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Comparative immunomudulating effects of five orally administrated bifidobacteria species in male albino rats

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Abstract

Bifidobacteria belong to effective probiotics in preventing and reducing the severity of some diseases by modulating the host immune response. Each probiotic species is unique, and thus its properties and effects have to be assessed separately. We investigate here the effects of *B.infantis*, *B.longum*, *B.adolescentis*, *B.bifidum*, *B.breve* and *B.bifidum* on immune parameters of albino rats orally administrated a yogurt fermented with one of the five bifidobacteria species for 30 days. *B.adolescentis* (adult-type bifidobacteria) induced a significant increase in pro-inflammatory cytokine secretion (IL-1, IL-4, IL-12 and TNF-alpha) relative to *B.breve* and *B.infantis* (infant-type bifidobacteria). *B.bifidum*, *B.longum* and *B.adolescentis* induced significantly high levels of the anti-inflammatory IL-10. *B.adolescentis* stimulated and increased the release of antioxidant enzymes (SOD, glutathione and catalase), and also stimulated secretion of high levels of total serum Ig. All studied bifidobacteria species, particularly *B. adolescentis*, caused significant reduction of the number of the pathogenic bacteria. In conclusion, *B. adolescentis* is superior to the other studied species of bifidobacteria in enhancing immune parameters.

Key words: Antioxidant enzymes, Bifidobacteria, Cytokines, Probiotics.

Introduction

Modulation of host immunity is one of the most common benefits of the administration of probiotics, live bacteria with clinically proven health effects in humans (Salminen *et al*. 2005). Probiotics have been shown to be useful in preventing certain disease conditions, as well as possibly promoting specific aspects of health (Rolfe 2000; Hamilton-Miller 2004). Good effects of probiotics have been reported in the management of diarrheal, inflammatory, and allergic diseases in infants (Salminen *et al*. 2005). In ulcerative colitis, probiotics offer a safe alternative to current therapy. Probiotics have been used to prevent urogenital tract infection and to reduce atopy in children (Macfarlane *et al*. 2002; Young *et al*. 2004). Neonatal application of probiotic bacteria inhibits subsequent allergic sensitization and airway disease in a murine model of asthma (Feleszko *et al.* 2007). Probiotics have been found to inhibit intestinal bacterial enzymes involved in the synthesis of colonic carcinogens (Rolfe 2000).

Generally, probiotics may counteract the inflammatory process by stabilizing the microbial environment and the permeability barrier of the intestine, and enhance the degradation of enteral antigens, altering their immunogenicity. They improve immunological response by increasing IgA synthesis (Salminen *et al*. 2005), and enhance intestinal health by inhibition of epithelial and mucosal adherence, inhibition of epithelial invasion and production of antimicrobial substances (Rolfe 2000). Different parts of the probiotic cell can modulate the immune system: cell wall material and cytoplasm have been suggested to elicit immune reactions (Stewart-Tull 1980; Ambrosini *et al*. 1998; Pessi *et al*. 1999); DNA sequences have been observed to affect the immune system (Klinman *et al*. 1996).

Bifidobacteria are considered an important part of the intestinal microflora in humans and other mammals. They are one of the specific bacterial components with a key impact on development of a healthy balanced infant microbiota (Salminen *et al*. 2005), associated with beneficial effects on the host, such as promotion of gut maturation and integrity, antagonism against pathogens and immune modulation (Blum *et al*. 2003). Dietary bifidobacteria have

been shown to enhance resistance to oral *Salmonella typhimurium* infection in mice (Shu 2000).

In a previous study, we found that bifidobacteria species, particularly *B.adolescentis,* protect rats against abnormal increase of atherogenic lipids. The present study compares the immune responses to five bifidobacteria species, necessary to select the most potent probiotic among these species.

