

Effects of seven potential probiotic strains on specific immune responses in healthy adults: a double-blind, randomized, controlled trial

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Received 13 November 2007; revised 26 February 2008; accepted 26 February 2008.

DOI:10.1111/j.1574-695X.2008.00413.x

Editor: Patrick Brennan

Keywords

probiotics; immune response; salivary and serum immunoglobulin; oral vaccine; healthy adults; randomized controlled trial.

Introduction

Probiotic bacteria have been suggested to confer a range of health benefits both in children (Van Niel *et al.*, 2002; Saavedra, 2007) and adults (Santosa *et al.*, 2006). Among the possible mechanisms explaining these effects is direct or indirect modulation of the intestinal immune system. Specific probiotic strains have indeed been shown to enhance local immunity through innate cell surface pattern recognition receptors or via direct lymphoid cell activation (Isolauro *et al.*, 2001; Cross, 2002). This stimulation seems to be strain specific, with differences between strains being due to the differences in their cell wall protein profiles (Galdeano & Perdigon, 2004) or CpG content of their DNA (Lammers *et al.*, 2003).

Abstract

This pilot study investigated the immunomodulatory properties of seven probiotic strains. Eighty-three healthy volunteers aged 18–62 years consumed 2×10^{10} CFU of bacteria or a placebo (maltodextrin) over 3 weeks (D0–D21). Subjects received an oral cholera vaccine at D7 and at D14; blood and saliva samples were collected at D0, D21 and D28. Serum samples were analyzed for specific IgA, IgG and IgM, and saliva samples were analyzed for specific IgA only, by ELISA. Statistical analyses were based on Wilcoxon's signed-rank test (intragroup analyses) and exact median *t*-test (intergroup analyses). Salivary analysis showed no difference in specific IgA concentrations between groups. Serum analysis indicated an effect of some of the tested strains on specific humoral responses. Between D0 and D21, IgG increased in two probiotic groups, for example, *Bifidobacterium lactis* BI-04 and *Lactobacillus acidophilus* La-14, compared with controls ($P = 0.01$). Trends toward significant changes in immunoglobulin serum concentrations compared with controls ($P < 0.1$) were found for six out of the seven probiotic strains. In conclusion, some strains of probiotics demonstrated a faster immune response measured with serum immunoglobulin indicators, especially IgG, although overall vaccination was not influenced. Specific strains of probiotics may thus act as adjuvants to the humoral immune response following oral vaccination.

Beyond intestinal immune modulation, probiotics may induce systemic immune responses (Gill *et al.*, 2001), explaining that probiotic consumption has been linked to nongastrointestinal disease prevention or treatment in previous trials. For instance, elderly subjects whom consumed a combination of probiotics (*Lactobacillus gasseri*, *Bifidobacterium longum* and *Bifidobacterium bifidum*) had shorter and less severe common cold episodes than controls (de Vrese *et al.*, 2006).

Many probiotic bacteria have been tested for their immunomodulatory properties, especially *Lactobacillus* sp. (Rinne *et al.*, 2005) and *Bifidobacterium* sp. (Gill *et al.*, 2001; Mullie *et al.*, 2004; Rinne *et al.*, 2005; de Vrese *et al.*, 2006). To measure the adjuvant effects of probiotics following vaccine administration, trials have been carried out in

Table 1. Characteristics of subjects at inclusion (D0)

