Eur J Nutr (2010) 49:173–179
DOI 10.1007/s00394-009-0063-5

ORIGINAL CONTRIBUTION

Effect of probiotic supplementation on immunoglobulins, isoagglutinins and antibody response in children of low socio-economic status

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Received: 20 May 2009 / Accepted: 29 September 2009 / Published online: 17 October 2009

Abstract
Background Antigen exposure is one of the major exogenous factors modulating human immunocompetence acquisition. Decline in family size and improvements in public health and hygiene in developed countries, may deprive the immune system of appropriate antigen input by diminishing infectious stimuli. Probiotics are a large group of microorganisms defined by their beneficial effects on human health and with stimulating effects on different functions of the immune system.

Aim of the study We conducted a double-blind, placebo-controlled trial to determine if probiotics maintain their immune-stimulating effects in a population of 162 children with a high index of natural exposure to microorganisms. Children were to ingest for at least 4 months one of two products, low-fat milk fermented by *Streptococcus thermophilus* (control product) or low-fat milk fermented by *S. thermophilus* and *Lactobacillus casei*, with *Lactobacillus acidophilus*, oligofructose and inulin added after the fermentation process (test product). According to their age, children were vaccinated with DTP-Hib vaccine or a 23-valent anti-pneumococcal vaccine.
Results Final analysis of results was done in 70 children in each group, showing that the rate of immunoglobulin and isoagglutinin acquisition was similar in both groups. There was no difference between groups in antibody levels neither before nor after vaccination. Days of fever and number of episodes of infection were not statistically different in either group.
Conclusions Supplementation of standard fermented milk with additional probiotics was not of benefit. The high natural rate of early microbial exposure in infants and children from a population of low socio-economic status living in a "less hygienic environment" may account for the absence of an additional immune-stimulating effect by supplementary probiotics.

Keywords Probiotics · Immune system stimulation · Antibody response · Hygiene hypothesis

This work was performed at the Hospital de Niños S.M. Ludovica, 14 y 65, La Plata, Argentina.

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Introduction

The immune response may be modified by changes in the type and degree of stimulation from the microbial

environment. In early infancy, intestinal colonisation has been linked with modulation of the immune system, apparently promoting the maturation of some responses [4, 10]. Repeated stimulation of the immune system by bacterial and viral infection induces a Th1 profile of the immune response [4, 18]. The "hygiene hypothesis" [19] suggests and predicts that declines in family size and improvements in public health and hygiene occurring in developed countries may favour a Th2 profile of the immune response by diminishing infectious stimuli.

Oral administration of probiotic bacteria has also been linked with a stimulatory effect of the immune system and with the ability to enhance antibody response in children and adults [4, 6, 13, 22].

Knowing the stimulatory effects of antigenic exposure in the immune response, we conducted the present double-blind, placebo-controlled trial to determine if the addition of probiotics to a standard fermented milk modifies immunoglobulin and isoagglutinin acquisition and post-vaccinal antibody responses (a useful marker to measure immunomodulation in nutrition intervention studies) [1] in infants and children from a population of low socio-economic status living in a "less hygienic environment" where there is a "natural" high index of early microbial exposure.

Method

Population

The study was conducted between June 2006 and December 2007 in the outpatient facilities of the Children's Hospital in La Plata, Argentina. Regarding living conditions, nutritional status and environment exposure, children were representative of the low-income population of the area of La Plata presenting with a high Townsend Material Deprivation Score [20] of 11.3. Written informed consent was obtained from parents after full explanation of the purposes of the study. The independent ethical committee and the corresponding authorities of the hospital approved the protocol.

Inclusion and exclusion criteria

All children aged 9 months to 10 years seen for health control at the outpatient facilities of the hospital were considered as candidates to enter the study. Children were not included in the protocol if they were breastfed at the time of their first visit or had a known chronic disease. Children who, according to their medical record, had been previously vaccinated with anti-pneumococcal vaccine (conjugated or not) were also excluded.

