POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
Pol. J. Food Nutr. Sci. 2003, Vol. 12/53, SI 1, pp. 20–23

IMMUNOSTIMULATIVE ACTIVITY OF PROBIOTIC *BIFIDOBACTERIUM* STRAINS DETERMINED *IN VIVO* USING ELISA METHOD

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Key words: *Bifidobacterium*, *Salmonella*, ELISA, probiotics, immunostimulation, IgA

The effect of *Bifidobacterium* strains: *B. longum* KN29.1 and KNA1, and *B. animalis* KSp4, on the level of bacterial antigen-specific IgA in serum blood of rats was studied using ELISA method. The experiment was carried out on 4 groups of young Wistar rats receiving daily for 14 days $\geq 10^9$ *Bifidobacterium* cells suspended in physiological saline (3 experimental groups) and bacteria-free physiological saline (control). Half the animals in each group were challenged orally with *S*. Enteritidis 458 as bacterial antigen, to which the level of the specific antibodies of the IgA class was determined by direct immunometric ELISA method. Briefly, the methodology comprised coating the microplates firstly with *S*. Enteritidis 458 in the amount of $2x10^8$ cells/100 μL/well, secondly with 100-fold diluted serum, conjugating with peroxidase-labelled goat anti-rat IgA conjugate (Nordic Immunology), and reading the absorbance at λ=450 nm. Application of the ELISA enabled comparison of the enhancement of the specific anti-S. Enteritidis IgA antibody response in serum blood of rats administered with probiotics. Those were significantly higher in all animal groups administered with bifidobacteria in comparison with the control group, both in non-infected and *Salmonella*-challenged animals. The obtained results indicate a direct stimulating effect of bifidobacteria on the immune system.

INTRODUCTION

Intestinal epithelial surfaces, lined with mucosa are the place of contact of the body with a variety of micro-organisms delivered by an oral route. They are an ideal site for initiation of infection by pathogenic bacteria, although not left without protection. The secretory immune system associated with mucosa plays a crucial role in the gut defence, producing secretory antibodies of IgA class (sIgA) against luminal antigens (toxins, pathogens, viruses) which prevent their interaction with epithelial surface. The process is called *immune exclusion* and affords protection at the mucosal barrier without inflammation as occurs with tissue defence [Delneste *et al*., 1998]. Apart from the secretion to the intestine, specific antibodies – produced in response to the luminal antigens – are transported to the circulation and *via* blood they appear on all mucosal surfaces of the body. That is why an exclusively breast-fed infant receives a very large dose of about 0.5 to 1 g of ready-to-use antibodies sIgA against a wide variety of antigens, effectively protecting it against infection [Hanson, 1998]. During the first months of life, intestinal microflora dominated by bifidobacteria plays an important immunomodulative role regarding maturation and moulding of intestinal and systemic immune mechanisms [Koletzko *et al*., 1998]. The study on one of the mechanisms of protective probiotic activity – enhancement of IgA secretion - was therefore undertaken. The ELISA method, based on the creating antibody-antigen complex as principle, was applied as appropriate for IgA determination. Immunoassay offers a specific, sensitive and rapid method to detect and quantify even trace amounts of allergens in relevant matrix (tissue, food or raw materials) and appears to be suitable for the determination of immunoglobulins and cytokins in biological and medical diagnostics.

The aim of the study was to determine immune stimulation by the selected probiotic *Bifidobacterium* strains, expressed as the level of the specific anti-*Salmonella* IgA in serum blood, by ELISA method.

