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A mixture of *Lactobacillus plantarum* CECT 7315 and CECT 7316 enhances systemic immunity in elderly subjects. A dose-response, double-blind, placebo-controlled, randomized pilot trial

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*Contributed equally to the work.

Abstract

**Background & aim:** Immunosenescence can increase morbi-mortality. Lactic acid producing bacteria may improve immunity and reduce morbidity and mortality in the elderly. We aimed to investigate the effects of a mixture of two new probiotic strains of *Lactobacillus plantarum* —CECT 7315 and 7316— on systemic immunity in elderly.

**Methods:** 50 institutionalized elderly subjects were randomized, in a double-blind fashion, to receive for 12 weeks 1) $5 \times 10^8$ cfu/day of *L. plantarum* CECT7315/7316 (“low probiotic dose”) (n = 13), 2) $5 \times 10^9$ cfu/day of the probiotic mixture (“high probiotic dose”) (n = 19), or 3) placebo (n = 15). Leukocyte subpopulations, and cytokine levels (IL-1, IL-10, TGF-$\beta_1$) were measured in venous blood at baseline, end of treatment (week 12), and end of follow-up (week 24). Infection and survival rates were recorded.

**Results:** After treatment, high probiotic dose resulted in significant increases in the percentages of activated potentially T-suppressor (CD8+CD25+) and NK (CD56+CD16+) cells, while low probiotic dose increased activated T-helper lymphocytes (CD4+CD25+), B lymphocytes (CD19+), and antigen presenting cells (HLA-DR+). Also, plasma TGF-$\beta_1$ concentration significantly decreased after treatment with both probiotic doses. Most of these changes remained 12 weeks after probiotic discontinuation. Incidence of infections during treatment showed a significant trend to be lower in the high probiotic dose group. In addition, there was a significant trend for morbi-mortality.

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tality to be greater in the placebo group vs. both probiotic groups.

Conclusions: Depending on the dose, L. plantarum CECT7315/7316 have different immune-enhancing effects in elderly subjects. These effects might result in a better clinical outcome.

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Key words: Immune cells. Cytokines. Aging. Probiotics. Lactobacillus plantarum.

Introduction

Some lactic acid bacteria strains are defined as probiotic as far as they are able to confer a variety of physiologic benefits to the host.1 Dietary supplementation with certain Bifidobacteria and Lactobacilli strains has been proven to be useful in the management of several gastrointestinal disorders such as acute infectious and antibiotic associated diarrhea in children2-3 and adults,4 gastrointestinal disorders such as acute infectious and antibiotic associated diarrhea in children2-3 and adults,4 ulcerative colitis,5,6 pouchitis,7,8 and irritable bowel syndrome.9,10 Also, they have been reported to be useful in protecting children from allergic illnesses,11,12 and in the prevention of bacterial, fungal or viral infections.13,14

Human studies have revealed that probiotic bacteria can have an influence on the host’s immune system. Some components of the immune response, including phagocytosis, natural killer (NK) cell activity and mucosal IgA production (especially in children), can be improved by some probiotic bacteria.15-17 Other components, including lymphocyte proliferation, the production of cytokines and antibodies other than IgA appear less sensitive to probiotics.18-19

It is well characterized that aging involves an involution and decreases the capacity to mediate effective immune responses to vaccination and invading pathogens.20,21 Immunosenescence has been associated to a decrease of mature T cell numbers, changes of NK and dendritic cell proportions, and loss of diversity of B cells in the blood of the elderly.22,23 Moreover, aging causes a decline in cell-mediated cytotoxic and phagocytic responses, and increases circulating levels of proinflammatory cytokines.24 Clinically, these changes potentially increase morbidity and mortality in elderly individuals through an increased rate of infections, malignancy, and autoimmunity-related disorders.25

Intervention trials in elderly subjects have shown that oral supplementation with Bifidobacterium lactis HN019 significantly increases the proportion of total, helper (CD4+) and activated (CD25+) lymphocytes in peripheral blood.26 In addition, Lactobacillus rhamnosus HN001 enhanced the ex vivo phagocytic capacity of polymorphonuclear leukocytes, as well as the tumoricidal activity of NK cells in these individuals.27 Furthermore, it has been shown that elderly people receiv-
products, 5) swallowing disturbances, and 6) use of antibiotics, probiotics, nutritional supplements and/or functional foods in the previous month.

