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The consumption of two new probiotic strains, *Lactobacillus gasseri* CECT 5714 and *Lactobacillus coryniformis* CECT 5711, boosts the immune system of healthy humans

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Summary. Orally ingested probiotic bacteria are able to modulate the immune system. However, differences exist in the immunomodulatory effects of different probiotic strains. Moreover, different regulatory effects, which depend on the health status of the consumer, have been identified. This work describes a randomized, double-blind, placebo-controlled human clinical trial to investigate the immune effects on healthy people of a fermented product containing two new probiotic strains, *Lactobacillus gasseri* CECT 5714 and *Lactobacillus coryniformis* CECT 5711, which was compared with another fermented product, a standard yogurt. Consumption of either the new product or yogurt increased the proportion of phagocytic cells, including monocytes and neutrophils, as well as their phagocytic activity. However, combination of the product containing the strains *L. gasseri* CECT 5714 and *L. coryniformis* CECT 5711 also induced an increase in the proportion of natural killer (NK) cells and in IgA concentrations. The effects were higher after two weeks of treatment than after 4 weeks, which suggests regulation of the immune system. In addition, the new product enhanced immunity in the participants to a greater extent than did the control standard yogurt. [Int Microbiol 2006; 9(1):47-52]

Key words: *Lactobacillus gasseri* · *Lactobacillus coryniformis* · probiotics · human clinical trial · immune response

Introduction

Endogenous microbiota has a modulatory effect on the mucosal and systemic immune response. In fact, the type of bacteria that colonize the intestine in newborns determines immunomodulation of the naive immune system [9]. The usual components of the gut microbiota, such as lactobacilli, modulate the immune system, causing, for instance, host resistance to infections, atopy, and gut inflammation [13,26,30]. While the immune mechanisms of such bacteria, called probiotics, have

not been totally defined, several clinical trials have studied the effects of probiotics consumption on human health. Most of these trials evaluated the effect of probiotics on patients suffering from diarrhea, *Helicobacter pylori* infections, intestinal inflammation, or atopy [4,10,14], and the results varied greatly. However, few data are available regarding the immune effects of probiotics in healthy people, the main target of such products currently on the market.

In a previous study, we reported that breast milk of healthy women is a major source of lactic acid bacteria to the infant gut, and that *Lactobacillus gasseri* is among the predominant

species [18]. The microbiota of newborns could be responsible for some of the health benefits observed in breast-fed compared to formula-fed infants [3,5,34]. A breast-milk strain of *L. gasseri* (CECT 5714) was selected based on its particular in vitro probiotic properties [19]. We also selected *L. coryniformis* CECT 5711 from an artisan goat's milk cheese; this strain exhibits peculiar probiotic properties, including the production of reuterin and cobalamin [20]. Also, in a randomized, double-blind, placebo-controlled human clinical trial, we investigated the effects of a fermented product containing the probiotic strains *L. gasseri* CECT 5714 and *L. coryniformis* CECT 5711 on the intestinal function of healthy adults and compared them with the effects of a standard probiotic with a long consumption history and demonstrated beneficial effects [1,21,25,29]. An increase in the concentration of fecal lactic acid bacteria and a beneficial effect on the bowel function of healthy adults volunteers consuming the new probiotic strain was observed. In this work, we describe the effects of the new probiotic strains on the immune system of healthy adults in that clinical trial.

Material and methods

Design of the trial. Thirty healthy adult human volunteers (15 females and 15 males) ranging in age from 23 to 43 years old were included in the study. Exclusion criteria and the protocol followed was described elsewhere [25].

Collection and analysis of fecal samples. After an overnight fast lasting at least 10 h, blood samples were withdrawn using EDTA-containing vacutainers (S-Monovette, Sarstedt, Germany) from the volunteers just before and after the treatment period. The proportion of neutrophils was analyzed using a Symex F-800 counter (Coulter Electronic, Luton, London, UK). Major leukocyte subset phenotypes were counted in EDTA-treated whole-blood samples by flow cytometry on a FACScalibur (Becton Dickinson, Oxford, UK) system and using the following fluorochrome-conjugated monoclonal antibodies (Becton Dickinson): anti-CD3+, -CD19+, -CD4+, -CD8+, -CD45RO+, -CD56+, -CD25+, -CD14+. The results were expressed as the percentage of positively staining mononuclear cells. Fecal samples were collected weekly, placed into pre-weighed bottles, and then homogenized in a peptone-saline solution (100 mg/ml) within 12 h.

