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MODULATION OF IMMUNOGLOBULIN A (IgA) RESPONSE BY OLIGOFRUCTOSE AND ITS SYNERGISTIC SETS WITH BIFIDOBACTERIA IN RATS

Elżbieta Biedrzycka, Maria Bielecka, Barbara Wróblewska, Lucjan Jędrychowski, Zenon Zduńczyk

Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland

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The previous study on rats showed that orally administered bifidobacteria stimulated the specific anti-*Salmonella* Enteritidis immunoglobulin A response (SE IgA R) in blood serum. The aim of the present study was to examine whether prebiotics and synbiotics could modulate SE IgA R as well. During a 2-week experiment, oligofructose as prebiotic (5% w/w in feed; OF group), and the synergistic sets of OF and *Bifidobacterium* strains ($\geq 10^9$ cells per rat, per day), including *B. longum* KN29.1, *B. longum* KNA1, and *B. animalis* KSp4, as synbiotics (OF+B1, OF+B2, OF+B3 groups, respectively), were administered to rats. Half the animals in each group were challenged orally with S. Enteritidis 458 as bacterial antigen. SE IgA R was determined by direct immunometric ELISA method. In non-infected rats, SE IgA R was slightly but significantly ($p \leq 0.05$) lower in OF and OF+B1 group, it showed a tendency to increase in OF+B2 group, and it was slightly but significantly higher ($p \leq 0.01$) in OF+B3 group, in comparison to the control. In *Salmonella*-infected rats, SE IgA R showed a tendency to increase in OF and OF+B1 groups, and was significantly higher in OF+B2 and OF+B3 groups (both $p \leq 0.01$) than in the control. Oligofructose alone did not stimulate SE IgA R, whereas synbiotics composed of OF and *Bifidobacterium* showed slight increase only. The results indicate that prebiotics and probiotics managed pathogenic activity in GI tract using different mechanisms – oligofructose possibly as a receptor analogue for pathogenic bacteria, and bifidobacteria by their immune-stimulating activity. Therefore, the SE IgA R obtained was not the effect of synergistic action of synbiotic components.

INTRODUCTION

Intestinal epithelial surfaces, lined with mucosa are the place of contact of the body with plenty of foreign antigens. The constituents of the complex intestinal ecosystem, *i.e.* resident and transient microflora as well as epithelial and immune cells, enter the interactions which play a significant role in development, maturation, maintenance and regulation of the gut-associated lymphoid tissue (GALT), a part of the immune system of the body [Blum *et al.*, 1999; Cebra, 1999]. The GALT produces large amounts of the secretory IgA, the essential component of mucosal immune defence which protects the host from bacterial, fungi and virus infections, as well as neutralises toxins. One of the major functions of IgA is to perform immune exclusion, the non-inflammatory mechanisms, that collectively limit the penetration of potentially harmful antigens through the mucosal epithelium to the interior of the body [Brandtzaeg, 1997].

Commensal microflora is able to regulate the intestinal barrier function and immunity [Hooper & Gordon, 2001]. Therefore, in the state of immunodeficiency, probiotics are recommended for enhancement of immune activity and improvement of the host defence against infectious agents. According to the definition recognised by the International Life Sciences Institute (ILSI), probiotic is a live microbial food ingredient that, when ingested in sufficient quantities, exerts health benefits on the consumer [Ashwell, 2002]. Two bacterial genera, *Lactobacillus* and *Bifidobacterium*, as characterised by entirely beneficial impact on host's health, are commonly used for probiotic recruitment [Gibson, 1998]. Bifidobacteria are regular colonic inhabitants. The inhibitory potential of bifidobacteria depends on their production of organic acids, as the increased production of acetic and formic acids may be of interest to the inhibition of intestinal pathogens such as *Escherichia coli* and *Salmonella* [Van der Meulen *et al.*, 2004]. However, in suckling mammals bifidobacteria play a role in maturation of immunity. The presence of *Bifidobacterium* sp. in the faecal flora of breast-fed children is associated with strong stimulation of the anti-rotavirus IgA response compared with what is observed in formula-fed children [Bourlioux *et al.*, 2003]. In adults with different problems related to the reduction of bifidobacterial populations, bifidobacteria, when administered as probiotics, may enhance gut immune functions. Yasui *et al.* [1992] were even screening the *Bifidobacterium* strains to find those capable of inducing large quantities of IgA which were further used to manage the cholera toxin in mice, influenza virus in mice, or rotavirus infection in infants [Yasui *et al.*, 1999a,b]. Medici *et al.* [2004] observed, among other effects, a significant increase in the number of IgA^+ producing cells in the small intestine and no significant differences in the large intestine as a result of administration of probiotic fresh cheese containing *Streptococcus thermophilus*, *Lactococcus lactis*, *Bifidobacterium bifidum*, *Lactobacillus acidophilus* and *L. paracasei* to mice. The important immunomodulating *B. bifidum*, *L. acidophilus*