Intervention

Included children were to ingest, once daily for at least 4 months, a (95 g) bottle of one of two products. Low-fat milk fermented by *Streptococcus thermophilus* strain F DVS STM 5 (1×10^8 cfu/ml) was the control product. Low-fat milk fermented by the same *S. thermophilus* (1×10^8 cfu/ml) and *Lactobacillus casei* strain CRL431 (1×10^6 cfu/ml), with *Lactobacillus. acidophilus* strain CRL730 (1×10^6 cfu/ml), oligofructose (950 mg/bottle), and inulin (240 mg/bottle) added after the fermentation process, was the test product. Both products were manufactured by the supplier (Sancor CUL Argentina). Daily cfu dose was therefore 95×10^8 cfu of *S. thermophilus* for the control group, and 95×10^8 cfu of *S. thermophilus*, 95×10^6 cfu of *L. acidophilus* and 95×10^6 cfu of *L. casei* for the experimental group.

Children were assigned to one of two groups (green or orange) in their first visit according to a computer-generated randomization list. Children were not randomized if they lived in the same household of a child who was already in the study (see below). The test product and the control product were packed in similar bottles of 95 g differing only in a mark (green or orange). Both products had the same nutrient composition (2.5 g protein, 12 g carbohydrates and 1.3 g fat per 95 g bottle in water solution). They differed in fibre content (0.3 and 1.4 g in control and test product, respectively). The products looked, smelled and tasted identical, and the colour code was not revealed to investigators. After completion of clinical and laboratory data evaluation, the code was opened. The green group had received the test product (experimental group) and the orange group had received the control product (control group).

Parents had the possibility of including in the study all their children of the age-range selected for the study and living in the same household. Sufficient product was provided to cover the required intakes of the respective product by all children, for the duration of the study. Provision to each family of the corresponding product was done weekly until the moment of obtaining the last serum sample, at the end of the study. All children living in the same household received the same product (green or orange) to avoid involuntary misadministration.

Procedures

At the moment of the first visit, a first (basal) blood sample and a questionnaire were obtained from each child entering the study. The following was recorded: height and weight for age, weight for height, and number of household contacts (adults and children). Children who were unable or

unwilling to sustain consumption of the product or to complete the other protocol requirements were excluded from the final analysis.

Children achieving 18 months of age received the fourth dose of DTP-Hemophilus influenzae type b (Hib) vaccine according to the national vaccine schedule (2–4–6 and 18 months of age) and tetanus antibody response was measured. Children above 18 months were proposed to receive the 23-valent anti-pneumococcal vaccine, and pneumococcal antibody response was considered. A final blood sample was obtained at the end of the study in both groups, after at least 4 months of product consumption. In the first and last sample, C-reactive protein (CRP), immunoglobulins (Ig) A, G, M, and E, isoagglutinins and peripheral blood counts were determined. Pre-vaccination antibody levels were obtained as follows: for children receiving the fourth dose of DTP-Hib, pre-vaccination tetanus antibody levels were determined with the first serum sample. In anti-pneumococcal vaccinated children, pre-vaccination pneumococcal antibodies were determined in an intermediate serum sample obtained immediately before vaccination. Post-vaccinal tetanus and pneumococcal antibodies were measured in the last sample, 30–40 days after vaccination.

A sample of stools was obtained from all children at the first and last visit.

Days of fever (more than 37.2°C axillary) and infectious episodes requiring medical attention were recorded weekly as the product for the next week was provided. The same visits were used to assess product consumption throughout the experimental period.

Weight for age, height for age and weight for height *Z* scores were calculated according to international standards [21].

The number of household contacts was determined in order to avoid a potential bias induced by different exposure rates between groups that may condition differences in the frequency of infectious episodes and even in immunoglobulin basal values. CRP was measured to monitor for acute inflammatory status.

Statistics

Immunoglobulin, PCR and particularly antibody data were not normally distributed; therefore, values are medians unless stated in the text or tables. For median comparison between groups, non-parametric tests (Wilcoxon) were used. For comparison between basal and final values, non-parametric tests for paired samples were used. Non-parametric (Spearman) or parametric (Pearson) correlation coefficients were used when adequate. Two-sided *P* values of 0.05 or less were considered statistically significant.

