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USE OF PROBIOTIC BACTERIA AS AN ADJUVANT FOR AN INFLUENZA VACCINE

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ABSTRACT: ***A double-blind, placebo controlled trial on 47 subjects showed that daily consumption of a probiotic bacterium, Lactobacillus fermentum strain VRI 003 (PCC®), prior to and four weeks after an intramuscular influenza vaccine injection significantly enhanced the serum hemagglutinin antibody inhibition titre to H1N1. The mean HAI titres to the two other antigens, H3N2 and FluB present in the vaccination were also slightly increased. The number of days of respiratory symptoms experienced by the subjects in the probiotic group was significantly less than the placebo group. Additionally, the probiotic group also had a much lower percentage of non-seroconverters (5.5% compared to 28% in the placebo group). This study provides suggestive evidence that oral consumption of a specific probiotic bacterium may provide a low cost and low risk adjuvant for influenza vaccines.***

KEY WORDS: Adjuvant, Influenza, Lactobacillus, Probiotic, and Vaccine

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INTRODUCTION

Influenza is a highly contagious respiratory disease that affects millions of people around the world each year (Langley and Faughnan, 2004). The major therapeutic approach is wide-scale immunization of the population. However, the efficacy of influenza vaccine is highly variable, ranging from 0 – 91% (Erickson et al., 2000). The success rate is somewhat lower in children and seniors. Methods are being sought to increase the efficacy of influenza vaccines. A significant focus has been on the development of novel adjuvants and on mucosal immunization.

Activation of the immune system to influenza virus infection in humans is known to be multifactorial. However, the precise contributions of the various arms of the immune system, including innate immunity, serum antibodies to viral antigens, local IgG and IgA, and Th1- and Th2-type immune responses have not yet been elucidated (Stephenson et al., 2006). There is some evidence that probiotic bacteria have potential to act as novel vaccine adjuvants in vitro and in vivo.

In 2005 Mohamadzadeh *et al*, showed that mature dendritic cells (MDCs) respond to certain strains of lactobacilli with inflammatory cytokines that lead to the development of Th1 immune responses (Mohamadzadeh et al., 2005). Both allogeneic T cell priming, as well as autologous T cell activation, was detected. All three species of lactobacilli were far more potent in priming $CD8^+$ T cells than was *E. coli* LPS. The authors concluded that because lactobacilli can activate MDCs, prime T cells, and induce Th1 cytokines, certain strains could be particularly advantageous as vaccine adjuvants, by promoting DCs to regulate T cell responses toward Th1 and Tc1 pathways. These findings added to the evidence indicating that intestinal bacteria and probiotics help maintain gut homeostasis by balancing pro-inflammatory and anti-inflammatory mucosal responses (Biancone et al., 2002).

In another study, Balb/c mice were immunised subcutaneously with a suboptimal dose of influenza antigen, such that no response upon priming was observed. Subsequently. *Bifidobacteria, L. plantarum,* or *L. casei* were administered intragastrically daily. Seven days after secondary immunisation, antigen-specific IgG was determined in serum. The higher dose of *L. casei* showed significant adjuvant activity, but none of the other probiotic doses did (Boersma et al, 2000). This indicates that not all strains of probiotic bacteria are capable of stimulating an immune response.

The potential of a strain of *L. fermentum* to act as an adjuvant for tetanus toxin vaccination was demonstrated in 2002 (Plant and Conway, 2002). Olivares et al (2007) showed that oral administration of a strain of *L. fermentum* (CECT5716) improves the immunologic response to influenza vaccination and may provide enhanced systemic protection from infection by increasing the T-helper type 1 response and virus-neutralizing antibodies.

The present study utilised a known immunostimulatory strain of *L. fermentum* (Prescott et al., 2005; Weston et al., 2005) to further test the hypothesis that oral ingestion of probiotics could boost the humoral immune response to a vaccine administered intra-muscularly in healthy human subjects. The vaccine chosen was the influenza vaccine.

MATERIALS AND METHODS

Probiotic

Hard gelatine capsules (size 1) containing either the freeze-dried probiotic *Lactobacillus fermentum* strain VRI 003 (Probiomics Ltd, Sydney Australia) (International Depositary Accession No. NM02/31074) at a concentration of 1 x 10^9 CFU with microcrystalline cellulose as the excipient, or a placebo (containing microcrystalline cellulose alone) were prepared. Screw cap glass bottles containing 60 capsules (placebo or probiotic) and a moisture-absorbing sachet were coded as either B or C to ensure that the identity of the samples was blinded from the subjects.

