

Effect of *Lactobacillus paracasei* subsp. *paracasei*, *L. casei* 431 on immune response to influenza vaccination and upper respiratory tract infections in healthy adult volunteers: a randomized, double-blind, placebo-controlled, parallel-group study^{1–4}

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ABSTRACT

Background: Probiotics can modulate the immune system in healthy individuals and may help reduce symptoms related to respiratory infections.

Objective: The objective of the study was to investigate the effect of the probiotic strain *Lactobacillus paracasei* subsp. *paracasei*, *L. casei* 431 (Chr. Hansen A/S) (hereafter, *L. casei* 431) on immune response to influenza vaccination and respiratory symptoms in healthy adults.

Design: A randomized double-blind, placebo-controlled trial was conducted in 1104 healthy subjects aged 18–60 y at 2 centers in Germany and Denmark. Subjects were randomly assigned to receive an acidified milk drink containing $\geq 10^9$ colony-forming units of *L. casei* 431 ($n = 553$) or placebo ($n = 551$) for 42 d. After 21 d, subjects received the seasonal influenza vaccination. The primary outcome was seroprotection rate (anti-influenza antibody titers by hemagglutination inhibition) 21 d after vaccination. Other outcomes were seroconversion rate and mean titers, influenza A-specific antibodies and incidence, and duration and severity of upper respiratory symptoms. Antibiotic use and use of health care resources were recorded.

Results: There was no effect of *L. casei* 431 on immune responses to influenza vaccination. Generalized linear mixed modeling showed a shorter duration of upper respiratory symptoms in the probiotic group than in the placebo group (mean \pm SD: 6.4 ± 6.1 vs. 7.3 ± 9.7 d, $P = 0.0059$) in the last 3 wk of the intervention period. No statistically significant differences were found for incidence or severity.

Conclusions: Daily consumption of *L. casei* 431 resulted in no observable effect on the components of the immune response to influenza vaccination but reduced the duration of upper respiratory symptoms. The trial was registered at www.isrctn.com as ISRCTN08280229.

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Keywords: immune function, probiotics, upper respiratory tract infection, *Lactobacillus paracasei* subsp. *paracasei*, vaccination response, *L. casei* 431

INTRODUCTION

Upper respiratory tract infection (URTI)⁵ is the most common illness for which patients seek medical care, and the financial costs related to URTI are substantial (1). Because there are no effective antiviral therapies for most URTIs, other remedies that could reduce susceptibility to URTIs or decrease symptom load and thereby

the number of lost school and work days are needed. Interventions that improve immune function could serve this purpose.

Studying the functionality of the immune system in healthy humans poses a special challenge. The immune system has a high degree of excess capacity and redundancy, which makes it difficult to detect and interpret small responses to interventions such as nutrition (2). Exploring immune parameters in healthy subjects not being challenged by a pathogen has so far not provided valuable insight into how the immune system may react (3, 4). Moreover, no biomarkers of immune function reliably reflect an individual's resistance to an infection (2, 5). The use of an in vivo infection or challenge model is therefore considered to provide the best method for exploring the response of the immune system in healthy humans (2, 6). One of the suggested methods is the use of a vaccine challenge (2, 7). The vaccine challenge model is also included in the guidelines on the scientific requirements for health claims related to gut and immune function from the European Food Safety Authority (8).

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit to the

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² Supported by Chr. Hansen A/S. *L. casei* 431 is a registered trademark of Chr. Hansen A/S.

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⁵ Abbreviations used: AE, adverse event; GMT, geometric mean titer; HAI, hemagglutination inhibition; ILI, influenza-like illness; *L. casei* 431, *Lactobacillus paracasei* subsp. *paracasei*, *L. casei* 431 (Chr. Hansen A/S); URTI, upper respiratory tract infection; WURSS-24, Wisconsin Upper Respiratory Symptom Survey–24.

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host" (9). They are known to have beneficial effects on the gastrointestinal and immune systems (9, 10). However, the observed beneficial effects are bacterial strain dependent (9, 10). *Lactobacillus paracasei* subsp. *paracasei*, L. casei 431 (Chr. Hansen A/S) (hereafter, L. casei 431) is a probiotic bacterial strain with immunomodulatory activity, as demonstrated in several in vitro and animal studies (11–13). The effect of L. casei 431 has been investigated in a dose-response study in healthy unchallenged subjects and in 2 vaccine challenge studies. In the dose-response study, no effect on immune parameters was found, confirming the need for studying the immune function after a challenge (4). The vaccine challenge studies demonstrated an effect of the L. casei 431 probiotic strain, shown by an increase in protective antibody titers toward polio (14) and an increase in influenza vaccine-specific antibodies (15). Smaller vaccine challenge studies using other probiotic strains have also demonstrated higher antibody responses than seen in the absence of probiotics (16–19), whereas one large recently published study showed no effect (20). To our knowledge, no studies have investigated the effect of L. casei 431 on susceptibility to URTI.

This randomized controlled trial in a large population of healthy adults assessed whether supplementation with the L. casei 431 probiotic strain 1) improved the immune response to an influenza vaccine, shown by an increase in antibody titers (through hemagglutination inhibition) and specific antibodies, and 2) decreased the susceptibility to and/or symptom load of URTIs.

METHODS

Study design

The study was a randomized, double-blind, placebo-controlled, 2-arm parallel-group study in healthy adult volunteers. Study products were consumed daily for 6 wk, 3 wk before and 3 wk after a challenge with the seasonal influenza vaccination. A 2-wk run-in period after screening and before randomization was used to ensure eligibility of subjects and to wash out potential prestudy probiotics. Efficacy evaluations were performed at 4 visits: randomization visit (day -21), vaccination visit (baseline, day 0), and primary efficacy visit (3 wk after vaccination, day 21). A follow-up visit was scheduled 9 wk after the end of the intervention period (12 wk after vaccination, day 84). Information on common cold and influenza-like illness (ILI) was collected during the intervention and follow-up periods.

