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Strain dependent protection conferred by *Lactobacillus* spp. administered orally with a *Salmonella* Typhimurium vaccine in a murine challenge model

M. Esvaran[a,b], P.L. Conway[a,b,*]

[a] Centre for Marine Bioinnovation, The University of New South Wales, Sydney, NSW 2052, Australia
[b] School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia

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ABSTRACT

Consumption of *Lactobacillus* spp. has been shown to enhance immune responses in mice. This study examined the immuno-adjuvant capacity of two strains: *Lactobacillus acidophilus* L10 and *Lactobacillus fermentum* PC2, in the induction of protective humoral immunity in a *Salmonella* Typhimurium vaccine challenge model. Briefly, BALB/c mice were divided into four groups. Three groups of mice received *S.* Typhimurium vaccine (10^8 colony forming units (CFU) per dose) on days 0 and 14. In addition to the vaccine, five doses (10^8 CFU per dose) of either *L. acidophilus* L10 or *L. fermentum* PC2 were also administered to a group. All mice were challenged with viable *S.* Typhimurium on day 28. On day 10 post challenge, the study was terminated and microbial and immunological parameters were assessed. Mice dosed with *L. fermentum* PC2 in addition to the vaccine had a significantly enhanced *S.* Typhimurium humoral response. The mice in this group had high levels of lactobacilli in the feces and in association with the Peyer's patches, no detectable levels of either lactobacilli or *S.* Typhimurium in the spleen, and no detectable weight loss. Mice given *L. acidophilus* L10 with the vaccine were unable to exhibit elevated *S.* Typhimurium specific humoral responses. However, there was no detectable *S.* Typhimurium in the spleens of this group. Interestingly, translocation of lactobacilli into the spleen was observed as well as a slight weight loss was noted in mice that received the *L. acidophilus* L10 with the vaccine. This study shows that, the *L. fermentum* PC2 had a greater capacity than the *L. acidophilus* L10 to act as an oral adjuvant in a *S.* Typhimurium oral vaccine program and afforded greater protection against a live *S.* Typhimurium challenge.

1. Introduction

Enteric infections are a leading global health problem in developing countries, causing around 3 million deaths annually [1]. To effectively combat such diseases, vaccination delivered *via* oral mucosal surfaces is needed. The advantages associated with oral vaccines are numerous. They are safe, relatively inexpensive and have the potential to be administered as single use vaccines [2]. In addition, the need for personnel and equipment requirements related to parenteral vaccines is virtually non-existent with mucosal immunization. Moreover the efficacy of certain existing vaccines (parenteral) can be enhanced by administration *via* the oral route [3] by stimulating both systemic and more importantly the mucosal immune system. Furthermore, ageing brings about changes in the structure and function of the gastrointestinal tract which result in a decline in competence and regulation of the immune system [4]. However, secretory IgA (sIgA) production does not appear to be affected by ageing [5]. Therefore existing parental vaccines can be administrated orally in older persons to elicit a greater immune response [2].

Despite the many advantages of oral administration of vaccines, few are currently available due to factors such as the need for high dosage and induction of oral tolerance to the fed antigen. Adjuvants confer many benefits when used in a vaccine formulation. They reduce the number of doses and the amount of antigen required per dose as well as enhance the magnitude and duration of the resulting immunity. Moreover, oral tolerance can also be abrogated by using appropriate mucosal adjuvants. Cholera toxin (CT) from *Vibrio cholera* and heat-labile enterotoxin (LT) from *Escherichia coli* are probably the most potent mucosal adjuvants currently known [6,7]. Though they are very powerful stimulators of both mucosal and systemic compartments, they are unsuited for use in humans due to their inherent toxicity and immunogenicity. However, non toxic derivatives of CT and LT and other molecules such as immune-stimulating complex (ICOMS) and compounds such as MF59, QS-21

* Corresponding author at: Centre for Marine Bioinnovation, The University of New South Wales, Sydney, NSW 2052, Australia. Tel.: +61 640293851593.
E-mail address: P.Conway@unsw.edu.au (P.L. Conway).

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and CpG ODN show great promise [1,8–12]. Although many oral adjuvants show promise, most are very expensive to manufacture and the search for a good oral adjuvant is still ongoing.

Lactobacillus spp. are 'generally regarded as safe' (GRAS) and are used extensively in the food industry. The role of the local immune response in protection against enteric bacterial infections has been known since the early 1900s [13]. *Lactobacillus*-fed mice have exhibited reduced severity of enteric infections such as *Salmonella* Typhimurium [14,15]. Protection in these studies has been correlated with an increase in both mucosal and serum specific humoral immune responses [16–18]. The capacity of lactobacilli to function in mice and humans as adjuvants has been demonstrated with a number of antigens, ranging from proteins to bacteria [19–21]. Moreover, adjuvant effects of lactobacilli are dependent on a number of parameters. Ibnou et al. showed that two strains which exhibited very similar properties *in vitro* displayed different Th biased responses *in vivo* [22]. The adjuvant effect of lactobacilli also appears to be influenced by the growth phase at which they are used [23]. Therefore strains should be chosen and cultured with attention to detail.

Infection of mice with *S.* Typhimurium results in a systemic infection. In mice, systemic dissemination produces a lethal disease that is similar to human typhoid fever caused by *S.* Typhi. As with most mucosal pathogens, the innate immune system is the first and crucial line of defense, restricting bacterial growth at epithelial surfaces and preventing systemic translocation of the pathogen. However, complete protection against a high dose of an enteric pathogen requires the contribution of a robust humoral immune response [24].