Phagocytic activity. In vitro phagocytic activity was determined by flow cytometry of whole-blood samples after the uptake of fluoresceinated *Escherichia coli* [7]. One hundred ml of heparin-treated whole blood was incubated at 37°C for 10 min with 10 ml (10^8 colony-forming units, cfu) of fluoresceinated bacteria. Erythrocytes were lysed with 100 ml of 4% formaldehyde and 1 ml of cool water. Samples were centrifuged at $2200 \times g$ for 5 min, suspended in 0.5 ml of 4% (w/v) formaldehyde in PBS, and analyzed by flow cytometry. The results were expressed as the percentages of monocytes and granulocytes showing phagocytic activity.

Total immunoglobulin and cytokines measurements. IgA, IgG, and IgE concentrations in serum and total IgA concentration in feces were measured by ELISA (Bethyl, Montgomery, TX, USA). Cytokine concentrations in sera were measured by ELISA (CytoSets, Biosource, Camarillo, CA, USA).

Statistical analysis. Data were analyzed using SPSS software (version 12.0, Chicago, IL, USA) as described in [25] for the Gaussian variables.

Results

Clinical observations. None of the volunteers voluntarily left the study. Only one had to leave at the end of the pre-test period, due to the use of antibiotics for treatment of an oral bacterial infection not related to this study.

Leukocyte subsets and proportions. Flow cytometric analysis of the proportion of monocytes (CD14+ cells) showed an increase after 2 weeks of consumption of either of the fermented products, but the differences were statistically significant only in the case of the group receiving the new probiotic product. At the end of the treatment, the proportions of monocytes were only slightly higher than the initial values (Table 1). After 2 weeks of treatment, there was also a significant increase in the neutrophil proportion in both groups, but at the end of treatment it was maintained only in the new probiotic group. The proportion of total lymphocytes and cells staining positively for CD3+ (T lymphocytes), CD8+ (cytotoxic T lymphocytes), CD4+ (T-helper lymphocytes), CD19+ (B lymphocytes), CD3+CD45RO+ (memory T lymphocytes), CD4+CD25+ (suppressor T lymphocytes), and CD56+ (natural killer, NK, cells) were in the ranges of those for hematologically normal Caucasian adults (Table 1). However, a significant decrease, more evident at the end of treatment, was detected in the proportions of lymphocyte and of CD3+ and CD19+ cells in both groups. Despite that decrease, we observed that these values had increased during the two-week pretreatment period, probably due to the absence of fermented product in the diet before the treatment, and were normalized after consumption of both fermented products (data not shown). The most outstanding result was the significant increase in the proportion of NK cells after 2 and 4 weeks of treatment in the group that had ingested the new probiotic strains (Fig. 1). In contrast, NK proportions in the control group decreased at the end of treatment and were significantly lower than in the group receiving the new probiotic preparation (Fig. 1). Subjects receiving the latter were ranked and stratified depending on their preintervention levels of NK cells into two groups: a group with high preintervention values (above the mean +10% of the preintervention proportion of NK cells) and a group with medium/low preintervention values (below the mean +10% of the preintervention proportion of NK cells). To study whether the effect of treatment was related to the initial proportion of NK cells, the relative increases in NK-cell proportions were calculated. In the group receiving the new probiotic, changes in NK-cell proportions occurred in volunteers who initially had normal/low values, with percentage increases of 209.49 ± 8.92 and 133.29 ± 12.83 , respectively, after 2 and 4 weeks of treatment. By contrast, there was no increase in the proportion of NK cells in subjects who had high initial values.

Table 1. Percentage of white blood cell subsets. Results are expressed as mean \pm s.e.m.

