

Effect of *Lactobacillus paracasei* NCC2461 on Antigen-Specific T-Cell Mediated Immune Responses in Aged Mice

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Abstract

Aging is associated with a reduced capacity to mount proper immune responses, in particular to vaccines. Probiotic lactic acid bacteria may improve the immune status of the elderly; however, there is little evidence showing an effect of these bacteria on humoral and cellular immune responses. In the present study, the immunomodulatory capacity of the probiotic *Lactobacillus paracasei* NCC2461 combined or not with a prebiotic composition, FOS/inulin, was examined in aged mice. Male C57BL/6J mice (21 months old) were allocated to one of three groups fed *ad libitum* for 44 days with different diets: a normal diet (control), a normal diet plus NCC2461 given in the drinking water, or a diet containing FOS/inulin plus NCC2461 in the drinking water. All mice were immunized on day 15 and challenged on day 22 with keyhole limpet hemocyanin (KLH). T helper (Th)1 cell-dependent immune responses (anti-KLH immunoglobulin G_{2a} [IgG_{2a}] levels and delayed type hypersensitivity response) were increased significantly in NCC2461-supplemented mice when compared to controls. Supplementation with FOS/inulin did not further improve the immune-enhancing effect mediated by the probiotic. Splenocyte proliferation, T cell subsets, systemic total IgG levels, and mucosal total IgA responses were not affected. Interestingly, supplementation with NCC2461 modulated the intestinal microbiota composition by increasing the numbers of bifidobacteria and lactobacilli. In conclusion, oral intake of *L. paracasei* NCC2461 by aged mice enhanced the specific adaptive immune response to *in vivo* antigenic challenge without altering other cellular and humoral immune responses. The poor responsiveness to antigenic challenge, frequently observed in elderly people, may be improved by supplementation with *L. paracasei* NCC2461.

Introduction

AGING IS ASSOCIATED WITH A NUMBER of alterations in the immune system, particularly a noted decline in cell-mediated immunity (diminished T cell functions) concomitant with humoral immune dysfunction (decreased antibody responses to foreign antigens; *e.g.* influenza vaccine, with increased levels of autoreactive antibodies) (for reviews, see refs. 1 and 2). Consequently, many elderly subjects are at increased risk of infectious and noninfectious diseases that contribute to morbidity and mortality. Whether the decline in immune function with aging is due to the aging process *per se* or is secondary to poor health or other lifestyle factors, in particular to suboptimal nutritional status, is still unclear. Nevertheless, several studies have demonstrated that nutritional supplementation may improve immune responses in the elderly.^{3–8}

The intestinal microbiota play a key role in defense mechanisms, but its composition is substantially altered during the aging process.^{9,10} It is possible that such changes may contribute to the attenuated host defense mechanisms in the elderly. Probiotics are defined as living microorganisms, which, when administered in sufficient numbers, confer health benefits to the host beyond that of inherent basic nutrition.¹¹ As such, the use of probiotics is an attractive means to improve the immune responses in the elderly.¹² Although the clinical significance of such use has been rarely evaluated, consumption of some probiotic strains by healthy elderly has been shown to improve host immunity and reduce the incidence of infections. Elderly subjects who consumed fermented yoghurt containing live bacteria had consistently lower levels of immunoglobulin E (IgE) antibodies and reported fewer nasal allergies than similarly aged subjects who did not consume yoghurt.¹³ Furthermore, a diet supple-

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mented with *Lactobacillus casei* strain Shirota enhanced the percentages of circulating T helper and natural killer (NK) cells in elderly patients suffering from colorectal cancer.¹⁴ Of interest, this probiotic strain had no immune-enhancing effect in adults aged from 40 to 65 years, even though the probiotic strain modulated the composition and the metabolic activity of the intestinal flora.¹⁵ More recently, a series of pre-post controlled trials showed that dietary supplementation with two probiotic strains from the lactobacilli and bifidobacteria genera *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019 were effective in promoting some aspects of cellular immunity in healthy middle-aged and elderly subjects. More specifically, increased blood cell phagocytosis and NK cell-mediated tumor-killing activity was observed.^{16–19} It has also been shown that milk fermented with yoghurt cultures and *L. casei* DN-114001 was effective in reducing the severity (duration and maximal temperature) but not the incidence of gastrointestinal and respiratory infections in healthy free-living elderly during the winter season.²⁰ Recently, it was reported that enteral administration of a fermented milk containing *L. johnsonii* NCC533 to elderly inpatients may reduce the risk of infections by improving nutritional status and reinforcing immune function—decreasing tumor necrosis factor- α (TNF- α) and increasing phagocytic activity.²¹