Ethics and subjects

The study was performed in accordance with the principles in the Declaration of Helsinki and Good Clinical Practice. Ethical approval was obtained from the National Committee on Health Research, Hillerød, Denmark (reference number H-1-2011-107) and Ethik-Kommission der Bayerischen Landesärztekammer, Munich, Germany (reference number 11075). The study was registered at www.isrctn.com as ISRCTN08280229. All individuals were informed about the study orally and in writing and gave their written informed consent to participate. The participants were identified through flyers and newspaper advertisements and enrolled at 2 centers: Harrison Clinical Research, Munich, Germany, and Department of Nutrition, Exercise and Sports, University of Copenhagen, Denmark, in September and October 2011.

Potential subjects were screened for eligibility and randomly allocated 1:1 into 2 groups. The study staff allocated randomization numbers consecutively to the subjects in the order in which they attended the randomization visit. Eligible subjects were healthy men and women aged 18–60 y with a BMI (in kg/m²) from 19 to 30. Exclusion criteria were the presence of acute disease requiring treatment, chronic immunologic diseases, cancers within the past 5 y, pregnancy or planning to become pregnant, intolerance of milk protein or lactose, hypersensitivity to any of the components of the vaccine, gastrointestinal surgery, antibiotic treatment or treatment with any drug known to affect the immune response within 1 mo before the trial, and consumption of probiotic products and fermented food during run-in. Furthermore, subjects were excluded if they had already received the seasonal influenza vaccine, were participating in another research study, had a fever at the time of recruitment, or had had influenza between August 2011 and the beginning of the study. Athletes who undertook intensive physical activity (≥ 14 h/wk) were also excluded.

Subjects were instructed to refrain from eating fermented dairy products in addition to products containing probiotics from screening until the end of the study.

Concomitant medication

Antimicrobial medication was prohibited from 1 mo before the screening visit until the end of the study. Subjects taking antibiotics during the run-in period were withdrawn from the study. If a randomly allocated subject needed a course of antibiotic treatment during the study, efforts were taken to separate the intake of the study product and the antibiotic treatment by at least 2 h, but the subject continued in the study and was included in the intention-to-treat population. Use of concomitant medication was documented from 1 mo before the screening visit until the end of the study.

Random assignment and blinding

The randomization list was provided by an independent statistician not involved in the study. A statistical program (SAS version 9.2; SAS Institute) was used to generate a list of random numbers in blocks of 8, which were assigned to the 2 treatment groups. Each product was labeled with a randomization number according to the randomization list, and the list was kept confidential during the study. Study subjects, the clinical team, statisticians, and the sponsor were blinded until the database had been locked. There was no difference in the packaging of the probiotic and the placebo products, and the labels differed only by randomization numbers. The blinding code was provided to the investigators after the statistical analyses were completed. Only the study supply coordinator at Chr. Hansen was unblinded to perform production and labeling of the study products.

Intervention/procedures

An acidified milk drink (100 mL) containing the probiotic strain L. casei 431 (ATCC55544) or a matching placebo, an acidified milk drink (100 mL) with no probiotic cultures, was provided to the subjects. Study products were manufactured at and provided by Chr. Hansen A/S. The probiotic milk drink contained a minimum of 1×10^9 CFUs, and subjects consumed 1 drink once daily for 6 wk. The placebo product was similar to



the active product in appearance, smell, and taste. Drinks were stored below 8°C after manufacture and at the study centers, and volunteers were asked to keep the products refrigerated.

Subjects received an intramuscular injection with 0.5 mL of influenza vaccine (Influvac; Abbott Health Care Products) 3 wk after the start of the intervention. The vaccine used was the seasonal influenza vaccine for the 2011–2012 season and contained antigens from A/California/7/2009-like virus, A/Perth/16/2009-like virus, and B/Brisbane/60/2008-like virus. At the vaccination visit, ear temperature was recorded to determine whether an acute febrile condition was present. If the subject had a temperature above 38°C, the visit was postponed for up to 4 d. If the elevated temperature persisted at the second visit, the subject was withdrawn from the study.

Endpoints

The primary efficacy variable was seroprotection rate defined, as the proportion of subjects with a hemagglutination inhibition (HAI) titer ≥ 40 at day 21 (3 wk after vaccination). HAI titers were determined for each of the 3 viral antigens in the vaccine (A/California/7/2009, A/Perth/16/2009, and B/Brisbane/60/2008).

Secondary variables were seroconversion rate, defined as the proportion of subjects with a prevaccination HAI titer < 10 and a postvaccination HAI titer ≥ 40 or subjects with a prevaccination HAI titer > 10 and at least a 4-fold increase in titer at day 21 (21). Additional secondary variables were geometric mean HAI titers (GMT) and influenza A-specific IgA in saliva at day 21.

Incidence, duration, and severity of common cold and ILI were recorded by subjects in a diary together with use of health care resources and reported as exploratory variables. Additional exploratory variables were influenza A-specific total IgG and subclasses IgG1 and IgG3 in serum at day 21 and HAI titers at day 84 (follow-up visit).

Information on adverse events (AEs) was collected from screening to the end of the study. An AE was defined as any untoward medical occurrence in a subject administered the test product, whether or not considered related to the study treatment or the vaccine. The study investigator rated relatedness of AEs to the study product or vaccine.

Blood sampling and laboratory methods

Serum was collected at days -21 , 0, 21, and 84 and frozen at -20°C until analysis. Saliva was collected by using a standardized saliva sampling kit (Saliva Collection System; Greiner Bio-One) and frozen at -80°C until analysis. HAI titers were determined at a WHO reference laboratory (Statens Serum Institut, Copenhagen, Denmark) by using a standard procedure. Influenza A-specific IgA in saliva was detected by using an ELISA method developed by Covance, Inc., in which IgA bound to influenza A antigen coated on microtiter plates was visualized by using anti-human IgA antibodies. Influenza A-specific total IgG in serum was determined by using a commercial kit according to the manufacturer's instruction (IBL-America). Influenza A-specific IgG1 and IgG3 in serum were determined by using a modification of the commercial kit for total IgG to allow for detection of the IgG subclasses 1 and 3. The analytic methods were validated and analyzed by Covance Inc. according to guidelines from International Conference of Harmonization (22).