Materials & Methods

Yogurt starter culture consisted of *Streptococcus salivarius* subsp *thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus*, obtained from the Cairo Microbiological Resource Center (Faculty of Agriculture Ain Shams University). Bifidobacteria strains were obtained from the American Type Culture Collection (Rockville, MD, USA) in a lyophilized form; their ATCC strain numbers are given below. All strains were maintained in modified MRS (Difico Laboratories, Detroit, MI) containing 5% lactose and 0.05% L-cystine HCL (Sigma Chemical Com, St, Louis, MO) as a reducing agent.

The yogurt was inoculated separately with 1.5% of the bifidobacterial strains at a concentration of 10^9 g^{-1} and gently mixed. It was supplied twice a day to the drinking water as 95 part plus 5 parts of the water (v/v) for 30 days. During this period, feed and water intakes were monitored. Rats drink about 10 ml water per 100 g body weight per day. Rats were fed on chow consisting of 8.2% protein, 2.6% fat, 8.6% ash, 62.2% carbohydrate, 7.6% fiber and 10.8% moisture (Hassan 2003).

A total of 30 adult male albino rats, each weighing 95-120 g, were purchased from the Biological Supply Center (Theodore Bilharz Research Institute, Cairo). They were divided into six groups of 5 rats, and treated as follows. The first group was orally administrated the yogurt starter culture (*Streptococcus salivarius* subsp *thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus*) for 30 days (control). Each of the other five groups was orally administered yogurt starter culture plus one of the bifidobacteria strains for 30 days: *B.infantis* (ATCC 11551); *B.longum* (ATCC 11549); *B.adolescentis* (ATCC 11550); *B.breve* (ATCC 11548); and *B.bifidum*. At the end of the experimental feeding, rats were fasted for about 16 h, weighed and then sacrificed and two blood samples (with and without anticoagulants) collected from each animal. The heparinized blood sample was used to investigate the total and the differential leukocytic count. The non-heparinized blood sample was used to obtain a serum for measuring the different cytokines, the antioxidant enzymes, the total protein and the total Ig. Serum was collected and preserved at -20 °C till use.

The total bacterial and bidfidobacterial counts were determined as described in the Difico manual (1973) using nutrient agar medium and incubated at 37°C for 48h. The bifidobacteria count used a modified MRS containing 5% lactose and 0.05% L-cystine (Sigma Chemical Com, Louis, Mo) as a reducing agent.

Interleukins and respiratory burst enzymes were measured using blood serum from each animal kept at –20 °C until processing. IL-1, IL-4, IL-10, IL-12 and TNF-alpha were measured using the corresponding rat immunoassay kits (BioSource International Inc. USA) according to the manufacturer's instructions. The respiratory burst enzymes (catalase, superoxide dismutase [SOD] and glutathione) were measured using kits (Biodiagnostica, Italy) according the manufacturer's instructions.

Statistical analysis used MINITAB (Version 13.1, 2002). All data were first tested for normality (Anderson-Darling test) and homogeneity of variances. Data were normally distributed, and variances were homogeneous, thus one-way ANOVA was used to determine overall effects of treatments, followed by individual comparison using Tukey's Pairwise comparison. Results are expressed as mean $\pm$ SD. Values of p>0.05 were considered statistically non-significant, while values of p<0.05 were considered statistically significant.

Results

To investigate the ability of bifidobacteria to affect the production of macrophages cytokines, pro-inflammatory (IL-1, IL-4, IL-12 and TNF-alpha) and anti-inflammatory (IL-10) cytokines were measured. *B.bifidum* significantly increased the levels of all cytokines compared to the control and to most of the other species (Figs. 1A,B; 2A-C). *B.adolescentis* also significantly induced the release of all the pro-inflammatory cytokines, and also a slight but significant increase in the level of the anti-inflammatory IL-10.

Results for the various antioxidants were similar, especially for catalase and SOD (Fig. 3A,B,C). *B.adolescentis* and *B.bifidum* induced significantly higher levels of catalase and SOD than those the control, *B.infantis, B.longum* and *B.breve* groups. *B. adolescentis* induced lower value of catalase than *B.bifidum*. *B.adolescentis* and *B.bifidum* induced significantly higher levels of glutathione than the control (Fig. 3C).

