

Applied nutritional investigation

Oral intake of *Lactobacillus fermentum* CECT5716 enhances the effects of influenza vaccination

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Abstract

Objective: We studied the coadjuvant capability of oral consumption of the breast-milk-isolated strain *Lactobacillus fermentum* (CECT5716) for an anti-influenza vaccine.

Methods: A randomized, double-blinded, placebo-controlled human clinical trial including 50 volunteers (31 male and 19 female) was performed to address the immunologic effects of an intramuscular anti-influenza vaccine in adults (33.0 ± 7.7 y old). Fifty percent of volunteers received an oral daily dose of methylcellulose (placebo) or probiotic bacteria (1×10^{10} colony-forming units/d) 2 wk before vaccination and 2 wk after vaccination.

Results: Two weeks after vaccination there was an increase in the proportion of natural killer cells in the probiotic group but not in the placebo group. The vaccination induced an increase in T-helper type 1 cytokine concentrations and in T-helper and T-cytotoxic proportions in both groups; however, the probiotic group showed a significant higher induction in some of these parameters. Regarding the humoral effects, induction of antibody response in the placebo group could not be detected. In the case of the probiotic group, a significant increase in antigen specific immunoglobulin A was detected. Although an increase in total immunoglobulin M was observed, changes in anti-influenza antigen specific immunoglobulin M were not observed. The incidence of an influenza-like illness during 5 mo after vaccination (October to February) was lower in the group consuming the probiotic bacteria.

Conclusion: Oral administration of the strain *L. fermentum* CECT5716 potentiates the immunologic response of an anti-influenza vaccine and may provide enhanced systemic protection from infection by increasing the T-helper type 1 response and virus-neutralizing antibodies. © 2007 Elsevier Inc. All rights reserved.

Keywords:

Lactobacillus; Immunologic response; Coadjuvant; Vaccine; Influenza

Introduction

Influenza is an acute viral respiratory infection that results in high morbidity and significant mortality mainly in older adults. Moreover, the economic burden of annual epidemics in the working population has been reported as important, with 10–20% of sick people leaving work for a

mean duration of 5–7 d in an influenza season of moderate impact [1,2]. Defense against influenza infection involves innate and adaptive immune responses. After infection most influenza viruses are detected and destroyed within a few hours by innate immune mechanisms. If influenza viruses escape these early defense mechanisms, they are detected and eliminated by adaptive immune mechanisms in which cytotoxic T lymphocytes and antibodies function as antigen-specific effectors to target the virus [3].

To control influenza, protective adaptive immunity must be induced in advance by the administration of a vaccine. However, the vaccine seems to show limited clinical effectiveness,

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ranging from 20% to 86%, as reflected in main studies in the previous 20 y [2,4,5]. To improve the effectiveness of the vaccine, coadministration of the inactivated virus with adjuvants such as cholera toxin or heat-labile enterotoxin has been used [6–8]. The mechanisms by which these molecules enhance the immune response against influenza viral antigens involve stimulation of the innate immune system [9]. However, the combination of the vaccine with these kinds of coadjuvants may not be clinically safe [10].

Oral administration of lactic acid bacteria has been reported to enhance innate and adaptive immunities in the host [11–15]. It has been previously demonstrated that consumption of these bacteria induces an increase in immunoglobulin A (IgA) related to the anti-infectious properties of lactic acid bacteria in diarrhea disease [16,17]. Moreover, innate immunity is enhanced by increasing the proportion and activity of phagocytic cells, such as monocytes and neutrophils [11–18]. The function of natural killer (NK) cells is also improved by consumption of some of these bacteria [14,19]. Therefore, lactic acid bacteria have been suggested as coadjuvants in a vaccination process to gain a more efficient protective response [20–22].

In a previous work, we described that breast milk of healthy women is an important source of lactic acid bacteria to the infant gut [23]. Breast feeding provides significant protection against infections in newborns and infants [24–27]. Breast milk components such as maternal immunoglobulins, lactoferrin, lactoperoxidase, lysozymes, and oligosaccharides have been involved in this activity [28,29], but, in addition, the presence of lactic acid bacteria with probiotic potential could contribute to the protective effect of breast milk [23,30].