MATERIALS AND METHODS

Experiment. The effect of the probiotic *Bifidobacterium* strains: *B. longum* KN29.1 and KNA1 as well as *B. animalis* KSp4, on the level of anti-*Salmonella*-specific IgA in rat blood serum was studied. The experiment was carried out on 4 groups of young Wistar rats (10 rats each), receiving daily for 14 days $\geq 10^9$ *Bifidobacterium* cells suspended in physiological saline (3 experimental groups) and bacteria-free physiological saline (control). Half the animals in each group received live cells of *Salmonella enterica* subsp. *enterica* ser. Enteritidis 458 (*S*. Enteritidis 458) as an antigen. On day 15 of the experiment, blood was collected and the levels of anti-*S*. Enteritidis 458 IgA antibodies were evaluated on the basis of the absorbance determined by

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indirect immunometric ELISA method in the obtained rat sera. Antibody response was evaluated by comparison of absorbance in the groups supplemented with bifidobacteria to that in the control, both in non-infected and *Salmonella*--challenged rats.

Experimental animals. Three-month-old males of Wistar rats with average body weight of 285 g were housed under conventional conditions of lighting and temperature, 5 individuals in each cage. The rats were fed with casein diet of Western type (13% protein, 10% fat) supplemented with 3% mineral (AIN-93G-MX) and 2% vitamin (AIN-93-VX) mixtures [Reeves, 1997], and drunk tap water *ad libitum.*

Bifidobacteria. The live cells of *Bifidobacterium longum* KN29.1 and *B. longum* KNA1 isolated from babies as well as of *B. animalis* KSp4 isolated from rat were administered to the animals. The strains were multiplying in 2 steps: (1) in semi-liquid modified nutrient Garche's agar medium [Rasic, 1990] (with bacto-casitone replaced by Peptobak, BTL, Łódź, Poland, and without lithium chloride), inoculated and incubated at 37°C/18 h in anaerobic conditions (pyrogallol plug); (2) surface growth on Garche's agar medium (incubation at 37°C/22-24 h in anaerobic jars equipped with Gas Pak Anaerobic System CO_2+H_2, Linegal Chemicals GmbH, Poland). The strain biofilms were washed, suspended in the physiological saline, and within maximum 0.5–1 h administered to animals with gastric tube, once a day, in the amount of av. $7.2x10^9$ ($5.7\text{-}9.4x10^9$) cells of *B. longum* KN29.1, $4.7x10^9$ ($1.8\text{-}8.5x10^9$) cells of *B. longum* KNA1 as well as $3.3x10^9$ ($1.8\text{-}6.5x10^9$) cells of *B. animalis* KSp4. *Bifidobacterium* counts were determined on modified Garche's agar medium after incubation at 37°C for 72 h under the anaerobic conditions (as mentioned above).

***Salmonella*.** *Salmonella enterica* subsp. *enterica* ser. Enteritidis 458 isolated from the ill person in the Sanitary and Epidemiology Station (Olsztyn, Poland) was cultivated under conditions described previously [Biedrzycka & Bielecka, 2002]. On day 2, 6, 10, and 12 of the experiment, the rats were challenged with live cells of *Salmonella* Enteritidis 458 in the amount of $3.7x10^3$, $3.7x10^6$, $1.8x10^8$, and $1.4x10^8$, respectively. Counts of *Salmonella* were determined using MacConkey agar medium, with Whitley Automatic Spiral Plater (Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK) for inoculation of Petri dishes.

Rat sera. The assay serum was prepared from rat blood obtained from abdominal artery, incubated at 37°C/1 h, and separated by centrifuging at 1500 x g for 10 min.

Determination of IgA level in serum blood with ELISA. Microplates coated with *S.* Enteritidis 458 cells suspended in 9 mmol/L carbonate buffer, pH 9.6, in the amount of $2x10^8$ /100μL/well, were incubated at 37°C/1 h, and washed (4x) with 10 mmol/L-phosphate buffered saline with Tween-20 (PBS-T), pH 7.4. The unbound sites of microplate were blocked with 1.5% gelatine solution in 9 mmol/L carbonate buffer, pH 9.6 (150 μL per well), incubated at 37°C/0.5 h and then washed (4x) with PBS-T. Rat serum was 100x diluted and applied in six repetitions by 100 μL/well, incubated at 37°C/1 h and washed (4x). Peroxidase-labelled goat anti-rat IgA conjugate (Nordic Immunology) was applied (100 μL/well), and after incubation at 37°C/1 h and washing (4x), tetra-metylbenzidine (TMB) was added (100 μL/well), followed by incubation at 37°C/0.5 h and the reaction was stopped with 50 μL of 2 mol/L H_2SO_4. Absorbance was read at λ=450 nm wave-length using reader Sunrise (Tecan).