Diary data

Subjects recorded data daily in a diary during the 6-wk intervention period (days -21 to 21) and the 9-wk follow-up period (days 22–84). The diary included the validated Wisconsin Upper Respiratory Symptom Survey (WURSS)–24, which was used to determine whether a subject had a URTI and to record the duration and severity of symptoms during a URTI episode. Once weekly, the diary also included the following question: “Have you been in contact with the health care system during the past week?” (answer yes/no).

The WURSS-24 is composed of 1 global severity item (“How sick do you feel today?”), 10 common cold-related symptom items, 3 ILI-related symptom items, 9 “interference with daily activity” items, and 1 global change item (23). A common cold episode was defined to start when “How sick do you feel today?” was answered as ≥ 1 (\geq “very mildly”) and ≥ 1 common cold symptom was present on 2 consecutive days and to last until the question “How sick do you feel today?” was answered “0” (not sick) for 2 consecutive days (24). An ILI episode was defined to start if 2 of the 3 symptoms (fever, body aches, and headache were present) or if fever plus one of the common cold symptoms (cough, sore throat, runny nose, plugged nose, feeling tired) were present for 2 consecutive days. Alternatively, one of the aforementioned criteria should have been fulfilled ≥ 1 d followed by days with symptoms of common cold, even if fever, body aches, or headache was not present after day 1. Incidence was defined as the number of episodes/total number of subjects in the intervention and follow-up periods, respectively.

Sample size calculation

The sample size was based on the primary endpoint, seroprotection rate for the 3 viral antigens in the vaccine. A significance level of 0.0166 was chosen to compensate for multiple testing of 3 primary efficacy endpoints. With a significance level of 0.0166, a placebo response rate of 30%, a difference of 10 percentage points between the probiotic and placebo groups, a 2-sided approach, and a potential dropout rate of 10%, a total of 1058 subjects were planned to be randomly allocated.

A separate sample size calculation was performed for the subgroup for the determination of influenza-specific IgA and IgG based on data from the study by Rizzardini et al. (15), and a subgroup of 178 subjects in each treatment arm was applied for the IgG and IgA assessments.

Statistical analysis

Data were analyzed by using appropriate statistical models, adjusting for relevant and preselected covariates. Age and sex were used as covariates in all models. Where a baseline value (antibody titers and antibody levels at day 0) was available, this was included in the model. A sensitivity analysis was performed, where applicable, by using the randomization date (day -21) as baseline. Center was included in all models, except for analysis of IgA and IgG, because samples for these parameters were obtained only at the German center. The binary primary endpoints were analyzed by using a logistic regression model. The output of this statistical analysis was a *P* value and OR (with 95% CI) for the chance of being seroprotected for probiotic vs.



placebo. Probiotic against placebo comparisons were performed for each viral strain separately.

A multivariate ANOVA was used for GMT, IgA, and IgG. Because data were nonnormally distributed, the ANOVA was performed on ranked data for the GMT analysis and on log-transformed data for the IgA and IgG analyses.

The following items were calculated from the WURSS-24 in subjects with common cold or ILI episodes: mean number of episodes, duration of each episode, and severity (questions 2–23 in WURSS-24). A mean score per episode was calculated based on the daily severity sum score for both intervention and follow-up periods.

An episode starting in the intervention period and continuing into the follow-up period was considered belonging to the intervention period. For each of the items, a linear mixed model was used to account for repeated measures and to test the effect of the probiotic strain on the chosen outcomes for each period. Period, sex, age, center, and treatment \times period interaction were included as covariates. Because normality of the data was not obtained, nonparametric analyses based on ranks were carried out.

A Cox proportional hazards regression model was used to analyze time to first common cold episode by using center, sex, and age as covariates.

All randomly allocated subjects who had consumed at least 1 dose of study product and had available efficacy data were included in the intention-to-treat analyses ($n = 1099$). The analyses of primary and secondary variables were also performed on the per-protocol population (subjects with no major protocol deviations, $n = 1060$).

The primary and secondary efficacy endpoints were adjusted for multiplicity by the Holm-Bonferroni method. None of the exploratory variables were adjusted for multiplicity. All statistical analyses were performed according to a written statistical analysis plan by using SAS version 9.2 (SAS Institute).

RESULTS

Subject disposition and compliance with study product

A total of 1104 subjects were eligible to participate in the study and were randomly allocated into the intervention groups. In total, 33 of the randomly allocated subjects discontinued the study (13 in the probiotic group and 20 in the placebo group). The most common reason for discontinuation was “subject withdrawal of consent for personal reasons” (11 of 33). A flowchart of participant involvement is shown in **Figure 1**. Scheduling of further randomization appointments was stopped when the total number of randomly allocated subjects had exceeded 1058.

The characteristics of the 2 study groups were similar at baseline (**Table 1**).

Treatment compliance was estimated by counting the number of returned unopened bottles during the intervention period. Compliance was $99.9\% \pm 2.5\%$ (mean \pm SD) in the probiotic group and $99.9\% \pm 2.5\%$ in the placebo group (NS).

Hemagglutination inhibition titers and influenza A-specific antibodies

There was no statistically significant difference in baseline seroprotection rates, GMT, IgA, or IgG between probiotic and placebo groups. Intention-to-treat analysis of the primary efficacy endpoint showed no significant difference between probiotic and placebo groups in protection rates at day 21 for any of the virus strains (**Table 2**). Analysis of seroconversion rates and GMT, IgA in saliva, and IgG and subclasses in serum also showed no differences between treatment groups (data not shown). Sensitivity analysis of the same parameters by using the per-protocol population or day -21 as baseline gave similar results. There was no heterogeneity in seroconversion rates or in any other endpoint between the 2 centers (data not shown).