In this work we describe the results of a human clinical trial performed to investigate the influence of consumption of a breast milk-isolated lactobacillus strain (*Lactobacillus fermentum* CECT5716) on the immune response induced by an influenza vaccine, as the primary endpoint of this study, in healthy adults.

Materials and methods

Volunteers and study design

The recruitment of volunteers was carried out in the medical service of Puleva Food S.A. (Granada, Spain) at the beginning of the vaccination program. Sixty-four healthy adult human volunteers were approached to participate in the trial. The exclusion criteria were frequent gastrointestinal disorders (frequent diarrhea, constipation episodes, or stomach acid), gastrointestinal surgery, metabolic diseases (diabetes, food allergy, or lactose intolerance), and/or antibiotic treatment during the trial. Fifty healthy adult human volunteers (19 female and 31 male) with an age range of 22 to 56 y (33 ± 7.7) were included in the study. The study was carried out according to the Helsinki Declaration. The study

Table 1
Recruitment and population

	Female	Male	Total
Approached	23	41	64
Declined	3	4	7
Excluded	1*	2†‡	3
Included	19	31	50
In placebo group	9	16	25
In probiotic group	10	15	25
Age (y)	31.1 ± 7.1	34.3 ± 7.9	33.0 ± 7.7
In placebo group	30.5 ± 6.0	33.6 ± 7.0	32.5 ± 6.7
In probiotic group	34.5 ± 8.6	34.1 ± 7.3	34.3 ± 7.7

* Excluded because of egg allergy.

† Excluded because of frequent stomach acid.

‡ Excluded because of antibiotic treatment.

protocol was approved by the ethical committee of Fundación Hospital Virgen de las Nieves (Granada, Spain) and informed written consent was obtained from all subjects. The volunteers were asked to exclude from their diet any kind of probiotic product and/or yogurt.

Volunteers were assigned to one of two groups randomized by gender and age, and the results of this randomization are summarized in Table 1. Those in the placebo group daily consumed a capsule containing 200 mg of methylcellulose. Those in the probiotic group daily consumed a capsule containing 1×10^{10} colon-forming units of the strain *L. fermentum* CECT5716 in a matrix of the same mix of methylcellulose. The study consisted of 28 d of probiotic treatment. The intramuscular vaccination was carried out at day 14 in the medical service of Puleva Food S.A. with a vaccine containing inactivated trivalent influenza (A/New Caledonia/20/99[H1N1], A/Fujian/411/2002[H3N2], B/Shanghai/361/2002[B]) for the vaccine campaign of 2004/2005 (Chiron S.r.l. Siena, Italy). All volunteers were vaccinated in the same week (third week of September 2004).

Clinical survey and diagnosis

The primary endpoint of the study was to evaluate the immune response induced by the vaccination process and its modulation by the consumption of probiotics. We especially focused on differences in lymphocyte subpopulations and immunoglobulin levels in blood.

In addition, a survey with items concerning the presence of fever ($>37^{\circ}\text{C}$ taken at the armpit), systemic symptoms (headache, myalgia, bone/joints pain, fatigue, anorexia, and digestive disorders), and respiratory symptoms (cough, nasal symptoms, and pharyngeal symptoms) was completed daily by the volunteers during the 5-mo (October to February) survey period. Volunteers were to report the development of any of these symptoms. Volunteers were instructed how to consider positive any symptom. A diagnosis of influenza-like illness (ILI) was based on the association of fever with any systemic symptom and at least one respiratory sign that lasted for at least 3 consecutive days. The episodes of ILI were added monthly for each group.

Collection of blood samples

After an overnight fast lasting at least 10 h, blood samples were taken from the volunteers at the beginning of the study (day 0), just before the vaccination (day 14), and at the end of treatment (day 28) using Vacutainers (S-Monovette, Sarstedt, Germany) containing ethylene-diaminetetra-acetic acid.

Analysis of leukocytes in blood

Major leukocyte subset phenotypes were counted in whole blood samples treated with ethylene-diaminetetra-acetic acid by flow cytometry in a FACScalibur (Becton Dickinson, Oxford, UK) by using the following fluorochrome-conjugated monoclonal antibodies (Becton Dickinson): anti-CD3⁺, anti-CD19⁺, anti-CD4⁺, anti-CD8⁺, anti-CD45RO⁺, and anti-CD56⁺. The results were expressed as the percentage of mononuclear cells that stained positively.