	<i>Bifidobacterium lactis</i> Bi-07 (n = 9)	<i>Bifidobacterium lactis</i> Bi-04 (n = 9)	<i>Lactobacillus acidophilus</i> La-14 (n = 9)	<i>Lactobacillus acidophilus</i> NCFM [®] (n = 9)	<i>Lactobacillus plantarum</i> Lp-115 (n = 9)	<i>Lactobacillus paracasei</i> Lpc-37 (n = 9)	<i>Lactobacillus salivarius</i> Ls-33 (n = 9)	Placebo (n = 20)
Age (years)*	35.3 (10.0)	38.0 (10.8)	34.5 (7.5)	40.6 (11.5)	35.0 (8.2)	44.5 (9.9)	35.5 (9.0)	34.5 (10.3)
Males (%)*	3/9 (33.3)	3/9 (33.3)	5/9 (55.5)	5/9 (55.5)	5/9 (55.5)	2/9 (22.2)	3/9 (33.3)	5/20 (25.0)
BMI (kg/m ²)*	23.8 (4.8)	23.4 (3.5)	22.5 (3.4)	24.3 (3.7)	21.8 (1.6)	23.9 (3.6)	21.9 (2.3)	22.6 (3.0)
Serum IgA (OD _{450 nm}) [†]	99.5 (0.0/378.3)	105.3 (26.0/353.3)	98.3 (50.0/639.3)	168.8 (55.3/1413.6)	85.0 (0.0/613.0)	127.6 (41.0/347.0)	85.3 (3.6/532.6)	50.6 (0.0/ 385.6)
Serum IgG (OD _{450 nm}) [†]	103.3 (13.5/1032.3)	134.3 (31.0/937.0)	136.3 (19.3/1276.3)	136.1 ^a (29.3/2027.6)	81.0 ^b (0.6/310.0)	80.5 ^a (17.0/377.6)	251.0 (3.6/2287.0)	566.3 (87.5/ 1887.3)
Serum IgM (OD _{450 nm}) [†]	61.6 (0.0/304.0)	75.0 (4.0/129.6)	74.0 (26.0/702.6)	55.1 (0.3/117.6)	56.0 (0.0/128.6)	51.6 (28.3/277.6)	105.0 (6.0/310.6)	49.6 (0.0/ 338.0)
Salivary IgA (OD _{450 nm}) [†]	0.08 (0.0/0.5)	0.20 (0.0/0.3)	0.08 (0.0/0.6)	0.14 (0.0/0.3)	0.14 (0.0/0.5)	0.02 (0.0/0.3)	0.10 (0.0/0.4)	0.11 (0.0/ 0.7)

*Data are presented as mean (SD).

[†]Data are presented as median (range).

^aP < 0.05 compared with placebo.

^bP < 0.01 compared with placebo (exact median t-tests).

children (Mullie *et al.*, 2004) and in adults (Link-Amster *et al.*, 1994; de Vrese *et al.*, 2005). Those trials demonstrate that specific probiotic strains are able to influence both local and systemic immune response and may improve immunization. Additional studies with new strains are still needed to confirm those results and better understand strain specificities.

In the current study, we compared the effects of seven probiotic strains, or a placebo, on the serum and salivary-specific immune responses after oral vaccination in healthy adults.

Materials and methods

Subjects

Eighty-three healthy volunteers aged 18–62 years participated in this randomized, double-blind, controlled study in eight parallel arms. Recruitment was performed by advertisements at the Lariboisière hospital, Paris, France. All participants gave their written informed consent upon inclusion. The study protocol was approved by the Ethics Committee of St-Germain-en-Laye (number 03052).

Inclusion criteria were: age between 18 and 65 years, normal clinical examination, no previous medical history related to allergy or immune disease, and affiliation to the French Health Care System. Premenopausal women had to use an effective contraceptive method to be eligible to the

study. Main exclusion criteria were: pregnant or breast-feeding women, previous history of vaccine allergy, vaccination against cholera or traveler's diarrhea in the previous 5 years, having a special diet or presenting gastrointestinal disorder likely to modify intestinal microbial communities, and taking medication regularly.

Subjects consuming products or supplements containing probiotics or prebiotics more than three times during the preinclusion period (D – 21–D0) were excluded from the analysis.

Bacterial strains

Seven freeze-dried industrial probiotics strains provided by Danisco Cultures (Paris, France) were used in the present study (Table 1), all of them being members of the genera *Lactobacillus* or *Bifidobacterium*. Subjects were to consume two capsules per day of the test product between D0 and D21. Each active capsule contained 1×10^{10} CFU of bacteria in a maltodextrin carrier. The control group received capsules containing only maltodextrin. All treatment packages were identical in presentation.