Influenza vaccine

An influenza vaccine, Fluvax (CSL, Melbourne, Australia), was used to immunize all subjects. The vaccine contained the following three influenza viral strain antigens:

- A (H1N1): an A/New Caledonia/20/99 (H1N1) - like strain, 15 μg HA per dose
- A (H3N2): an A/California/7/2004 (H3N2) - like strain, 15 μg HA per dose
- B: a B/Malaysia/2506/2004 - like strain, 15 μg HA per dose

Study Design and Protocol

The study protocol was approved by the Human Research Ethics Committee of St Vincent's Hospital, Sydney (Approval No. H05/123). The study design was a randomized, double blind, placebo-controlled trial.

Initially it was planned to recruit 100 healthy adults ages 18 to 49, who had not had a previous influenza vaccine, and without a history of adverse reactions to vaccines, from a corporate health clinic in Sydney. The site was chosen because they routinely administered influenza vaccines to healthy subjects. Pregnant women or women planning to become pregnant within the duration of the study were excluded. However, due to slow recruitment, only 47 subjects were recruited by the end of the influenza season in Sydney (November 2006). It was decided to end the trial and analyse these subjects. The St Vincent's Hospital Sydney Human Research Ethics Committee also approved this decision.

Subjects were required to attend the clinic three times over a period of six weeks. Participants were randomly assigned to receive either the probiotic or the placebo capsules. Randomization into two groups was carried out using a random allocation program called Allocator (http://home.clara.net/sisa/randmiz.htm). At the first visit the subject's eligibility was checked and a medical history was taken by a registered nurse who allocated each subject an ID number and a bottle of capsules with instructions to take one capsule daily.

All patients who met eligibility criteria were randomized into the trial. At the second visit, 14 days after the first visit, a 10 ml blood sample was taken for baseline serum HAI levels prior to administration of a standard influenza vaccine (FluVax from CSL, Melbourne, Australia) intramuscularly. At the third visit, four weeks post-vaccination, a second blood sample was taken. A questionnaire on intestinal and general health issues and any side effects or adverse reactions to the injection was given to all participants at the second visit, and collected at the third.

Primary Outcome – HAI Titre

Serum from each subject was separated and stored at –20°C until the end of the study. The samples were then sent to the Institute of Medical and Veterinary Sciences in Adelaide, Australia for analysis. Three antigens present in the vaccine – H1N1, H3N2 and FluB - were tested using the hemagglutinin antibody inhibition (HAI) assay by standard methods (Ellis and Zambon, 1997). Serum sample dilutions ranged from 1 in 10 to 1 in 1,024 in serial two-fold dilutions. The titer is expressed as the reciprocal (1/titre) of the highest dilution of serum that completely inhibited hemagglutination.

The results of the HAI levels were analysed by a statistician under blinded conditions. The code was broken once the statistician had concluded the analysis of any significant differences between the groups.

Secondary Outcome - Patterns of Illness

Subjects were required to keep a weekly symptom log for the duration of the study to record any symptoms of illness (and side effects) including local and general reactions to the injection (pain, inflammation, itching, numbness, stiffness); acute illness; and respiratory symptoms (runny nose, sore throat, cough). The results of the illness patterns were analysed by an independent biostatistician for significance.

Titres to Measles and Varicella zoster antigens

To test the specificity of the response, the serum samples were titrated against two common viral antigens, measles and varicella zoster. The VZ/ measles were tested using Enzygnost kits from Dade Behring to detect IgG antibody specific for the measles or VZ viruses.

RESULTS

A total of 47 subjects were recruited by the end of the winter season in Sydney. This consisted of 26 in the placebo group and 21 in the probiotic group. The average age of the subjects in the placebo group was 32, and 64% were female. The average age in the probiotic group was 31, and 53% were female. Four subjects did not complete the study and were lost to follow-up - one in the placebo group, and three in the probiotic group.

There was no statistical difference in sex distribution between the placebo and probiotic groups (chi-squared = 0.58, df = 1, two-tailed p = 0.447). Similarly, there was no difference in mean age between the placebo and probiotic groups (t = 0.33, two-tailed p = 0.742).

1. HAI Titres

H1N1

The following table shows that the H1N1 titre variable was distinctly skewed in both groups at baseline and was still positively skewed post-vaccination, though less so. Table 1 shows the skewness, means and medians for the two groups in terms of their H1N1 titres. It was decided to use the median for comparison as it more accurately reflects the serum titres and to use nonparametric statistical methods.

At baseline there was no significant difference between the medians of the two groups as demonstrated by the Mann-Whitney U test (U = 222.0, p = 0.668).

