples did not have a normal distribution or did not show homoscedasticity, non-parametric tests were employed: Mann-Whitney´s U-test or Kruskal-Wallis ANOVA plus Dunn's post-hoc tests. Graphical and statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Values were considered significantly different at * $P<0.05$, ** $P<0.01$, or *** $P<0.001$.

3. Results

Intragastric administration with live *E. faecalis* CECT7121 increases production of specific antibodies

Specific antibody titres against *P. multocida* and *M. haemolytica* were determined on days 0, 10, 27 and 38. Two groups of animals were immunised with a BRD vaccine administered in three doses (days 0, 15 and 27); one of them was also inoculated with *E. faecalis* CECT7121 (Ef/Vac group) according to a scheme of administration previously established (Castro *et al.*, 2012) while the other received PBS (Vac group). Immunisation with the BRD vaccine induced the synthesis of high levels of specific IgG in both groups of immunised animals. No specific antibodies were detected in non-immunised animals.

The treatment with *E. faecalis* CECT7121 potentiated the specific immune response generated by vaccination (Figure 2). At the end of the study, the sera from mice belonging to the Ef/Vac group presented higher IgG titres against PM than mice from the Vac group (* $P<0.05$; Figure 2A). Regarding the anti-MH IgG titres, and from day 10, antibody levels of mice from the Ef/Vac group were higher than those of the Vac group. On day 27, the Ef/Vac group presented significantly higher levels than non-treated immunised mice (*$P<0.05$). On day 38 this tendency was sustained, although without reaching a statistical significance ($P=0.0652$; Figure 2B).

The antibody production kinetics were different for both microorganisms. For PM, the kinetics of IgG secretion in both immunised groups were similar until day 27 (two BRD immunisations). Only after receiving the third vaccine inoculation were the specific IgG titres for the Ef/Vac group higher than those of the Vac group (Figure 2A). In contrast, for MH, the IgG levels measured in sera from *E. faecalis* CECT7121-treated mice rapidly increased after the first immunisation, as it can be seen on day 10 (Figure 2B).

As markers of Th1 and Th2 responses, the levels of specific IgG2a and IgG1 were determined, respectively. Both anti-PM specific antibody isotypes were higher in the *E. faecalis* CECT7121-treated and immunised mice on day 38 (* $P<0.05$; Figure 3A, 3B). The treatment with *E. faecalis* CECT7121 exerted a 1.8 and 4.3-fold increase in IgG2a and IgG1, respectively.