B.adolescentis, B.longum and *B.bifidum* significantly elevated the level of the total Ig in the serum. No significant changes in total protein were induced by any of the tested groups (Table 1). An increase relative to the control in total WBC counts was evident in all treatment groups, although these were not statistically significant (Table 2). Differential leukocytic counts showed that the proportion of monocytes was significantly elevated in the *B.adolescentis* group, but the change in lymphocyte proportion in this group was not significant.

Bifidobacteria, *Escherechia coli* and the total bacteria were counted at the end of the experimental period. This count was planned to determine the comparative tolerance of different bifidobacteria species and the effect of each of them on pathogenic bacteria. All studied bifidobacteria species, particularly *B.adolescentis*, showed significant reduction of the number of pathogenic bacteria compared to the control (Table 3): rats administrated this species showed the highest bifidobacteria count. This result indicates that this species has the most significant tolerance to gastrointestinal tract conditions.

Table 1: Total protein and total immunoglobulin in the serum of albino rats orally administered a yogurt fermented with one of five bifidobacteria species.

	Control	*B. infantis* group	*B. Longum* group	*B. adolescentis* group	*B. breve* group	*B. bifidum* group
Total protein g/dl	6.12 ± 0.625	6.08 ± 1.636	8.4 ± 1.636	4.08 ± 0.988	6.3 ± 1.4	8.44 ± 1.22
Ig mg/dl	640 ± 90.6	708 ± 86.7	$1144* \pm 274$	$1462* \pm 344$	816 ± 76.7	$1418* \pm 159.9$

Values are mean $\pm$ SD of five replicates.
Statistical significance (*$P < 0.05$) compares each bifidobacteria species with the control.

Table 2: Total and differential leukocytic count induced in albino rats after 30 days of orally administered yogurt fermented with one of five bifidobacteria species.

	Total WBC x10^3	Lymphocytes %	Neutrophils %	Monocytes %
Control	3.90 ± 0.12	72.68 ± 12.43	24.61 ± 7.81	5.18 ± 0.89
B. infantis	4.14 ± 0.83	75.68 ± 11.01	21.27 ± 8.21	9.82 ± 3.40
B. longum	4.33 ± 1.02	64.14 ± 5.08	30.11 ± 4.5	9.98 ± 3.22
B. adolescentis	4.46 ± 0.64	69.96 ± 2.86	18.96 ± 1.05	$11.04 \pm 2.80*$
B. breve	3.98 ± 0.36	69.92 ± 5.53	23.40 ± 4.72	5.94 ± 1.63
B. bifidum	4.61 ± 0.34	68.32 ± 2.68	25.82 ± 1.5	5.82 ± 1.83

Values are mean $\pm$ SD of five replicates.
Statistical significance (*$P < 0.05$) compares each bifidobacteria species with the control.

Table 3: Numbers of *Escherichia coli*, bifidobacteria and total bacteria in the feces of albino rats after 30 days of orally administered yogurt fermented with one of five bifidobacteria species.

	Control group	*B. infantis* group	*B. longum* Group	*B. aldolescentis* Group	*B. breve* group	*B. bifidum* group
Escherichia coli x 10^6	116.6 ± 1.14	$12.6^* \pm 2.3$	$58.4^* \pm 3.44$	$6.8^* \pm 2.168$	$92.4^* \pm 3.8$	$25.8^* \pm 3.96$
Bifidobacterium x 10^6	15.4 ± 2.07	$255.8^* \pm 2.77$	$51^* \pm 5.57$	$256^* \pm 8.79$	$45.2^* \pm 2.86$	$181.8^* \pm 10.06$
Total bacterial count x 10^8	125.8 ± 3.96	$6.2^* \pm 3.56$	$5.8^* \pm 2.95$	$4.6^* \pm 1.517$	$79^* \pm 6.2$	$11.2^* \pm 1.924$