Vaccine

The vaccine used was a commercial oral preparation of cholera vaccine composed of inactivated *Vibrio cholerae* antigenic fractions (Dukoral[®]; SBL Vaccin AB, Stockholm, Sweden). It contains fractions from three strains of *V.*

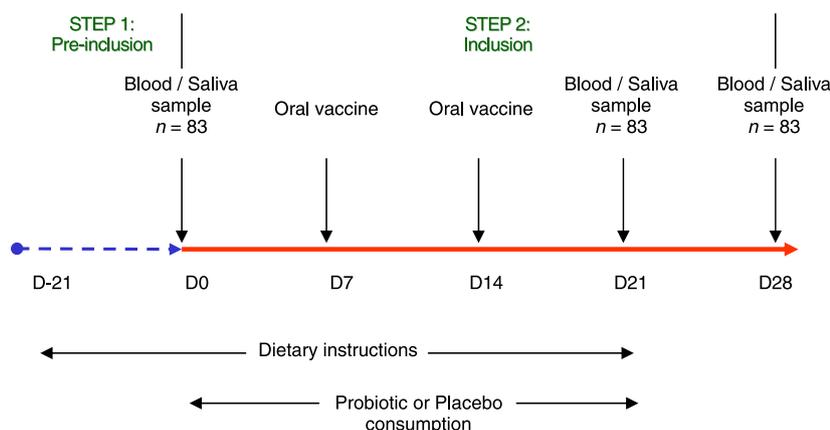


Fig. 1. Study protocol.

cholerae (Ogawa, Inaba and Inaba Eltor) and recombinant cholera toxin B; one vaccine dose consists of $c. 10^{11}$ vibriions.

Protocol

This study was carried out at the Therapeutic Research Unit, Lariboisière Hospital. Subjects were selected in two steps; preinclusion period (D – 21) and inclusion period (D0) (Fig. 1). At the preinclusion visit (D – 21), the investigator checked for inclusion/exclusion criteria and recorded the subject's demographic characteristics. Eligible subjects were instructed on how to avoid foods containing probiotics and prebiotics during the period D – 21 to D21, such as yogurt, yogurt-like products, cheese, raw sausages and fermented vegetables. They were also asked not to consume dietary supplements containing probiotics or prebiotics. All subjects were given a diary to assess compliance with these instructions and to record concomitant medication.

At the inclusion visit (D0), inclusion/exclusion criteria were checked again. Eligible subjects received their treatment pack consisting of two capsules per day to be consumed during 21 days. Compliance to ingestion of capsules was assessed using a specific questionnaire. Blood samples and saliva samples were collected at D0 (inclusion visit).

Four visits were planned at D7, D14, D21 and D28. Oral vaccination occurred at D7 and D14; subjects were to attend the study center without eating or drinking for at least 1 h before vaccination. Blood and saliva samples were collected at D21 and D28 for antibody determination (serum IgA, IgG and IgM, as well as salivary IgA). Samples were stored at -20°C until analysis.

Blood and saliva sample collection

Blood samples were collected using two 5-mL vacutainer tubes, and subsequently centrifuged at 1200 g for 10 min at 4°C . Four aliquots of serum were collected using cryotubes

(CRYOVIAL[®] CRY1JS 1.2 mL) and were stocked at -20°C until analysis.

Two salivary samples of at least 1 mL each were collected in 5-mL tubes. The liquid fraction was transferred to two cryotubes (CRYOVIAL[®] CRY1JS 1.2 mL) and was stored at -20°C until analysis.

Determination of specific immunoglobulin

An ELISA method adapted from the GM1 dosage was used to determine serum-specific IgA, IgG and IgM and salivary-specific IgA (Svennerholm & Holmgren, 1978; Svennerholm & Wiklund, 1983; Berquist *et al.*, 1997). This method is based on the binding of cholera toxin to solid-phase-coupled GM1, utilizing the known affinity of this toxin for the cholera toxin receptor ganglioside GM1.

Plates were coated with a GM1 solution (Calbiochem, San Diego, CA), before being incubated for 2 h at room temperature with cholera toxin ($0.5\ \mu\text{M mL}^{-1}$). Two hundred microliters of GM1 solution were added in each well ($2\ \mu\text{g mL}^{-1}$ concentration) and incubated at room temperature overnight. All plates were coated at the same time, with the same GM1 solution. Plates were washed three times with 200 μL of phosphate-buffered saline (PBS; Calbiochem) and were blocked with 200 μL of 0.1% bovine serum albumin (BSA; Sigma-Aldrich Corp., St Louis, MO) in PBS (Calbiochem) by incubation for 1 h at 37°C .