L. paracasei NCC2461, a probiotic strain from the Nestlé probiotic collection, was selected on the basis of its safety, its industrial properties (yield, stability), and its *in vitro* antimicrobial activity. Previously, it has been shown to produce antagonist metabolites and antioxidants and to stimulate cells of the immune system.²² Interestingly, oral administration of a supplement containing *L. paracasei* NCC2461 combined with prebiotics (FOS/inulin) and a cocktail of micronutrients has been shown to reduce the incidence of winter infections in free-living elderly, possibly by improving natural host defense mechanisms such as NK activity and the interferon- γ (IFN- γ) response to influenza antigen stimulation.²³ Although the same group failed to demonstrate modulation of immune function by the prebiotics alone in the same target population,²⁴ other preclinical and clinical trials have shown that these prebiotics improve immune parameters.^{25–28}

The aim of the present study was to determine the efficacy of *L. paracasei* NCC2461, when combined or not with prebiotics (FOS/inulin) to support the results previously obtained in free-living elderly²³ and to extend the study by examining the effect of the supplementation on the humoral and cellular immune functions of aged mice.

More specifically, antigen-specific serum IgG_{2a} and IgG₁ levels, measured after immunization with keyhole limpet haemocyanin, were used as markers of T helper (Th)1- and Th2-dependent humoral responses, respectively. Delayed type hypersensitivity (DTH) response was used to evaluate effects on cellular Th1-dependent immunity. Finally, major bacterial species were investigated in fecal samples to examine whether changes in immune parameters correlated with a change in the composition of the microbiota.

Methods

Animals and housing

Specific pathogen-free male C57BL/6J mice purchased at 4 weeks of age from Charles River Laboratories (France),

were housed in a temperature- and light-controlled environment (12 h light/dark cycle, temperature 22°C, humidity 56%). Animals had *ad libitum* access to water and the semi-synthetic Kliba 3434 diet until initiation of the study, and they were provided with shredded paper bedding for social activity. Mice were housed 5 per cage until the age of 5 months, after which time, they were housed individually. Because C57BL/6 male mice have a long life span of about 26–28 months and are the most widely used for gerontological studies, 21-month-old mice of this strain were used as model of aging.

Dietary and experimental conditions

At 21 months of age, mice were randomized into one of three groups of 10 or 15 animals. Control mice (H₂O, $n = 10$) and *L. paracasei* NCC2461-supplemented mice (NCC2461, $n = 15$) received a complete semisynthetic diet containing 14% proteins and 5% cellulose (following the American Institute of Nutrition, AIN-93 protocol). Mice supplemented with probiotic combined with prebiotics (NCC2461 + FOS/inulin, $n = 15$) received the same semisynthetic diet in which the cellulose was replaced by 5% of a FOS and inulin mix (70/30). A spray-dried powder of the probiotic *L. paracasei* NCC2461 (Nestec Culture Collection, Nestle Ltd., Konolfingen, Switzerland) was dissolved in sterilized mineral water as drinking water. Water was replaced every other day with new suspensions of the probiotic. Bacterial intake was estimated at $\sim 1 \times 10^9$ colony-forming units (CFU) of *L. paracasei* NCC2461 per animal per day. Throughout the 44-day study period, mice were allowed free access to diet and drinking water. Individual mouse weights were recorded once a week. At the end of the study, *i.e.*, 44 days after starting the experimental diets, all mice were euthanized under anesthesia.

Immunization protocol

The T cell-dependent humoral response (antigen-specific antibody production) was measured *in vivo* in mice after immunization with keyhole limpet hemocyanin (KLH), an innocuous protein isolate that generates a strong T cell-mediated antibody response and has been used extensively in animals. All aged mice were immunized on day 15 with a subcutaneous injection of 100 μ g of KLH (Brenntag Biosector, Frederikssund, Denmark) emulsified in 1% alum. Blood samples were collected on days 0, 15, 29, and 44 for serum or plasma preparation.