Total immunoglobulin and cytokine measurements

Total IgA, IgG, and IgM concentrations in plasma were measured by enzyme-linked immunosorbent assay (ELISA) quantitation kits (Bethyl, Montgomery, TX, USA). Cytokine concentrations in plasma were measured by ELISA quantitation kits (CytoSets, Biosource, Camarillo, CA, USA).

Specific immunoglobulins were also measured by ELISA. Briefly, 96-well plates were coated with 500 ng/mL of the vaccine suspension in coating buffer (0.5 M Na₂CO₃). After overnight incubation at 4°C, plates were washed three times with wash solution (50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin). Then plasma samples were added to the plates and incubated for 1 h at room temperature. Plates were washed three times, and 100 µL of goat anti-human IgG, IgA, or IgM (Bethyl) was added for 1 h at room temperature. Staining was performed with 3,3',5,5'-Tetramethylbenzidine (Sigma Chemical, St. Louis, MO, USA) for 20 min at room temperature in the dark. The reaction was stopped with 0.1 N H₂SO₄, and plates were read at 450 nm.

Statistical analysis

The data were analyzed with SPSS 12.0 (SPSS, Inc., Chicago, IL, USA). For the gaussian variables, the longitudinal effect of the treatment within each group at different time points of the study was analyzed by one-way repeated measures analysis of variance followed by paired *t* test (within-group comparison). Two-way repeated measures analysis of variance was used to analyze statistical differences produced by the treatment followed by independent *t* test to assess in which time points the groups differed.

The incidence of ILIs in the placebo and probiotic groups was compared using non-parametric, independent, two-

sample tests (Mann-Whitney U test). Statistical significance was defined as *P* < 0.05.

Results

Tolerance and clinical observations

Throughout the entire study the capsules were well tolerated by all volunteers and none reported any adverse effect associated with its consumption. No one had or voluntarily decided to abandon the study. Compliance with the probiotic treatment was followed by fecal detection of the probiotic strain (data not shown). The detection of *L. fermentum* CECT5716 was followed by polymerase chain reaction in the feces. The bacterium was present in 92% of the volunteers (23 of 25) in the probiotic group and in 12% of the placebo group (3 of 25).

Effects on lymphocyte subsets

Flow cytometric analysis showed in all cases that cells staining positively for CD3⁺ (T lymphocytes), CD8⁺ (cytotoxic T lymphocytes), CD4⁺ (T helper lymphocytes), CD19⁺ (B lymphocytes), CD3⁺CD45RO⁺ (memory T lymphocytes), and CD56⁺ (NK cells) were within the ranges for hematologically normal Caucasian adults (Table 2). Nevertheless, in both groups an increase in T-helper and T-cytotoxic lymphocytes was observed 2 wk after vaccination. In the case of memory T lymphocytes, the increase observed in both groups did not depend on the treatment or vaccination process because the effect was detected before vaccination. The vaccination did not cause significant changes in NK cells in the placebo group, but the consumption of probiotic bacteria induced a significant increase in the proportion of NK cells at the end of the study (Table 2).

Effects on cytokine concentration

Tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), and interleukin (IL) 12 and IL-10 cytokines were measured in plasma (Table 3). The vaccination process induced an increase in serum IL-12. In the probiotic group, an increase was observed before vaccination, after 2 wk of probiotic treatment. After vaccination, although an induction was also observed and values were still higher than those in the placebo group, differences did not reach statistical significance. In the case of TNF- α , vaccination induced an increase in the cytokine concentration in both groups. However, the consumption of probiotic bacteria induced a significantly higher increase. Regarding IFN- γ and the immunoregulatory cytokine IL-10, no significant differences were detected. However, in the probiotic group, a trend to increased IFN- γ blood levels was already observed after 2 wk of probiotic consumption (*P* = 0.1; Table 3).