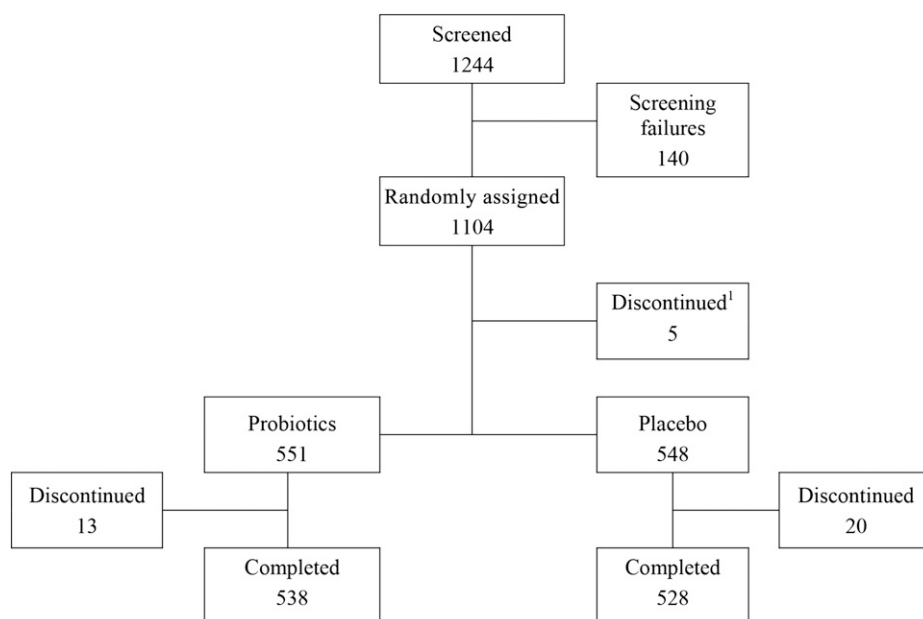


FIGURE 1 Disposition of the subjects. Values are expressed as the number of participants. ¹The subjects were discontinued before any efficacy data were obtained, and 2 were discontinued before receiving any study product. The main reasons for discontinuation were the following: subjects' wish for personal reasons (probiotics, $n = 6$; placebo, $n = 9$), terminated by the investigator because of infection at the vaccination visit or subjects' general condition (probiotics, $n = 1$; placebo, $n = 5$), or adverse events or serious adverse events (probiotics, $n = 3$; placebo, $n = 4$).

TABLE 1
Demographic and baseline characteristics of the subjects (intention-to-treat population)

Characteristic	Placebo (<i>n</i> = 551)	Probiotics (<i>n</i> = 548)
Age, y	31.3 (18.2–60.5) ¹	31.6 (18.2–60.8)
Men, <i>n</i> (%)	213 (38.9)	240 (43.6)
BMI, kg/m ²	23.8 (18.7–30.4)	23.7 (18.6–30.4)
Current smoker, <i>n</i> (%)	149 (27.0)	149 (27.0)
Influenza vaccination in 2010–2011, <i>n</i> (%)	32 (5.8)	37 (6.7)
Ethnic group, Caucasian/other, <i>n</i> (%)	524 (95.6)/24 (4.4)	539 (97.8)/12 (2.2)

¹Mean; range in parentheses (all such values).

Incidence, duration, and severity of common cold and ILI; use of antibiotics; and health care resources

The number of subjects with episodes and the total number of episodes for common cold and ILI are summarized in **Table 3**. An effect of the *L. casei* 431 probiotic strain on the number of episodes of common cold and ILI was not found (Table 3). A post hoc time-to-event analysis showed no significant impact of the probiotic strain on the risk of common cold (**Figure 2**). The preplanned analysis showed a trend for a shorter duration of common cold in the probiotic group in the intervention period (weeks 1–6) (mean \pm SD days; probiotics: 6.3 \pm 7.3; placebo: 7.1 \pm 8.5; *P* = 0.10). Therefore, a post hoc analysis was performed to investigate treatment effects in weeks 1–3, 4–6 (intervention period), 7–9, 10–12, and 13–15 (follow-up period) on incidence, duration, and severity of common cold and ILI episodes. The 3-wk periods were chosen based on the assumption that it takes \sim 3 wk for the probiotics to modulate the antiviral immune defense through the gut. The post hoc analysis of WURSS-24 scores in 3-wk periods showed a significant effect of the *L. casei* 431 probiotic strain on the duration of common cold and ILI in the second half of the intervention period (days 1–21) (**Table 4**). During the intervention period, the mean severity score for the common cold was 12% lower in the probiotic group compared with placebo, although not statistically significant (*P* = 0.12), whereas no effect was found on the severity of ILI.

TABLE 2
Seroprotection rates at day 21 (3 wk after vaccination) (intention-to-treat population)¹

Time point, strain	Placebo (<i>n</i> = 542) ²	Probiotics (<i>n</i> = 531) ²	OR ^{3,4} (95% CI), day 21
Day 0 (baseline), H1N1	116 (21.9)	108 (19.9)	
Day 21, H1N1	445 (83.8)	448 (82.7)	0.99 (0.70, 1.39)
Day 0 (baseline), H3N2	338 (63.7)	343 (63.3)	
Day 21, H3N2	526 (99.1)	536 (98.9)	0.81 (0.22, 3.01)
Day 0 (baseline), B	75 (14.1)	73 (13.5)	
Day 21, B	465 (87.6)	475 (87.6)	0.99 (0.69, 1.44)

¹Seroprotection rate is defined as proportion of subjects with a hemagglutination inhibition titer \geq 40. Day 0 (vaccination visit) was used as the baseline. Values are given as the number of seroprotected subjects (% of total). B, B/Brisbane/60/2008; H1N1, A/California/7/2009; H3N2, A/Perth/16/2009.

