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Basic nutritional investigation

Supplementation with *Bifidobacterium longum* Bar33 and *Lactobacillus helveticus* Bar13 mixture improves immunity in elderly humans (over 75 years) and aged mice

Alberto Finamore Ph.D. [a,*], Marianna Roselli Ph.D. [a], LorenzoMaria Donini M.D. [b], Dr. Elisa Brasili Ph.D. [a], Rita Rami [a], Paola Carnevali M.Sc. [c], Lorenza Mistura M.Sc. [a], Alessandro Pinto M.D. [b], AnnaMaria Giusti M.Sc. [b], Elena Mengheri Ph.D. [a]

[a] *CREA Research Centre for Food and Nutrition, Rome, Italy*
[b] *Sapienza University, Department of Experimental Medicine, Rome, Italy*
[c] *R&D Advanced Research Microbiology, Barilla G&R f.lli SpA, Parma, Italy*

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ABSTRACT

Objective: Aging induces several physiologic and immune changes. The usefulness of probiotics in ameliorating age-related disorders remains largely unexplored. The aim of this study was to evaluate the effectiveness of a *Bifidobacterium longum* Bar33 and *Lactobacillus helveticus* Bar13 mixture in improving the physiologic status and immunity of older adults (over 75 years). Furthermore, the possible role of such mixture in ameliorating gut immunity in aged mice was investigated.
Methods: A randomized, double-blind, placebo-controlled trial was conducted with 98 adults (84.6 ± 7.8 y), supplemented for 30 d with a biscuit containing a probiotic mixture of *B. longum* Bar33 and *L. helveticus* Bar13 (1:1), or no probiotics, as placebo. Blood was collected for analysis of biochemical parameters, lymphocyte subpopulations, natural killer activity, and cytokine release. Aged Balb/c mice received the same probiotic mixture or placebo daily for 28 d, then blood and intestinal lymphocyte subpopulations were analyzed.
Results: The probiotic mixture ameliorated immune response in older adults by increasing naive, activated memory, regulatory T cells, B cells, and natural killer activity and decreasing memory T cells compared with placebo ($P < 0.05$). The biochemical parameters did not change after probiotic supplementation. In the gut of old mice, the two probiotics modulated cells crucial for gut immune homeostasis by increasing regulatory T (Treg and Tr1) and decreasing $\gamma\delta$ T cells compared with control mice ($P < 0.05$). In addition, B cells increased in the gut and blood of probiotic-treated mice.
Conclusion: Results from the present study data indicated that *B. longum* Bar33 and *L. helveticus* Bar13 improve immune function at intestinal and peripheral sites in aging.

Introduction

A progressive decline in cell function occurs during aging, causing several health complications such as chronic inflammation, higher susceptibility to infections, autoimmune disorders, and cancers [1,2]. These diseases have been ascribed to changes in both innate and adaptive immune systems, which determine a status termed *immunosenescence*, characterized by a decline in naive T-cell production, accumulation of CD8$^+$ and memory T cells, reduction in CD4$^+$ T and B cells, and decreased natural killer (NK) cell activity [3–5]. An immune risk phenotype (IRP) associated with mortality, which included high CD8$^+$, low CD4$^+$, and low CD19$^+$, cells, poor T-cell proliferative response, and seropositivity to

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[*] Corresponding author: Tel.: +39 6 51494580; Fax: +39 6 51494550.
E-mail address: Alberto.finamore@crea.gov.it (A. Finamore).

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cytomegalovirus, was identified in the Swedish OCTO and NONA studies in an older population [6,7]. However, the 6-y follow-up of the same OCTO and NONA participants revealed that the surviving nonagenarians and centenarians did not maintain the IRP characteristics [8]. Another study conducted on centenarians found a cluster of individuals with longer 5-y survival showing higher naive, activated memory, and effector memory T cells [9]. Recently, the term *immunosenescence* has been reconsidered to immunoadaptation or immunoremodeling, in which the aging-associated immune changes are viewed as adaptive rather than solely detrimental [2].

Perturbations of gut microbiota composition are associated with aging, and these changes favor the growth of pathogens and increase the susceptibility to gut-related diseases [10]. Members of the health-promoting bacteria, namely the probiotic bacteria, are affected by aging. In particular, a reduction of numbers and species of bifidobacteria has been reported in older persons [11,12]. Probiotics exert several beneficial effects for the host by protecting against pathogenic bacteria and viruses, enhancing immune function, counteracting intestinal inflammatory diseases, and improving metabolic functions and the lipid profile [1,13–16]. Thus, interventions with such bacteria have been proposed to improve aging-associated alterations. However, to our knowledge few studies have analyzed the efficacy of different probiotic strains in improving immunosenescence and other age-related dysfunctions [17–19], reporting sometimes contrasting results [20,21], whereas no studies have compared the ability of probiotics in enhancing innate and adaptive immunity in both older individuals and older mice.

In a previous study conducted on an Italian cohort of old individuals, a modulation of gut microbiota with a reduction of pathogenic bacteria was found by feeding a biscuit containing the probiotics *Bifidobacterium longum* Bar33 and *Lactobacillus helveticus* (previously named *L. acidophilus*) Bar13 [22]. In the present study, we evaluated the same cohort to determine whether the probiotics contained in the biscuit could improve the innate and adaptive immune response and the physiologic status. In addition, we investigated the possible role of such a probiotic mixture in ameliorating gut immunity in older mice. Indeed, the intestinal immune system plays an important role in the local and systemic immune response, protecting against colonization and invasion by pathogens and maintaining health and functional status of the host [23].

Materials and methods

Study participants

Ninety-eight men and women with an average age of 84.6 ± 7.8 y were recruited at the Villa Le Querce nursing home of Nemi (Rome, Italy) for this study. At enrollment, these individuals were characterized for health status (medical, physical, cognitive status, and presence or absence of frailty), nutritional status (including estimation of dietary intake of several key nutrients), anthropometric parameters, lifestyle, and diet and medications using standardized protocols. The exclusion criteria were severe clinical conditions (grade 4 of the Cumulative Illness Rating Scale), severe cognitive impairment assessed by the Short Portable Mental Status Questionnaire (SPMSQ), artificial nutrition, and obesity with body mass index (BMI) >30 kg/m^2. The recruited participants showed good physical health conditions and did not use antibiotics and probiotics or yogurt for a 1-mo period before sampling.