The hypothesis was that the probiotic group would have a significantly higher H1N1 median titre after vaccination than the placebo group (rather than just a significantly different median). Therefore one-tailed tests of significance rather than two-tailed tests were used.

TABLE 1. Analysis of H1N1 serum titres in subjects in the placebo group and in the probiotic group at baseline and at four weeks post-vaccination

GROUP	PARAMETER	BASELINE	4 WEEKS POST-VACCINATION
Placebo (n=25)	Skewness	4.6	4.7
	Mean 1/titre	20	404
	Median 1/titre	10	160
Probiotic (n=19)	Skewness	2.0	1.7
	Mean 1/titre	21	2073
	Median 1/titre	10	320

TABLE 2. Comparison of median H1N1 serum titers pre- and post-vaccination

PLACEBO GROUP	WILCOXON Z = -4.107	ONE-TAILED P < 0.001
Probiotic group	Wilcoxon z = -3.823	one-tailed p < 0.001

TABLE 3. Difference scores (median H1N1 titers post-vaccination - baseline)

GROUP	SKEWNESS	MEDIAN 1/TITRE
Placebo	2.0	140
Probiotic	0.7	300

In both groups the H1N1 median titre after vaccination was significantly higher than baseline as demonstrated by the Wilcoxon matched pairs signed ranks test (Table 2). After vaccination the median for the probiotic group was significantly higher than the median for the placebo group (Mann-Whitney U = 163.5, one-tailed p = 0.038). As well as comparing before and after H1N1 scores we also examined the difference score of the titres (post-vaccination titre minus baseline titre). The difference scores were distinctly skewed. The median difference was significantly higher in the probiotic group than in the placebo group (Mann-Whitney U = 152.5, one-tailed p = 0.022). The overall difference in level of H1N1 (after Fluvax compared to before Fluvax) did not differ significantly by sex (male median = 135, female median = 150, Mann-Whitney U = 225.5, two-tailed p = 0.839). A subject with a rise in titre of <4 fold post-vaccination is considered to be a non-seroconverter (Stephenson et al., 2006). There were 7 such subjects (28%) in the placebo group (Fig. 1A), and only one (5.5%) in the probiotic group (Fig. 1B).