Subjects were randomized to receive, in a double-blind fashion, either 1) 5·10^8 cfu/day of the *L. plantarum* CECT7315/7316 mixture in 20 g of powdered skimmed milk (“low probiotic dose”), 2) 5·10^9 cfu/day of the probiotic mixture in the same vehicle (“high probiotic dose”), or 3) vehicle alone (placebo). Randomization was performed in separate lists for each participating center. Both probiotic preparations and placebo were presented in identical vacuum sealed sachets, and administered as single daily dose, diluted in 200 mL of water or other cold drink, for 12 weeks. Subjects were then followed for a period of 12 additional weeks (follow-up period).

Medical visits were performed at baseline, and every 4 weeks until the end of the follow-up period (week 24). Clinical anamnesis (with particular reference to infections) and physical examination were carried out at every visit. Infection was defined as either 1) a febrile episode with a definite clinical focus and/or microbiologically confirmed etiology, or 2) a febrile episode without clinical focus or positive culture, but requiring empirical antibiotic therapy.

The body mass index (BMI), and the Barthel index to assess the functional capacity for daily living activities, were measured at baseline, and weeks 12 (end of the treatment period) and 24 (end of follow-up). Also, fasting venous blood samples were obtained at these time points for routine laboratory analysis, and immunological parameters evaluation (see below).

Blood immunological parameters

The effect of the probiotic mixture on blood immunological parameters was the primary end point of this trial. As mentioned, they were measured at baseline, at the end of the treatment period (week 12), and at the end of the follow-up period (week 24).

Assessment of blood leukocyte subpopulations

Leukocytes were obtained from heparinized whole blood samples by means of Lymphoprep™ (Axis-Shield, Oslo, Norway) gradient centrifugation. After being washed in cool PBS, freshly isolated leukocytes were incubated for 15 min. in darkness with fluorochrome-conjugated mouse antibodies in two separate tubes. In the first one, different subsets of T cells, active or not, were labeled with anti-CD3-PECy5, anti-CD4-FITC, anti-CD8-APC and anti-CD25-PE (BD Biosciences, San José, CA, USA). In the second tube, total T cells, NK cells, B cells and antigen-presenting cells were stained by anti-CD3-FITC, anti-CD56/16-PE, anti-CD19-APC, and anti-HLA-DR-PECy5 (BD Biosciences, San José, CA, USA), respectively. Pheno-typic assessment was performed by flow cytometry using a FACScalibur cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Plasma cytokine measurements

Plasma concentrations of IL-1 and IL-10 were measured by BDTM Cytometric Beat Array Human Soluble Proteins Flex Set assays (BD Biosciences, San Diego, USA) according to the manufacturer’s protocols. The beat analyses were resolved in FL3 and FL4 channels of the BD FACScalibur flow cytometer (BD Biosciences, San José, CA, USA). Analyses of sample data were performed using FCAP Array™ software (BD Biosciences, San José, CA, USA).

Plasma concentrations of TGF-β1 were evaluated using a commercially available ELISA kit (Deltaclon, Madrid, Spain) following the manufacturer’s instructions. Plasma samples were acidified with HCl 1 M, and diluted to 1:50 prior to analysis. All measurements were performed in duplicated.

Ethical considerations

All subjects gave their informed consent to participate in the study before randomization. The trial was performed under the norms of the Helsinki’s Declaration, and was approved by the Ethical Committees of the Institute for Research in Biomedical Sciences Germans Trias i Pujol, and the participating centers.

Statistical analysis

Data are expressed as median plus interquartile range (IQR) for quantitative parameters, and as frequencies for qualitative variables.

Comparisons of quantitative variables among groups were performed by means of the Kruskal-Wallis non-parametric test (with post-hoc Mann-Whitney U test). Quantitative variables among groups were compared with the Chi^2 test. Changes in quantitative variables within groups were assessed by means of the Wilcoxon rank-sum test for repeated measures. All statistical analyses were performed using the SPSS 12.0 package for Windows (SPSS, Chicago, IL, USA). P-values below 0.05 were considered as significant.