Plates were washed three times with 200 μL of PBS, and were incubated with the B-subunit of cholera toxin (CTB) (Dukoral, SBL Vaccin AB, Stockholm, Sweden), $0.5\ \mu\text{g mL}^{-1}$ ($100\ \mu\text{L wells}^{-1}$), 2 h at room temperature. Then plates were washed three times with 200 μL of PBS-Tween 20 0.2% (Calbiochem, San Diego, CA). Serum or salivary samples were added and plates were incubated 2 h at room temperature. Plates were washed again three times in 200 μL of PBS-Tween 0.2%. Plates were then incubated with

HRP-conjugated goat-anti-human secretory component (Nordic Immunological Laboratories, Tilburg, the Netherlands) or HRP-conjugated goat anti-human IgA (Jackson Immuno Research, West Grove) or HRP-conjugated goat anti-human IgG (Fc γ -specific) (Jackson Immuno Research) or HRP-conjugated goat anti-human IgM (Jackson Immuno Research), in order to measure, respectively, specific salivary IgA, serum IgA, serum IgG and serum IgM. All those reagents were diluted in PBS, with a dilution of 1:5000 except for goat-anti-human secretory component (1:2000). Plates were washed four times in PBS-Tween 0.2%. The plates were developed using 100 μ L solution of *o*-phenyldiamine (OPD, Calbiochem) and H₂O₂ (Calbiochem): one tablet of OPD was diluted in 12.5 mL citrate buffer (50 mmol L⁻¹) and 12.5 μ L of 30% H₂O₂ was added. Plates were incubated 20 min at room temperature. The reaction was stopped by addition of 50 μ L of H₂SO₄ (1.8 N) and plates were read at 450 nm. For each plate, we used two positive controls using serum samples kindly provided by the Pasteur Institute (Paris, France).

Statistical analyses

The sample-size calculation for this study was based on the previously reported effects of probiotics on specific humoral response following oral vaccination (Link-Amster *et al.*, 1994). We aimed at comparing each intervention group against the control group. With a two-sided 0.05 significance level ($\alpha = 0.05$) and serum-specific IgA change as the primary outcome measure, studying seven subjects in each intervention group and 17 subjects in the control group makes it possible to detect a significant difference at 80% power. To account for an expected 15% dropout rate, we decided to recruit nine subjects in each intervention group and 20 subjects in the control group (total 83 subjects). We intentionally allocated a higher number of subjects in the control group in order to maintain the global type 1 error (α) at the 0.05 level.

Statistical analyses were all conducted using the SAS statistical program (version 8.2) (SAS Institute Incorporated, Cary, NC). Changes in categorical variables were assessed with χ^2 -test or Fisher's exact tests. Intragroup comparisons for continuous variables were performed using Wilcoxon's signed-rank tests on medians, because data showed non-normal distribution (Kolmogorov-Smirnov test) and/or nonhomogeneity of variances (Levene's test). Intergroup comparisons were performed using exact median *t*-test. We decided to compare groups using medians rather than means in order to account for non-normal distribution of data.

Baseline characteristics are reported as means \pm SDs or medians (range). Changes throughout the study are reported as medians with ranges.

Results

Subjects

All volunteers completed the study. Consumption of unauthorized foods was very limited (only four out of 83 subjects consumed unauthorized foods at least once during the period D-21/D21), with no differences between the probiotic groups and the control group. Similar results were found regarding medication: respectively four and six subjects consumed unauthorized drugs during the period D-21/D0 and during the period D0-D21, with no differences compared with controls. All subjects showed excellent compliance to the treatment consumption; only one subject did not consume its two capsules for 1 day.

Groups did not differ for gender, age or BMI at inclusion (Table 1). Regarding immunoglobulin measurements at inclusion, a lower concentration was found for serum IgG in three groups compared with controls: *Lactobacillus acidophilus* NCFM[®] ($P = 0.03$), *Lactobacillus plantarum* Lp-115 ($P < 0.001$) and *Lactobacillus paracasei* Lpc-37 ($P = 0.01$). All other immunoglobulin measurements were similar in probiotic groups compared with the control group.

Immunoglobulin evolution following vaccine administration

Blood samples were obtained from all subjects at D0, D21 and D28. Changes in serum immunoglobulin during the periods D0-D21 (early response) and D21-D28 (late response) are presented in Table 2. During the early response, serum IgG tended to increase during probiotic consumption, with significant increases compared with controls in subjects consuming *Bifidobacterium lactis* Bl-04 and *L. acidophilus* La-14 ($P = 0.01$). No significant differences were found for changes in serum IgA and IgM. Change in salivary IgA was smaller in the group consuming *Lactobacillus salivarius* Ls-33 compared with placebo ($P = 0.01$).