Laboratory determinations

Immunoglobulins and CRP (normal value <8 mg/l) were determined by nephelometry (Array 360 System, Beckman Coulter, California. USA). The last serum dilution producing agglutination of A and/or B red blood cells was considered to be the isoagglutinin titre. Anti-tetanus and anti-pneumococcal antibodies were determined by ELISA using IgG PCP and IgG Tetanus (The Binding Site Ltd, Birmingham, UK) according to the manufacturer's instructions.

As a control of adequate product consumption, the presence of *L. casei* in the final stool sample was determined by fluorescence in situ hybridization and performed by one of us (GS) only before the code was open. The proportion of aerobes/anaerobes was also determined in both, initial and final stool samples. Briefly, for aerobic cultures, brain heart infusion agar was used. For anaerobic flora, trypticase soy-yeast extract agar, supplemented with 5% rabbit blood and previously stored for 4 days in anaerobic glove boxes, was used. After 3 and 7 days of culture, respectively, total aerobic and anaerobic flora was counted.

Results

One hundred and 62 children (72 female and 90 male) were recruited to initiate the protocol and were randomly assigned to groups as previously stated. The experimental and the control group were constituted by 84 and 78 children, respectively.

Twenty-two children, 14 from the experimental and 8 from the control group did not receive the product as established in the protocol (interruption or premature discontinuation). Their initial data was considered but they were excluded from the final analysis of the results that was done in 70 children in each group. They consumed the product for a median of 124 days (range 90–274) before vaccination, and 34 days (range 24–69) after vaccination. Parents of 15 children decided against anti-pneumococcal vaccination.

As seen in Table 1, nearly all basal physical and laboratory parameters were comparable between groups. The exception was CRP, that was more elevated in the experimental group. Both groups were similar in number of adult and children household contacts.

Immunological parameters and infectious episodes

As shown in Table 2, basal IgM values had a tendency to be more elevated in the experimental group, in the limits of significance ($P < 0.051$). After product consumption,

Table 1 Patient characteristics

	Control	Experimental	*P*
Age (months)	45.5 (24.0/74.5)[a]	46.5 (23.0/77.0)	0.782
Sex (m/f)	46/32	44/40	0.390
Household contacts			
Children	2.00 (1.00/3.00)	2.00 (1.00/4.00)	0.526
Adults	2.00 (2.00/3.00)	2.00 (2.00/3.00)	0.228
Weight for age *Z* score	−0.23 (−0.89/0.43)	−0.03 (−0.72/0.61)	0.110
Height for age *Z* score	−0.60 (−1.19/−0.24)	−0.29 (−1.18/0.28)	0.054
Weight for height *Z* score	0.42 (−0.14/0.91)	0.37 (−0.18/1.19)	0.655
C-reactive protein (mg/l)	3.36 (1.88/6.55)	4.20 (3.14/7.65)	0.016
Leukocytes/mm^3	7,600 (6,000/9,400)	7,400 (6,025/9,575)	0.993

[a] In parentheses, 25/75 centiles

Table 2 Pre- and post-treatment serum levels of immunoglobulins and isoagglutinins

	Control	Experimental	*P*
IgG (g/l)			
Pre-treatment	10.03 (8.64/12.10)[a]	10.45 (8.46/12.52)	0.924
Post-treatment	11.30 (9.95/12.60)	10.90 (9.68/12.87)	0.599
IgA (g/l)			
Pre-treatment	0.87 (0.69/1.33)	0.88 (0.48/1.23)	0.228
Post-treatment	1.04 (0.74/1.57)	0.93 (0.66/1.26)	0.085
IgM (g/l)			
Pre-treatment	1.03 (0.85/1.36)	1.16 (0.94/1.66)	0.051
Post-treatment	1.20 (0.94/1.47)	1.29 (1.05/1.90)	0.082
IgE (iu/l)			
Pre-treatment	101 (24/236)	85 (25/199)	0.760
Post-treatment	127 (52/384)	161 (61/380)	0.964
Isoagglutinins (titre)			
Anti-A			
Pre-treatment	64	32	0.549
Post-treatment	64	32	0.185
Anti-B			
Pre-treatment	32	32	0.136
Post-treatment	32	32	0.360

[a] In parentheses, 25/75 centiles

median values of immunoglobulins and isoagglutinins were comparable between groups.