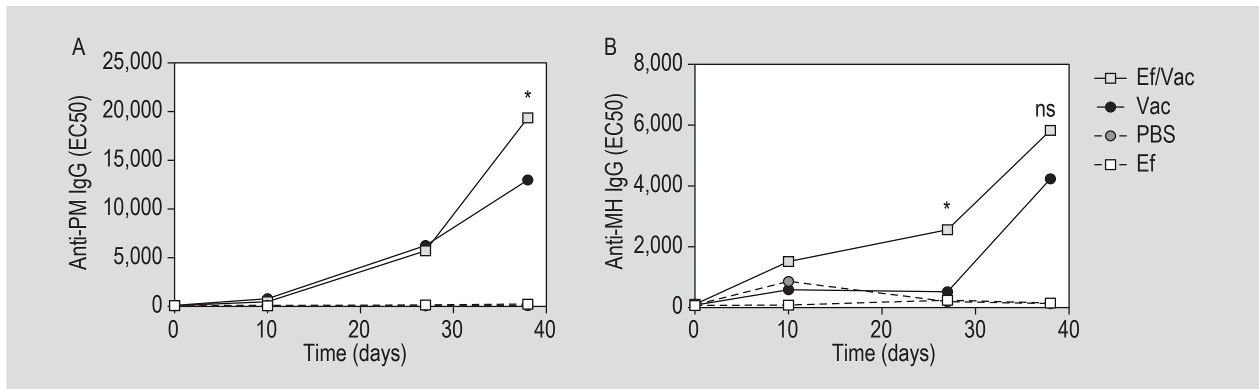

Figure 2. Effect of intragastric administration of *Enterococcus faecalis* CECT7121 on serum specific immunoglobulin G (IgG). Animals belonging to the positive control group were inoculated with saline (Vac) and those of the experimental group (Ef/Vac) were intragastrically inoculated with an *E. faecalis* CECT7121 suspension (3.0×10⁸ cfu/ml). Non-immunised mice were used as controls (Ef and PBS). (A) Levels of anti-*Pasteurella multocida* (PM) IgG. (B) Levels of anti-*Mannheimia haemolytica* (MH) IgG. Each point represents the means (n=8). * *P*<0.05, Mann-Whitney's *U*-test.