During the late response, no differences were found regarding changes of immunoglobulin in the probiotic groups compared with placebo. Intragroup analysis showed that serum IgA and IgM increased in subjects consuming *L. acidophilus* NCFM[®] (respectively $P = 0.03$ and 0.02). Salivary IgA increased in the placebo group during this period ($P = 0.04$).

The overall vaccination titer was not influenced by the administration of the probiotics during the vaccination protocol, Table 3. Although the group consuming *B. lactis* Bl-04 and *L. plantarum* Lp-115 showed a lower level of serum IgM, $P = 0.09$ and 0.01, respectively.

Table 2. Ig evolutions during the periods D0–D21 and D21–D28*

	Absolute changes in immunoglobulin between D0 and D21				Absolute changes in immunoglobulin between D21 and D28			
	Serum IgA (OD _{450 nm})	Serum IgG (OD _{450 nm})	Serum IgM (OD _{450 nm})	Salivary IgA (OD _{450 nm})	Serum IgA (OD _{450 nm})	Serum IgG (OD _{450 nm})	Serum IgM (OD _{450 nm})	Salivary IgA (OD _{450 nm})
<i>Bifidobacterium lactis</i>	−13.8	8.9 ^d	5.7	0.08	−0.3	2.8	−5.8	0.06
Bi-07	(−71.3/7.3)	(−666.0/125.3)	(−14.6/54.6)	(−0.3/0.2)	(−139.6/147.3)	(−300.6/442.3)	(−60.6/102.0)	(−0.3/0.7)
<i>Bifidobacterium lactis</i>	6.0	29.0 ^{a,b}	2.3	0.06	0.3	−4.3	−2.6	−0.01
Bl-04	(−108.0/358.6)	(−15.1/1771.0)	(−31.0/53.3)	(−0.2/0.4)	(−140.3/445.1)	(−149.0/29.0)	(−135.0/29.6)	(−0.3/0.4)
<i>Lactobacillus acidophilus</i>	−30.0 ^{d,e}	32.3 ^{a,e}	−19.6 ^e	0.00	−11.0 ^d	8.6	3.6	0.08 ^c
La-14	(−176.3/76.0)	(−84.3/450.0)	(−261.3/18.5)	(−0.1/0.2)	(−84.3/64.6)	(−355.0/477.3)	(−23.3/125.6)	(−0.0/0.4)
<i>Lactobacillus acidophilus</i>	−46.5	−1.0	−2.8	0.00	50.6 ^{c,d}	14.3	15.8 ^c	0.02
NCFM [®]	(−309.3/390.3)	(−426.0/164.6)	(−41.6/94.6)	(−0.0/0.3)	(−6.0/440.0)	(−16.1/728.3)	(−5.5/465.0)	(−0.3/0.0)
<i>Lactobacillus plantarum</i>	24.6	40.6 ^{d,e}	4.3	−0.02	−16.6	−1.3	−3.6	0.03
Lp-115	(−206.0/616.6)	(−19.3/616.6)	(−47.3/61.0)	(−0.1/0.2)	(−241.6/59.3)	(−141.3/76.0)	(−146.6/27.3)	(−0.1/0.6)
<i>Lactobacillus paracasei</i>	−3.3	−2.3	9.6	0.02	−17.6	24.6	−11.0 ^{d,f}	0.01
Lpc-37	(−125.3/235.3)	(−117.6/61.1)	(−47.3/73.3)	(−0.0/0.6)	(−167.6/392.0)	(−26.6/439.8)	(−61.6/443.3)	(−0.0/0.0)
<i>Lactobacillus salivarius</i>	31.0	−10.3	17.6	−0.04 ^a	0.0	−7.6	15.6	0.00
Ls-33	(−56.3/953.0)	(−284.6/203.6)	(−82.6/376.3)	(−0.2/0.0)	(−902.0/310.6)	(−304.0/73.5)	(−386.3/178.6)	(−0.0/0.3)
Placebo	0.6	−14.0	11.6	0.06 ^b	3.1	33.6	5.6	0.05 ^c
	(−136.0/25.0)	(−594.6/383.3)	(−60.3/60.8)	(−0.3/0.3)	(−64.8/599.3)	(−225.6/1210.0)	(−64.5/166.0)	(−0.5/0.6)

*Data are presented as median (range).