Results

A total of 60 individuals were assessed for eligibility and randomized (n = 20 for each therapeutic group). Unfortunately, however, 10 subjects withdrew their consent within the first 72 hours after randomization. Thus, 50 subjects were finally included in the study (fig 1). There were no differences among the therapeutic
groups at baseline, regarding demographics, BMI, Barthel index, and routine laboratory parameters (table I). No adverse events attributable to the trial supplements were recorded.

**Immunological parameters**

Immunological parameters — the main end-point of the study — could be only assessed in those individuals surviving the treatment period (15 in the placebo group, 13 in the low-dose probiotic group, and 19 in the high-dose probiotic group) (fig. 1).

**Blood leukocyte subpopulations**

Baseline percent values of the different cell phenotypes were similar among the three therapeutic groups (table II). *L. plantarum* CECT 7315/7316 induced different changes in blood leukocyte subpopulations depending on the dose of probiotic administered. At the end of the treatment (week 12) high dose resulted in significant increases in the percentages of activated potentially T-suppressor (CD8+CD25+) and NK (CD56+CD16+) cells, while low dose induced increases in activated T-helper lymphocytes (CD4+CD25+), B lymphocytes (CD19+), and antigen presenting cells (HLA-DR+) (table II). Of note, most of these changes remained at the end of the follow-up period (week 24), 12 weeks after probiotic treatment cessation (table II).

**Plasma cytokine concentrations**

Plasma concentrations of both IL-1 and IL-10 were undetectable at every time point in all therapeutic groups. Plasma TGF-β1 levels were similar at baseline among the three groups. A significant decrease in TGF-β1 concentration was observed after treatment with both probiotic doses, and at the end of follow-up period, while no change was observed in the placebo group (fig. 2).

**Clinical outcomes**

Seven subjects of the placebo group developed infections: 4 of them during the 12-week therapeutic period (3 fatal cases of pneumonia, one case of urinary tract infection), and 3 during the follow-up period (acute bronchitis in 2 cases, urinary tract infection in one case). Five individuals of the group treated with low probiotic suffered from infections: 3 during the treatment period (pneumonia in 2 cases, acute bronchitis in one) and 2 during the follow-up (acute bronchitis, middle otitis). Three subjects in the high probiotic dose suffered from infections during the follow-up (acute bronchitis in 2, urinary tract infection in one). The...
The infection rate during the treatment period showed a significant trend to be lower in the high probiotic dose group, while there were no differences in the follow-up period (table III).

As mentioned, the 3 cases of pneumonia in the placebo group were the only deaths occurring in the study. Thus, there was a significant trend for mortality to be greater in the placebo group as compared to the probiotic groups (table III). No case of mortality occurred during the follow-up.

No significant change in the BMI, the Barthel index, and the routine laboratory test was observed in the survivors of the three groups, either during the treatment or the follow-up periods (data not shown).

### Discussion

In the last two decades, growing evidence has been produced stressing the role of the intestinal microbiota in the development of both local and systemic immunity.30,31 This regulatory activity involves intestinal epithelial cells, macrophages, dendritic cells, and T-lymphocytes. In fact, axenic mice have been shown to possess fewer dendritic cells in the gut-associated lymphoid tissue, smaller amounts of T-cells in the spleen, and decreased activation of CD4+ cells than animals with normal intestinal microbiota.32-34

Only a few of the vast number of *L. plantarum* strains identified to date have well established immunomodulatory properties. *L. plantarum* CECT 7315 and 7316 strains were identified as probiotics as a result of extensive studies on different bacterial strains isolated from 0-5 year-old children mostly fed with vegetables.27 The results of the present study with (institutionalized) healthy elders show that supplementation with the *L. plantarum* CECT 7315/7316 combination is effective in enhancing systemic immunity in humans, resulting in increased numbers of B lymphocytes (CD19+), NK (CD56+CD16+) cells, and antigen presenting cells (HLA-DR+), in addition to enhanced activation (CD25 expression) of CD4+ and CD8+ T-
cells. These results are in agreement with previous studies which have shown that the intake of lactic acid producing bacteria increases the CD4+, CD25+, CD19+ and CD56+ phenotypes in peripheral blood cells from elderly volunteers.23,35 Taken together, these findings prove the usefulness of probiotic bacteria to cope with the process of immunosenescence in the elderly.