The Ethics Committee of the Polyclinic Umberto I of Rome approved the study. All participants gave a written informed consent upon recruitment.

Study design and intervention

This was a randomized, double-blind, placebo-controlled trial conducted from 2011 to 2015. The participants were randomized into two groups consuming one biscuit containing probiotics (PRO group) or no probiotics as placebo (PL group) daily for 30 d. The probiotics consisted of a mixture (1:1) of *B. longum* Bar33 and *L. helveticus* Bar13 (Barilla culture collection), added at 1×10^9 colony-forming unit (CFU) in lyophilized form to the chocolate cream covering the biscuit (Barilla, Parma, Italy). Bacterial strain survival within the chocolate cream was preliminarily verified with a storage test, and their concentrations remained unmodified for 6 mo [22]. For each individual enrolled in the study, peripheral blood was collected before (T0) and after 30 d of intervention (T30) and immediately processed for analyses. The placebo and probiotic groups at T30 were named T30-PL and T30-PRO, respectively.

Anthropometric parameters, questionnaires, and characteristics

Nutritional risk assessment was performed using the Mini Nutritional Assessment (MNA) consisting of 18 questions grouped into the following four parts: 1) anthropometry (BMI), weight loss, mid upper arm circumference (AC), calf circumference; 2) clinical status (medications, mobility, pressure sores and skin ulcers, lifestyle, psychological stress or neuropsychological diseases); 3) dietary assessment (autonomy on feeding, quality and number of meals, fluid intake); and 4) self-perception of health status and nutrition, as previously described [24]. The total Mini Nutritional Assessment score ranged from 0 to 30 points: <17 points = malnutrition; 17 to 23 = risk for malnutrition; ≥ 24 = good nutritional status. The following anthropometric parameters were collected: body weight, height, AC, triceps skinfold thickness, calf circumference, BMI (body weight [kg]/height [m^2]), muscle arm circumference [$MAC = (AC - \text{triceps skinfold thickness}) \times \pi$]. The anthropometric data collection was preceded by an interassessor training session and was performed using a SECA scale 86 (200 kg, to the nearest 0.1 kg, certified and homologated as class III), a flexible metallic tape (200 cm, to the nearest 1 cm), a telescopic stadiometer (200 cm; 49 cm of telescopic arm), and an Holtain Tanner Whitehouse Plicometer. Dietary anamnesis was performed to evaluate energy intake by a dietitian using the weighted average of the major food categories of macronutrients.

A multidimensional geriatric assessment was performed as previously described [25] using the following:

1. Cumulative Illness Rating Scale to evaluate comorbidities that included comorbidity index and severity index;
2. Short Portable Mental Status Questionnaire to identify cognitive impairment;
3. Geriatric Depression Scale; and
4. Instrumental Activities of Daily Living scale and Activities of Daily Living test to evaluate current functional ability.

Biochemical parameters in blood

Blood samples were collected from the participants in the morning after a 12-h overnight fast at baseline and at the end of the study. White blood cells, red blood cells, lymphocytes number, hemoglobin, C-reactive protein, glucose, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triacylglicerides, alanine, transaminase, insulin, albumin, uric acid, bilirubin, iron, transferrin, and fibrinogen, were measured using commercially available kits supplied by ABX Italy (Rome) using COBAS-MIRA biochemistry analyzer (Roche, Manheim, Germany).

Flow cytometry analysis of lymphocyte subpopulations

The phenotypic analysis was performed on heparinized whole blood within 3 h from blood withdrawal. Surface staining was performed using the following antibodies (BD Biosciences, Milan, Italy): FITC anti-CD4 (clone RPA-T4), PE anti-CD8 (clone HIT8 a), PE-Cy5 and FITC anti-CD3 (clone HIT3 a), PE-Cy5 anti-CD45 (clone HI30), PE anti-CD19 (clone HIB19), PE anti-CD16/56 (clones B73.1 and NCAM16.2), PE anti-CD127 (clone HIL-7 R-M21), PE-Cy5 anti-CD25 (clone M-A251), FITC anti-CD28 (clone CD28.2), and PE anti-CD95 (clone DX2). Isotype-matched antibodies were used to set fluorescence markers and to identify non-specific binding. Whole blood (100 μL) was labeled with appropriate mixtures of saturating amount of the antibodies and incubated at 4°C for 30 min in the dark. After lysis of blood cells with FACS Lysing Solution (BD Biosciences, Milan, Italy) in ice, samples were centrifuged at 650*g* and the cell pellet, containing leukocytes, was resuspended in FacsFlow for flow cytometry analysis. Lymphocytes were identified by forward and side scatter parameters, and the percentage of T helper and cytotoxic cells was calculated on lymphocyte gate (CD3$^+$). After the CD3 gate, CD4$^+$ helper and CD8 bright$^+$ cytotoxic T cells were gated into naive (CD28$^+$CD95$^-$), effector memory (CD28$^-$CD95$^+$) and activated memory (CD28$^+$CD95$^+$) subsets, as previously published [4]. B lymphocytes and NK cell subsets were calculated on leukocyte gate (CD45$^+$). The percentage of regulatory T (Treg, CD4$^+$CD25$^+$CD127$^-$) cells was calculated on lymphocyte CD4$^+$ gate. Acquisition and analysis were performed on a FACSCalibur instrument (BD Biosciences) using Cell Quest software (BD Biosciences), and ≥10 000 events were acquired. To obtain the absolute numbers, total lymphocyte count from quality certified clinical laboratory analysis was used. The absolute number of total CD4$^+$, CD8$^+$, B, NK, and Treg cells per mm^3 of peripheral blood was calculated multiplying the lymphocyte count by the frequency of different subsets in the lymphocyte gate [9].