^a $P < 0.05$ compared with placebo (intergroup analysis, exact median t -tests).

^b $P < 0.05$ compared with D0 (intragroup analysis, Wilcoxon's signed-rank tests).

^c $P < 0.05$ compared with D21 (intragroup analysis, Wilcoxon's signed-rank tests).

^d $0.05 < P < 0.1$ compared with placebo (intergroup analysis, exact median t -tests).

^e $0.05 < P < 0.1$ compared with D0 (intragroup analysis, Wilcoxon's signed-rank tests).

^f $0.05 < P < 0.1$ compared with D21 (intragroup analysis, Wilcoxon's signed-rank tests).

Table 3. Immunoglobulin evolutions during the periods D0–D28*

	Absolute changes in immunoglobulin between D0 and D28			
	Serum IgA (OD _{450 nm})	Serum IgG (OD _{450 nm})	Serum IgM (OD _{450 nm})	Salivary IgA (OD _{450 nm})
<i>Bifidobacterium lactis</i>	3.8	1.2	3.0	0.16
Bi-07	(−186.0/153.6)	(−223.6/93.3)	(−41.6/146.3)	(−0.08/0.53)
<i>Bifidobacterium lactis</i>	−25.0	24.6	−13.0 ^a	0.00
Bl-04	(−124.0/424.0)	(−88.0/1793.0)	(−81.6/61.0)	(−0.35/0.69)
<i>Lactobacillus acidophilus</i>	−16.0	39.0	−6.0	0.14
La-14	(−242.3/56.0)	(−16.6/816.1)	(−223.6/136.0)	(−0.01/0.64)
<i>Lactobacillus acidophilus</i>	−4.8	38.3	8.6	0.01
NCFM [®]	(−297.3/830.3)	(−15.3/394.6)	(−5.8/423.3)	(−0.07/0.24)
<i>Lactobacillus plantarum</i>	−38.0	11.0	−12.0 ^b	0.13
Lp-115	(−186.0/375.0)	(−88.0/550.6)	(−85.6/5.3)	(−0.15/0.46)
<i>Lactobacillus paracasei</i>	2.0	15.6	11.6	0.08
Lpc-37	(−163.6/348.3)	(−93.0/396.0)	(−39.3/396.0)	(−0.12/0.61)
<i>Lactobacillus salivarius</i>	32.6	−65.6	18.3	−0.00
Ls-33	(−87.0/254.3)	(−435.0/102.5)	(−116.0/215.3)	(−0.19/0.21)
Placebo	0.3	5.33	12.83	0.10
	(−91.0/613.6)	(−327.3/615.3)	(−34.0/105.6)	(−0.19/0.66)

*Data are presented as median (range).

^a $P < 0.05$ compared with placebo (intergroup analysis, exact median t -tests).

^b $0.05 < P < 0.1$ compared with placebo (intergroup analysis, exact median t -tests).

Discussion

This pilot study was designed to obtain preliminary results regarding the effects of seven probiotic strains on immune

response following oral vaccination in healthy adults. In the present study, we have shown that probiotic supplementation may influence systemic antibody response, with strain-specific effects.

We aimed at measuring the effects of probiotics both on local and systemic responses. The oral vaccine we used is suggested to have a local effect, which may be measured in saliva or intestinal fluids (Langevin-Perriat *et al.*, 1988). This is also indicated by the increase in salivary IgA in the placebo group and the fact that the other groups, with the exception of *L. salivarius* Ls-33, had a similar increase in salivary IgA titer. However, we also determined serum IgA, IgM and IgG, the latter being particularly important as it leads to immune memory. Previous studies using oral cholera vaccination have indeed considered salivary IgA as potential indicators for intestinal IgA response (Jertborn *et al.*, 1986, 1984). However, our results indicate that measurement of specific salivary IgA has technological difficulties. In a previous trial, Forrest also found that specific salivary IgA is a poor indicator of specific intestinal IgA response after oral vaccination (Forrest, 1992).

We did not find significant effects of probiotics on specific serum IgA or IgM. This may be related to insufficient immunogenicity of the oral vaccine, as suggested by the low immune response in the placebo group, receiving vaccine only. This, despite that the methodology used measured toxins A and B (through GM1 binding), which are the most antigenic components of the vaccine.