Values are mean ± SD of five replicates.
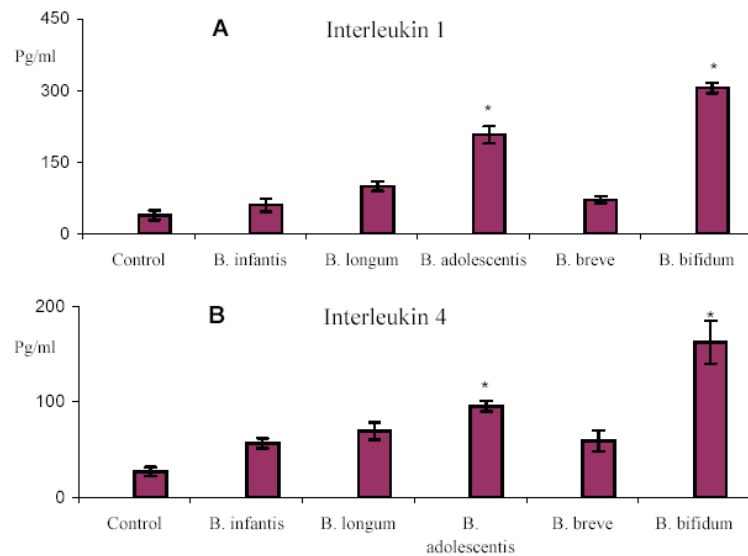Statistical significance (*$P < 0.05$) compares each bifidobacteria species with the control.

Fig. 1: Effect of the five different bifidobacteria species on levels of the pro-inflammatory cytokines, IL1 (A) and IL4 (B) in the blood serum of albino rats.
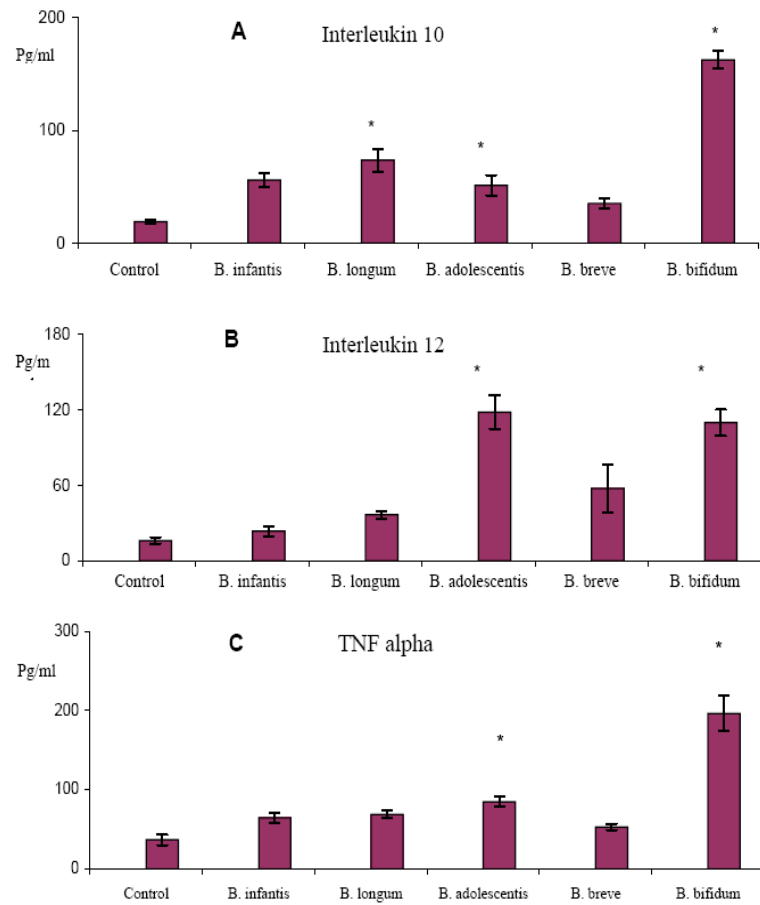
Fig. 2: Effect of the five different bifidobacteria species on levels of the anti-inflammatory IL-10 (A) and the pro-inflammatory IL12 (B) and TNF-alpha (C) in the blood serum of albino rats.