Previously, we showed using a mouse model that oral co-administration of *Lactobacillus fermentum* KLD with vaccine antigens enhanced both systemic and mucosal immune responses against the fed antigen [25]. In the current study, we extend those findings by examining the protection afforded by the inclusion of two lactobacillus strains, *L. fermentum* PC2 and *Lactobacillus acidophilus* L10 in a *S.* Typhimurium vaccine challenge model. Furthermore, we examined if the observed protection correlated with enhanced *S.* Typhimurium specific humoral immunity. Immunogenic doses of heat-*killed S.* Typhimurium (HKST) alone are capable of generating sufficient immunity [26]. Hence, in this study, a subimmunogenic dose of HKST was used to evaluate the impact of inclusion of lactobacilli as adjuvants in the vaccine program.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *Lactobacillus* strains, *L. acidophilus* L10 (FII 545 600), *L. acidophilus* (FII 529 500), *L. acidophilus* (FII 529 600), *Lactobacillus paracasei* L26 (FII 542 300), *L. fermentum* PC2 (FII 548 700), and the *S.* Typhimurium (UNSW 078 300) were obtained from the culture collection in the School of Biotechnology and Biomolecular Sciences, the University of New South Wales. All bacterial stock cultures were stored at −80 °C in 30% glycerol. *Lactobacillus* strains were grown in de Mann Rogosa Sharpe (MRS) broth in an anaerobic chamber maintained at 37 °C. The *S.* Typhimurium strain is streptomycin resistant and was grown aerobically in Tryptone Soya Broth (TSB) with streptomycin at a concentration of 2 mg ml^{-1} at 37 °C. Glycerol stocks of *S.* Typhimurium and *Lactobacillus* strains were inoculated (1%) into respective media, and propagated at appropriate conditions for 18 h prior to use as a primary culture.

2.2. Mice

Female specific pathogen free BALB/c mice, 6–8 weeks of age were obtained from the Animal Research Center (Perth, Australia). They were housed in plastic cages (10 mice per cage) in the Biotechnology and Biomolecular Sciences Animal facility at the University of NSW, and fed *ad libitum* a commercial diet (Gordon's Specialty Stockfeed, Sydney, Australia) and autoclaved water. All animal experiments were approved by the University of NSW animal ethics committee.

2.3. In vitro adhesion to murine Peyer's patches

Mice were sacrificed by CO_2 asphyxiation, and the entire small intestine was removed and flushed with 5 ml of cold phosphate buffered saline (PBS, pH 7.4), to remove debris. The Peyer's Patches were carefully excised and placed into wells of a 24 well tissue culture plate (4–6 pieces per well) (Nunc, Denmark). The adhesion assay was carried out as detailed previously [27]. Briefly, primary cultures of *Lactobacillus* spp. were inoculated (1%) in fresh MRS broth supplementing with [methyl 1′, 2′-3H] thymidine (ICN; 10 μCi ml^{-1}). After incubation at 37 °C for 18 h, the bacterial cultures were harvested by centrifugation at 2500 × *g*, for 10 min. The pellets were washed twice with PBS and resuspended to an optical density (OD_{600}) of 0.5. These suspensions were immediately placed on ice, until use. Aliquots of the cell suspensions (0.5 ml) were added to each well of the 24 well tissue culture plate and, incubated with gentle shaking for 20 min at 37 °C. The tissue pieces were then washed four times with PBS (1 ml per wash). All washings were conducted at room temperature. After the final wash, each tissue piece was placed into individual glass scintillation vials. Aliquots of perchloric acid (150 μl) and hydrogen peroxide (300 μl) were added to each vial prior to incubation at 70 °C for 18 h. At the end of incubation, the vials were allowed to cool to room temperature, scintillation fluid (10 ml) was added to each vial, and the disintegrations per min determined using a liquid scintillation counter (Beckman). The degree of adhesion of each strain was determined by calculating the number of radioactively labeled bacteria per mg wet weight of tissue per vial.

2.4. In vivo experimental design

The mice were randomly divided into 4 groups of 10 mice per group. After acclimatization for a week, three of the groups of mice were orally immunized with HKST (10^7 colony forming units (CFU) per mouse) on days 0 and 14. In addition to the vaccine, the mice were given 5 doses (on days 2, 4, 6, 8 and 10 during the vaccination program) of either live *L. acidophilus* L10 or *L. fermentum* PC2 (10^8 CFU per dose) by oro-gastric intubation. The negative control group was only given PBS instead of the vaccine. The vaccine control group received the vaccine alone. Two weeks after the second immunization, all four groups of mice were challenged oro-gastrically with live *S.* Typhimurium (10^6 CFU per mouse) in PBS (100 μl) containing 10% sodium bicarbonate. The mice were sacrificed on day 10 post-challenge by CO_2 asphyxiation. Blood, intestines and spleens were collected for microbial and immunological analysis. Blood samples were held overnight at 4 °C prior to being centrifuged at 10,000 × *g* for 1 min. The sera were stored in sterile tubes at −80 °C until assayed for IgG. Intestinal fluid samples were obtained by flushing the lumen of the entire small intestine with 2 ml PBS containing 1 mM phenylmethylsulfonylflouride (PMSF) (Sigma, USA). The washings were centrifuged at 10,000 × *g* for 10 min and, the supernatant stored at −80 °C until assayed.

2.5. Preparation of S. Typhimurium lysate for use in ELISA

A stationary-phase culture of *S.* Typhimurium was washed twice in ice-cold PBS. The washed whole cell pellet was resuspended in PBS containing 1 mM (PMSF; Sigma) and adjusted to an absorbance of 0.8 at OD_{600}. This OD corresponded to a viable