²Samples were missing or titers not available from 9 subjects in the placebo group and 17 in the probiotic group.

³OR of being seroprotected.

⁴None of the ORs were statistically significant when analyzed with logistic regression, including baseline, age, sex, and center as covariates in the model.

A significant effect was seen when use of health care resources was analyzed. During the follow-up period, 154 (28.2%) subjects had answered yes to the question “Have you been in contact with the healthcare system during the past week?” in the placebo group compared with 122 (22.1%) in the probiotic group (*P* = 0.028). There was no significant difference in the use of health care resources in the intervention period. The use of concomitant antibiotic medication during the study was lower in the probiotic group, in which 22 courses of antibiotics were taken, compared with 38 in the placebo group (*P* = 0.036).

Adverse events

A total number of 2212 AEs in 914 subjects were reported during the study. Of these, 41 events in 34 subjects (21 in the probiotic group and 20 in the placebo group) were assessed as study product related. The most prevalent of the product-related AEs were gastrointestinal disorders (48% of events) and nasopharyngitis (29%). In total, 373 events in 344 subjects (186 in the probiotic group and 187 in the placebo group) were assessed as vaccine related. Five AEs were defined as serious; none of these were assessed to be related to the study product or vaccine.

DISCUSSION

In the present study, we investigated the effect of *L. casei* 431 on the immune response to vaccination and on symptoms related to URTIs. Analyses of the primary and secondary efficacy variables showed no significant difference between probiotic and placebo groups; however, data showed a shorter duration of common cold and ILI episodes in weeks 4–6 in subjects who had consumed the probiotic product. Moreover, use of health care resources and number of antibiotic courses were both lower in the probiotic group.

To our knowledge, this is the first large randomized controlled trial investigating the effect of a probiotic strain on immune response to vaccination in immunocompetent healthy adults. Several smaller studies have reported a positive effect of probiotic strains on antibody response to vaccination (16–19). There are 2 reports of a positive effect of *L. casei* 431 from vaccine challenge studies, one using a polio vaccine (14) and another using an influenza vaccine in a similar design as the present study (15). The latter found that subjects who received the *L. casei* 431 probiotic strain had significantly increased antigen-specific immune response compared with subjects who received placebo. A recent study in >700 elderly subjects failed to show the effect of *L. casei* Shirota on immune response to influenza vaccination or on the susceptibility to URTI (20). The authors

TABLE 3

Number of common cold and ILI episodes during the intervention and follow-up periods (intention-to-treat population)¹

Illness, period	Placebo (n = 551)		Probiotics (n = 547)			
	n ²	% of total	E ³	n ²	% of total	E ³
Common cold						
Intervention	314	57.4	489	315	57.2	505
Follow-up	275	50.3	412	261	47.4	375
ILI						
Intervention	108	19.7	124	114	20.7	133
Follow-up	130	23.8	153	115	20.9	142

¹No significant differences were found when data were analyzed with a nonparametric linear mixed model, including period, sex, age, center, and treatment × period interaction as covariates. ILI, influenza-like illness.

²Number of subjects with an episode in the given period.

³Number of episodes in given period.

speculated whether the immune system of the elderly included in the study may not be sensitive for stimulation, which could be the reason for the lack of effect of the probiotic intervention.

The effectiveness (i.e., the observed protection rates) after a seasonal influenza vaccine varies from year to year (25). This could explain the differences between the results obtained in the current study using the 2011–2012 influenza vaccine and the study published by Rizzardini and coworkers (15) using the influenza vaccine for the 2008–2009 season. The results obtained in the current study revealed that the 2011–2012 vaccine was un-

expectedly efficacious, with seroprotection rates ranging from 82% to 99%. Even the B strain, which historically has resulted in seroprotection in ~35% of vaccinated individuals, had unexpectedly high postvaccination response rates (26). With response rates up to 99%, it might not have been possible to further increase protection rates and therefore not possible to detect any difference between the probiotic group and the placebo group. Alternatively, one could speculate that the potential effect exerted by the *L. casei* 431 probiotic strain could be detected by using other measures or biomarkers of the immune system.

The data from our study showed a shorter duration of symptoms during common cold and ILI episodes in the probiotic group compared with the placebo group. This is supported by data from other probiotic intervention trials showing that probiotics are capable of reducing the duration and potentially severity of upper respiratory symptoms (27–31). In these studies, the probiotic effect on URTI incidence is not so clear, which is also in line with our data, in which no effect on the incidence of URTI or ILI and no effect on time to URTI was found. In contrast, a Cochrane review that assessed the efficacy of probiotics for the prevention of acute URTIs found that probiotics were better than placebo in reducing the number of URTI episodes, whereas no effect was found on duration of episodes (32). This apparently conflicting evidence may be due to different effect and mechanism of action of different probiotics, as well as a high level of design heterogeneity and a generally low quality of the evidence included in the Cochrane review (33). In addition, 2 of the above-mentioned studies showing a positive effect of probiotics on the duration of