DTH response

The DTH response was used as an *in vivo* measure of cellular immunity. The response was calculated as the difference in ear thickness before and after challenge with a recall antigen. Briefly, on day 22 of the study, *i.e.*, on day 7 after immunization with KLH, ear thickness of the mice was measured with a micrometer. Immediately thereafter, the DTH response was elicited by intradermal injection of KLH (10 μ L of 0.5 μ g/ml) into the right ears. The left ear, injected with vehicle alone (saline = phosphate-buffered saline [PBS]), served as internal control for each animal. Twenty four hours postelicitation, and for 6 days thereafter, the thickness of both the control (left ear) and the treated ear (right ear) was measured. The DTH responses (KLH-PBS) were expressed

as the magnitude of ear swelling, using the following formula: Δ in ear thickness = [treated ear (right, KLH) ear thickness – control (left, PBS) ear thickness], where Δ in ear thickness = [post-elicitation – pre-elicitation ear thickness].

Blood sampling

Blood collected from the tail vein on days 0, 15, and 29 of the study was allowed to clot overnight at 4°C. The serum was separated by centrifugation at $13,000 \times g$ for 10 min at 4°C. At sacrifice (day 44), blood was collected into 1.5-mL tubes containing 60 μ L of 50 mM EDTA; the plasma was retrieved after a 5-min centrifugation at 1,500 rpm. Aliquots of serum and plasma were stored frozen at –20°C until analysis.

Fecal sampling for analysis of the microbiota

Fresh fecal pellets were collected on day 37 after the introduction of experimental diets, directly homogenized in Ringer medium containing 10% glycerol, and then frozen at –80°C until analysis of the microbiota. The enterobacteria, lactobacilli, bifidobacteria, and enterococci were enumerated after plating on semiselective media according to the method previously described,²⁵ and their identity was confirmed by microscopy. The detection limit for all of the assessed bacteria was dilution 10^{-3} . Bacterial counts were expressed as the mean of Log₁₀ of CFU/gram of feces $\pm$ standard error of the mean (SEM). The identification of bifidobacteria was confirmed by PCR using species-specific primers as previously described.²⁹

Proliferative response of splenocytes

Spleen was collected post mortem, i.e., 44 days after beginning the experimental diets. Splenocytes were resuspended at 10^6 cells/mL in RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin (Sigma), 1% HEPES (Gibco), 0.1% 2-mercaptoethanol (Gibco), and 10% heat-inactivated fetal calf serum (FCS; Amimed). Cell viability was confirmed by Trypan Blue exclusion. Cell proliferation assay, in the absence or the presence of 10 μ g/mL concanavalin A (ConA; Sigma, St. Louis, MO), 5 μ g/mL lipopolysaccharide (LPS from *E. coli* serotype 055:B5; Sigma), or 100 μ g/mL KLH was performed using [³H]thymidine, as previously described.³⁰ All samples were set up in duplicate and the mean counts per min (cpm) calculated. The stimulation index (SI) was expressed as the cpm of stimulated cells over the cpm of nonstimulated cells.

Flow cytometry analysis

Single-cell suspensions of splenocytes were analyzed by flow cytometry (FACScalibur equipped with CellQuest analysis software, Becton Dickinson, Franklin Lakes, NJ) for cell-surface antigen expression using the following antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 (clone GK1.5; Pharmingen, Becton Dickinson, Franklin Lakes, NJ), FITC-conjugated anti-mouse CD3 (clone 17A2; Pharmingen), FITC-conjugated anti-mouse CD8 (clone 53-6.7; Pharmingen), phycoerythrin (PE)-conjugated anti-mouse CD44 (clone IM7; Pharmingen), and PE-conjugated anti-NK cells (NK1.1, clone PK136; Cedarlane, Ontario, Canada). Unlabeled cells were used as negative controls.

Determination of KLH-specific IgG concentrations in sera and plasma

The levels of KLH-specific total IgG, IgG₁, and IgG_{2a} in the sera were determined by enzyme-linked immunoassay (ELISA). Briefly, microtiter plates were coated with KLH (50 μ L/well at 5 ng/mL and 100 ng/mL for total IgG, and IgG₁ and IgG₂, respectively) and incubated at 37°C for 3 h. Free binding sites were blocked with ELISA buffer for 1 h at 37°C. Serum samples were then added and the plates were incubated at 4°C overnight. Bound antibodies were detected following incubation with a biotin-conjugated goat anti-mouse IgG(H+L), IgG₁ (γ 1 chain specific), or IgG_{2a} (γ 2a chain specific) from Southern Biotechnologies (Birmingham, USA) for 1 h at 37°C with shaking. Plates were read at 450 nm after the addition of the TMB peroxidase substrate from KPL. Anti-KLH IgG (H+L) titers were expressed as the reciprocal of the highest dilution that yielded an optical density at 454 nm (OD_{450nm}) that was twice that of preimmunized mice. Anti-KLH IgG₁ and IgG_{2a} were expressed as mean OD_{450nm} values.