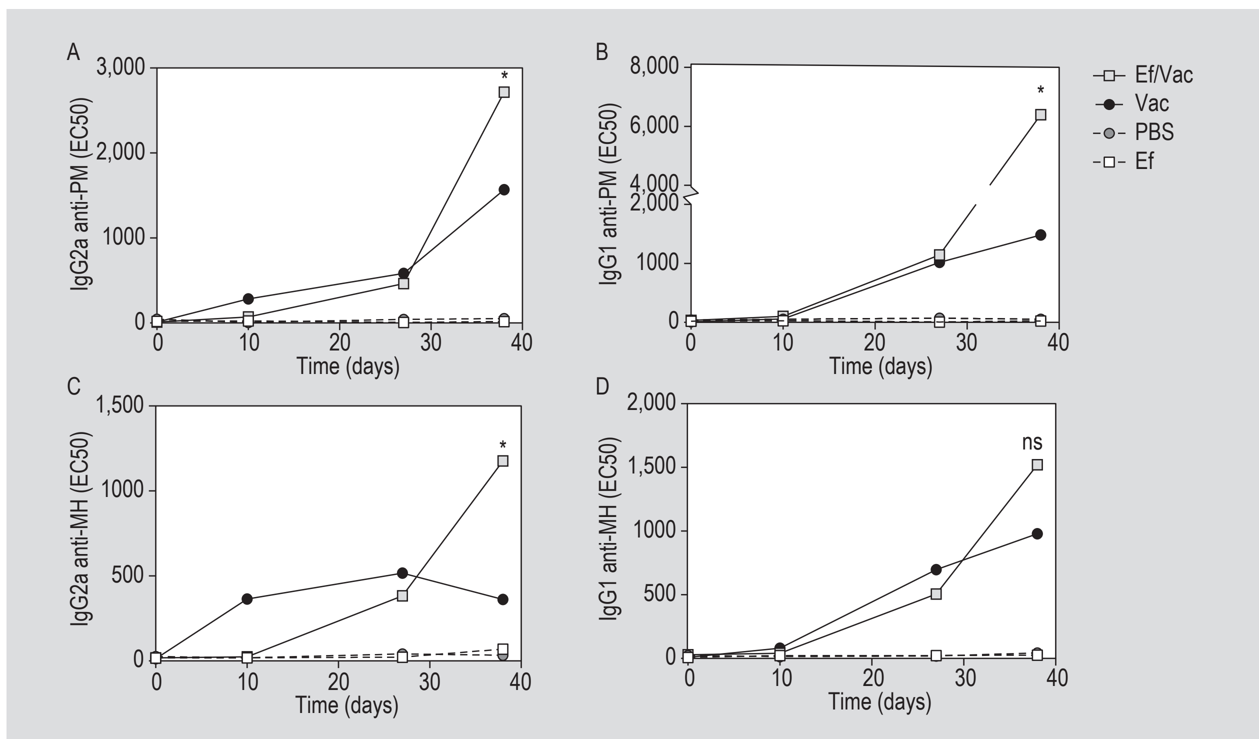

Figure 3. Determination of specific IgG1 and IgG2a titres in serum samples by ELISA after bovine respiratory disease immunisation of naïve BALB/c vs mice treated with *Enterococcus faecalis* CECT7121. The positive control group was inoculated with saline (Vac) and the experimental group (Ef/Vac) was inoculated i.g. with an *E. faecalis* CECT7121 suspension (3.0×10⁸ cfu/ml). Non-immunised mice were used as controls (Ef and PBS). Levels of anti-*Pasteurella multocida* (PM): IgG2a (A) and IgG1 (B). Levels of anti-*Mannheimia haemolytica* (MH): IgG2a (C) and IgG1 (D). Each point represents the means (n=8). **P*<0.05, Mann-Whitney's *U*-test.

MH-specific IgG2a titres were also higher in the Ef/Vac group (3.25-fold increase; *P<0.05; Figure 3C). Regarding the levels of anti-MH IgG1, after treatment with *E. faecalis* CECT7121, a trend was observed towards higher values when compared to those that had only been immunised (1.55-fold increase; P=0.0878; Figure 3D).

Administration of *E. faecalis* CECT7121 increases avidity of specific antibodies

To study the capacity of *E. faecalis* CECT7121 to improve the quality of the specific immune response, an avidity assay was developed. At the lower dilution (1/1000), the concentrations of 6 M and 8 M urea were suitable for

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sufficient dissociation of the immune complexes. This procedure allowed the observation of differences in serum avidities between the two immunised groups, showing higher RAI and AI for the *E. faecalis* CECT7121-treated group (Figure 4). For specific antibodies against PM, the optimal concentration of the chaotropic agent to compare between groups was 6 M; whereas for MH, 6 M and 8 M urea were both useful to detect differences in avidity. When the avidity of specific antibodies against PM was analysed, a higher binding capacity in the Ef/Vac group was detected, as assessed by the RAI and AI (*$P<0.05$; Figure 4A, 4B). For MH, the *E. faecalis* CECT7121-treated group also presented higher values of RAI and AI than animals belonging to the control immunised group (*$P<0.05$; **$P<0.01$; Figure 4C, 4D). Values of RAI and AI are also presented in the Table 1.

Administration of *E. faecalis* CECT7121 stimulates the production of pro-inflammatory cytokines

The levels of interleukin (IL)-2, interferon-γ (IFN-γ) and IL-12 were determined in spleen cell culture supernatants stimulated *ex vivo* with heat-killed bacteria as cellular immune response markers. In all cases, the specific cytokine secretion was low while the positive control (ConA) induced high cytokine production (data not shown). No significant changes in the IL-2 levels were observed after the specific stimulus *versus* basal conditions (Figure 5A).

Table 1. Relative avidity index (RAI) and avidity index (AI) of sera from mice immunised with the bovine respiratory disease vaccine and treated or not with *Enterococcus faecalis* CECT7121 (Ef/Vac group and Vac group, respectively).

Group[1]			RAI	AI
Pasteurella multocida				
	6M urea	Ef/Vac group	83.5±15.4	159.5±62.9
		Vac group	71.9±12.1	97.4±49.1
		P-value	<0.05	<0.05
Mannheimia haemolytica				
	6M urea	Ef/Vac group	83.8±9.7	100.5±26.8
		Vac group	65.6±17.9	66.65±40.4
		P-value	<0.05	<0.05
	8M urea	Ef/Vac group	77.4±18.5	95.6±43.3
		Vac group	45.3±24.2	44.9±38.9
		P-value	<0.01	<0.05

[1] *P. multocida* or *M. haemolytica* were employed as coating agent and urea as chaotropic agent.