Table 2
Percentage of lymphocyte subsets*

	Control group			Probiotic group		
	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4
T lymphocytes	60.16 ± 2.8	63.32 ± 2.1	58.72 ± 2.9	63.47 ± 2.9	63.91 ± 1.6	59.39 ± 2.3
T-helper lymphocytes	30.34 ± 1.7	29.47 ± 1.8	34.18 ± 1.8 ^{†‡}	31.67 ± 1.5	30.46 ± 1.3	36.27 ± 1.5 ^{†‡}
T-cytotoxic lymphocytes	19.19 ± 1.2	18.56 ± 1.2	25.08 ± 1.2 ^{†‡}	21.48 ± 1.1	22.21 ± 1.2	26.40 ± 1.3 ^{†‡}
Memory T lymphocytes	21.07 ± 2.0	29.98 ± 2.2 [†]	33.18 ± 1.3 [†]	22.55 ± 1.7	29.57 ± 1.8 [†]	31.40 ± 1.9 [†]
Natural killer cells	17.03 ± 1.6	17.41 ± 1.7	18.62 ± 1.1	16.80 ± 1.7	18.11 ± 1.6	21.64 ± 1.5 [†]
B lymphocytes	07.36 ± 0.7	07.72 ± 0.7	07.78 ± 0.5	07.51 ± 0.4	07.95 ± 0.6	07.72 ± 0.6

* Data presented as mean ± SEM.

[†] Statistically significant difference with respect to week 0, $P < 0.05$.

[‡] Statistically significant difference between week 2 and week 4, $P < 0.05$.

Effects on immunoglobulin concentrations

Total and anti-influenza-specific IgGs, IgAs, and IgMs were measured in plasma by ELISAs. Two weeks after vaccination, an increase in antibody response in plasma of the placebo group could not be detected but a significant decrease in IgG concentration was observed (Table 4). In contrast, in the case of the probiotic group, there was a significant increase in specific anti-influenza IgA in serum after vaccination. In addition, in the probiotic group, a

significant increase in total IgM was observed, which did not reach statistical significance in the case of the specific anti-influenza IgM (Table 4).

Incidence of ILI

Episodes of ILI (defined as described in MATERIALS AND METHODS) were recorded daily by the volunteers and added monthly for each group during the 5-mo survey period (Fig. 1). During this period the number of ILI episodes in the

Table 3
Cytokine concentrations*

	Control group			Probiotic group		
	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4
IL-10 (pg/mL)	109.17 ± 20.01	115.20 ± 12.44	122.26 ± 13.66	108.00 ± 95.15	111.14 ± 20.59	129.45 ± 15.85
IL-12 (pg/mL)	65.84 ± 7.03	72.01 ± 8.16	87.65 ± 9.70 ^{†‡}	63.35 ± 6.96	89.48 ± 12.55 [†]	102.80 ± 12.98
TNF- α (pg/mL)	57.48 ± 8.19	73.70 ± 9.44 [†]	84.20 ± 10.04 ^{†‡}	59.54 ± 8.84	110.15 ± 17.59 ^{†§}	117.56 ± 16.11 [§]
INF- γ (pg/mL)	23.65 ± 4.77	23.03 ± 4.11	23.98 ± 4.30	23.94 ± 5.88	25.25 ± 5.40	25.47 ± 5.96

IL, interleukin; INF, interferon; TNF, tumor necrosis factor

* Data presented as mean ± SEM.

[†] Statistically significant difference with respect to week 0, $P < 0.05$.

[‡] Statistically significant difference between week 2 and week 4, $P < 0.05$.

[§] Statistically significant difference between control and probiotic group, $P < 0.05$.

^{||} Statistically significant difference with respect to week 0, $P < 0.01$.

Table 4
Immunoglobulin concentrations*

	Control group			Probiotic group		
	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4
IgG (mg/dL)	814.39 ± 127.63	720.25 ± 90.15	448.28 ± 98.22 [†]	825.00 ± 96.92	696.92 ± 107.85	824.04 ± 112.17 [§]
IgA (mg/dL)	155.13 ± 17.06	145.03 ± 22.86	146.57 ± 20.49	147.41 ± 10.90	145.95 ± 15.78	144.31 ± 16.28
IgM (mg/dL)	260.36 ± 41.12	229.69 ± 31.31	212.66 ± 31.91	250.71 ± 24.96	241.75 ± 25.17	320.16 ± 32.90 ^{†‡§}
IgG-sp (OD)	—	0.623 ± 0.05	0.549 ± 0.05	—	0.590 ± 0.04	0.538 ± 0.05
IgA-sp (OD)	—	0.445 ± 0.04	0.440 ± 0.03	—	0.488 ± 0.03	0.577 ± 0.06 ^{‡§}
IgM-sp (OD)	—	0.264 ± 0.01	0.266 ± 0.00	—	0.268 ± 0.01	0.279 ± 0.00

sp, specific; Ig, immunoglobulin; OD, optical density at 450 nm

* Data presented as mean ± SEM.