Statistical analyses

Data were expressed as the median $\pm$ standard error (SE) of the median (based on the estimator Sn of Rousseeuw) or as the mean $\pm$ SEM. The various parameters at each time point were analyzed using the Kruskal–Wallis test, followed by the Wilcoxon test for comparison of two specific groups. The percentage of fecal samples with detectable bifidobacteria was compared using the Fisher exact test. Probability values of less than 5% were considered significant. Analysis was performed with the software R 2.6.1 (<http://www.R-project.org>).

Results

Effect of *L. paracasei* NCC2461 supplementation on body weight of aged mice

As shown in Fig. 1, there was no significant difference in the body weights of the different groups. All median body weight values were slightly increased in all groups at the initiation of the study, between day 1 and day 37 (H₂O, 35.2 ± 2.4 to 36.7 ± 1.3 ; NCC2461, 37.2 ± 1.5 to 39.3 ± 1.4 ; NCC2461 + FOS/inulin, 35.9 ± 1.7 to 38.9 ± 1.7). This effect was probably due to the switch in diet before and during the study period (Kliba-3436 and AIN-93 diet, respectively).

Effect of *L. paracasei* NCC2461 supplementation on humoral responses in aged mice

To assess the effect of dietary supplementation with *L. paracasei* NCC2461 $\pm$ FOS/inulin on B cell functions in aged mice, total and antigen-specific antibody levels were measured in immunized aged mice fed with the different experimental diets. Total immunoglobulin levels at systemic and mucosal sites (i.e., total IgG in sera and total IgA in feces, respectively) were not affected by the dietary intervention (data not shown), indicating that supplementation of aged mice with the probiotic *L. paracasei* NCC2461, with or without FOS/inulin, did not alter nonspecific humoral immune responses. Significant levels of antigen-specific, anti-KLH IgG(H+L), were induced in aged mice after immunization, indicating that aged mice are capable of producing

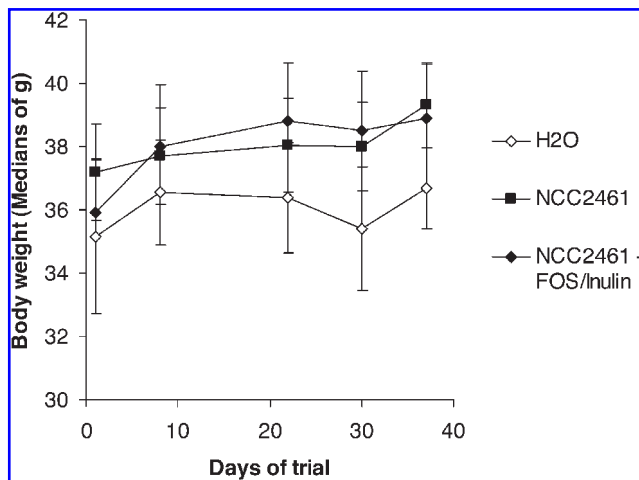


FIG. 1. Body weight of C57BL/6J male aged mice (21 months old) fed a normal diet and control drinking water (H_2O , $n = 10$), or drinking water supplemented with *L. paracasei* NCC2461, without or with prebiotic FOS/inulin in the diet (NCC2461, $n = 15$; NCC2461 + FOS/inulin, $n = 15$, respectively) for 44 days. Values are medians $\pm$ SE of medians.

an antigen-specific B cell response; however, the antibody titers were not statistically different between the groups (data not shown). As shown in Fig. 2, the levels of KLH-specific IgG₁ and IgG_{2a} were increased in the supplemented mice when compared to the nonsupplemented control mice. Although not statistically significant, when compared to the control group (H_2O), a trend toward increased anti-KLH IgG₁ production was found in the two NCC2461-supplemented groups on day 44 of the study ($p = 0.096$ for NCC2461 vs. H_2O and $p = 0.072$ for NCC2461 + FOS/inulin vs. H_2O) (Fig. 2A). Enhanced anti-KLH IgG_{2a} production was significant for both NCC2461-supplemented groups at both day 29 and day 44 of the study (day 29, $p < 0.001$ for NCC2461 vs. H_2O and $p = 0.005$ for NCC2461 + FOS/inulin vs. H_2O ; day 44, $p = 0.013$ for NCC2461 vs. H_2O and $p = 0.002$ for NCC2461 + FOS/inulin vs. H_2O , respectively) (Fig. 2B). Of note, this increase in anti-KLH IgG_{2a} was not significantly different between the group fed NCC2461 alone and the group fed NCC2461 + FOS/inulin.