One of the aims of the study was to assess which of two probiotic dosages had the greatest immune enhancing effects. Unexpectedly, however, we found not quantitative but qualitative differences in the immunological effects between the two evaluated dosages. Supplementation with 5·108 cfu/day of the \textit{L. plantarum} CECT7315/7316 mixture (low dose) was associated with changes in the blood cell subsets suggesting an enhanced immunoregulatory and/or Th2 polarized response (increased CD4+CD25+, CD19+, and HLA-DR+ cells).36-38 In contrast, using a daily probiotic dose ten times greater (high dose) resulted in a significant increase of potentially cytotoxic (CD8+CD25+, CD56+CD16+) cell phenotypes39 in peripheral blood.

These findings open the possibility to use different probiotic dosage for different indications. For instance, one could anticipate that low doses might be useful as coadjuvant therapy to vaccinations40,41—as far as they promote acquired humoral immune responses—, while higher doses might be useful to prevent infections17—as they promote more immediate and unspecific cellular responses.

The observed decrease in plasma TGF-β1 concentration associated to \textit{L. plantarum} CECT 7315/7316 supplementation, no matter which dose was administered, deserves special comment. TGF-β1 belongs to a superfamily of cytokines which regulate a plethora of developmental processes, and a disruption of their activity has been involved in a variety of human diseases ranging from fibrotic diseases to the progression of many cancers.42 Immunological actions of TGF-β1 include inhibition of dendritic cell maturation and NK activity.43 More recently, its fundamental role in the polarization of the Th17 response has been related to highly pro-inflammatory T cell subset and to some autoimmune processes,44,45 which could be particularly relevant in the elderly.46

All these immunomodulatory actions of \textit{L. plantarum} CECT 7315/7316 might have a positive impact on clinical outcome. Indeed, in spite that the present trial is clearly underpowered to assess clinical end points, a