[†] Statistically significant difference with respect to week 0, $P < 0.05$.

[‡] Statistically significant difference between week 2 and week 4, $P < 0.05$.

[§] Statistically significant difference between control and probiotic groups, $P < 0.05$.

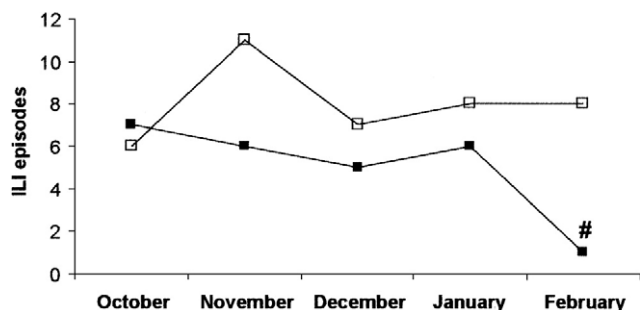


Fig. 1. Episodes of ILI were recorded monthly for the placebo group (white squares) and the probiotic group (black squares). #Statistically significant difference for control versus probiotic group ($P < 0.05$). ILI, influenza-like illness.

probiotic group was smaller than that in the placebo group, but significant differences were observed only in February. Forty ILI episodes were recorded in the placebo group, whereas 25 episodes were reported in the probiotic group. The vast majority of volunteers recorded only one episode of ILI during the 5 mo, although 3 of 25 volunteers in the placebo group and 1 of 25 in the probiotic group recorded as many as four ILI episodes during the study. Further, 36% (9 of 25) and 40% (10 of 25) of the volunteers in the placebo and probiotic groups, respectively, reported no ILI episode during the study.

Discussion

Influenza vaccination is currently recommended especially in populations at risk to prevent flu complications; however, in some annual campaigns the vaccine coverage is low [31], which calls for the requirement of new alternatives or adjuvant approaches to improve it. Cholera toxin and heat-labile enterotoxin have been used as coadjuvants because these molecules enhance the adaptive response induced by influenza vaccines by mechanisms involving stimulation of the innate immune system [6–9]. However, as previously mentioned, the use of these coadjuvants may not be clinically safe [10]. Thus, the use of other, efficient, safer coadjuvants is needed. Very recently, the capability of some lactobacilli strains to act as coadjuvants by enhancing the antibody response after polio virus vaccination has been reported [22]. Therefore, we evaluated the effect of *L. fermentum* CECT5716 during a flu vaccination process.

Two considerations must be made before discussing the results obtained. First, the population size was determined in order to obtain differences between groups regarding immune cellular and molecular parameters such as lymphocyte populations or immunoglobulin and cytokine levels, which correspond to the primary endpoint of this study. Second, the use of only two study groups (placebo and probiotic), all vaccinated 2 wk after the initiation of the study, does not allow us to clearly state whether some of the

observed effects were mainly due to the vaccination process per se or to the treatment with probiotics. We assigned a probiotic effect in those differences observed between both groups, especially if they were already observed at day 14. The effect of the vaccination process per se will correspond to the differences observed in the placebo group between weeks 2 and 4. The differences in this same period that were detected only in the probiotic group in comparison with the placebo will correspond to the adjuvant effect of *L. fermentum* during the vaccination protocol.

During a natural viral infection, innate immune mechanisms constitute the first barrier against influenza infection through effector cells, molecules, and factors involved in the restriction of viral spread. For example, NK cells are detected in pulmonary lymphocytes 48 h after influenza virus infection producing IFN- γ and limiting the viral spread by virus-infected cell lysis [3,32]. In this sense, the oral administration of *L. fermentum* CECT5716 induced an increase in NK cells 2 wk after vaccination, which could not be observed in the placebo group. Vaccination induced the expression of TNF- α and IL-12 in both groups, although the increase was higher in those volunteers who consumed the probiotic bacteria. Because IL-12 is involved in NK and T-helper type 1 lymphocyte activation [33], these differences could explain the increased amount of NK cells observed in the probiotic group. Moreover, NK cells in turn are producers of IFN- γ , a fact that also correlates with the levels of this cytokine observed in the probiotic group.