	Control group			Probiotic group		
	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4
Monocytes (%)	07.29 \pm 0.58	09.03 \pm 1.26	08.11 \pm 0.94	07.02 \pm 0.53	11.00 \pm 1.40*	08.45 \pm 1.62
Neutrophils (%)	50.55 \pm 1.75	57.48 \pm 2.81*	54.28 \pm 2.06	50.90 \pm 1.98	57.34 \pm 2.37**	58.66 \pm 1.37**
Lymphocytes(%)	45.81 \pm 0.93	37.49 \pm 2.42*	42.50 \pm 2.19	43.97 \pm 1.81	37.13 \pm 2.61**	38.13 \pm 1.33*
T lymphocytes (%)	67.65 \pm 2.33	64.95 \pm 2.09	61.15 \pm 2.87	62.90 \pm 2.36	56.48 \pm 2.68*	56.49 \pm 2.64**
T helper (%)	37.56 \pm 1.80	33.14 \pm 1.89	36.68 \pm 2.72	39.25 \pm 1.43	36.75 \pm 1.12	39.31 \pm 1.62
T cytotoxic (%)	25.21 \pm 1.73	25.45 \pm 2.02	26.32 \pm 2.41	23.74 \pm 1.08	22.24 \pm 1.40	23.33 \pm 0.97
T suppressor (%)	17.52 \pm 1.44	19.41 \pm 2.09	16.15 \pm 0.96	18.42 \pm 1.36	18.54 \pm 1.81	17.75 \pm 1.36
T memory (%)	39.32 \pm 2.33	39.28 \pm 4.10	36.49 \pm 2.82	35.10 \pm 2.01	37.31 \pm 2.69	32.36 \pm 1.91*
B lymphocytes (%)	11.38 \pm 1.62	11.01 \pm 1.42	09.24 \pm 1.70**	09.48 \pm 0.72	08.74 \pm 0.84	08.23 \pm 0.71*

Statistically significant difference with respect to week 0: * $p < 0.05$, ** $p < 0.01$.

Phagocytic activity. The percentages of both mononuclear and polymorphonuclear cells showing phagocytic activity in vitro increased significantly during the period in which the two fermented products were consumed (Fig. 2). Although the increase was higher in the group that had ingested the new probiotic preparation, the differences between the groups were not statistically significant.

Effects on cytokine expression pattern and immunoglobulin production. The cytokines tumor necrosis factor (TNF)- α , interleukin (IL)-12, IL-10 and IL-4 were measured in serum. In the group consuming the new probiotic product, IL-10 and IL-4 increased significantly after 2 weeks of treatment. However, at the end of the treatment period, the IL-4 levels no longer differed from those at

the beginning of treatment, while IL-10 levels were higher, but not significantly (Table 2). A significant increase in IgA concentrations was measured in the blood of volunteers who

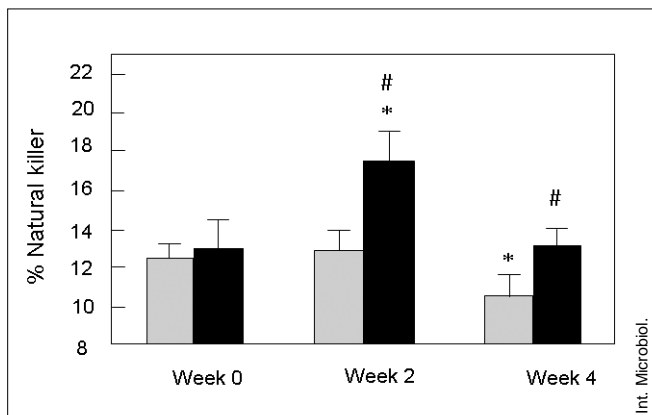


Fig. 1. Mean (\pm s.e.m.) of the percentage of lymphocytes staining positively for CD56+ (natural killer cells) in the blood of volunteers who received the new probiotic product (black bars) and in the control group (gray bars). * Statistically significant difference (* $p < 0.05$) respect to week 0. # Significant difference ($p < 0.05$) between control and treatment group.