Inoculation with *E. faecalis* CECT7121, before the BRD immunisation, induced an increase in the levels of IFN-γ, a typical Th1 cytokine, by spleen cells stimulated with PM and MH *versus* non-stimulated cells (*$P<0.05$ and **$P<0.01$,

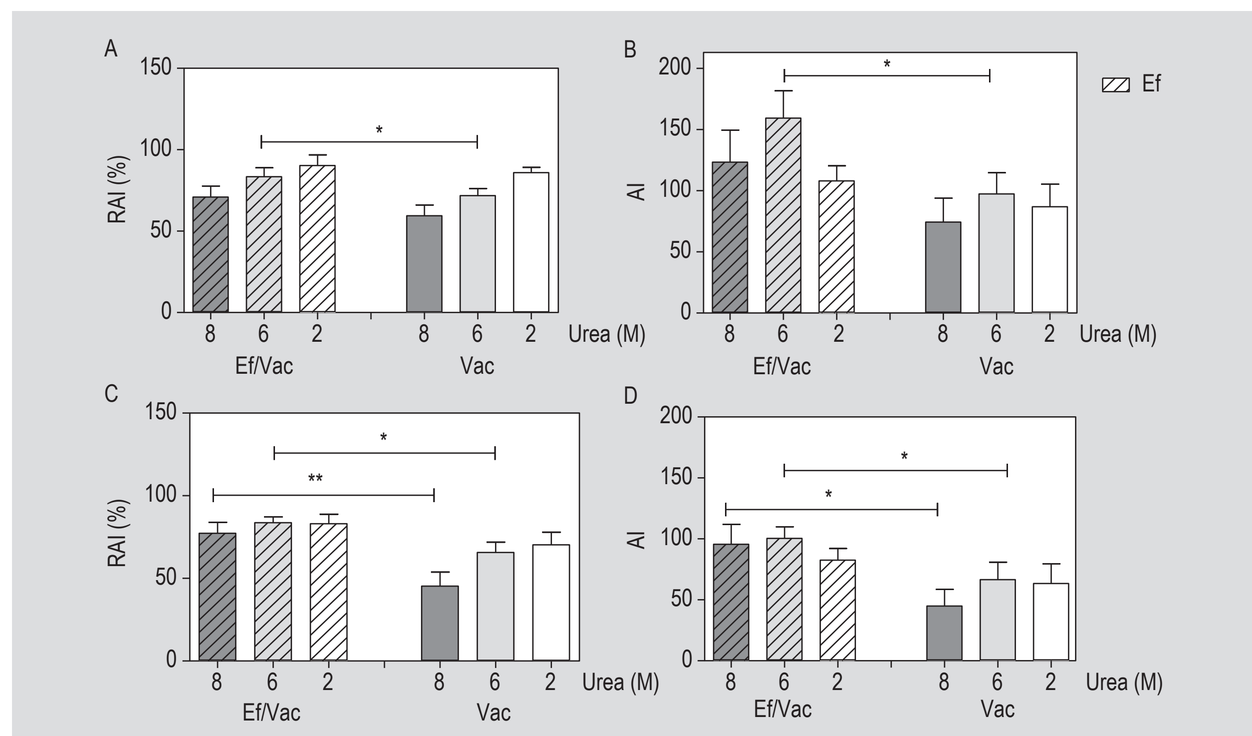

Figure 4. Comparison of antibody responses detected as avidity by ELISA expressed as relative avidity index (RAI) or avidity index (AI). (A) RAI and (B) AI for anti-*Pasteurella multocida* (PM) antibodies. (C) RAI and (D) AI for anti-*Mannheimia haemolytica* (MH) antibodies. Results are expressed as mean ± standard error of the mean of murine sera from animals of the same experimental group. * *P*<0.05 and ** *P*<0.01; unpaired Student's t-test.