With a more potent vaccine, clearer differences might thus have been obtained. This hypothesis is supported by explanatory analyses. When analyzing data using a two-sided 0.1 significance level ($\alpha = 0.1$), we indeed found that all tested strains, except *L. salivarius* Ls-33, had significant effects on serum immunoglobulin evolutions compared with controls (Table 2). Previous trials also increases in specific serum immunoglobulin following a combination of probiotics and oral vaccination against *Salmonella* Typhi (Link-Amster *et al.*, 1994), rotavirus (Isolauri *et al.*, 1995), polio (de Vrese *et al.*, 2005) and *Haemophilus influenzae* (Kukkonen *et al.*, 2006).

In the current study, two out of the seven strains (*B. lactis* Bl-04 and *L. acidophilus* La-14) increased IgG in an early response compared with controls 7 days after second vaccine administration. In addition, another *B. lactis* strain (Bi-07) and *L. plantarum* Lp-115 also tended to induce an increase ($P = 0.09$) in specific serum IgG. These findings are interesting, since IgG are involved in immune memory and thus may contribute to disease prevention in the long term.

Regarding serum IgA, *L. acidophilus* La-14 tended to decrease serum IgA during the early response ($P = 0.09$), and during the late response ($P = 0.05$); this may be related to the concomitant increase observed in serum IgG. On the contrary, *L. acidophilus* NCFM[®] tended to exhibit a late increased response in serum IgA ($P = 0.09$), while no early response was observed for this parameter by this group. A late decreased response in serum IgM was observed in

subjects consuming *L. paracasei* Lpc-37 compared with controls ($P = 0.09$). This may related to the, albeit nonsignificant, increased early response in serum IgM from and the increased late response in serum IgG.

The improved early response may indicate that the probiotic strains tested actually cause a faster response to the vaccination. But did not cause a change in overall vaccination response in the control group compared with the group not receiving probiotics.

We found that different strains from the same species (*B. lactis* and *L. acidophilus*) behave different. This finding confirms strain-specific effects of probiotics on immunity, which have been linked to e.g. specific bacterial cell wall protein profiles (Galdeano & Perdigon, 2004). Similar findings have been reported in previous trials involving several strains or species.

The mechanisms by which probiotics modulate the immune response are not fully understood. The adjuvant effects of probiotics may be mediated by components of the protoplast and the cell wall-like lipoteichoic acid and polysaccharide-peptidoglycan complexes (de Vrese *et al.*, 2005). For instance, muramyl peptides, which are components of peptidoglycans, are known to stimulate endogenous secretion of cytokines. Lipoteichoic acid and polysaccharide-peptidoglycan complexes are ligands of toll-like receptors, particularly TLR2, which are expressed by intestinal epithelial cells and immunocytes.

This pilot study indicates that several probiotic strains may modulate immune response to oral vaccination and induce a faster increase in serum IgG concentrations. It confirms that specific serum immunoglobulin, but not specific salivary immunoglobulin are relevant indicators of immunomodulation by probiotics. Further randomized, controlled studies including a larger number of subjects could be performed to understand the immunomodulatory effects of selected probiotics and its consequences in terms of disease prevention. Such a study would also benefit from the inclusion of a probiotic strain with known effects on specific immunity from an earlier vaccination trial.

Acknowledgement

We would like to thank Jean-Michel Fournier (Cholera and Vibriosis Unit, Pasteur Institute, Paris, France) for his contribution to data interpretation.

References

- Berquist C, Johansson EV, Lagergard T, Holmgren J & Rudin A (1997) Intranasal vaccination of humans with recombinant cholera toxin B subunit induces systemic and local antibody responses in the upper respiratory tract and the vagina. *Infection Immun* 65: 2676–2684.