NK cytotoxicity

NK cytotoxicity was assayed as previously described by Houh et al. [26]. NK cells were isolated from blood (5 mL) of the participants at baseline (T0) and after supplementation with probiotics (T30-PRO) by immunomagnetic selection using the Miltenyi MACS separator instrument (Miltenyi Biotec, Bologna, Italy). The lytic activity of NK cells was evaluated as the ability to induce apoptosis using K562 (NK-sensitive leukemic cells) as target cells cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in an atmosphere of 5% carbon dioxide and 95% air at 90% relative humidity. All cell culture reagents were from Euroclone (Milan, Italy). For NK activity assay, 1×10^6 target cells, harvested in log phase, were stained with 1 μM carboxyfluorescein diacetate succinidyl ester (CFDA-SE; Molecular Probes Inc., Milan, Italy) for 5 min at 37°C, washed, and then cultured with NK cells isolated from the subjects at 37°C for 4 h. Different NK (effector, E)–to-K562 (target, T) ratios were tested (50, 25, and 12.5:1 using 5×10^5 NK effector cells) and the best results were obtained with the 50:1 ratio. After incubation, target cells were stained with 0.05 mg/mL 7-aminoactinomycin D (7-AAD; BD Pharmingen, Milan, Italy), a fluorescent marker for apoptosis, at room temperature for 5 min to detect lysed cells. Cytotoxicity of NK against K562 cells was analyzed based on regions showing double-positive staining for CFDA-SE and 7-AAD using a FACSCalibur instrument (Becton Dickinson, Milan, Italy). The lytic activity of NK cells isolated at T30 was compared with that of the same individuals at T0. NK cytotoxicity was calculated using the following formula:

$$\text{cytotoxicity}\ (\%) = (\mathrm{E}-\mathrm{ES}-\mathrm{TS})/(\mathrm{TM}-\mathrm{TS}) \times 100$$

where E is experimental data release, ES is effector spontaneous cell death, TS is target spontaneous death, and TM is target maximum death.

Cytokine analysis

The levels of cytokines in serum were analyzed using the multiparametric Bioplex/Luminex technology (human magnetic Luminex screening assay, Labospace, Milan, Italy). The following cytokines were simultaneously detected in 50 μL undiluted samples: interleukin (IL)-8, IL-1 β, IL-6, IL-10, tumor necrosis factor (TNF)-α, and IL-12 p70.

Animals

Balb-c aged mice (20 mo old) were kept at 23°C with a 12-h light/dark cycle and fed ad libitum with standard laboratory diets (4 RF21, Mucedola, Milan, Italy). Animals were housed individually in stainless steel cages and randomly divided into two groups (10 mice per group) to receive an oral mixture (1:1) of *B. longum* Bar33 and *L. helveticus* Bar13 (1×10^9 colony-forming unit; probiotic group) or PBS (Control group) daily for 4 wk. The two probiotics were the same strains used in the human study. Mice had free access to food and water. Body weight and food intake were recorded every week and every other day, respectively. No differences in food intake and body weight were observed between the two groups during the experimental period (data not shown). Animals were anesthetized with intraperitoneal injection of pentobarbital (10 mg/kg) and small intestines were excised and placed in cold phosphate-buffered saline while blood was drawn via cardiac puncture. Animal studies were performed under conditions approved by the National Health Ministry, Department of Food, Nutrition and Animal Health.

Bacterial strains for animal experiments

B. longum Bar33 and *L. helveticus* Bar13 were stored and lyophilized at −20°C. Viability of bacteria was verified in preliminary experiments by agar plating of appropriate dilutions of the bacterial cultures, obtained by refreshing the lyophilised strains in MRS medium on DeMan Rogosa Sharpe (MRS, Difco, Milan, Italy). The two bacteria were daily resuspended, mixed (1:1) in 75 μL saline and given to mice with little amounts of diet to ensure complete intake.

Lymphocyte preparation from mouse small intestine and flow cytometry analysis of T and B cell phenotypes in the gut and blood

The phenotypic analysis was performed in intraepithelial lymphocytes (IELs), *lamina propria* lymphocytes (LPLs), and blood of old mice. IELs and LPLs were prepared from small intestine, as previously described [15]. IELs and LPLs (1×10^6 cells) were preincubated with anti-CD16/CD32 to block Fc receptors, thus avoiding non-specific binding. Cells were then washed and labeled with appropriate mixture of monoclonal antibodies for cell surface staining, centrifuged and resuspended in FacsFlow as previously described [27]. For circulating lymphocytes, 100 μL of whole blood were labeled with antibodies and treated as described for the older adults. To exclude dead or dying cells and therefore non-specific antibody-binding cells, all lymphocytes were gated according to forward and side scatter parameters. The percentage of B lymphocytes was calculated on leukocyte gate ($CD45^+$), whereas the $CD4^+$ and $CD8^+$ subsets, and $TCR\gamma\delta^+$ and $TCR\alpha\beta^+$, were calculated on $CD3^+$ gate. Acquisition and analysis were performed as previously described.

The analysis of $CD4^+CD25^+Foxp3^+$ (Treg) cells was performed in IELs and LPLs with a specific Kit (eBioscience, San Diego, CA, USA) staining CD4 (FITC), CD25 (PE), and transcription factor Foxp3 (PE-Cy5), according to manufacturer's instructions. Lymphocytes were stained for surface markers CD4 and CD25, then permeabilized and stained for intracellular Foxp3. Cells were analyzed by flow cytometry and the percentage of $CD25^+Foxp3^+$ cells was calculated on $CD4^+$ gate. For analysis of $Foxp3^-$ Tr1 cells, the antibodies FITC anti-CD4, PE anti-IL-10, and PE-Cy5 anti-Foxp3 were used. As for Tregs, cells were stained with CD4 surface marker, then permeabilized and stained for intracellular IL-10 and Foxp3. The percentage of $Foxp3^-$ Tr1 cells was calculated on $CD4^+$ gate.

Statistical analysis

Before analysis, normal distribution and homogeneity of variance of all variables were assumed with Shapiro–Wilk's and Levene's tests, respectively. Statistical significance was evaluated by one-way analysis of variance followed by post hoc Tukey honestly significant difference test. Differences with $P < 0.05$ were considered significant. Statistical analysis was performed with the SPSS version 17 (SPSS, Chicago, IL, USA).