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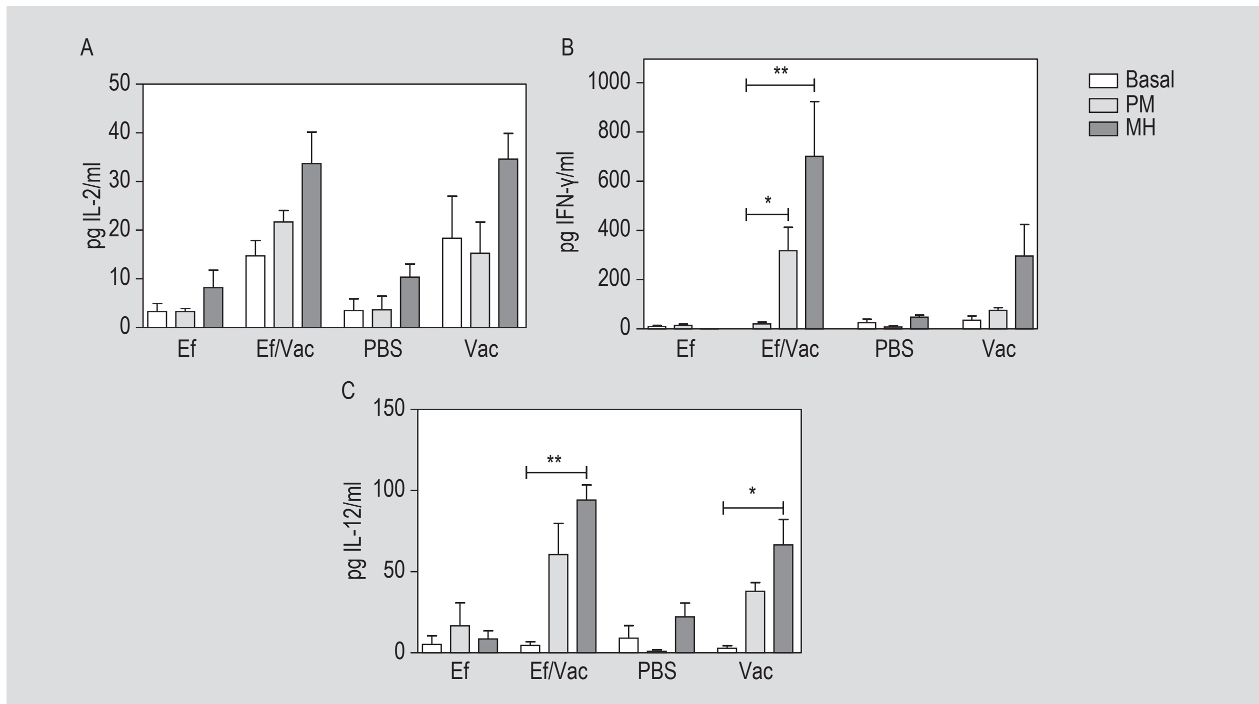

Figure 5. (A) Interleukin (IL)-2, (B) interferon (IFN)-γ and (C) IL-12 production measured by ELISA in splenocyte culture supernatants after incubating with culture medium (Basal), *Pasteurella multocida* (PM) or *M. haemolytyca* (MH) for 72 h. Eight mice from each group were analysed in each assay and results were expressed as pg/ml. **P*<0.05, ***P*<0.01, Kruskal-Wallis ANOVA followed by the Dunn's multiple comparison tests with selected data pairs.

respectively; Figure 5B). However, no statistical differences between vaccinated groups were detected. A significant increase in the secretion of IL-12 after MH stimulation was detected in both immunised groups; however, the levels of this cytokine were not different between groups (Figure 5C).

4. Discussion

Vaccines contribute to health improvement and welfare. In practice, co-administration of adjuvants is the traditional approach to enhance the immunogenicity of vaccine antigens. There is evidence that certain probiotic administered around the time of vaccination have similar effects in potentiating the immune response (Boge *et al.*, 2009; Castro *et al.*, 2008; Olivares *et al.*, 2007; Wen *et al.*, 2012). BRD is a general term for the respiratory disease syndrome. In the veterinary area, it is known that BRD vaccines only partially prevent infections caused by *P. multocida* and *M. haemolytica* (Roier *et al.*, 2013). Consequently, renewed efforts and innovative strategies are required to improve the specific immune response elicited by commercially available vaccines to this pathology. Bacterial strains with immunostimulatory effects could be useful to enhance antigen-specific immune responses as novel vaccine adjuvants (Licciardi and Tang, 2011). In this work, *E. faecalis* CECT7121 administration was evaluated as a potential strategy to enhance the specific immune response elicited by the BRD vaccine.