Regarding cellular-specific immune responses, the vaccination induced an increase in T-helper ($CD4^+$) and T-cytotoxic ($CD8^+$) lymphocytes. T-cytotoxic lymphocytes play an important role in defense against influenza infection by killing the virus-infected cells and producing IFN- γ that inhibit virus replication [34,35]. No other clinical relevant differences in lymphocyte subtypes were observed due to the vaccination protocol or the consumption of probiotics. The differences observed in memory T lymphocytes in both groups and before the vaccination process must be related to immune modulation due to the restriction diet (volunteers were not allowed to consume fermented products during the study) [36].

The major humoral protective immunity induced by influenza virus infection is provided by S-IgA and IgG antibodies. However, parenteral inactivated vaccines have been reported to mainly induce serum IgG antibodies that are weakly cross-protective across drift viruses within a subtype [37]. Surprisingly, in this study we detected an increase in specific anti-influenza IgA antibodies in plasma of the probiotic group, whereas no increase was observed in specific IgG or IgM antibodies. A potential explanation for this could be the low response triggered by the vaccine of this current campaign. Moreover, two facts could explain the differences observed in IgA-specific antibodies. First, IgA antibodies react not only to homologous viruses but also to variant viruses in the same subtype in contrast to IgG antibodies that react mainly to homologous viruses [3].

Second, reinfection results in a secondary IgA antibody response, which is characterized by a rapid rise in the IgA antibody titer. Thus, IgA antibodies triggered by a previous natural infection or vaccinations could cross-react with the vaccine and induce a greater IgA response. Due to the high incidence of flu and the increasing number of influenza vaccination campaigns in Spain, it is impossible to obtain a test adult population without previous contact with this antigen.

Conversely, a significant increase in total IgM was detected but only in the probiotic group. Because significant changes in specific IgMs were not observed, this increase could be mainly due to the immunologic response triggered by the probiotic bacteria.

Thus far, our results suggest that the parenteral inactivated vaccine used in this study seems to induce a T-lymphocyte cell proliferation or maturation but poorly induces a complete antibody response. We have also demonstrated that the consumption of a *L. fermentum* strain during a period around vaccination could enhance the immunologic effects of the vaccine by inducing the production of specific anti-influenza antibodies and by increasing the production of T-helper type 1 cytokines and other factors involved in viral defense. The mechanisms by which lactic acid bacteria could modulate the immune response are not fully understood; however, this is not surprising because the immune system associated to the gut mucosa represents the larger immune compartment of the body [38]. In this sense, important immune disturbances have been reported to occur in germ-free animals [39].

Human studies have shown that gram-positive bacterial species are strong inducers of monocyte-derived IL-12 [40], a powerful signal to activate NK cells [41,42]. Monocytes and macrophages, together with dendritic cells, play a crucial role in the innate immune response, which in turn leads to activation of the adaptive immune system [43]. These cells recognize conserved molecular patterns of bacterial components through Toll-like receptors, the activation of which triggers the production of cytokine mediators in the development of T-cell differentiation [44]. Thus, the significantly higher values of NK cells and T-helper type 1–promoting cytokines (IL-12, IFN- γ , and TNF- α) detected in probiotic group could have led to the enhancement of the specific response against influenza triggered during the vaccination protocol. In this respect, there are several reports describing the effects of lactic acid bacteria on IgA production in rodents and humans [41].

The greater immune response observed in the probiotic group in comparison with the placebo group seems to correlate with a lower incidence of ILI during 5 mo of survey. However, these clinical data have to be taken in perspective due to the small population of this study, and more clinical studies are required to demonstrate the clinical efficacy of using probiotics in a coadjuvant approach to viral infections.

Conclusion

In this work we have demonstrated that the use of oral probiotic strains is an efficient, safe, and easy method to improve the protective immune response triggered by influenza vaccination.

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