- Cross ML (2002) Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. *FEMS Immunol Med Microbiol* **34**: 245–253.
- de Vrese M, Rautenberg P, Laue C, Koopmans M, Herremans T & Schrezenmeir J (2005) Probiotic bacteria stimulate virus-specific neutralizing antibodies following a booster polio vaccination. *Eur J Nutr* **44**: 406–413.
- de Vrese M, Winkler P, Rautenberg P *et al.* (2006) Probiotic bacteria reduced duration and severity but not the incidence of common cold episodes in a double blind, randomized, controlled trial. *Vaccine* **24**: 6670–6674.
- Forrest BD (1992) Indirect measurement of intestinal immune responses to an orally administered attenuated bacterial vaccine. *Infect Immun* **60**: 2023–2029.
- Galdeano CM & Perdigon G (2004) Role of viability of probiotic strains in their persistence in the gut and in mucosal immune stimulation. *J Appl Microbiol* **97**: 673–681.
- Gill HS, Rutherford KJ, Cross ML & Gopal PK (2001) Enhancement of immunity in the elderly by dietary supplementation with the probiotic *Bifidobacterium lactis* HN019. *Am J Clin Nutr* **74**: 833–839.
- Isolauri E, Joensuu J, Suomalainen H, Luomala M & Vesikari T (1995) Improved immunogenicity of oral D × RRV reassortant rotavirus vaccine by *Lactobacillus casei* GG. *Vaccine* **13**: 310–312.
- Isolauri E, Sutas Y, Kankaanpää P, Arvilommi H & Salminen S (2001) Probiotics: effects on immunity. *Am J Clin Nutr* **73**: 444S–450S.
- Jertborn M, Svennerholm AM & Holmgren J (1984) Gut mucosal, salivary and serum antitoxic and antibacterial antibody responses in Swedes after oral immunization with B subunit-whole cell cholera vaccine. *Int Arch Allergy Appl Immunol* **75**: 38–43.
- Jertborn M, Svennerholm AM & Holmgren J (1986) Saliva, breast milk, and serum antibody responses as indirect measures of intestinal immunity after oral cholera vaccination or natural disease. *J Clin Microbiol* **24**: 203–209.
- Kukkonen K, Nieminen T, Poussa T, Savilahti E & Kuitunen M (2006) Effect of probiotics on vaccine antibody responses in infancy – a randomized placebo-controlled double-blind trial. *Pediatr Allergy Immunol* **17**: 416–421.
- Lammers KM, Brigidi P, Vitali B, Gionchetti P, Rizzello F, Caramelli E, Matteuzzi D & Campieri M (2003) Immunomodulatory effects of probiotic bacteria DNA: IL-1 and IL-10 response in human peripheral blood mononuclear cells. *FEMS Immunol Med Microbiol* **38**: 165–172.
- Langevin-Perriat A, Lafont S, Vincent C, Revillard JP, Mazert MC, Gerfaux G & Tron F (1988) Intestinal secretory antibody response induced by an oral cholera vaccine in human volunteers. *Vaccine* **6**: 509–512.
- Link-Amster H, Rochat F, Saudan KY, Mignot O & Aeschlimann JM (1994) Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunol Med Microbiol* **10**: 55–63.
- Mullie C, Yazourh A, Thibault H, Odou MF, Singer E, Kalach N, Kremp O & Romond MB (2004) Increased poliovirus-specific intestinal antibody response coincides with promotion of *Bifidobacterium longum*-*infantis* and *Bifidobacterium breve* in infants: a randomized, double-blind, placebo-controlled trial. *Pediatr Res* **56**: 791–795.
- Rinne M, Kalliomaki M, Arvilommi H, Salminen S & Isolauri E (2005) Effect of probiotics and breastfeeding on the bifidobacterium and lactobacillus/enterococcus microbiota and humoral immune responses. *J Pediatr* **147**: 186–191.
- Saavedra JM (2007) Use of probiotics in pediatrics: rationale, mechanisms of action, and practical aspects. *Nutr Clin Pract* **22**: 351–365.
- Santosa S, Farnworth E & Jones PJ (2006) Probiotics and their potential health claims. *Nutr Rev* **64**: 265–274.
- Svennerholm AM & Holmgren J (1978) Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM1-ELISA) procedure. *Curr Microbiol* **1**: 19–23.
- Svennerholm AM & Wiklund G (1983) Rapid GM1-enzyme-linked immunosorbent assay with visual reading for identification of *Escherichia coli* heat-labile enterotoxin. *J Clin Microbiol* **17**: 596–600.
- Van Niel CW, Feudtner C, Garrison MM & Christakis DA (2002) *Lactobacillus* therapy for acute infectious diarrhea in children: a meta-analysis. *Pediatrics* **109**: 678–684.