Statistical analysis. The results of absorbance are expressed as the means and standard deviation of the values for five animals. The statistical significance of the difference between experimental and control groups was determined by Student's t-test.

RESULTS

In the control group of animals, the specific IgA antibody response (IgA-AR) expressed as A_{450} was 0.484±0.032 (Figure 1a). In groups that received probiotic *Bifidobacterium* strains – *B. longum* KN29.1, *B. longum* KNA1, and *B. animalis* KSp4 – IgA-ARs were 0.648±0.109; 0.612±0.068; 0.641±0.127, respectively, and in all groups they were significantly higher than in the control at the significance level of p≤0.05; 0.01 and 0.05, respectively. In the control group of rats challenged with *Salmonella,* IgA-AR was 0.416±0.026. In the experimental groups, IgA-ARs were respectively 0.589±0.064; 0.616±0.091; 0.633±0.086, significantly higher at respectively p≤0.001, 0.01 and 0.001, than in the control group of rats challenged with *Salmonella* (Figure 1b). The level of immune response was not significantly differentiated either between the groups of animals administered with *Bifidobacterium* or between the groups additionally challenged with *Salmonella*. In the control group of animals challenged with *Salmonella,* IgA-AR was slightly lower (by 0.068, p≤0.01) than in the control group of non-infected rats, whereas IgA--ARs were not different between the experimental groups with or without *Salmonella*.

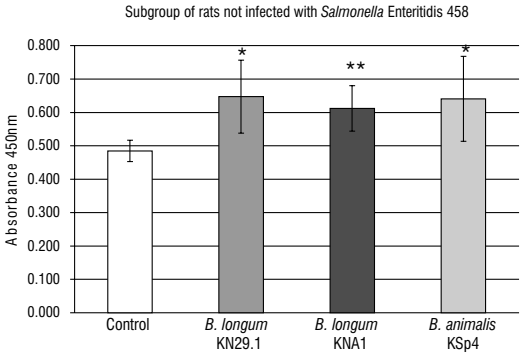

FIGURE 1a. Changes of the specific anti-*Salmonella* Enteritidis IgA antibody response in serum blood of rats administered with *Bifidobacterium*. The IgA level significantly different from the control at the significance level of * p≤0.05, ** p≤0.01.

DISCUSSION

Immunoglobulin A is the main secretory immunoglobulin appearing on all mucosal surfaces of a body. Mucosal sIgA prevents bacterial adherence, which is

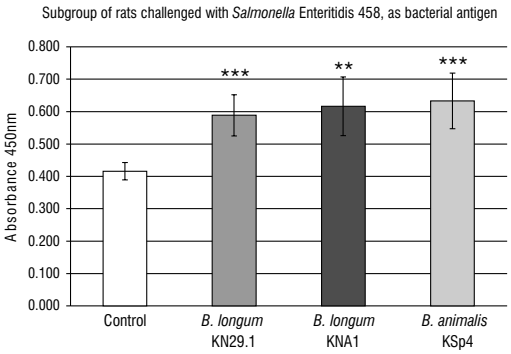

FIGURE 1b. Changes of the specific anti-*Salmonella* Enteritidis IgA antibody response in serum blood of rats administered with *Bifidobacterium* and challenged with *S*. Enteritidis 458. The IgA level significantly different from the control at the significance level of ** $p \leq 0.01$, *** $p \leq 0.001$.