Author's address for correspondence: Elżbieta Biedrzycka, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, ul. Tuwima 10, 10-747 Olsztyn, Poland, e-mail: elabied@pan.olsztyn.pl

and *L. paracasei* effects in the gut were concluded. Our previous studies showed increased specific anti-*S*. Enteritidis IgA antibody response in rats administered with bifidobacteria [Biedrzycka *et al.*, 2003].

Colonic bifidobacterial populations may be strengthened by prebiotics, *i.e.* non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health [Gibson & Roberfroid, 1995]. Unfortunately, the immunomodulatory effects of carbohydrates had little attention so far [Vanderhoof, 1998]. The research on the immunostimulating activity of prebiotics and related health effects, *e.g.* in prevention of pathogen infection, is similarly scarce. Duggan *et al.* [2003], while evaluating the effect of prebiotic oligofructose 0.55 g daily dose on the prevalence of diarrhoea in infants aged 6–12 months in a shantytown community near Lima, did not observed any change in diarrhoea prevalence, use of health care resources, or response to *H. influenza* type B immunisation. However, infants and young children who continued to be breast-fed might not benefit from prebiotic supplementation, especially in such a low dose.

Another possibility in immunomodulation is the use of synbiotics which constitute the combination of probiotics and prebiotics [Gibson & Roberfroid, 1995], however, their use was not extensively studied up-to-date. It seems worth examining whether the specific substrate may improve the immunostimulative capability of probiotics, just like it could improve the survival of the probiotic organism. To act synergistically, the major mechanisms of protective immune action against pathogens of probiotic and prebiotic compound should be consistent. While *Bifidobacterium* strains were proved to stimulate immune IgA responses against pathogenic bacteria and viruses, oligosaccharides were suggested to function as receptor analogues to block viral and pathogenic bacterium adhesion [Zopf & Roth, 1996]. In the case of the restricted contact of pathogenic bacteria with gut immune system it is difficult to expect the increase of the IgA as the primary effectory immune response stimulated in the gut. Following that, the interesting matter is IgA-modulating effect of synbiotics – being synergistic or something more or less halfway between probiotic and prebiotic. Both those hypotheses need verification. Therefore the aim of the study was to examine whether prebiotic and synbiotics could modulate the immune response of the specific anti-*Salmonella* Enteritidis immunoglobulin A (SE IgA R).

MATERIALS AND METHODS

Experiment. The effect of oligofructose (prebiotic) and its synergistic sets (synbiotics) with *Bifidobacterium* strains: *B. longum* KN29.1, *B. longum* KNA1, and *B. animalis* KSp4, on the level of the specific anti-*Salmonella* IgA in rat blood serum was studied. The experiment was carried out on 5 groups of young Wistar rats (10 rats each), receiving daily for 14 days: OF group – oligofructose and bacteria-free physiological saline; OF+B1, OF+B2, and OF+B3 groups - oligofructose and $\geq 10^9$ *Bifidobacterium* cells suspended in physiological saline; or – control group (C) – bacteria-free physiological saline. 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 indirect immunometric ELISA method in the obtained rat sera. SE IgA R was evaluated by comparison of absorbance in the groups supplemented with prebiotic and synbiotic 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*.