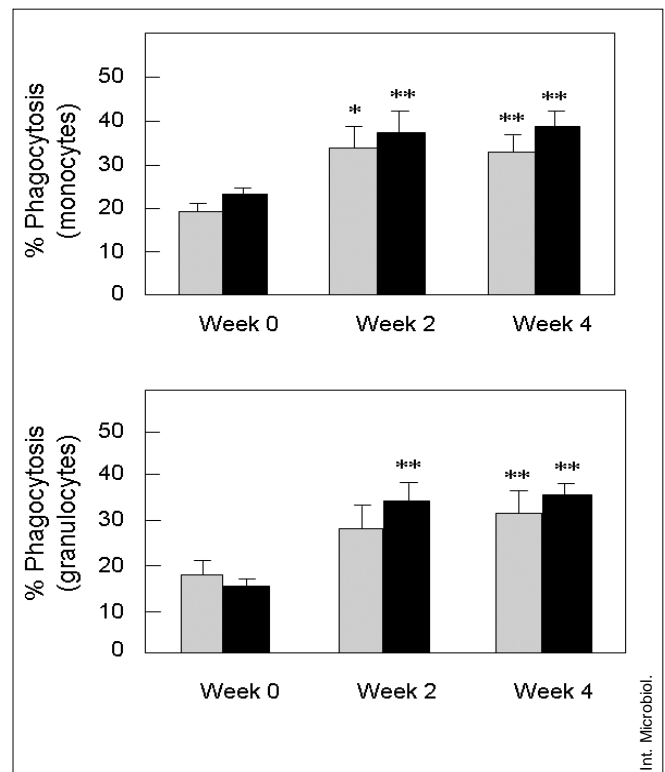


Fig. 2. Monocytes and granulocytes were differentiated by size and complexity by flow cytometer. Phagocytic activity of monocytes (A) and granulocytes (B) before and after 2 and 4 weeks of treatment is expressed as the mean (\pm s.e.m.) of the percentage of leukocytes containing fluoresceinated *Escherichia coli* after in vitro incubation of the bacteria with fresh blood. Black bars, probiotic group; gray bars, control group. *, ** Statistically significant difference (* $p < 0.05$, ** $p < 0.01$) respect to week 0.

Table 2. Cytokine concentrations in blood. Results are expressed as pg (mean \pm s.e.m.) per ml of serum

	Control group			Probiotic group		
	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4
TNF- α	38.49 \pm 13.25	36.14 \pm 13.14	37.49 \pm 15.69	38.37 \pm 9.24	46.57 \pm 11.16	35.74 \pm 11.54
IL-10	56.38 \pm 27.03	60.71 \pm 28.69	72.24 \pm 35.68	64.27 \pm 31.75	84.62 \pm 39.29*	81.80 \pm 54.25
IL-12	21.41 \pm 5.57	23.17 \pm 5.90	33.06 \pm 9.40	21.43 \pm 5.25	23.06 \pm 5.78	22.03 \pm 6.44
IL-4	30.96 \pm 9.39	43.79 \pm 17.52	47.27 \pm 15.79	28.26 \pm 9.93	37.96 \pm 10.22*	25.7 \pm 15.36

TNF, Tumor necrosis factor; IL, interleukin. Statistically significant difference with respect to week 0: * $p < 0.05$.

were given the new probiotic product (Table 3). In feces, there was a slight increase in IgA levels, but it was not significant. Serum IgG levels remained stable in both groups throughout the study. In addition, after 2 weeks there was a significant decrease in the serum IgE levels of volunteers in the new-probiotic group and in the control group; however, at the end of treatment the decreases were not significant (Table 2).

Discussion

Modulation of the immune response is one of the most acclaimed health benefits attributed to probiotic strains [6]. Previous studies in humans reported the effects of oral administration of probiotic strains on innate and specific immunity [2,31,32,37]. Although studies of the mechanisms by which probiotics exert this immunomodulatory effect are still in progress, recent data have shown differences in the immunomodulatory effects of different probiotic strains. Moreover, different regulatory effects have been detected in healthy subjects and in patients with inflammatory diseases [12]. These results suggest that the specific immunomodulatory properties of probiotic bacteria should be specifically characterized on target populations.

Our results showed that the consumption of either the new probiotic product or a standard yogurt preparation resulted in an increase in phagocytic cells, including monocytes and neutrophils, and enhanced phagocytic activity. These results are

consistent with those of previous studies on other probiotic strains [31,32]. Although the new probiotic strains seemed to be more effective, consumption of the standard yogurt produced similar results. In fact, the effect of *L. delbrueckii* subsp. *bulgaricus* on monocytes and macrophages function was previously reported [29].