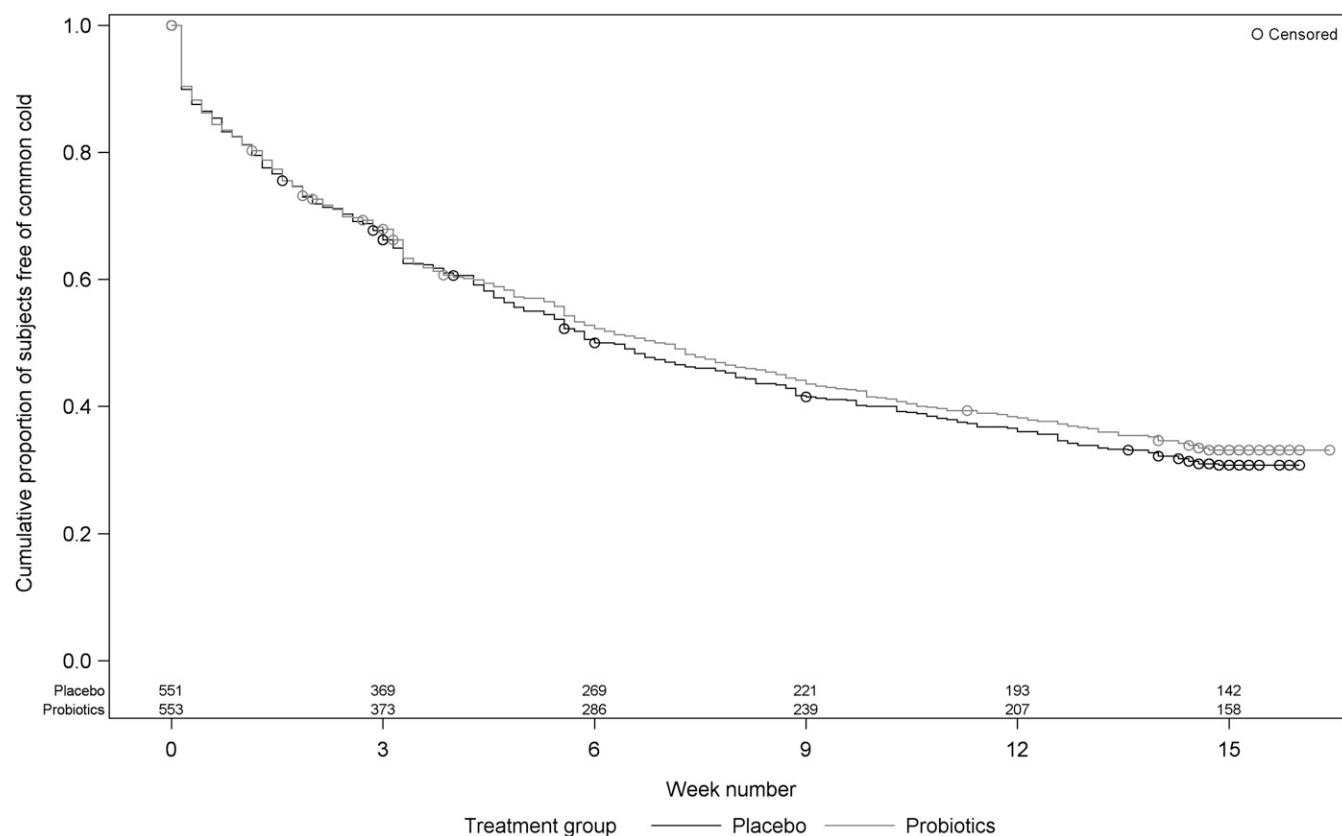


FIGURE 2 Time to common cold (intention-to-treat population). No statistically significant difference between placebo and probiotics was found when data were analyzed in a Cox proportional hazards regression model adjusted for center, sex, and age (HR: 0.97; 95% CI: 0.84, 1.12; $P = 0.67$). Weeks 1–6 were the intervention period, and weeks 7–15 were the follow-up period. Subjects at risk in the placebo and probiotics groups are provided at the bottom of the figure.

TABLE 4

Duration of common cold and influenza-like illness episodes in the 6-wk intervention period (days -21 to 21) (intention-to-treat population)

Illness	Placebo (n = 551)		Probiotics (n = 547)	
	Mean ± SD	Median (range)	Mean ± SD	Median (range)
Common cold				
Weeks 1-3 (days -21 to 0)	7.3 ± 9.7	4 (1-106)	6.4 ± 6.1	4 (2-50)
Weeks 4-6 (days 1-21)	6.8 ± 7.1	5 (2-67)	6.1 ± 9.5	4 (2-81) ¹
Influenza-like illness				
Weeks 1-3 (days -21 to 0)	7.8 ± 13.8	5 (1-106)	7.2 ± 5.5	6 (2-31)
Weeks 4-6 (days 1-21)	8.1 ± 10.5	4 (2-67)	4.8 ± 3.1	4 (2-15) ²

¹Generalized linear mixed modeling (post hoc) of duration of the common cold in weeks 4-6 of the intervention as the response variable showed a significantly lower duration in the probiotic group than in the placebo group ($P = 0.0059$ for the treatment effect). The model included period, sex, age, center, and treatment × period interaction as covariates, and the analysis was performed on ranks because data were not normally distributed.

²Generalized linear mixed modeling (post hoc) of duration of influenza-like illness in weeks 4-6 of the intervention as the response variable showed a significantly lower duration in the probiotic group than in the placebo group ($P = 0.017$ for the treatment effect). The model included period, sex, age, center, and treatment × period interaction as covariates, and the analysis was performed on ranks because data were not normally distributed.

URTIs were not included in the Cochrane review (27, 28). Furthermore, the clinical diagnosis of URTI is based on symptoms. Because healthy adults are unlikely to seek medical care for mild and uncomplicated URTIs, the symptoms are usually self-reported. Collecting valid subject-reported data is a challenge, and different methods for collecting data on URTI symptoms may also give rise to differences between studies. We chose to use the WURSS, which we believe is the best currently available and the only validated questionnaire for self-reported symptoms of URTI.

It is generally considered that symptoms related to URTI do not result from the infectious agents themselves but rather from the inflammatory response of the host toward the agent (34). Current knowledge suggests that probiotics work by stimulating the innate viral defense mechanisms (35), thereby potentially reducing the degree of inflammation in the host. This is supported by evidence of modulation of the inflammatory response in probiotic clinical studies. For example, high numbers of cytotoxic/suppressor T cells (CD8⁺) and T-helper cells (CD4⁺) in combination with lower disease duration were found in the probiotic group compared with the placebo group by de Vrese et al. (31). It is likely that the effect on URTI duration observed in this study is through modulation of the host inflammatory response by the *L. casei* 431 probiotic strain. Future research is needed to establish the effect of the *L. casei* 431 probiotic strain on biomarkers of the immune system related to anti-inflammation to elucidate the mechanism of action of this probiotic strain on URTI duration. Such biomarkers may include regulatory T cells, which reduce inflammation and can be induced by probiotic strains (36, 37). In support of this, Johnstone et al. (38) recently demonstrated that regulatory T cells are associated with reduced risk of viral respiratory infection in the elderly.