Results

Baseline characteristics and effects of probiotics on anthropometric, immune, and biochemical parameters of older adults

Of the 98 individuals randomized to the groups receiving probiotics (PRO) or placebo (PL), 2 died, 4 refused to continue, and 14 patients were excluded for illness or low adherence to the protocol. The remaining 78 individuals (45 PRO and 34 PL) completed the trial. No adverse events owing to the probiotic consumption were reported. The baseline characteristics are shown in Table 1. The estimated calorie, protein, fat, and carbohydrate intakes and

Table 1
Baseline characteristics of the participants to the study

Variables		
Number, n (%)		98
Male/female, n		69/29
Age, y, mean ± SD		84.6 ±7.8
Lifestyle		
Smokers, n (%)		5 (5.1)
Daily alcohol consumption, n (%)		2 (2.4)
Anthropometric features		
Weight, kg, mean ± SD		63.1 ± 17.4
Height, cm, mean ± SD		154.4 ± 10.2
Waist circumference, cm, mean ± SD		93.6 ± 15.6
BMI, kg/m^2, mean ± SD		26.3 ± 6.4
Multidimensional geriatric evaluation		
CIRS scale	High comorbidity index (>4) (%)	27.8
MNA	Score, mean ± SD	17.1 ± 3.9
	Malnutrition (score <17) (%)	39.5
	Risk of malnutrition (score 17–23.5) (%)	56.3
	Normal nutritional status (score ≥24) (%)	5.3
ADLs	Score mean ± SD	5.5 ± 4.4
	No. of lost functions/6, mean ± SD	1.6 ± 2.2
	>3 lost functions (%)	25.6
IADL scale	Score, mean ± SD	2.2 ± 2.8
SPMSQ	Score, mean ± SD	3.3 ± 1.9
	Moderate/severe cognitive status impairment (>7/10) (%)	26.7
GDS scale	Score, mean ± SD	6.1 ± 2.2
	Depression (score ≥10/15) (%)	4.4

ADL, activities of daily living; BMI, body mass index; CIRS, Cumulative Illness Rating Scale; GDS, Geriatric Depression Scale; IADL, instrumental activities of daily living; MNA, Mini Nutritional Assessment; SPMSQ, Short Portable Mental Status Questionnaire.

Table 2
Immune and biochemical parameters of elderly at baseline (T0) and after 30 d (T30) of placebo (PL) or probiotic (PRO) supplementation

Parameters	T0	T30-PL	T30-PRO
WBC (number x $10^3/mm^3$)	5.96 ± 1.8	6.09 ± 1.53	5.81 ± 1.52
RBC (number x $10^6/mm^3$)	4.01 ± 0.5	3.98 ± 0.59	3.99 ± 0.53
Hb (g/dL)	12.02 ± 1.6	11.94 ± 1.68	11.88 ± 1.56
Lymphocytes (cells/mm^3)	1.77 ± 0.59	1.73 ± 0.48	1.87 ± 0.65
IgA (mg/dL)	258.13 ± 113.2	264.57 ± 119.86	236.01 ± 119.75
IgG (mg/dL)	1232.22 ± 399.1	1352.90 ± 371.33	1226.40 ± 384.78
hs-CRP (mg/L)	11.61 ± 18.7	12.35 ± 20.26	21.31 ± 85.65
Fibrinogen (mg/dL)	400.36 ± 87.6	395.74 ± 76.83	359.04 ± 69.06
Total cholesterol (mg/dL)	184.22 ± 42.2	171.18 ± 39.93	189.85 ± 38.67
LDL cholesterol (mg/dL)	113.16 ± 42.9	88.73 ± 52.43	106.53 ± 50.22
HDL cholesterol (mg/dL)	41.89 ± 11.5	32.55 ± 3.86	44.17 ± 2.42
Triacylglycerols (mg/dL)	118.54 ± 52	113.68 ± 40.70	124.02 ± 48.85
Glucose (mg/dL)	82.45 ± 17	84.14 ± 26.95	79.81 ± 17.39
Insulin (μU/mL)	7.49 ± 4.9	13.42 ± 25.19	9.27 ± 10.63
HOMA-IR index	1.58 ± 1.7	3.57 ± 7.78	1.80 ± 2.89
Albumin (g/dL)	3.54 ± 0.4	3.42 ± 0.53	3.56 ± 0.41
Uric acid (mg/dL)	4.75 ± 1.5	4.53 ± 1.42	4.95 ± 1.44
Bilirubin (mg/dL)	0.51 ± 0.4	0.39 ± 0.13	0.48 ± 0.24
Iron (μg/dL)	54.05 ± 24.1	46.09 ± 7.32	57.88 ± 5.67
Transferrin (mg/dL)	200.65 ± 55.3	178.85 ± 22.43	219.09 ± 25.14
Ferritin (ng/mL)	145.24 ± 163.3	170.90 ± 171.93	109.31 ± 132.62
CHE (U/L)	6784.02 ± 1983.0	6434.05 ± 2282.38	6860.32 ± 2079.23

CHE, cholinesterase; Hb, hemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; hs-CRP, high-sensitivity C-reactive protein; Ig, immunoglobulin; LDL, low-density lipoprotein; RBC, red blood cells; WBC, white blood cells.
Data are means ± SDs.

physical activity levels did not significantly differ between the two groups at T0 and T30 (data not shown).

The anthropometric, immune, and biochemical parameters are shown in Table 2. No significant changes were observed in any of the blood parameters at the end of the probiotic or placebo supplementation periods compared with those at T0.

Effect of probiotic supplementation on immune phenotype of the elderly

Immune phenotyping of peripheral blood lymphocytes is shown in Table 3. At the end of probiotic supplementation (T30-PRO) there was a significant increase in B lymphocytes and Treg cells compared with T0 and T30-PL groups, whereas no differences were observed in $CD4^+$ helper and $CD8^+$ cytotoxic T cells and NK cells. In addition to these basic immune parameters, other important, more elderly-specific lymphocyte subsets, such as naive ($CD28^+CD95^-$), effector memory ($CD28^-CD95^+$) and activated memory ($CD28^+CD95^+$) $CD4^+$ and $CD8^+$ T cells were analyzed (Table 4). Data are reported as percentage of the ratio of each lymphocyte subpopulation at T30 over T0 (% T30-PL/T0 and % T30-PRO/T0), gated on $CD4^+$ or $CD8^+$ T cells. The results show that the percentages of $CD4^+$ and $CD8^+$ naive T cells increased, whereas $CD4^+$ effector memory T cells decreased after probiotic supplementation. In addition, the percentage of $CD8^+$ activated memory subpopulations were significantly enhanced by probiotic supplementation.