Monocytes and macrophages, together with dendritic cells, play a crucial role in the innate immune response against microbial antigens, which in turn leads to activation of the adaptive immune system [15]. These cells recognize conserved molecular patterns of bacterial components through toll-like receptors (TLR), leading to activation of a variety of transcription factors, which triggers cytokine production [36]. The ability of these cells to recognize conserved structures present in large groups of bacteria enables them to effectively detect a wide range of pathogens, and this response is strengthened even when the increase in the phagocytic function is induced by non-pathogen bacteria, such as those in probiotic preparations. NK cells are also involved in the innate response and play a major role in recognizing and killing both virus-infected cells and tumor cells. In our study, the percentage of NK cells increased in the new-probiotic group but decreased in the control group. Moreover, the increase in the proportion of NK cells was restricted to volunteers in the new-probiotic group that initially had normal/low numbers of NK cells, while there was no change in subjects with initially higher NK-cell counts. In elderly people, in individuals with poor lifestyle habits, such as

Table 3. Immunoglobulin concentrations in serum. Results are expressed as mean \pm s.e.m.

	Control group			Probiotic group		
	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4
IgA(mg/g feces)	200.94 \pm 37.92	225.13 \pm 73.20	214.66 \pm 42.49	181.21 \pm 37.33	214.95 \pm 45.11	248.36 \pm 55.91
IgA(mg/dl serum)	139.39 \pm 16.98	151.89 \pm 30.32	126.51 \pm 23.23	137.08 \pm 20.37	158.12 \pm 19.51	159.29 \pm 23.00*
IgG(mg/dl serum)	1045.5 \pm 169.0	1317.1 \pm 154.4	1141.0 \pm 118.8	1144.1 \pm 203.6	1303.2 \pm 277.5	1114.5 \pm 169.6
IgE(mg/dl serum)	190.40 \pm 39.26	161.48 \pm 35.25*	195.33 \pm 52.95	192.58 \pm 48.07	147.64 \pm 39.61**	169.53 \pm 39.21

Statistically significant difference with respect to week 0: * $p < 0.05$, ** $p < 0.01$.

smoking, or in those with mental stress, a decrease in the proportions of NK cells has been reported [11,24,33]. Since probiotics seem to offer benefits to people whose health could be improved, they could also be effective in people with typically low levels of NK function. The positive effect of probiotics on NK cells has been previously reported for other lactobacillus strains [8,23], and their stimulation of the immune system and antitumor effects in animal models of cancer have been reported [17,35]. The lack of an increase in the proportion of NK cells in the control group may have been due to a lower adherence of the starter strain, *L. delbrueckii* subsp. *bulgaricus*, to both the gut mucosa and Peyer's patches, which would have made contact with immune cells difficult [27]. In fact, we did not detect an increase in fecal lactic acid bacteria in the control group [25]. Therefore, the consumption of the new probiotic product might have increased the innate immune response. Innate immunity is activated very quickly after infection when acquired immunity has not yet developed. Thus the improvement of innate immunity would strengthen the response against possible infections or cell transformations. The effects were in generally higher after 2 weeks of treatment than after 4 weeks, suggesting that they involved regulation of the immune system. The effect of treatment on the response capability against stimuli occurring weeks after the end of the treatment finished is currently being investigated.

The level in serum of the main cytokines involved in the regulation of the immune response were also analyzed in order to determine whether the acquired immune response was also affected. The increase in IL-10 and IL-4 concentrations suggests a role for a Th2 response. However, the serum IgE concentration was lower in the new-probiotic group than in controls, which suggests that other cytokines should be involved in IgE switching. IgE is involved in the allergy processes, so a decrease in the levels of this immunoglobulin could be beneficial for allergic subjects. In fact, the beneficial effect of a strain of *L. rhamnosus* in the prevention of atopy has been reported [14,15].