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### Table II

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Baseline&lt;sup&gt;a&lt;/sup&gt;</th>
<th>End of treatment (week 12)</th>
<th>End follow-up (week 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+</td>
<td>Placebo 67.8 (51.9-73.5)</td>
<td>61.0 (59.6-71.8)</td>
<td>64.3 (56.2-69.0)</td>
</tr>
<tr>
<td>Low-dose probiotic 71.1 (56.7-73.1)</td>
<td>67.7 (56.6-75.6)</td>
<td>64.7 (58.6-73.3)</td>
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</tr>
<tr>
<td>High-dose probiotic 66.8 (59.1-75.6)</td>
<td>71.6 (58.0-76.4)</td>
<td>66.5 (59.5-72.1)</td>
<td></td>
</tr>
<tr>
<td>CD4+</td>
<td>Placebo 37.7 (32.3-45.0)</td>
<td>34.1 (27.8-43.3)</td>
<td>37.5 (32.6-40.4)</td>
</tr>
<tr>
<td>Low-dose probiotic 37.5 (27.4-46.8)</td>
<td>36.1 (30.1-44.9)</td>
<td>36.1 (30.8-45.2)</td>
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<tr>
<td>High-dose probiotic 35.9 (24.9-42.8)</td>
<td>30.9 (26.3-38.9)</td>
<td>30.0 (24.9-38.4)</td>
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<tr>
<td>CD4+CD25+</td>
<td>Placebo 13.9 (10.3-22.4)</td>
<td>14.2 (13.3-26.4)</td>
<td>15.3 (12.4-22.3)</td>
</tr>
<tr>
<td>Low-dose probiotic 12.2 (8.9-18.0)</td>
<td>17.0 (11.1-22.2)</td>
<td>14.3 (10.2-21.9)</td>
<td></td>
</tr>
<tr>
<td>High-dose probiotic 11.6 (10.6-20.2)</td>
<td>15.4 (11.0-21.3)</td>
<td>14.5 (9.8-20.1)</td>
<td></td>
</tr>
<tr>
<td>CD8+</td>
<td>Placebo 21.3 (12.2-26.3)</td>
<td>18.5 (12.9-26.2)</td>
<td>21.7 (13.2-28.4)</td>
</tr>
<tr>
<td>Low-dose probiotic 20.6 (13.3-28.4)</td>
<td>19.1 (12.3-30.4)</td>
<td>19.4 (12.9-30.1)</td>
<td></td>
</tr>
<tr>
<td>High-dose probiotic 22.2 (17.7-36.4)</td>
<td>23.4 (21.7-36.9)</td>
<td>23.5 (20.6-37.7)</td>
<td></td>
</tr>
<tr>
<td>CD8+CD25+</td>
<td>Placebo 3.3 (1.6-4.8)</td>
<td>3.2 (2.2-4.5)</td>
<td>3.1 (2.5-4.1)</td>
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<tr>
<td>Low-dose probiotic 3.4 (2.2-4.9)</td>
<td>3.7 (2.6-6.3)</td>
<td>3.9 (2.3-4.9)</td>
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</tr>
<tr>
<td>High-dose probiotic 2.9 (2.1-4.9)</td>
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<td>3.9 (2.4-6.1)</td>
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<tr>
<td>CD19+</td>
<td>Placebo 6.3 (6.0-8.3)</td>
<td>6.4 (6.2-10.7)</td>
<td>6.5 (5.9-10.4)</td>
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<tr>
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<td>6.7 (4.5-7.8)</td>
<td></td>
</tr>
<tr>
<td>CD56+CD16+</td>
<td>Placebo 18.1 (10.3-28.6)</td>
<td>18.4 (14.3-27.1)</td>
<td>18.4 (15.5-28.9)</td>
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<td>Low-dose probiotic 17.2 (11.4-25.5)</td>
<td>19.5 (11.4-27.9)</td>
<td>17.2 (14.4-26.9)</td>
<td></td>
</tr>
<tr>
<td>High-dose probiotic 16.9 (10.4-24.4)</td>
<td>19.8 (10.4-32.8)</td>
<td>18.0 (12.5-33.5)</td>
<td></td>
</tr>
<tr>
<td>HLA-DR+</td>
<td>Placebo 6.8 (6.3-8.3)</td>
<td>6.1 (5.8-7.4)</td>
<td>7.3 (5.9-8.6)</td>
</tr>
<tr>
<td>Low-dose probiotic 6.5 (6.2-9.3)</td>
<td>7.5 (7.2-9.2)</td>
<td>7.5 (6.4-9.7)</td>
<td></td>
</tr>
<tr>
<td>High-dose probiotic 6.3 (5.6-8.1)</td>
<td>6.3 (5.6-7.0)</td>
<td>6.0 (5.0-6.9)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>No differences among the three groups were observed at baseline for none of the phenotypes (Kruskal-Wallis test).

<sup>p</sup>P < 0.05 vs. Baseline (Wilcoxon rank-sum test/Independent/Mildly dependent/Partly dependent/Severely dependent/Partly dependent/Fully dependent.

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\textit{Lactobacillus plantarum} CECT 7315 y CECT 7316 en ancianos
positive effect of probiotic supplementation on both infection rate and survival is suggested. Nevertheless, these promising data must be confirmed in larger trials specifically designed to assess clinical outcomes.

Conflict of interest statement

JM, MAG, JE, JC, SA, MAB, and EC share the authorship of the patent involving the probiotic strains used in this trial. JE, JC, SA, and MAB are affiliates of AB-BIOTICS, the company that developed the probiotic strains.

Statement of authorship

JM and EP contributed to the design of the trial, immunological measurements and drafting of manuscript. VL performed the immunological studies. MAG contributed to the design of the trial. JE, SA, and MAB contributed to the design of the trial and provided significant advice on the properties of the probiotic strains used. JC collected clinical data and performed the statistical analysis. EC contributed to the design of the study, collection of data, statistical analysis and drafting of the manuscript. The final version of the manuscript has been approved by all the authors.

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References


Table III

| Infections and mortality during the treatment and follow-up periods in the three therapeutic groups |
|----------------------------------|----------------|----------------|----------------|
| Placebo                          | Low-dose probiotic | High-dose probiotic | p for trend |
| Infections during treatment period | 4/18            | 3/13            | 0/19        | 0.049 |
| Infections during follow-up period | 3/15            | 2/13            | 3/19        | NS    |
| Mortality during treatment period | 3/18            | 0/13            | 0/19        | 0.037 |
| Mortality during follow-up period | 0/15            | 0/13            | 0/19        | NS    |

Chi-square test.