Serum cytokines in elderly

The level of circulating IL-8 resulted significantly increased, whereas proinflammatory TNF-α was reduced in probiotic group T30-PRO compared with T0 and T30-PL groups. No differences were observed in IL-6 secretion owing to the high variability of data (Table 5). IL-1 β, IL-10, and IL-12 p70 resulted undetectable.

Effect of probiotic supplementation on NK activity in elderly

Although the number of NK cells did not change after probiotic supplementation, the cytotoxic activity of these cells increased,

Table 3
Immune cell percentages in blood of elderly at baseline (T0) and after 30 d (T30) of placebo (PL) or probiotic (PRO) supplementation

Subpopulations	T0	T30-PL	T30-PRO
Helper $CD4^+$ T cells ($CD3^+CD4^+CD45^+$)	69.02 ± 13.8	69.02 ± 13.6	70.64 ± 12.1
(cells/mm^3)	1111.7 ± 319.1	1128.6 ± 385.9	1328.3 ± 55.1
Cytotoxic $CD8^+$ T cells ($CD3^+CD8^+CD45^+$)	24.78 ± 12.8	24.78 ± 12.2	24.70 ± 11.2
(cells/mm^3)	474.1 ± 320.5	405.2 ± 259.4	483.8 ± 305.2
B cells ($CD19^+CD3^-CD45^+$)	5.62 ± 2.1	5.21 ± 1.2	8.56 ± 2.7*,†
(cells/mm^3)	86.2 ± 29.3	84.2 ± 40.1	154.5 ± 77.6*,†
NK cells ($CD3^+CD56^+CD16^+CD45^+$)	16.24 ± 8.5	17.45 ± 7.9	14.92 ± 8.3
(cells/mm^3)	294.9 ± 120.9	300.2 ± 72.2	288.7 ± 186.4
Treg ($CD4^+CD25^+CD127^-$)	2.82 ± 1.3	2.82 ± 1.4	4.60 ± 1.2*,†
(cells/mm^3)	34.4 ± 18.5	33.9 ± 19.4	55.6 ± 26.3*,†

Data are the means ± SDs.
* $P < 0.05$ vs T0.
† $P < 0.05$ vs T30-PL.

Table 4
Lymphocyte subsets in blood of elderly at baseline (T0) and after 30 d (T30) of placebo (PL) or probiotic (PRO) supplementation

	Subpopulations	T30-PL/T0 (%)	T30-PRO/T0 (%)	*P*-value
	Naive ($CD28^+CD95^-$)	71.60 ± 13.94	201.34 ± 85.70	0.01558
CD4	Effector memory ($CD28^-CD95^+$)	105.90 ± 31.68	77.39 ± 19.29	0.0314
	Activated memory ($CD28^+CD95^+$)	101.54 ± 9.29	94.63 ± 4.90	NS
	Naive ($CD28^+CD95^-$)	109.57 ± 56.15	214.73 ± 6.76	0.0167
CD8	Effector memory ($CD28^-CD95^+$)	108.17 ± 21.37	117.92 ± 28.77	NS
	Activated memory ($CD28^+CD95^+$)	71.36 ± 21.92	166.54 ± 83.86	0.00917

NS, not significant.
Data are means ± SDs.

reaching >73% cytotoxicity in the T30-PRO group compared with the T30-PL and T0 groups, which showed about 41% cytotoxicity, with 50:1 E-to-T ratio (Fig. 1).

Changes in lymphocyte subsets in blood and gut of old mice

Considering the importance of the intestinal immune system for the host health, additional experiments were conducted in old mice to investigate whether *B. longum* Bar33 and *L. helveticus* Bar13 mixture could improve the age-related alterations of gut lymphocyte subsets.

The lymphocyte populations isolated from the two compartments of the intestinal immune system, namely IELs and LPLs, were analyzed for the presence of helper $CD4^+$ and cytotoxic $CD8^+$ T cells, $CD19^+$ B cells, $TCR\gamma\delta^+$ cells, and $TCR\alpha\beta^+$ T cells. These last two T-cell subpopulations are abundant at intestinal sites. The immunophenotype of IELs of probiotic mice did not differ from that of control mice (Fig. 2A), whereas significant differences were observed in LPLs, where $CD4^+$ and B cells were higher, and $TCR\gamma\delta^+$ T cells were lower in probiotic than control mice (Fig. 2B). The different lymphocyte populations were also analyzed in blood, where a significant increase in B cells was found in probiotic mice but not in control mice, whereas T-cell subpopulations did not change (Fig. 2C). The actual cell numbers (expressed as cell/mm^3 of blood lymphocyte subpopulations of probiotic-treated mice ($CD4^+$: 2.10 ± 0.31 × 10^6; $CD8^+$: 0.53 ± 0.06 × 10^6; $TCR\alpha\beta^+$: 6.94 ± 0.02 × 10^6; $TCR\gamma\delta^+$: 0.10 ± 0.04 × 10^6) did not differ from those of control mice ($CD4^+$: 2.2 ± 0.58 × 10^6; $CD8^+$: 0.84 ± 0.24; $TCR\alpha\beta^+$: 6.38 ± 0.75 × 10^6; $TCR\gamma\delta^+$: 0.14 ± 0.06 × 10^6). B-cell number significantly increased in probiotic-treated mice compared with control (1.76 ± 0.38 × 10^6 versus 2.9 ± 0.22 × 10^6; $P < 0.05$).