The finding of a shorter duration of illness in the probiotic group is supported by the observation that subjects in this group had less use of health care resources and fewer antibiotic courses probably due to less severe URTI symptoms. The data on duration should be interpreted with some caution because the endpoint was qualified as exploratory and the analysis was performed post hoc with no correction for multiplicity. However, considering that the clinical effect has been found consistently in several randomized controlled trials of probiotics, these findings are likely to

be valid. A confirmation in an intervention trial with duration and severity of upper respiratory symptoms as the primary outcome is warranted.

One strength of the current study is that it was a highly controlled and fully blinded study performed according to good clinical practice. A further strength is that it was conducted as a 2-center study with no heterogeneity between outcomes at the 2 centers. A final strength is the large sample size of the current study. It may be considered a weakness of the study that the antibody response to the vaccination was so strong in both groups that there was little dynamic range to actually observe a difference between the groups with respect to the primary outcome. Furthermore, only the humoral immune response was studied. Helper T-cell activity is needed for antibody production against influenza, but the potential effects of the study product on antigen-specific cytotoxic T-cell function were not investigated in this study. However, relating individual aspects of the immune response to antibody production is complex because of the many cell types involved in generating an antibody response and to the many interactions among those cells, as discussed elsewhere (36). Probiotics may affect the activity of certain immune cell types and not others, with these effects seeming to be probiotic strain specific (36). Hence, directly linking immunologic measurements with antibody responses to vaccines is likely to be challenging, especially given the paradoxical observations that probiotic strains may induce regulatory T cells, as demonstrated in animal models (36), which could impair antibody production.

In conclusion, in the present study, *L. casei* 431 did not show an effect on antibody titers and influenza A-specific antibodies 3 wk after influenza vaccination. The data suggest that the *L. casei* 431 probiotic strain may reduce the duration of common cold and ILI episodes in healthy adults. This could point toward a positive effect of the *L. casei* 431 probiotic strain on the immune system despite no effect being observable by using the vaccination model.