Most vaccines mediate protection through the induction of specific IgG serum antibodies (Maidens *et al.*, 2012) which directly correlates with protection and is described as the 'gold-standard' to determine the influence of probiotics on immunity (Redondo *et al.*, 2017). In our murine model, the commercial BRD vaccine induced the synthesis of IgG against both *P. multocida* and *M. haemolytica*. The *E. faecalis* CECT7121 treatment was able to potentiate the response generated by vaccination, as evidenced by an increase in anti-PM and anti-MH IgG levels, compared to the Vac group. It is believed that probiotics should be continually supplied to exert their effects (Maldonado Galdeano *et al.*, 2007); however, despite the discontinued administration of *E. faecalis* CECT7121, the immunodulatory effect continues, surely because this strain remains in the murine intestinal mucosa without altering the pre-existing microbiota for at least 18 days (Castro *et al.*, 2007, 2008).

Immune responses are characterised by a rapid appearance of low IgG antibody titres after first antigen exposure and then, the booster exposure to antigens reactivate the immune memory and results in a rapid increase in the IgG antibody titre. In this work, we observed different kinetics of antibody production against the bacterial immunogens. For PM, specific antibody levels increased according to the immunisation plan, and in this case, the adjuvant effect of *E. faecalis* CECT7121 was detected on the final day

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of the assay. Instead, for MH, higher specific antibody levels were observed earlier, on day 10, and the greatest difference between immunised groups was observed on day 27. This observation suggests that, for some antigens, the immunomodulatory effect may occur during the primary vaccine antigen sensitisation, and a posterior effect would be achieved through the stimulation of a population of central memory cells that would respond more efficiently upon subsequent encounters with the antigen. On the other hand, it could be possible that for MH an additional reinforcement in the *E. faecalis* CECT7121 administration was necessary to continue evidencing the difference in antibody titers. The effect of probiotic microorganisms on the priming of naïve B cells has already been reported (Bandeira Roosa *et al.*, 2012; Benyacoub *et al.*, 2003; Maassen *et al.*, 2003). However, studies assessing the effect of probiotics from *Enterococcus* species on the specific humoral immune response are scarce. Working with a dog model, Benyacoub *et al.* (2003) demonstrated that the oral administration of *Enterococcus faecium* (SF68) induced a higher canine distemper virus (CDV) vaccine-specific circulating IgG production than control immunised dogs.

Aluminium-based adjuvants generally induce a Th2-biased response (Pulendran and Ahmed, 2011). In mice, the activation of the Th2 lymphocyte subset is associated with the production of IgG1 and IgE while the activation of the Th1 lymphocyte subset, induces the production of IgG2a (Calder, 2007; Inoue *et al.*, 2009; Zhu and Paul, 2010). In our work, both immunised groups presented an increase in specific IgG1 titres; it is in line with the reported Th2 activity of the aluminium adjuvants. IgG2a levels were also increased as the immunisation plan progressed. Altogether, the pre-treatment with *E. faecalis* CECT7121 induced higher titres of both immunoglobulin isotypes as compared to the control immunised mice. This effect was more marked when specific IgG2a levels were analysed, which is consistent with the broad pro-Th1 activity reported for this bacterium (Castro *et al.*, 2007, 2010; Molina *et al.*, 2015).