Oligofructose. A highly purified preparation of oligofructose – Raftilose P95 (Orafti, Belgium) was added to feed in the amount of 5% (w/w).

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 under 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. 7.2×10^9 ($5.7–9.4\times10^9$) cells of *B. longum* KN29.1, 4.7×10^9 ($1.8–8.5\times10^9$) cells of *B. longum* KNA1 as well as 3.3×10^9 ($1.8–6.5\times10^9$) 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 *S*. Enteritidis 458 in the amount of 3.7×10^2, 3.7×10^6, 1.8×10^8, and 1.4×10^8, 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. The analyses were done with ELISA method, as described earlier [Biedrzycka *et al.*, 2003]. Briefly, the methodology comprised coating the microplates firstly with *S*. Enteritidis

458 in the amount of 2×10^8 cells/100 μL/well, secondly with 100-fold diluted serum (100 μL/well), conjugating with peroxidase-labelled goat anti-rat IgA conjugate (Nordic Immunology), and reading the absorbance at $\lambda=450$ nm.

Statistical analysis. The results of absorbance were expressed as the means and standard deviation of the values for five animals. The statistical significance of the differences was determined by Student's t test.

RESULTS

In the control group of animals, the specific anti-*Salmonella* IgA antibody response (SE IgA R) expressed as A_{450} was 0.484±0.032 (Figure 1). In the group that received prebiotic (OF), SE IgA R was 0.434±0.026, whereas in the groups administered with synbiotics consisting of oligofructose and *Bifidobacterium* strains – *B. longum* KN29.1 (OF+B1), *B. longum* KNA1 (OF+B2), and *B. animalis* KSp4 (OF+B3) – SE IgA Rs were 0.439±0.040; 0.524±0.045; 0.548±0.016, respectively. Upon OF and OF+B1 supplementation, SE IgA Rs were slightly but significantly lower (both $p\leq0.05$), in OF+B2 group – it showed an ascending tendency (not significant), and only in OF+B3 group it was slightly but significantly increased by 0.064 ($p\leq0.01$) in comparison to the control.

In the control group of rats challenged with *Salmonella*, SE IgA R was 0.416±0.026 (Figure 2). In the experimental groups, SE IgA Rs were: 0.442±0.036 in OF group, and 0.435±0.020; 0.569±0.077; 0.573±0.078, in OF+B1, OF+B2, and OF+B3 group, respectively. Oligofructose alone and synbiotic containing *B. longum* KN29.1 showed a tendency to a slight increase (not significant) of SE IgA Rs in comparison to the control. In the other synbiotic groups, OF+B2 and OF+B3, slight but significant increases (both $p\leq0.01$) of SE IgA Rs were observed.

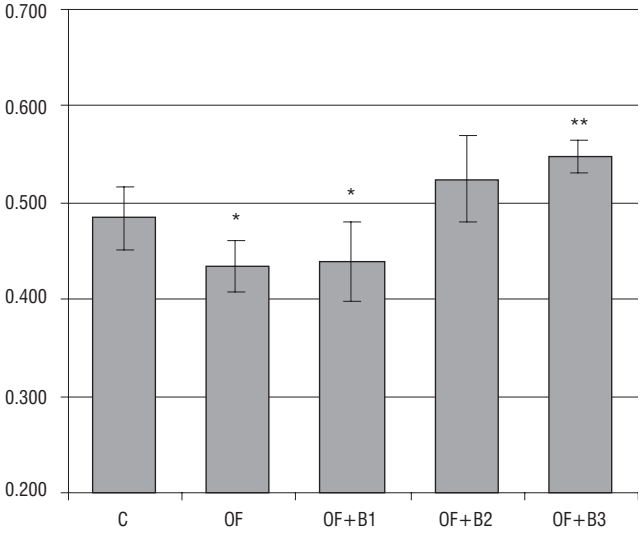

FIGURE 1. Changes of the specific anti-*Salmonella* Enteritidis IgA antibody response (A_{450}) in blood serum of rats administered with oligofructose[1)] alone or in synbiotic form with *Bifidobacterium*[2)] ([1)] OF – oligofructose; [2)] B1 – *B. longum* KN29.1, B2 – *B. longum* KNA1, B3 – *B. animalis* KSp4. The IgA level significantly different from the control at the significance level of * $p\leq0.05$, ** $p\leq0.01$).