Discussion

Besides clinical findings, different studies have shown the influence of probiotic bacteria on the immune response in various diseases. The mechanism may be based on modulating pro- and anti-inflammatory cytokines, since the former play an important role in gut inflammation (Fiocchi 1998; Fukushima *et al.* 1999; Schultz *et al.* 1999; Sartor 2004). Our data showed that bifidobacteria species significantly stimulated both the pro- and/or the anti-inflammatory cytokines. Similar results were found by Helwig *et al.* (2006). Variable cytokine profiles are obtained by different bifidobacteria strains (Juntunen 2001). The species is of fundamental importance in the selection of strains for probiotic use (Perdigon *et al.* 2003). Therefore the unique properties of the different species remains an important issue for clarification.
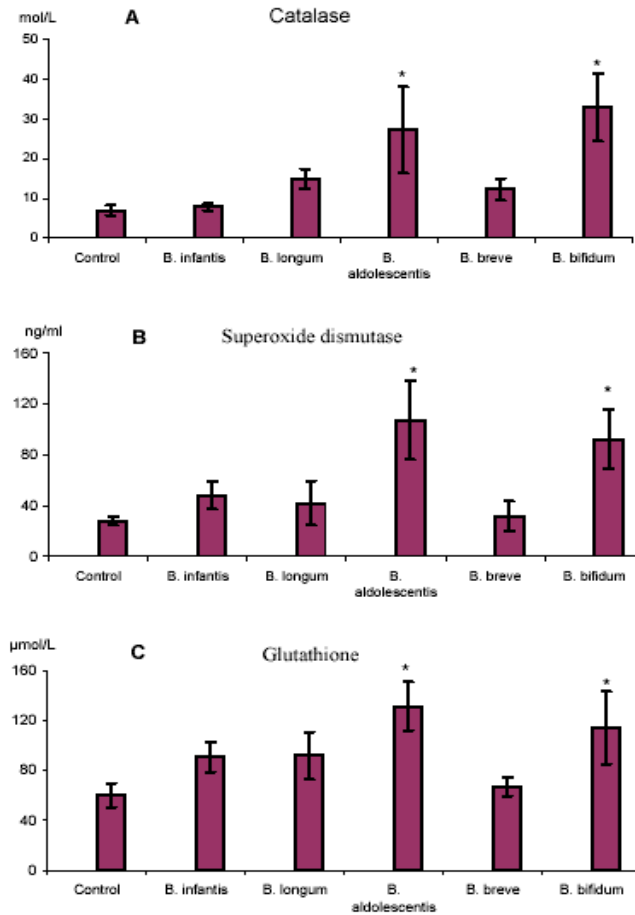
Fig. 3: Effect of the different bifidobacteria species on levels of the blood anti-oxidant enzymes (A) catalase; (B) superoxide dismutase; and (C) glutathione.

It has previously been found that strains isolated from healthy infants mainly stimulate the pro-inflammatory cytokines (Salminen *et al*. 2005). In contrast, the present data showed that the adult-type *B.adolescentis* induced significantly greater pro-inflammatory cytokine secretion than the infant-type *B.breve* and *B.infantis*. He *et al*. (2002) suggested that *B.adolescentis* elevated the pro- and inhibited the anti-inflammatory cytokines. However, in our experiments, this species recorded a slight but significant increase in the level of IL-10. Increases in IL-10 levels may cause a balance between the pro- and anti-inflammatory cytokines. The probiotic effects may be mediated via control of the balance between pro- and anti-inflammatory cytokines (Havenaar *et al*. 1992; Salminen *et al*. 1998; Hemmi *et al*. 2000). Therefore this increase in the level of IL-10 may be necessary, since IL-10 prevents inflammatory reactions such as toxic colitis (Madsen *et al*. 2000; Steidler *et al*. 2000; Fiorucci *et al*. 2002). Increases in IL-10 correspond to positive effects on inflammatory bowel disease (Helwig, 2006). In case of *B.bifidum* a high value of IL-10 was induced and at the same time IL-12 and other cytokines were significantly high. This indicates that *B. bifidum* can also achieve a balance between pro- and anti-inflammatory cytokines.