Expansion of regulatory T cells in the gut of old mice

Because of the crucial role of regulatory T cells in the immune response, the effect of probiotic supplementation on the two main subsets of regulatory T cells, namely $Foxp3^+$ Treg ($CD4^+CD25^+Foxp3^+$) and $Foxp3^-$ Tr1 ($CD4^+Foxp3^-IL\text{-}10^+$) cells, was studied in intraepithelial and *lamina propria* compartments. As shown in Figure 3, both $Foxp3^+$Treg and $Foxp3^-$ Tr1 cells were increased in LPLs of probiotic mice compared with control mice, and $Foxp3^-$ Tr1 cells were expanded by probiotics also in IELs.

Table 5
Serum cytokine levels in elderly at baseline (T0) and after 30 d (T30) of placebo (PL) or probiotic (PRO) supplementation

Cytokine (pg/mL)	T0	T30-PL	T30-PRO
IL-8	160.59 ± 91.90	72.08 ± 47.93	1000.11 ± $381.913^{*,\dagger}$
IL-6	523.52 ± 497.07	341.89 ± 225.57	316.07 ± 153.23
TNF-α	210.89 ± 190.45	484.52 ± 259.19	53.29 ± $28.17^{*,\dagger}$

IL, interleukin; TNF, tumor necrosis factor.
Data are means ± SDs.
*$P < 0.05$ vs T0.
†$P < 0.05$ vs T30-PL.

Discussion

The effectiveness of probiotics in ameliorating aging-related disorders is still debated, and their ability to influence inflammation and immunosenescence has been poorly investigated. In addition, according to the concept of strain-dependent effects, there is still a need to define and characterize probiotic strains able to offer benefits in aging processes. Mixtures of probiotic strains appear to be more effective than single strains in ameliorating several pathologies and immune function [28,29]. This can be explained by the fact that the different strains and species present in a mixture may have a broader spectrum of activities and synergistic effects. In particular, a recent review recommended the use of a two-strain combination for human studies [30]. In our previous study, we demonstrated that a *L. helveticus* Bar13 and *B. longum* Bar33 mixture prevented experimental colitis through modulation of immune response [15]. Furthermore, this probiotic mixture used in the present study given to the same cohort of older adults improved the microbiota composition [22]. Here, we provided an assessment of the ability of this mixture to improve innate and adaptive immune response in elderly adults and aged mice. We did not find significant changes in biochemical parameters. This result can be due to the fact that before the intervention, the participants did not suffer from particular diseases, and the values of blood glucose, lipid, hormones, and other proteins were in the normal range. Consistent with our findings, previous studies observed improvement in immunity in elderly adults without changes in the physiologic conditions after probiotic supplementation [31,32]. Furthermore,

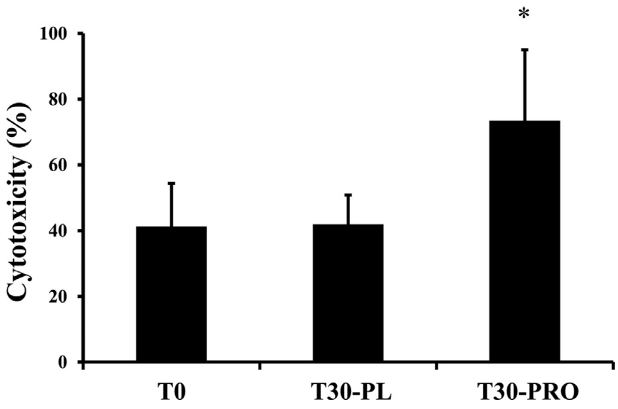

Fig. 1. Increased NK cytotoxicity after probiotic supplementation. NK activity was assayed in blood of elderly at baseline (T0) and after 30 d (T30) of placebo (PL) or probiotic (PRO) supplementation by flow cytometry. E-to-T ratio = 1:50. Data are the means ± SDs. *$P < 0.01$ vs T0. NK, natural killer.

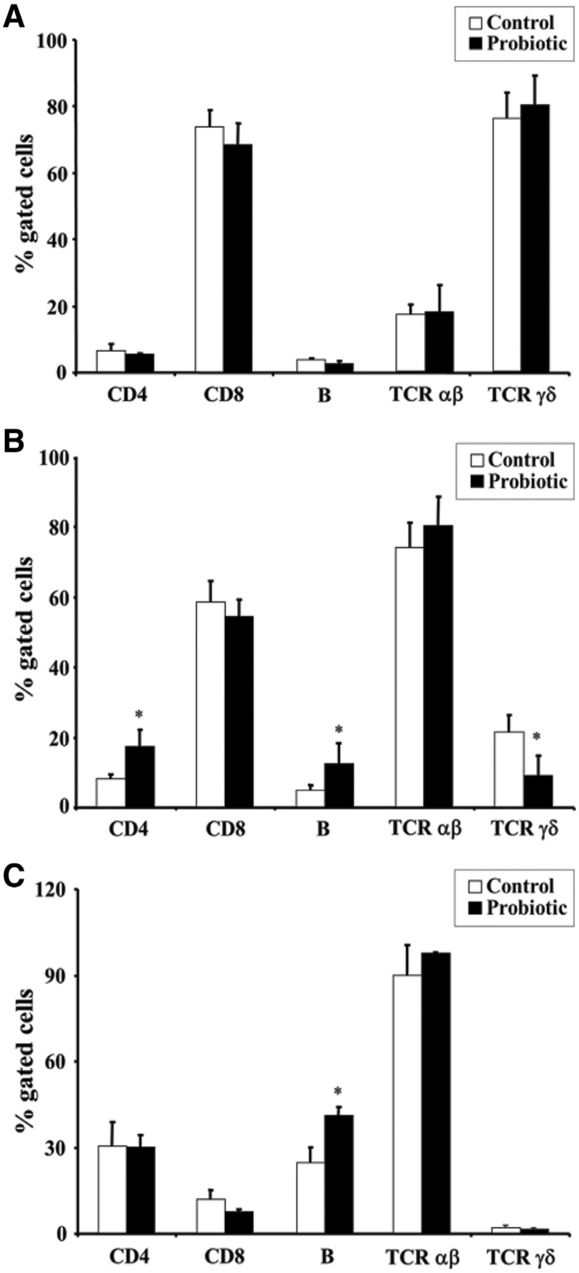

Fig. 2. Effect of probiotics on lymphocyte populations of aged mice. Mice received orally PBS (control) or *B. longum* Bar33 and *L. helveticus* Bar13 mixture (probiotic) for 4 wk. (**A**) Intestinal intraepithelial lymphocytes (IELs); (**B**) Intestinal *lamina propria* lymphocytes (LPLs). (**C**) Blood. Cell percentages were analyzed by flow cytometry. The percentage of $CD4^+$, $CD8^+$, $TCR\alpha\beta^+$, and $TCR\gamma\delta^+$ cell subsets were calculated on lymphocyte gate ($CD3^+$), whereas B cells were calculated on leukocyte gate ($CD45^+$). Data are the means ± SDs. $^*P < 0.05$ vs control.