The avidity describes the net force by which antibodies and multivalent antigens bind. Low-avidity IgG antibodies are elicited after the first immunological challenge. Somatic hypermutation of antibody binding sites leads to the selection of high-affinity antibodies (affinity maturation); thus, over time, antibody avidity matures (Han *et al.*, 2015). IgG antibody avidity tests have been used in the serodiagnosis of primary and chronic infections (Akingbade *et al.*, 2003; Gonzaga *et al.*, 2011; Han *et al.*, 2015; Rudzińska *et al.*, 2017) and to evaluate vaccine efficiency and failures (Gulati *et al.*, 2005; Longworth *et al.*, 2002; Mercader *et al.*, 2012; Park *et al.*, 2007). An IgG avidity ELISA, based on the dissociation of immune complex with urea, was developed for the determination of IgG avidity. Under our experimental conditions, the relative avidity indexes obtained from animals in both immunised groups were higher than 60%, which is a value that is considered a marker of high avidity (Özkul *et al.*, 2008). For both bacteria, when 6 M urea was employed, sera from *E. faecalis* CECT7121-treated mice showed a higher binding capacity than that obtained in the vaccinated group, thus demonstrating that this bacterial strain is able to improve the quality of the specific humoral immune response. Remarkably, although no statistical differences were found in the titres of IgG against MH on day 38 between treated and control BRD immunised mice, sera belonging to the Ef/Vac group showed higher avidity than the Vac group. Even when 8 M urea was employed, serum IgG from *E. faecalis* CECT7121-treated mice retained their antigen binding capacity.

In this work, cytokine production was also studied. Considering that *E. faecalis* CECT7121 usually promotes Th1 immune responses (Castro *et al.*, 2007, 2010; Molina *et al.*, 2015), the secretion of IFN-γ was of special interest. Only in the Ef/Vac group, splenocytes stimulated with both heat-killed bacteria secreted higher levels of IFN-γ than non-stimulated splenocytes. Nevertheless, no statistical differences were found in the production of this cytokine between the vaccinated groups. BALB/c mice are known for its tendency to establish Th2 responses (Shimada *et al.*, 2005) and its difficulty to establish persistent Th1 responses, mainly when high concentrations of aluminium as adjuvant are used. The latter fact might account for the lack of differences between groups, considering that the observed induction of IgG2a antibodies can be explained in a Th1-rich scenario triggered by *E. faecalis* CECT7121. IL-2, cytokine involved in T cell proliferation, is a good marker to indicate whether a memory T cell response was generated. Unfortunately, no specific IL-2 production was found in the immunised groups. It is possible that our experimental conditions were not optimal to ensure the secretion of appropriate amounts of this cytokine. However, a T cell response must have been elicited because, for the isotype switch to occur, T and B cell collaboration is required.

Overall, our results provide new evidences of the immunomodulatory action of *E. faecalis* CECT7121. To sum up, *E. faecalis* CECT7121 was able to improve the quantity (antibody titres) as well as the quality (antibody avidity) of the specific humoral immune response elicited by the BRD vaccine. Besides, the probiotic bacteria stimulated the production of the Th1 cytokine IFN-γ by splenocytes in *M. haemolytica* and *P. multocida* immunised animals.

Previously, we have observed that this probiotic strain was able to potentiate the cellular immune response (Castro *et al.*, 2008). In this work and for the first time, the effect of *E. faecalis* CECT7121 on the specific antibody production was demonstrated. Considering that the use of probiotic microorganisms as adjuvants is a promising original approach to enhance vaccine immunogenicity, our

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findings support the use of *E. faecalis* CECT7121 as an adjuvant strategy to potentiate vaccine immune responses. Further studies performed will be necessary to evaluate the immunostimulatory effect in the bovine host.

Conflict of interest statement

The authors have no conflict of interest.

Acknowledgements

This work was supported by grants from Universidad de Buenos Aires (20020130200012BA) and CONICET (11420110100381). Authors are grateful to Ms. Ana Carolina Mourelle and Ms. María Angélica Miranda for their valuable technical help and animal care.

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