considered to be one of the most important defence mechanisms against mucosal bacterial invasion [Marcotte & Lavoie, 1998]. With reference to concentration, IgA is the second immunoglobulin in human blood serum, however its biological role has not been completely explained so far. Studies on animals reviewed by Kasper [1998] have shown that orally applied LAB and fermented milks modulate the immune response. The similar effect was shown in human studies by Link–Amster *et al.* [1994], using direct ELISA. The authors observed over 4-fold increase in the specific serum IgA titre to *Salmonella typhi* Ty21a in the group administered with fermented milk containing *L. acidophilus* and bifidobacteria for 3 weeks and challenged with attenuated cells of *S. typhi* Ty21a (simulation of enteropathogen infection) as well as an increase in the total serum IgA. They indicated that LAB may act as an adjuvant and increase humoral immune responses. Perdigon *et al.* [1995] showed that single strains of *L. casei, L. acidophilus* and yoghurt enhanced the number of IgA producing cells, and *L. casei* significantly increased concentrations of sIgA specific for *S. typhimurium* in mice fed with that strain and challenged with *Salmonella*. An interesting relationships between LAB and immunity were observed by De Ambrosini *et al*. [1998]. They stated that *L. casei* CRL 431 was able to stimulate phagocytosis without changes in IgA, whereas *L. acidophilus* CRL 730 produced an increase in the levels of IgA without modifying phagocytosis.

Immunoadjuvant activity of LAB was investigated in several studies, whereas that of the selected probiotic strains of bifidobacteria in ours. Immunostimulative activity of *Bifidobacterium* has been accidentally studied up to now, whilst it is obvious that they constitute a great and important part of intestinal microflora and their implantation in the intestine by ingestion of bio-yoghurt or probiotic preparations is possible and widely used. Oral administration of all three probiotic *Bifidobacterium* strains to rats has been demonstrated to increase systemic specific antibody response (increased specific anti-*S*. Enteritidis-IgA antibody response in the blood serum), as reflecting enhancement of specific mucosal immunity, in both rats non-infected and challenged with *Salmonella.* Unfortunately, the level of stimulation cannot be compared to other studies because different models of experiments and different single strains were used. Ko *et al.* [1999] reported that *B. bifidum* Bb11 increased IgA synthesis as well as the number of IgA-secreting cells. Its mitogenic activity was ascribed to the threefold increased proliferation of spleen cells and further induction of spleen B cells to be reactive to TGF-β1 and IL-5, which resulted in an increased surface of IgA expression and total IgA production. Using murine model, Tejada-Simon *et al*. [1999] showed the adjuvant activity of yoghurt containing *L. acidophilus* and *Bifidobacterium* spp. demonstrated by generating a strong gut mucosal and systemic IgA anti-cholera toxin response in comparison to the yoghurt containing only yoghurt bacteria and to the control. The immunoadjuvant potential of *Bifidobacterium* was also appreciated by Yasui *et al.* [1992] who developed the screening test for detection of strains inducing large quantities of IgA using the murine Peyer's patch cell culture and ELISA method for measuring the quantities of IgA antibody in the culture supernatants. Next, one of the selected strains, *B. breve* YIT 4064, administered to mice along with cholera toxin, significantly increased production of IgA antibodies specific against the toxin used. Moreover, in the subsequent experiments its capability for activation of humoral immune system has been confirmed – augmented anti-rotavirus IgA production in mice [Yasui *et al.*, 1999].

CONCLUSIONS

The obtained results indicate a direct stimulating effect of the selected probiotic *Bifidobacterium* strains on the immune system. The determined lack of *S.* Enteritidis 458 influence on the level of the specific anti-*Salmonella* IgA may show some immunity of the experimental rats to the bacterial antigen used.

ACKNOWLEDGEMENTS

The work was in part supported by the EU project Centre of Excellence CA1-CT-2000-70017.

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