Effect of *L. paracasei* NCC2461 supplementation on cellular immune responses in aged mice

DTH response was examined to assess the effect of dietary supplementation with *L. paracasei* NCC2461 $\pm$ FOS/inulin mix on T cell functions in aged mice in vivo. As shown in Fig. 3, consumption of *L. paracasei* NCC2461, with or without FOS/inulin, significantly increased the DTH response when compared to the controls ($p \leq 0.001$ at days 1, 2, 6, and 7, and $p = 0.024$ at day 3 for NCC2461 vs. H_2O ; $p < 0.001$ at days 3, 6, and 7 and $p = 0.002$ at days 1 and 2 for NCC2461 + FOS/inulin vs. H_2O). Of note, the DTH responses of *L. paracasei* NCC2461-supplemented mice and of NCC2461 + FOS/inulin-supplemented mice were not significantly different, although a slight but nonsignificant difference was observed on day 2 ($p = 0.080$ for NCC2461 vs. NCC2461 + FOS/inulin).

Effect of *L. paracasei* NCC2461 supplementation on T cell subsets and proliferative response in splenocytes of aged mice

To evaluate further the effect of dietary supplementation with *L. paracasei* NCC2461 $\pm$ FOS/inulin on T cells in aged mice, the proportion of CD3⁺, CD4⁺, CD8⁺, CD44^{high} (memory T cells), CD44^{low} (naïve T cells), and NK cells in splenocytes were measured. The proportions of these cell types were not significantly affected by NCC2461 supplementation with or without prebiotics (data not shown).