Rigby *et al*. (2005) found that intestinal dendritic cells can produce both IL-12 and IL-10 following bacterial stimulation by *B.longum*. However, in the present study we found that IL-10 increased after application of *B.longum*, whereas levels of pro-inflammatory cytokines remained low. Reduction of release of IL-12 from APC by increasing IL-10 (Bluelens *et al*. 1997; Morel *et al*. 1997; Fickenscher *et al*. 2002) may inhibit native T cells from differentiating to Th1 (Huang *et al*. 2001; Abu-El-Saad & Abdel-Moneim, 2005). This may be taking place in the case of *B.longum*. In contrast, *B.adolescntis* and *B.bifidum* might stimulate the increase of Th1 cells relative to Th2 under the influence of high IL-12 and other cytokines.

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The result of the differentiation of T cells to Th1 is an increased secretion of IgA and a reduced production of IgE (Kiriavainen *et al.* 1999), which leads also to a reduced allergic response (Ouwehand *et al.* 2002). Yasui *et al.* (1992) mentioned that bifidobacteria are able to enhance immune function by increasing several factors, including IgA synthesis. Olas *et al.* (1999) concluded that IgA has both a pro-inflammatory and an anti-inflammatory capability, and this dual function might contribute to the feedback mechanisms maintaining a balance between pro-inflammatory and anti-inflammatory activities. Here, *B.adolescentis* induced the most significant change in total Ig. The increase in total Ig may indicate an increase in the IgA as well.

In innate immunity, neutrophils and monocytes play an important role in host defense by killing invading microorganisms. One of their important aspects is the ability to synthesize pro- and anti-inflammatory cytokines and growth factors that modulate the inflammatory response (Cassatella 1999). Therefore the significant increase in the proportion of monocytes caused by *B.adolescentis* may explain the corresponding increase in the level of all cytokines.

The increased values of the antioxidant enzymes indicated that administration of bifidobacteria species has activated them. A strain of *B.bifidum* is known to have a significant antioxidant action, able to protect the intestinal lining from lipid peroxidation in iron-overloaded mice (Ito *et al.* 2001). Mn-SOD can be induced by a lot of stimuli, including cytokines (TNF alpha, IL-6, IFN alpha, IL-10, IL-1), LPS, inflammatory and ischaemic stress (Raineri *et al.* 1996; Nishioka *et al.* 1998; Sugino *et al.* 1998; Pang *et al.* 2000). Accordingly, because the most studied cytokines were elevated by *B.adolescentis*, SOD was also higher in this group relative to other groups.

Increased glutathione level was induced by *B.adolescentis* and it is believed that glutathione peroxidase plays an important role in cellular antioxidant defense by reducing hydrogen peroxide and various hydroperoxides (Wendel 1980). Unlike glutathione and SOD, catalase was lower in the *B.adolescentis* group than in the *B.bifidum* group. Spolarics *et al.* (1996) suggested that a decrease in catalase activity may be due to inactivation by its own substrate. In addition, catalase plays a significant role in the elimination of H_2O_2, especially under conditions where H_2O_2 reaches high intracellular concentrations when catalase handles approximately half of the generated H_2O_2 (Gaetani *et al.* 1994).

Probiotics can stimulate synthesis and secretion of IgA which coats mucosal surfaces against harmful bacterial invasion. This reduces the number of pathogenic bacteria (Forchielli & Walker 2005). Furthermore, probiotics can competitively exclude pathogens directly or hinder the adhesion of pathogens to receptors on the gastrointestinal surface (Dai & Walker 1999). This can explain the sharp reduction in the number of *Escherichia coli* in the bifidobacteria-treated groups.