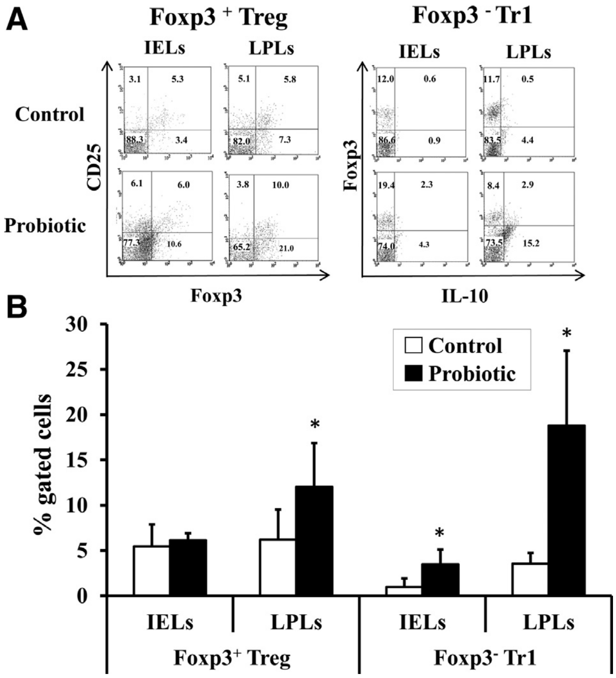

Fig. 3. Increased regulatory T cells of aged mice after probiotic supplementation. Mice received orally PBS (control) or *B. longum* Bar33 and *L. helveticus* Bar13 mixture (probiotic) for 4 wk. $Foxp3^+$ Treg ($CD4^+CD25^+Foxp3^+$) and $Foxp3^-$ Tr1 ($CD4^+Foxp3^-IL\text{-}10^+$) cells were analyzed by flow cytometry in intestinal intraepithelial lymphocytes (IELs) and *lamina propria* lymphocytes (LPLs). $Foxp3^+$T reg and $Foxp3^-$ Tr1 cell percentages were calculated on T-lymphocyte gate ($CD4^+$). (**A**) Representative dot plots of cell distribution after labeling with anti-CD25 and anti-Foxp3 ($Foxp3^+$ Treg) or with anti-Foxp3 and anti-IL-10 ($Foxp3^-$ Tr1). Numbers inside the quadrants represent cell percentages. (**B**) Histograms of the mean values of percentages ± SD. $^*P < 0.05$ vs control.

centenarians with a longer 5-y survival displayed immune changes independently from their health and functional status [9].

The main characteristics of age-related immune alterations of T-cell populations are a reduction of naive and increase in memory T cells. As a consequence of low percentage of naive T cells, the T-cell repertoire is not replenished, leading to immune defects and increased susceptibility to infectious diseases [2]. Differently from naïve T cells, memory T cells respond to previously encountered antigens, and their increase during aging is thought to be due to lifetime exposure to different aggressors. Healthy aging, as that of centenarians, is characterized by a less marked decrease in naive cells and an increase in memory cells compared with those found in younger populations [9,33]. In our study, we found that the probiotic mixture was able to ameliorate the immunosenescence by inducing an increase in both $CD4^+$ and $CD8^+$ naive T cells and a decrease in effector memory $CD4^+$ T cells in peripheral blood. These findings are in line with previous results showing increased naive and decreased memory T cells in elderly supplemented with *L. delbrueckii* spp. bulgaricus 8481 [18]. However, other studies did not find changes in $CD4^+$, naive, and memory T cells after supplementation with *L. gasseri* TMC0356 to older individuals [31]. We also showed that the probiotic mixture raised the percentage of $CD8^+$ activated memory ($CD28^+CD95^+$) T cells. A progressive loss of CD28 expression in $CD8^+$ T cells has been shown to be associated with aging [34]. CD28 plays a critical role for T-cell activation, thus the increase in activated memory T cells induced by *L. helveticus* Bar13 and *B. longum* Bar33 mixture can be considered a beneficial effect. In agreement with our results, some authors showed that treatment of elderly individuals with a mixture of *L. plantarum* CECT 7315 and CECT 7316 or *B. lactis* HN019 resulted in significant increases in activated $CD8^+$ T cells ($CD25^+$) [17,35]. In addition, Miyazawa et al. [31] found a reduced loss of CD28 expression in $CD8^+$ T cells in elderly supplemented with *L. gasseri* TMC0356. Interestingly, a study on Italian centenarians reported that individuals with a longer 5-y survival showed high values of all T-cell populations including $CD8^+$ activated memory T cells [9].

NK cells are important components of the innate immune response against virus infection and tumors and may also contribute to activate other cellular components of innate and adaptive immunity by secreting cytokines and chemokines [36]. Aging may induce several alterations to NK cells, with a decrease in NK cell number and a decline in NK cell activity, that are associated with increased incidence of infectious diseases and mortality risk [37,38]. Some studies conducted in centenarians reported a preservation of NK cell number and activity associated with healthy aging [39]. According to these results, low NK cytotoxicity may be considered a marker of increased morbidity or mortality owing to infections, whereas enhancement of NK cell activity can be associated with healthy aging. In the present study, we showed that the probiotic treatment did not change the total number of NK cells but increased NK activity, thus improving the innate immune response. These results are in agreement with those of previous studies in old subjects. For instance, administration of *B. longum* SP 07/3 and *L. casei* Shirota enhanced NK activity [40,41], and supplementation with *B. lactis* HN019 increased both the number and activity of NK cells [17], whereas *L. delbrueckii* subsp. bulgaricus 8481 induced an increased proportion of NK cells [18]. However, a lack of effect on NK cell number and activity was also found after administration of *L. gasseri* TMC0356 [31] or *B. longum* bv. infantis CCUG 52486 in old individuals [42].