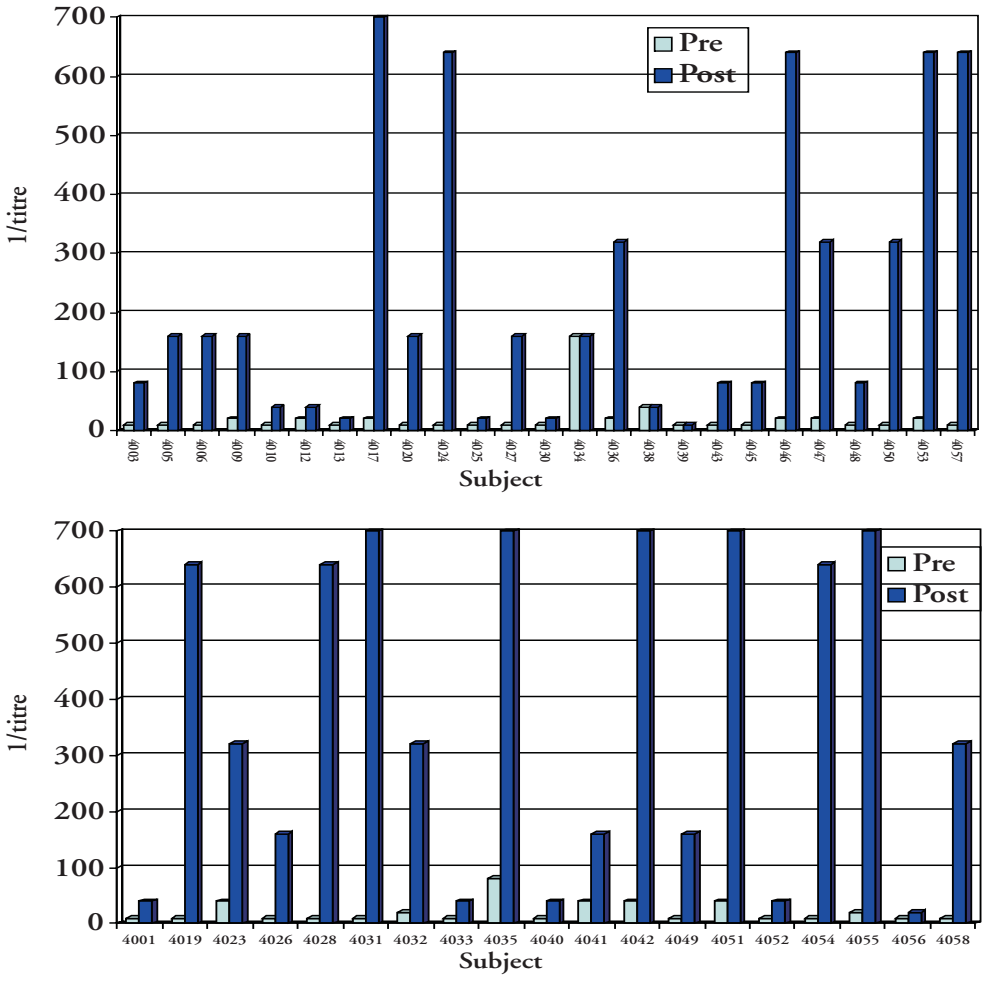
H3N2 and FLU B

There was a more modest enhancement of the HAI titre to the other two antigens in the vaccine, H3N2 and Flu B, than for H1N1. There were no significant differences between the serum HAI titres for these antigens, (p= 0.390 and 0.283 respectively); although the probiotic group recorded a higher mean titre for both antigens (Tables 4 and 5). The median titre in the probiotic group was higher than placebo for FluB, but lower than placebo for H3N2.

MEASLES and VARICELLA ZOSTER

There was no significant difference between the IgG titres at baseline and at 4 weeks post-vaccination to these two antigens in either the probiotic or placebo groups.

FIGURE 1. Pre- and post-vaccine titres in individual subjects in the placebo (A) and probiotic (B) groups.



Generally the post-vaccination titre tended to be slightly lower than the pre-vaccination titre.

TABLE 4. Comparison between placebo and probiotic H3N2 1/titres pre and post-vaccination

GROUP	MEAN BASELINE	MEAN POST-VACCINATION	MEDIAN BASELINE	MEDIAN POST-VACCINATION
Placebo	17	69	10	80
Probiotic	11	80	10	40

TABLE 5. Comparison between placebo and Flu B 1/titres pre and post-vaccination

GROUP	MEAN BASELINE	MEAN POST-VACCINATION	MEDIAN BASELINE	MEDIAN POST-VACCINATION
Placebo	12	60	10	20
Probiotic	12	92	10	40

TABLE 6. Analysis of respiratory symptoms and duration in subjects consuming the probiotic capsules compared to those consuming placebo capsules

WEEK	SUBJECTS WITH NO SYMPTOMS (%)		SUBJECTS WITH SYMPTOMS (%)		DAYS OF SYMPTOMS		SYMPTOM DAYS/ SUBJECT / WEEK	
	Probiotic	Placebo	Probiotic	Placebo	Probiotic	Placebo	Probiotic	Placebo
1	61	56	39	44	15	34	0.83	1.36
2	89	72	11	28	5	31	0.28	1.24
3	100	80	0	20	0	18	0.00	0.72
4	89	72	11	28	3	19	0.17	0.76
TOTAL/ AVERAGE	85%	70%	15%	30%	23	102	0.32	1.02

2. Reaction to the Injection

There was no significant difference between the groups in terms of adverse localised reactions to the vaccination. There were no severe episodes reported in either group, with a minority (16% in both groups) reporting the occasional mild reaction (temporary pain or redness). None of the subjects suffered a febrile illness.

3. Respiratory symptoms

Over all subjects the number of days of respiratory symptoms (per person) ranged from 0 to 21 with a median of 1. 42% of subjects had no symptom days.

There was no difference between the placebo group and the probiotic group in the percentage of subjects who did not have any days of symptoms (36% and 50% respectively; chi squared = 0.84, two-tailed p = 0.359). However, when the hypothesis that, among those who did have symptoms, the average number of symptom days will be lower in the probiotic group than in the placebo group was tested, there was a significant difference. The medians were 2 and 5 respectively and this was statistically significant (Mann-Whitney U = 38.5, one-tailed p = 0.027). Furthermore, looking at the number of days on which symptoms were experienced as a percentage of the total exposure days possible (i.e. number of subjects x 28 days), the placebo group had a 3-fold higher percentage of symptom days (14.6%) compared to the probiotic group (4.6%).

There was no difference between the two groups in the prevalence of symptoms in the first week (chi squared = 2.35, exact two-tailed p = 0.992). However, in weeks 2 to 4 the percentage of subjects with no symptom days was significantly higher in the probiotic group than in the placebo group (chi squared = 3.88, one-tailed p = 0.024) (Table 6).