The increase in Th2 cytokines, including IL-4, could also affect IgA switching. In fact, a significant increase in the total IgA concentration in serum was detected, but only in the group that received the new probiotic product. IgA is the main immunoglobulin involved in mucosal defense. Thus, an increase in its concentration enhances its protection against pathogens. It has been reported that increases in IgA are related to the anti-infectious properties of probiotics in the treatment of diarrheal disease [37,38].

Although both probiotic products—the new preparation and the control yogurt—induced immune effects, consumption of the former, containing two lactobacilli strains, *L. gasseri* (CECT 5714) and *L. coryniformis* (CECT 5711), enhanced immunity in healthy people to a greater extent than the standard yogurt consumption.

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El consumo de dos nuevas cepas probióticas, *Lactobacillus gasseri* CECT 5714 y *Lactobacillus coryniformis* CECT 5711, estimula el sistema inmunitario de individuos sanos

Resumen. La ingestión oral de bacterias probióticas puede modular el sistema inmunitario. Sin embargo, existen diferencias en los efectos inmunomoduladores de diferentes cepas probióticas. Además, se han identificado distintos efectos reguladores, que dependen del estado de salud del consumidor. Este trabajo describe un ensayo clínico aleatorizado, con ocultación doble (*double blind*) y con control de placebo llevado a cabo en humanos para estudiar los efectos inmunitarios del consumo de un producto fermentado que contiene dos nuevas cepas probióticas, *Lactobacillus gasseri* CECT 5714 y *Lactobacillus coryniformis* CECT 5711, comparados con los efectos producidos por otro producto fermentado, un yogurt clásico. El consumo del nuevo producto o del yogurt clásico aumentó la proporción de células fagocitarias, como monocitos y neutrófilos, así como su actividad fagocitaria. Sin embargo, la combinación del producto que contenía las cepas *L. gasseri* CECT 5714 y *L. coryniformis* CECT 5711 indujo además un aumento en la proporción de linfocitos citotóxicos naturales (células NK) y en las concentraciones de IgA. Estos efectos fueron más acusados después de dos semanas de tratamiento que después de 4 semanas, lo que sugiere una regulación del sistema inmunitario. Además, el nuevo producto reforzó la inmunidad en las personas que participaron en el ensayo más que el yogurt clásico usado como control. [*Int Microbiol* 2006; 9(1):47-52]

Palabras clave: *Lactobacillus gasseri* · *Lactobacillus coryniformis* · probióticos · ensayo clínico en humanos · respuesta inmunitaria

O consumo de duas novas cepas probióticas, *Lactobacillus gasseri* CECT 5714 e *Lactobacillus coryniformis* CECT 5711, estimula o sistema imunitário de indivíduos sãos

Resumo. A ingestão oral de bactérias probióticas pode modular o sistema imunitário. No entanto, existem diferenças nos efeitos imunomoduladores de diferentes cepas probióticas. Além disto, foram detectados diferentes efeitos reguladores dependentes do estado de saúde do consumidor. Este trabalho descreve um ensaio clínico aleatorizado, com ocultação dupla (*double blind*) e com controle de placebo levado a cabo em humanos para estudar os efeitos imunitários do consumo de um produto fermentado que contém duas novas cepas probióticas, *Lactobacillus gasseri* CECT 5714 e *Lactobacillus coryniformis* CECT 5711, comparados com os efeitos produzidos por outro produto fermentado, um iogurte clássico. O consumo tanto do novo produto como do iogurte clássico aumentou a proporção de células fagocitárias, como monócitos e neutrófilos, assim como sua atividade fagocitária. No entanto, a combinação do produto que continha as cepas *L. gasseri* CECT 5714 e *L. coryniformis* CECT 5711 induziu também um aumento na proporção de linfócitos citotóxicos naturais (células NK) e nas concentrações de IgA. Estes efeitos foram mais aguçados após duas semanas de tratamento do que após 4 semanas, o que sugere uma regulação do sistema imunitário. Além disto, o novo produto reforçou de forma mais acentuada a imunidade das pessoas que participaram do ensaio do que o iogurte clássico usado como controle. [*Int Microbiol* 2006; 9(1):47-52]

Palavras chave: *Lactobacillus gasseri* · *Lactobacillus coryniformis* · probióticos · ensaio clínico em humanos · resposta imunitária