The development of the microbiota and the process of competitive exclusion would depend on the specificity of the bacteria and bacterial adhesions for the receptors and the relative concentration of the competing bacteria (Miettinen *et al.* 2000; Isolauri *et al.* 1991). The effects of gastrointestinal conditions such as pH, bile, and digestive enzymes, on the survival of probiotic bacteria are also significant factors (Ouwehand *et al.* 2001). Various bacteria show different levels of tolerance to gastrointestinal conditions. In the present study, counting provided an indication on the tolerance of the different species to gastrointestinal conditions. *B.adolescentis* recorded the highest count, implying that it has a high tolerance for gastrointestinal conditions. At the same time it might adhere and colonize in the gastrointestinal conditions in a better rate than did other competing species.

Study of the genome of *B.longum* indicates that this strain has specific gene sequences that promote adherence to intestinal mucosa, especially in the colon (Ouwehand *et al.* 2002). The gene may predispose some strains and species (Salminen *et al.* 2005), and it seems likely that it may also be present in *B.adolescentis* which appears to be more tolerant than *B.longum*.

In conclusion, the present results clearly showed that *B.adolescentis* was the most potent of the five investigated bifidobacteria species in enhancing the measured immune parameters. At the same time, it decreased the number of pathogenic bacteria, indicating a promising role in preventing bacterial infections. Furthermore, *B.adolescentis* was effective in reducing lipid atherogenic factors (data under publication). Although this study clearly showed the superiority of *B.adolescentis*, the role of other mechanisms such as competitive inhibition and increased acidity affecting the activity and composition of gut microflora and others must be taken into account in the selection of particular probiotics. Because of species-specific responses, specificity has to be investigated for each species separately.

Acknowledgements

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الملخص العربى

مقارنة التأثير المناعي لخمسة أنواع من البيفيدوبكتيريا التي يتم اعطائها عن طريق الفم لذكور الجرزان البيضاء.

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تعد البيفيدوبكتيريا من البروبيوتك probiotics التي لها تأثير واضح في منع و التقليل من بعض الأمراض. و من المفيد دراسة تأثير كل نوع من هذه البكتيريا علي حدا. لذلك تهدف هذه الدراسة الي مقارنة تأثير خمسة أنواع من هذه البكتيريا علي بعض المؤشرات المناعية في ذكور الجرزان البيضاء. قسمت الجرزان الي ستة مجاميع, ظلت واحدة منها كمجموعة ضابطة, أما الباقي فأعطيت كل مجموعة نوع واحد فقط من أنواع البيفيدوبكتيريا .B *infantis, B. longum, B.*) (adolescentis, *B. bifidum, B. breve* and *B. bifidum* لمدة 30 يوما. أظهرت النتائج تميز واضح لصالح بكتيريا ال *B. adolescentis* حيث سجلت زيادة معنوية واضحة في مستوي pro-inflammatory cytokines مقارنة بمعظم الأنواع الأخري. كذلك كان لهذا النوع أثر واضح في رفع مستوي ال anti-inflammatory cytokine IL-10. من ناحية أخري ساهم هذا النوع في زيادة الأنزيمات المضادة للأكسدة (SOD, glutathione and catalase), كذلك معدل الأجسام المضادة في السيرم. علاوة علي ذلك أظهرت النتائج أن *B. adolescentis* لها قدرة أعلي من مثيلاتها علي تحمل ظروف القناة الهضمية من خلال تواجدها بأعداد كبيرة. في نفس الوقت أثر هذا النوع علي معدل تواجد البكتيريا الممرضة و بدا ذلك في الأنخفاض الحاد لبكتيريا الأشريشياكولاي. وخلصت هذه الدراسة الي أن *B. adolescentis* هي أفضل من الأنواع المنافسة من حيث المؤشرات التي تم دراستها.