The group of children in whom anti-tetanus antibodies were measured after the fourth dose of DTP-Hib vaccine was very small because many children aged 1 year or less were excluded from the study because of the very long period of sustained product consumption required before vaccination at 18 months of age, and by the high frequency of breastfeeding (Table 3).

The two groups had similar median values of pre-vaccinal anti-tetanus and anti-pneumococcal antibody levels. Nearly all children responded satisfactorily to vaccination, with significant differences in pre- versus post-vaccinal antibody levels. Proportions of responders versus non-responders were similar (data not shown). There was no difference between groups in antibody levels after vaccination (Table 3). There was no correlation between nutritional status and post-vaccinal antibody levels (data not shown).

Days of fever and number of episodes of infection were not statistically different in both groups of children (Table 3).

As stated before, basal values of CRP and IgM seemed to be more elevated in the experimental than in the control group, but they were similar in the intermediate and the last sample. When this fact was analysed, a significant positive correlation was observed between basal CRP and IgM values ($P < 0.001$), perhaps linked to a subclinical acute infection that was more frequent in the experimental group. A significant correlation between IgM and CRP was also present for the intermediate and the last pair of samples ($P = 0.008$ and $P < 0.001$, respectively), where no differences either in CRP or IgM values between groups were present.

In faeces collected at the end of the study, *L. casei* was present in 83% of children in the experimental group and it was detected in less than 5% of children in the control group. Aerobes and anaerobes were equally represented in both groups at the beginning and at the end of the study (data not shown).

The same comparisons of immunoglobulins, antibody levels and infectious episodes were done after exclusion of children with a weight for age *Z* score below −2 in order to exclude malnutrition as a cause of non-response [15, 16]. Again, no differences between groups were present for all variables tested (data not shown).

Discussion

Probiotics are a large group of microorganisms defined by their beneficial effects on human health. The choice of

Table 3 Antibody response, days of fever and infectious episodes

	Control	Experimental	*P*
Tetanus antibodies (iu/ml)			
Pre-vaccination	0.43 (6)[a]	0.46 (5)	0.537
	[0.24/1.10][b]	[0.28/4.98]	
Post-vaccination	7.00 (6)	8.34 (5)	0.913
	[6.33/15.39]	[3.63/16.66]	
Pneumococcal antibodies (mg/l)			
Pre-vaccination	31.25 (52)	35.00 (56)	0.614
	[11.95/68.75]	[14.60/69.90]	
Post-vaccination	268.00 (52)	279.00 (56)	0.671
	[166.50/491.75]	[164.25/525.25]	
Infectious episodes (absolute number)			
Upper respiratory tract infections	34	40	0.882
Gastroenteritis	9	5	0.326
Varicela	0	2	0.476
Pneumonia	1	2	1.000
Days of fever	56	77	0.235

[a] In parentheses, total number of subjects tested

[b] In brackets, 25/75 centiles

L. acidophilus and *L. casei* was made because they are among the most explored probiotic bacteria [9, 14–16]. *L. Casei* strain CRL431 has been able to stimulate post-vaccinal antibody production [6] and both *L. Casei* CRL431 and *L. acidophilus* CRL730 exert a beneficial effect on persistent diarrhoea [7, 8]. The stimulatory effect of probiotics on different functions of the immune system is a well-described but poorly understood phenomenon [9]. When administered to laboratory animals or humans, probiotics associate to an augmentation of immunoglobulins (particularly IgA) and antibodies against vaccines received while consuming the probiotic bacteria [2, 4–6, 13, 22]. However, some of these immunologic effects are also evoked by food antigens or proteins [23], and many differences were the rule when immunomodulatory effects of different probiotic bacteria were compared. Moreover, the same probiotics may have opposite immunomodulatory effects when administered to autoimmune or allergic-prone individuals, compared to normal populations [12].

Some recent placebo-controlled clinical trials suggest a better post-vaccinal antibody response in children receiving probiotics when vaccinated against tetanus toxoid and protein-conjugated polysaccharides [13, 22]. Antibody response is considered a "marker with high suitability" when measuring immunomodulation in human nutrition intervention studies [1]. Tetanus antibody response measures ability to respond against proteins. Antibody production after non-conjugated pneumococcal vaccine reflects ability to respond against polysaccharides.