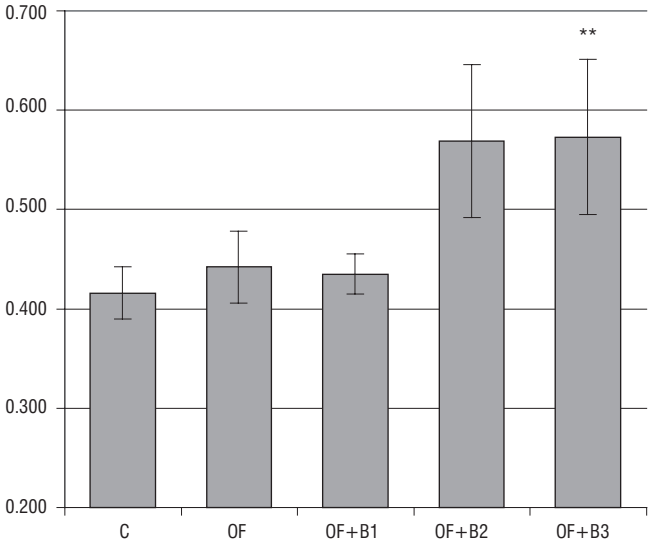

FIGURE 2. Changes of the specific anti-*Salmonella* Enteritidis IgA antibody response (A_{450}) in blood serum of rats administered with oligofructose [1)] alone or in synbiotic form with *Bifidobacterium* [2)], and challenged with *S*. Enteritidis 458 ([1)] OF – oligofructose; [2)] B1 – *B. longum* KN29.1, B2 – *B. longum* KNA1, B3 – *B. animalis* KSp4. The IgA level significantly different from the control at the significance level of ** $p\leq0.01$).

The level of immune response was not significantly different between OF and OF+B1 groups, as well as between OF+B2 and OF+B3 groups, whereas it was significantly different ($p\leq0.01$ and $p\leq0.001$) in the groups of animals administered with the prebiotic OF and synbiotic OF+B1 in comparison to those receiving synbiotics OF+B2 and OF+B3, both in non-infected and *Salmonella*-infected rats. In the control group of animals challenged with *Salmonella*, SE IgA R was slightly lowered (by 0.068, $p\leq0.01$) than in the control group of non-infected rats, whereas the other SE IgA Rs were not different in the experimental groups with or without *Salmonella*.

DISCUSSION

Immunoglobulin A is the main secretory immunoglobulin appearing on all mucosal surfaces of the body. Bifidobacteria exert various effects on immune system related functions, such as modification of the mitogenic activity and adjuvant activity, promotion of macrophages, antitumor effects and also stimulation of antibody production [Bornet & Brouns, 2002]. Our previous report showed that they are capable of increasing the specific anti-*S*. Enteritidis IgA antibody response in rats [Biedrzycka *et al.*, 2003]. Following that, we wondered about stimulation of their immunomodulating action by use of a prebiotic component. Upon probiotic supplementation, the significant increases in SE IgA R were determined on the level of ~0.13–0.16 and ~0.17–0.22, respectively, in non-infected and *Salmonella*-infected rats, whereas, in the present study, they occurred in two synbiotic groups on the level of ~0.06 and ~0.15-0.16, respectively. The comparison shows much higher stimulation of the specific IgA response against *Salmonella* by probiotics than by synbiotics.