DISCUSSION

Our study provides independent confirmation of the report (Olivares et al., 2007) that an orally ingested probiotic bacterium can be effective as an adjuvant for a vaccine administered parenterally. Given that the increase in H1N1 titer was on average 4.5 fold higher in those consuming the probiotic, and the number of seroconverters was also 4.5 fold higher, this has enormous significance for vaccine adjuvant development in general and for influenza vaccines specifically. This response appeared to be specific to the vaccinated antigen, as titers of two common viral antigens, measles and varicella zoster, were not elevated in either group.

There is increasing evidence that some probiotics can enhance antibody responses to specific antigens. Orally consumed *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* CRL43 have been shown to enhance the neutralizing antibody response in humans to an orally delivered (polio virus) vaccine by 2 - 4-fold (de Vrese et al., 2005). In chickens, consumption of probiotic bacteria induced an enhanced antibody response to some but not all antigens tested (Haghighi et al., 2005). In the present study, we investigated the adjuvant activity of a probiotic bacterium, *Lactobacillus fermentum* VRI 003, in combination with a vaccine administered intramuscularly to a group of healthy human subjects.

The results demonstrated that consumption of *L. fermentum* VRI-003 prior to and after vaccination induced a significantly enhanced neutralising serum antibody response to one of the antigens in the vaccine (H1N1) compared to consumption of placebo capsules. Furthermore the percentage of H1N1 non-responders (as defined by a post-vaccination titre of <1:40) in the probiotic group was four-fold less than that in the placebo group. The placebo group level of 72% seroconversion is in line with other studies that have looked at the effectiveness of influenza vaccines (Langley and Faughnan, 2004). There was a mild response to the other two antigens in both placebo and probiotic consumers, and whilst there was an elevated response to both antigens in those groups consuming the probiotic, this was not statistically significant, possibly due to the low level of the response.

The increased H1N1 titre in the group consuming the probiotic was also associated with a significant reduction in days of respiratory tract symptoms compared to the placebo group as reported in a self-assessment questionnaire. It is not known if these were specific symptoms relating to influenza infection, or general respiratory tract infections.

Whilst previous studies have shown that probiotics can enhance immune responses to antigens delivered to mucosal surfaces, it is somewhat surprising that orally consumed probiotic bacteria can have a significant effect on stimulation of an immune response to an antigen delivered intramuscularly. This study was not designed to establish a mechanism for such an effect. However, there is considerable data that demonstrate the influence that probiotic and commensal bacteria have on the immune response generally.

The commensal microbial flora of the intestinal tract comprises both Gram-positive and Gram-negative bacteria that may be involved in homeostasis of gut-associated immunity (Ahrne et al., 1998; Erickson and Hubbard, 2000; Guarner and Schaafsma, 1998), and recent studies have highlighted the effects of probiotic bacteria on immune competent cells (Ahrne et al., 1998; Erickson and Hubbard, 2000; Grangette et al., 2001; Mercenier et al., 2003; Reid et al., 2003). Commensal bacteria present in the gut microbiota are in close contact with cells of the immune system, in particular dendritic cells (DC) in the gut lamina propria. DCs directly sample the gut lumen by projecting their dendrites through the tight junctions of the epithelial cells lining the surface of the intestine (Resigno et al., 2001). DCs contain several different Toll-like receptors (TLR) on their surface. TLRs are pattern recognition receptors that function to detect the presence of foreign microbes and generate rapid host defence responses (Rakoff-Nahoum et al., 2004). Binding of components of probiotic bacteria to TLRs can lead to the activation and maturation of DC (Christensen et al., 2002). When activated, DC and other antigen-presenting cells release pro-inflammatory cytokines (including TNF-α, interleukin (IL)-1, IL-6, IL-8, and IL-12), and other molecules that are involved in induction of the adaptive immune response, where both T and B-lymphocytes and antibody production play a crucial role (Kabelitz et al., 2006).

It is possible therefore that the effect seen in this study of enhancement of serum antibody response to H1N1 and other antigens is a result of components of *L. fermentum* VRI 003 activating one or more TLR which ultimately enhance the adaptive immune response generally, and in particular to specific antigens in the influenza vaccine which mimic invading pathogens. However, this effect, if present, did not appear to enhance the inflammatory response at the injection site, as both groups reported similar low levels of mild reaction to the injection.

Clearly the enhancement of the immune response to influenza vaccine in this small trial is worth further investigating in a trial with larger numbers, as it has the potential to provide a low-cost and low risk solution to improving the efficacy of population-based influenza vaccination.

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CONFLICT OF INTEREST STATEMENT

The authors were both officers of Probiomics Ltd, the sponsor and the company that produced Lactobacillus fermentum VRI-003 at the commencement of the study. Dr French left the company prior to analysis of the data. Professor Penny was a Director of the company throughout the duration of the study.

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