In the double-blind placebo-controlled trial by West et al. [22] children receiving *Lactobacillus F19* showed a better antibody response against tetanus toxoid. However, no differences were found in anti-Hib antibody response. Conversely, Kukkonen et al. [13] were able to show differences in post-vaccinal serum levels of Hib IgG antibodies but not in the anti-tetanus antibody response in children who, as well as their mothers before delivery, had been supplemented with *L. rhamnosus*, *Bifidobacterium*, *Propionibacterium* and galacto-oligosaccharides.

West et al. [22] and Kukkonen et al. [13] included breastfed children, and children in their studies had received a Hib-conjugated vaccine. Unlike the mentioned authors, we excluded from our work breastfed children and we measured anti-pneumococcal antibodies after administration of a non-conjugated vaccine in an older group of children. We decided to exclude breastfed children because of the well-known protective effect of maternal milk from infection and its effect on intestinal microflora [10], that may act as confounding factors. The study population did not regularly consume probiotics or other fermented milk-containing products.

Our findings did not support a role for the addition of prebiotics (oligofructose and inulin) or probiotics (*L. acidophilus* strain CRL730 and *L. casei* strain CRL431) to standard fermented milk in the reduction of the frequency or duration of infectious episodes, or in the systemic post-vaccinal antibody response of children receiving tetanus toxoid or pneumococcal vaccines. Our control product was milk fermented using *S. termophilus*, and it may be argued that the negative results we obtained may be due to the immune-stimulation effects of this probiotic agent (Reviewed in [12]). However, addition of *L. casei* DN-114 001 to standard yoghurt fermented by *L. bulgaricus and S. termophilus* may significantly reduce the incidence of acute diarrhoea [14]. The dose of oligofructose (950 mg/day), and inulin (240 mg/day) utilised was

probably very low to obtain a "prebiotic effect", taking into account the failure of a 6 g/day dose, to stimulate post-vaccinal antibody production [3].

Methodological differences and the known differential effects of different probiotic bacteria may account for these apparently contrasting results on infections and post-vaccinal antibody response in children. However, a role for other factors cannot be easily excluded. Acquisition of full immunological competence in humans is a complex process by which a relatively "immature" immune system at birth reaches the complexity and characteristics found in adult life. This "maturation" takes place from birth and continues during the first years of life. A role for intestinal microflora and other sources of antigen stimulation has been suggested [4, 10, 23]. A decrease in the frequency of daily contact with germs and the reduction in the frequency of childhood infections may deprive their immune system of an important source of information in order to function correctly [11]. Not having a direct manner to quantify microbial exposure, we used the Townsend material deprivation score because, as suggested in the "hygiene hypothesis", decline in family size and improvements in public health and hygiene in developed countries may reduce natural rates of microbial exposure [11, 17].

From the point of view of immunological stimuli, probiotics are perhaps a safer way to receive "our daily germs dose" [17], providing appropriate antigen inputs by replacing artificially an increasingly limited environmental flora.

The intensity of "natural" exposure to environmental flora may potentially modify the relative stimulatory role of probiotics acting as a source of stimuli, and the high natural rate of exposition to infectious agents in our low-income population may account for the absence of an additional stimulation by supplementary probiotics.

Acknowledgments The work was supported by Sancor CUL Argentina, IDIP and CICPBA. NP formulated the hypothesis that the high index of microbial exposure in low socioeconomic environments may reduce or eliminate the immune-stimulating effects of probiotics. He participated in the design of the study, the analysis of results and the final writing of the manuscript. JCI conducted field work with patients and families, participated in the design of the study, the analysis of results and the final writing of the manuscript. CG-B, AV and LD coordinated sample taking, performed immunological laboratory determinations and participated in the analysis of results. MA participated in the design of the study and the statistical analysis of data. SG participated in the design of the study, the analysis of results and performed determinations in faecal flora. JCP, DV and RC participated in the design of the study, the preparation of products and the analysis of data.

Conflict of interest statement Pernas J, Vicentin D, and Cravero R are members of the staff of Sancor CUL Argentina. The participation of Iannicelli J. and Girard-Bosch C. was partially supported by Sancor.

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