Treg cells are an important component of the adaptive immune system and play a central role in immunologic homeostasis, suppressing inflammation, maintaining peripheral tolerance, and inhibiting autoimmunity [43]. Alterations in frequency, phenotype, and activity of blood Treg cells have been found in advanced age and have been suggested to contribute to inflammation and increased risk for autoimmune disease in the elderly [44]. Several studies observed an increase in Treg population during aging [45–47], but there is also evidence of a decrease in these cells [48–50]. Recent data suggest that natural Treg cells accumulate with age, whereas inducible Treg cells (iTregs), namely T cells that become Treg cells after antigenic stimulation in the periphery, appear reduced [44]. Although it is not clear if the function of Tregs is affected by aging, it is thought that an expansion of these cells in elderly may lead to increased risk for cancer and infections, although it may be beneficial in treating the age associated inflammation [44]. The results of recent studies are in favor of beneficial effects deriving from high Treg population in the elderly by showing a significant reduction in Treg frequency in older individuals with ischemic stroke [51] and enhanced Tregs in older adults with a reduced risk for respiratory infections [52]. Probiotics are able to reduce inflammation and autoimmune diseases by inducing Tregs [53–55]. In the present study, we found an increase in Treg cells after supplementation with *L. helveticus* Bar13 and *B. longum* Bar33 mixture in blood of old individuals. Consistent with the previous findings, Tregs may be upregulated to suppress the aging associated inflammatory signals. This is supported by the finding of a reduced secretion of TNF-α after *L. helveticus* Bar13 and *B. longum* Bar33 supplementation. We can hypothesise that the population of iTregs was the target of the probiotic supplementation. Indeed, iTregs derive from naive CD4$^+$ T cells that upregulate Foxp3 in the periphery [56], and in our study, the naive CD4$^+$ T cells were increased after supplementation with *L. helveticus* Bar13 and *B. longum* Bar33. Notably, the intestinal *lamina propria* is a site of Tregs expansion [57]. These cells migrate from mesenteric lymph nodes (MLNs) to intestinal *lamina propria* and may reenter the MLNs from the lamina or re-enter blood circulation [58,59]. Therefore, we investigated whether *L. helveticus* Bar13 and *B. longum* Bar33 affected the percentage of Treg population in the gut of aged mice. We analyzed two subsets of regulatory T cells, namely Foxp3$^+$ Treg and Foxp3$^-$ Tr1, and we found an increase in gut *lamina propria* of Foxp3$^+$ Treg and Foxp3$^-$ Tr1 cells. These results indicate that supplementation with *L. helveticus* Bar13 and *B. longum* Bar33 leads to an increase in Tregs both in the periphery and in the gut, likely maintaining noninflammatory immune responses. In agreement with our results, a previous study conducted on accelerated aging mice showed that *L. plantarum* WCFS1 and *L. casei* BL23 induced an increase in MLN Tregs [60]. To our knowledge there are no data on Treg modulation by probiotics in both old individuals and aged mice.

We also found an increase in circulating levels of IL-8 in the elderly after supplementation with the two probiotics. Other studies reported a stimulation of IL-8 secretion by probiotics in aging. For instance, *B. adolescentis* induced an increase in IL-8 in aged rats [61], and a supplementation with a synbiotic containing *L. rhamnosus* and oligofructose reported that inflammatory cytokines in serum, in particular IL-8, were increased in older adults [62]. Because IL-8 is a potent chemoattractant, its upregulation induced by *L. helveticus* Bar13 and *B. longum* Bar33 might have provided protection in inflammatory processes. Interestingly, a recent study [63] showed that Tregs may stimulate the release of cytokines including IL-8 from basophils, which are cells involved in autoimmune and other inflammatory diseases. Another recent study showed that naive CD4$^+$ T cells expressed high levels of IL-8, which was active in inducing neutrophil migration [64]. These data suggest that in our study the increased level of IL-8 derived from the enriched population of naive T cells or Treg cells in older adults.

Still debated is the effect of immunosenescence on $\gamma\delta$ T cells that are T lymphocytes bearing TCR $\gamma\delta$ receptor and responding fast to toxins, infections, cancer, or epithelial injury [65]. A recent study showed that TCR$\gamma\delta$ in *lamina propria* compartment express IL-17 and IL-10 [66], in agreement with the notion that TCR$\gamma\delta$ lymphocytes are a major source of proinflammatory IL-17 [67]. Aging may affect both frequency and function of circulating $\gamma\delta$ T cells [68,69], but no studies investigated whether aging could affect intestinal $\gamma\delta$ T cells or whether probiotics could regulate these cells in the gut. In our previous work conducted on mice supplemented with the same probiotic mixture used in the present study, we showed that the prevention of experimental colitis was associated with expansion of intraepithelial $\gamma\delta$ T cells within IELs and a decrease in these cells within LPLs [15]. Here, we show that in aged mice, the probiotic mixture induced an increase in CD4$^+$ T cells and B cells, and a decrease in $\gamma\delta$ T cells in the *lamina propria* compartment, but did not affect the different T-cell populations of the IELs. Together, these data indicate that *L. helveticus* Bar13 and *B. longum* Bar33 are effective in improving the intestinal immune response in aging.

Conclusions

The present study reported the health benefits of *L. helveticus* Bar13 and *B. longum* Bar33 mixture in aging, showing novel aspects of probiotic activities in such context. We show that the two probiotics are able to improve the innate and adaptive immune response in the elderly by increasing the naive and activated memory T cells, decreasing memory T cells, enhancing Treg population and NK activity, and modulating cytokine secretion. The results on aged mice show that the two probiotics induce an increase in regulatory T cells and $\gamma\delta$ T cells in the gut, which are crucial for the protection against infections and inflammation and for the immune homeostasis. The data indicate the effectiveness of *L. helveticus* Bar13 and *B. longum* Bar33 mixture in improving the immune function both at intestinal and peripheral sites in aging.

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