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REFERENCES

- Footitt J, Johnston SL. Cough and viruses in airways disease: mechanisms. *Pulm Pharmacol Ther* 2009;22:108–13.
- Albers R, Antoine JM, Bourdet-Sicard R, Calder PC, Gleeson M, Lesourd B, Samartin S, Sanderson IR, Van Loo J, Vas Dias FW, et al. Markers to measure immunomodulation in human nutrition intervention studies. *Br J Nutr* 2005;94:452–81.
- Lomax AR, Calder PC. Probiotics, immune function, infection and inflammation: a review of the evidence from studies conducted in humans. *Curr Pharm Des* 2009;15:1428–518.
- Christensen HR, Larsen CN, Kaestel P, Rosholm LB, Sternberg C, Michaelsen KF, Frokiaer H. Immunomodulating potential of supplementation with probiotics: a dose-response study in healthy young adults. *FEMS Immunol Med Microbiol* 2006;47:380–90.
- Calder PC. Immunological parameters: what do they mean? *J Nutr* 2007;137:773S–80S.
- Burleson GR, Burleson FG. Influenza virus host resistance model. *Methods* 2007;41:31–7.
- Cummings JH, Antoine JM, Azpiroz F, Bourdet-Sicard R, Brandtzaeg P, Calder PC, Gibson GR, Guarner F, Isolauri E, Pannemans D, et al. PASSCLAIM—gut health and immunity. *Eur J Nutr* 2004;43(Suppl 2): III18–73.
- European Food Safety Authority Panel on Dietetic Products, Nutrition and Allergies. Guidance on the scientific requirements for health claims related to gut and immune function. *EFSA J* 2011;9:1984.
- FAO/WHO. Guidelines for the evaluation of probiotics in food: report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. London, Ontario, Canada: WHO/FAO; 2002.
- Rijkers GT, Bengmark S, Enck P, Haller D, Herz U, Kalliomaki M, Kudo S, Lenoir-Wijnkoop I, Mercenier A, Myllyluoma E, et al. Guidance for substantiating the evidence for beneficial effects of probiotics: current status and recommendations for future research. *J Nutr* 2010;140:671S–6S.
- Villena J, Barbieri N, Salva S, Herrera M, Alvarez S. Enhanced immune response to pneumococcal infection in malnourished mice nasally treated with heat-killed *Lactobacillus casei*. *Microbiol Immunol* 2009;53:636–46.
- Haro C, Villena J, Zelaya H, Alvarez S, Agüero G. *Lactobacillus casei* modulates the inflammation-coagulation interaction in a pneumococcal pneumonia experimental model. *J Inflamm (Lond)* 2009;6:28.
- Marranzino G, Villena J, Salva S, Alvarez S. Stimulation of macrophages by immunobiotic *Lactobacillus* strains: influence beyond the intestinal tract. *Microbiol Immunol* 2012;56:771–81.
- de Vrese M, Rautenberg P, Laue C, Koopmans M, Herremans T, Schrezenmeier J. Probiotic bacteria stimulate virus-specific neutralizing antibodies following a booster polio vaccination. *Eur J Nutr* 2005;44:406–13.
- Rizzardini G, Eskesen D, Calder PC, Capetti A, Jespersen L, Clerici M. Evaluation of the immune benefits of two probiotic strains *Bifidobacterium animalis* ssp. *lactis*, BB-12 and *Lactobacillus paracasei* ssp. *paracasei*, *L. casei* 431 in an influenza vaccination model: a randomised, double-blind, placebo-controlled study. *Br J Nutr* 2012;107:876–84.
- Boge T, Remigy M, Vaudaine S, Tanguy J, Bourdet-Sicard R, van der Werf S. A probiotic fermented dairy drink improves antibody response to influenza vaccination in the elderly in two randomised controlled trials. *Vaccine* 2009;27:5677–84.
- Davidson LE, Fiorino AM, Snyderman DR, Hibberd PL. *Lactobacillus GG* as an immune adjuvant for live-attenuated influenza vaccine in healthy adults: a randomized double-blind placebo-controlled trial. *Eur J Clin Nutr* 2011;65:501–7.
- Olivares M, Diaz-Ropero MP, Sierra S, Lara-Villoslada F, Fonolla J, Navas M, Rodriguez JM, Xaus J. Oral intake of *Lactobacillus fermentum* CECT5716 enhances the effects of influenza vaccination. *Nutrition* 2007;23:254–60.
- French PW, Penny R. Use of probiotic bacteria as an adjuvant for an influenza vaccine. *Int J Probiotics Prebiotics* 2009;4:175–80.
- Van Puyenbroeck K, Hens N, Coenen S, Michiels B, Beunckens C, Molenberghs G, Van Royen P, Verhoeven V. Efficacy of daily intake of *Lactobacillus casei* Shirota on respiratory symptoms and influenza vaccination immune response: a randomized, double-blind, placebo-controlled trial in healthy elderly nursing home residents. *Am J Clin Nutr* 2012;95:1165–71.
- Schmidt-Ott R, Schwarz T, Haase R, Sander H, Walther U, Fourneau M, Htun-Myint L, Sanger R, Schuster V. Immunogenicity and re-actogenicity of a trivalent influenza split vaccine in previously unvaccinated children aged 6–9 and 10–13 years. *Vaccine* 2007;26:32–40.
- International Conference on Harmonisation. Validation of analytical procedures: text and methodology Q2 (R1). ICH Harmonised Tripartite Guideline CPMP/ICH281/95; 2005. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1_Guideline.pdf.
- Barrett B, Hayney MS, Muller D, Rakel D, Ward A, Obasi CN, Brown R, Zhang Z, Zgierska A, Gern J, et al. Meditation or exercise for preventing acute respiratory infection: a randomized controlled trial. *Ann Fam Med* 2012;10:337–46.
- Barrett B, Brown RL, Mundt MP, Thomas GR, Barlow SK, Highstrom AD, Bahrainian M. Validation of a short form Wisconsin Upper Respiratory Symptom Survey (WURSS-21). *Health Qual Life Outcomes* 2009;7:76.
- Osterholm MT, Kelley NS, Sommer A, Belongia EA. Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis. *Lancet Infect Dis* 2012;12:36–44.
- Xie H, Jing X, Li X, Lin Z, Plant E, Zoueva O, Yang H, Ye Z. Immunogenicity and cross-reactivity of 2009-2010 inactivated seasonal influenza vaccine in US adults and elderly. *PLoS ONE* 2011;6:e16650.
- Smith TJ, Rigassio-Radler D, Denmark R, Haley T, Touger-Decker R. Effect of *Lactobacillus rhamnosus* LGG(R) and *Bifidobacterium animalis* ssp. *lactis* BB-12(R) on health-related quality of life in college students affected by upper respiratory infections. *Br J Nutr* 2013;109:1999–2007.
- Guillemard E, Tondou F, Lacoïn F, Schrezenmeier J. Consumption of a fermented dairy product containing the probiotic *Lactobacillus casei* DN-114001 reduces the duration of respiratory infections in the elderly in a randomised controlled trial. *Br J Nutr* 2010;103:58–68.
- Turchet P, Laurenzano M, Auboiron S, Antoine JM. Effect of fermented milk containing the probiotic *Lactobacillus casei* DN-114001 on winter infections in free-living elderly subjects: a randomised, controlled pilot study. *J Nutr Health Aging* 2003;7:75–7.
- Pregliasco F, Anselmi G, Fonte L, Giussani F, Schieppati S, Soletti L. A new chance of preventing winter diseases by the administration of synbiotic formulations. *J Clin Gastroenterol* 2008;42(Suppl 3 Pt 2): S224–33.
- de Vrese M, Winkler P, Rautenberg P, Harder T, Noah C, Laue C, Ott S, Hampe J, Schreiber S, Heller K, et al. Effect of *Lactobacillus gasseri* PA 16/8, *Bifidobacterium longum* SP 07/3, *B. bifidum* MF 20/5 on common cold episodes: a double blind, randomized, controlled trial. *Clin Nutr* 2005;24:481–91.



32. Hao Q, Lu Z, Dong BR, Huang CQ, Wu T. Probiotics for preventing acute upper respiratory tract infections. *Cochrane Database Syst Rev* 2011;9:CD006895.
33. Sæterdal I, Underland V, Nilsen ES. The effect of probiotics for preventing acute upper respiratory tract infections. *Glob Adv Health Med* 2012;1:124–5.
34. Kirchberger S, Majdic O, Stockl J. Modulation of the immune system by human rhinoviruses. *Int Arch Allergy Immunol* 2007;142:1–10.
35. Bron PA, van Baarlen P, Kleerebezem M. Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. *Nat Rev Microbiol* 2012;10:66–78.
36. West NP, Cripps AW. Are vaccination models suitable to determine whether probiotics have beneficial health effects in the general population? *Hum Vaccin Immunother* 2013;9:621–4.
37. de Roock S, van Elk M, van Dijk ME, Timmerman HM, Rijkers GT, Prakken BJ, Hoekstra MO, de Kleer IM. Lactic acid bacteria differ in their ability to induce functional regulatory T cells in humans. *Clin Exp Allergy* 2010;40:103–10.
38. Johnstone J, Parsons R, Botelho F, Millar J, McNeil S, Fulop T, McElhaney J, Andrew MK, Walter SD, Devereaux PJ, et al. Immune biomarkers predictive of respiratory viral infection in elderly nursing home residents. *PLoS ONE* 2014;9:e108481.