Spleen cell proliferative responses to ConA (T cell mito-

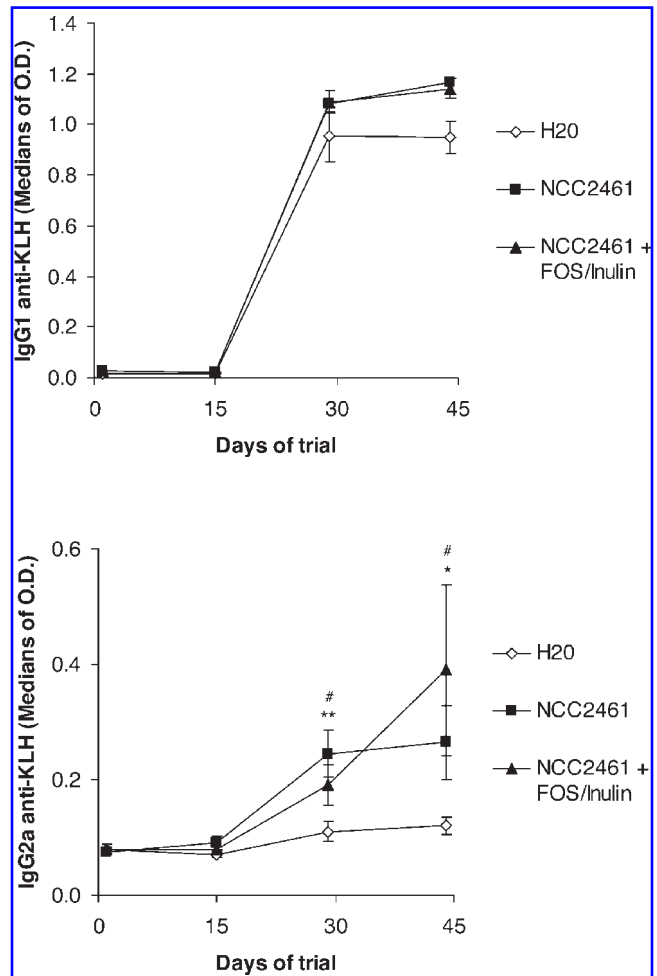


FIG. 2. Anti-KLH IgG₁ and IgG_{2a} isotype responses in aged mice (21 months old) fed a normal diet and control drinking water (H_2O , $n = 9$) or drinking water supplemented with *L. paracasei* NCC2461, without or with prebiotic FOS/inulin in the diet (NCC2461, $n = 14$; NCC2461 + FOS/inulin, $n = 13$, respectively). Probiotic bacteria were given in the drinking water and prebiotics were incorporated in the diet. Antibody levels were determined by ELISA, as described in Methods, on blood samples collected at different days of the study, before and after s.c. injection of KLH, and then diluted 1/1,000 for IgG₁ and 1/100 for IgG_{2a} for analysis. Values are medians of measured OD_{450nm} $\pm$ SE of medians for each group of mice. (**) $p < 0.001$; (*) $p < 0.05$ comparing NCC2461 to control H_2O ; (#) $p < 0.01$ comparing NCC2461 + FOS/inulin to control H_2O .

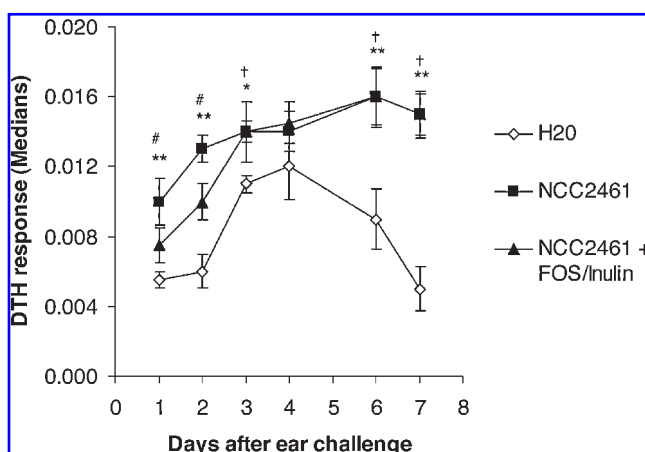


FIG. 3. DTH responses in aged mice fed a normal diet and control drinking water (H₂O, $n = 10$) or drinking water supplemented with *L. paracasei* NCC2461, without or with prebiotic FOS/inulin in the diet (NCC2461, $n = 15$; NCC2461 + FOS/inulin, $n = 14$, respectively). DTH responses were measured as described in Methods. The DTH response was calculated by measuring the difference in ear thickness between the right (KLH) and the left ear (PBS). Values represent medians $\pm$ SE of medians. (**) $p \leq 0.001$; (*) $p < 0.05$ comparing NCC2461 to control H₂O; (†) $p < 0.001$; (#) $p < 0.01$ comparing NCC2461 + FOS/inulin to control H₂O.

gen) and LPS (B cell mitogen) were similar in all groups (data not shown). Interestingly, while a cellular proliferative response to KLH was observed for all groups *ex vivo*, no effect of dietary treatment was observed (data not shown). In addition, the production of Th1- (IL-2 and IFN- γ) and Th2- (IL-4) like cytokines by ConA-stimulated splenocytes of aged mice *ex vivo* was similar for all groups (data not shown).

Effect of *L. paracasei* NCC2461 supplementation on the microbiota of aged mice

Key members of human intestinal microbiota such as enterobacteria, enterococci, lactobacilli, and bifidobacteria were analyzed in mouse feces at day 37 of the study. All aged mice were colonized with enterobacteria and enterococci (data not shown) and lactobacilli (Table 1), whereas no bifidobacteria were detected (Table 1). Dietary supplementation with *L.*

paracasei NCC2461, with or without FOS/inulin, had no effect on the enterobacteria and enterococci counts in aged mice (data not shown). However, a significant increase in fecal lactobacilli counts was observed in the two NCC2461-supplemented groups (Table 1). Furthermore, a marked increase in bifidobacteria counts, and as such, the percentage of mice colonized with bifidobacteria, was observed in the two NCC2461-supplemented groups (Table 1), particularly in the group also receiving FOS/inulin.

Discussion

In the present study, we investigated the effects of oral administration of the probiotic *L. paracasei* strain NCC2461, with or without prebiotics FOS/inulin, on the immune system of aged mice. Supplementation with *L. paracasei* NCC2461 enhanced Th1 cell-dependent immune responses. More specifically, it increased the DTH response and reinforced adaptive humoral responses to specific antigen. There was no significant difference between the group receiving the probiotic alone and the group also receiving prebiotics, indicating that the prebiotic FOS/inulin did not further improve the beneficial immune modulatory action of the probiotic.