The weaker action of synbiotics was determined by the prebiotic component. Oligofructose alone slightly de-

creased or had no significant impact on SE IgA R. The results obtained for oligofructose are consistent with those of Qiao *et al.* [2002] who studied the immune responses in rhesus rotavirus-challenged Balb/c mice treated with bifidobacteria and prebiotic supplements, including arabinogalactan, short-chain fructo-oligosaccharides, or iso-maltodextrins. The authors observed rotavirus-specific IgA responses in serum in rotavirus-infected litters in the group administered with a synbiotic containing fructooligosaccharides on the level below or comparable to the control, which was much lower than in all the other synbiotic groups and probiotic group as well. The strongest specific IgA protection occurred in probiotic group, like in our studies, whereas the addition of the selected prebiotics did not improve the results over bifidobacteria treatment alone. However, the clinical effect of diarrhoea symptoms was significantly diminished in the experimental litters treated with both bifidobacteria and prebiotic compounds, and not substantially different from that observed in pups treated with bifidobacteria alone. Roller *et al.* [2004], studying modulation of intestinal immune functions in rats by inulin enriched with oligofructose in combination with probiotic *Lactobacillus rhamnosus* and *Bifidobacterium lactis*, also concluded that the combined application of probiotic and prebiotic does not simply result in additive or synergistic effects.

The possible explanation could be the mechanism of oligofructose action, different from simple enhancement of *Bifidobacterium* growth and IgA stimulation. Oligofructose might possibly block the contact of *Salmonella* with the intestinal epithelial cells and further with GALT, therefore the initiation of immune response did not occur. Pathogen could be cleared this way from a mucosal surface and failed to find a host [Zopf & Roth, 1996]. That hypothesis can be strengthened by the observation of no symptoms of diarrhoea or abnormal animal behaviour in the experimental group treated with either probiotics, or prebiotic, or synbiotics.

Secondary structure of oligosaccharides allows them to act as receptor analogues for cell surface sites in the gut epithelium, preventing adhesion of microbes to the gut mucosa [Uauy & Araya, 2004]. In past few decades, carbohydrate-binding proteins used by pathogens to recognise and adhere to cell surfaces have been described with increasing frequency [Zopf & Roth, 1996]. Several dozens of bacterial pathogens are able to initiate specific binding to human cells by recognising the specific oligosaccharide surface configurations, and some viruses, yeasts and protozoa as well. Oligosaccharides are thought to be assembled by glycosyltransferases which synthesise cell surface glycoconjugates that are often used as receptors by pathogens [Newburg, 1996]. Most adhesin combining sites accommodate oligosaccharide segments consisting of three, four, or five monosaccharides. The greatest evidence was accumulated for maternal milk oligosaccharides which are able to protect babies from many infectious agents. Some milk oligosaccharides have been documented to protect the nursing infant by acting as receptor homologues, inhibiting the binding of pathogens to their host receptors, *e.g.* enteropathogenic *Escherichia coli* and *Campylobacter jejuni* [Cravioto *et al.*, 1991; Cervantes *et al.*, 1995]. Morrow *et al.* [2004] have found that 2-linked fucosylated oligosaccharides, which when present in maternal milk at high level, effectively prevented either *Campylobacter* or calicivirus diarrhoea in breast-fed infants. The same mechanism of binding-blocking functioned in the protection against heat-stable enterotoxin of *Escherichia coli* [Crane *et al.*, 1994].

The results of up-to-date studies on pathogen-binding oligosaccharides in the gastrointestinal tract were related mainly to oligomers of mannose and galactose, therefore further studies with oligofructose are needed to confirm our hypothesis. Proving that possible oligofructose action would be attractive in prevention of enteric infections.

CONCLUSIONS

Oligofructose alone did not appear to increase the specific anti-*S*. Enteritidis IgA response. Therefore, synbiotics constructed of IgA-not-stimulating oligofructose and IgA-stimulating *Bifidobacterium* did not synergistically improve that parameter. The results seem to confirm the hypothesis that oligofructose, like some other short-chain oligosaccharides may act as receptor analogue for pathogenic bacteria, preventing their binding to the gut epithelium, which incriminates in lesser antigen receptor binding, recognition, processing and generation of IgA antibodies, even if compared to probiotics. However that hypothesis needs further confirmation.

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