Few other studies have reported the influence of probiotics on immunity in aged mice. It has been suggested that the oral administration of heat-killed *Lactococcus lactis* subsp. *cremoris* H61 in senescence-accelerated mice SAMP-6 mice may enhance the Th1-type immune response, as spleen cells from mice fed H61 strain were found to produce more interleukin-12 [IL-12] and IFN- γ than those from control mice³¹; however, no other immunological analysis was performed in this study. It has also been reported that oral administration of the probiotic *L. casei* strain Shirota improves influenza viral infection in aged mice, most likely via activation of cellular immunity, as shown by increased NK activity and production of IFN- γ and TNF- α by nasal lymphocytes.³² Recently, it has been demonstrated that intake of the probiotic *L. johnsonii* La1 reinforces intestinal and systemic immunity in aged mice by respectively, increasing fecal IgA antibodies to diphtheria toxin (DT) and restoring the levels of serum anti-DT IgA and IgG as well as spleen cell proliferation and CD8⁺ T cells numbers in aged mice with protein-energy malnutrition (PEM).³³ Interestingly, the systemic effect was seen in aged mice with PEM and not in aged mice receiving the control diet, suggesting that *L. johnsonii*

TABLE 1. LACTOBACILLI AND BIFIDOBACTERIA COUNTS IN FECAL SAMPLES OF AGED MICE THAT RECEIVED THE DIFFERENT EXPERIMENTAL DIET CONDITIONS FOR 37 DAYS

	<i>Lactobacilli</i>		<i>Bifidobacteria</i>	
	% of mice ^a	Counts ^b	% of mice ^a	Counts ^b
H ₂ O ($n = 9$)	100	8.2 $\pm$ 0.3	0	3
NCC2461 ($n = 14$)	100	9.4 $\pm$ 0.1**	28.6	8.2 $\pm$ 0.3
NCC2461 + FOS/Inulin ($n = 14$)	100	9.2 $\pm$ 0.2*	64.3 [†]	9.0 $\pm$ 0.6

Lactobacilli and bifidobacteria present in the feces of C57BL/6J aged mice after 37 days on the experimental diet conditions were counted as described in Methods. Only fecal samples from mice presenting more than 3 Log₁₀ were considered.

^aPercent of colonized mice.

^bValues represent mean of Log₁₀ of CFU/g $\pm$ SEM; limit of detection 3 Log₁₀. Statistically significant difference from the control group (H₂O): ** $p < 0.001$; * $p = 0.001$; and [†] $p = 0.003$.

La1 exerts a beneficial effect in a context of compromised immune condition.³³

In this study, we used a model of healthy aged mice. It has been shown that aging is associated with several dysfunctional stages of lymphocyte activation, particularly with progression of lymphocytes to a state of immune unresponsiveness to antigens and to an increased incidence of autoimmune disease.³⁴ The major changes are attributed to altered T lymphocyte function. Such age-related changes include thymic involution and a decrease in T cell proliferation as it could be reflected by a decrease in DTH response,^{35–38} or *in vitro* in lymphocytes cultures stimulated with mitogen or antigens.^{39,40} A shift in T cell subsets, with a decrease in the proportion of naïve T cells and an increase in memory/activated T cells with aging,^{41,42} would account for the decrease in IL-2 secretion, T cell proliferation, and DTH responses observed in the aged population. Hence, the age-associated impairment in cell-mediated immunity is mainly characterized by the failure of elderly subjects to mount an efficient DTH response.^{35–37} This response is a reliable indicator of the cell-mediated immune response *in vivo*. DTH is a type IV hypersensitivity reaction mediated by antigen-specific effector Th1 cells that release cytokines, which then act on vascular endothelium and cause a transient inflammatory reaction. A positive DTH reflects an effective immune response of the host to antigen challenge. Low, or no, DTH responses were shown to be associated with increased morbidity and mortality.^{43–46} In the present study, we found that *L. paracasei* NCC2461 intake markedly enhanced the DTH response in aged mice and, as such, may improve host immune competence.

In general, the B cell compartment of the immune system is only affected to a minor extent by senescence. Nevertheless, antigen-specific responses to vaccination are known to be altered in aging.⁴⁷ In agreement with previous observations,⁴⁸ we found that the ability of aged B cells to respond appropriately to an exogenous T-dependent antigen such as KLH, was preserved in old mice. Results demonstrated that *L. paracasei* NCC2461 reinforced antigen-specific immunoglobulin production. More specifically, anti-KLH IgG levels were increased in the serum of aged mice supplemented with *L. paracasei* NCC2461, whereas systemic total IgG and mucosal total IgA levels were not affected. Thus, oral supplementation with *L. paracasei* NCC2461 may improve immune responses in the aged host, without altering immune homeostasis.

It is widely accepted that the balance between Th1 and Th2 cells is critical for determining the outcomes of various diseases. The relative production of IgG_{2a} and IgG₁ isotypes can be used as a marker of the induction of Th1-like and Th2-like immune responses, respectively. In the present study, Th1-mediated humoral responses (anti-KLH IgG_{2a} levels) were enhanced in *L. paracasei* NCC2461-supplemented aged mice when compared to nonsupplemented control mice. Of note, the C57BL/6 aged mice immunized with KLH generated an anti-KLH IgG₁ response that was much higher than the anti-KLH IgG_{2a} response (10-fold factor), but this profile was not affected by probiotic supplementation. This observation correlates with the age-associated shift toward a Th2-type immune response.⁴⁹ Indeed, there is an age-related decrease in Th1-associated cytokines (e.g., IL-2 and IFN- γ) with a parallel increase in Th2-associated cytokine secretion (e.g.,

IL-4).^{49–51} In the present study, IL-4, IFN- γ , and IL-4 production, which was measured *ex vivo*, in culture supernatant of splenocytes stimulated with the T cell mitogen ConA was the same whether or not the mice were supplemented mice, further suggesting that immune homeostasis was not altered by probiotics.

The human intestinal environment changes substantially with aging. There is a decrease in bifidobacteria fecal levels^{9,10,52} that might have important health consequences.^{53,54} In the present study, we found that the microbiota of aged mice appear comparable to that of elderly subjects. In an independent study with young adult mice of the same strain, we observed that the proportion of mice with bifidobacteria in their feces was higher than that in aged mice (30% versus 0%, with mean values of 8.1 versus <3, respectively). The fact that aged mice are poorly colonized with bifidobacteria is in agreement with the reduced number of bifidobacteria reported with aging in human feces and intestinal contents.^{9,10,52} In the present study, a significant increase in fecal lactobacilli and bifidobacteria counts was observed in the *L. paracasei* NCC2461-supplemented groups when compared to controls. These increased levels of lactobacilli are attributed, at least in part, to the presence of the probiotic *L. paracasei* NCC2461 and reflect its capacity to resist passage through the gastrointestinal tract. Although the specific levels of *L. paracasei* NCC2461 were not investigated in fecal samples in this study, in a previous similar study, live *L. paracasei* NCC2461 was detected in 100% of fecal samples 2 weeks after the beginning of oral administration.

Interestingly, in our study, the intake of *L. paracasei* NCC2461 also increased the bifidobacteria counts and, consequently, the percentage of aged mice colonized with bifidobacteria. Of note, the rates of positive mice and the level of CFU counts were similar to those observed previously in nonsupplemented young adult mice. Furthermore, in agreement with the well-reported prebiotic property of the FOS/inulin to stimulate the growth of bifidobacteria in the intestine,^{55,56} the intake of *L. paracasei* NCC2461 combined with FOS/inulin further increased the bifidobacteria counts and the percentage of aged mice colonized with bifidobacteria. The present study demonstrates for the first time in aged mice that intake of the probiotic *L. paracasei* NCC2461, with or without prebiotic FOS/inulin, modulated the microbiota composition by increasing the numbers of bifidobacteria and lactobacilli. This supports the use of this single probiotic strain to counteract the decreased levels of these genera that is seen with aging.

Several studies have shown that supplementation with prebiotics can improve immune function in the aged population.^{25,27} We hypothesized that a probiotic effect on immune response could be further improved by using prebiotics, but no apparent synergistic effect was observed in the present study. However, it is acknowledged that an effect of a prebiotic alone was not investigated, and that we cannot exclude the fact that our model was not sensitive enough to highlight a synergistic effect of the combined ingredients.

Although the mechanisms underlying the probiotic effect are not fully elucidated, the present results suggest that feeding of the probiotic *L. paracasei* NCC2461, with or without prebiotic FOS/inulin, in the elderly strengthens cell-mediated immune responses that might improve vaccine responsiveness and resistance to infectious diseases. In conclusion,

intake of the probiotic *L. paracasei* NCC2461 might be beneficial in counteracting the immune defects that occur with aging.

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