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Probiotic bacteria down-regulate the milk-induced inflammatory response in milk-hypersensitive subjects but have an immunostimulatory effect in healthy subjects

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Summary

Background Probiotic bacteria can influence immune responses both specifically by stimulating antibody production and nonspecifically by enhancing phagocytosis of pathogens and modifying cytokine production.
Objective The authors hypothesized that probiotic bacteria can alleviate hypersensitivity by influencing phagocytes. The modulation of phagocytes may be different in healthy subjects compared with hypersensitive subjects.
Subjects and methods In a double-blind, cross-over study, challenges with milk in milk-hypersensitive and healthy adults with or without an intestinal bacterial strain, *Lactobacillus GG* (ATCC 53103) were performed. The challenge-induced immunoinflammatory response was recorded by measuring the expression of phagocytosis receptors prior to and after the challenge using flow cytometry.
Results In milk-hypersensitive subjects, milk challenge increased significantly the expression of CR1, FcγRI and FcαR in neutrophils and CR1, CR3 and FcαR in monocytes. Milk with *Lactobacillus GG* prevented the increase of the receptor expression. In healthy subjects, milk challenge did not influence receptor expression while milk with *Lactobacillus GG* increased significantly the expression of CR1, CR3, FcγRIII and FcαR in neutrophils.
Conclusion Probiotic bacteria appear to modulate the nonspecific immune response differently in healthy and hypersensitive subjects. This is seen as an immunostimulatory effect in healthy subjects, and as a down-regulation of immunoinflammatory response in milk-hypersensitive subjects.

Keywords: immune regulation, immunostimulation, immunosuppression, milk hypersensitivity, phagocytosis, probiotics

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Introduction

Probiotic bacteria which beneficially influence the host by improving its microbial balance [1] have various clinical effects on patients and on healthy subjects. For instance *Lactobacillus GG* (ATCC 53103), an extensively studied probiotic strain, can prevent [2,3] and promote recovery from diarrhoea [4,5]. Immunological mechanisms behind this may include stimulation of specific antibody-secreting cell response [4], enhancement of pathogen phagocytosis [6] and modification of cytokine production [7]. Consequently, probiotic bacteria may influence both specific and nonspecific immune responses.

Dietary antigens, such as proteins in cow's milk, can induce inflammation in food hypersensitive subjects. In the intestine, oral antigen challenge can enhance the release of inflammatory mediators, such as histamine and eosinophil cationic protein, and cause a leakage of albumin and hyaluronan [8,9], and increase intestinal permeability [10–12]. Recently, these inflammation-induced alterations have successfully been treated by probiotic bacteria [13],

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even though most studies report stimulation of immune responses following probiotic intake [4,6,7,14].

The authors hypothesized here that the oral introduction of a probiotic strain can influence milk-induced inflammatory response and that the inflammatory response may be different in healthy and hypersensitive subjects. For this purpose, in a double-blind, cross-over study, milk-hypersensitive adults with normal lactose tolerance but with gastrointestinal reactions following consumption of milk products and healthy control subjects were exposed to a milk challenge with or without probiotic bacteria. Challenge-induced immunological changes were recorded by measuring the expression of phagocytosis receptors prior to and after the challenge. Phagocytosis mediated by phagocytosis receptors is responsible for the early activation of the inflammatory response before antibody production and it is therefore suitable for studying challenge-induced hypersensitivity reactions.

Materials and methods

Subjects

The study participants were 17 students and staff members (13 women, four men, aged 22–50 years, mean 28 years) from the Department of Biochemistry and Food Chemistry, University of Turku, Finland. Inclusion criteria required that they were free from signs and symptoms of acute infections in the beginning of the study. Subjects with mild, untreated infections during the study were not excluded. Three subjects had atopic dermatitis. On the basis of clinical history, lactose tolerance test with ethanol [15] and the double-blinded, placebo-controlled milk-challenge, participants were divided into two groups. Nine subjects were milk-tolerant (control group, including two subjects with atopic dermatitis). Eight subjects were nontolerant to milk with unequivocal gastrointestinal reactions such as abdominal bloating, flatulence, abdominal pain and diarrhoea (milk-hypersensitive group, including one subject with asthma and atopic dermatitis). All subjects had normal lactose tolerance.

Study design

Double-blind, cross-over study comprised two one-week challenge periods preceded and followed by a one-week washout period totally free from milk protein. The subjects were challenged with milk (commercial pasteurized and homogenized milk with 10 g/L fat and 34 g/L protein, Valio Ltd, Turku, Finland) with or without *Lactobacillus GG*-bacteria (ATCC 53103, from Dr Maija Saxelin, Valio Ltd, Helsinki, Finland). The sequence of the oral challenges was randomized. Each challenge period lasted one week, and 200 mL of milk was taken twice a day. *Lactobacillus GG* dose was 2.6×10^8 colony forming units (c.f.u.) per day during the challenge period. Since the concentration of bacteria decreases during storage, fresh milk preparation was delivered twice a week to each subject.

Reagents for receptor analysis

Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} ions (pH 7.4) was made without phenol red and supplemented with 0.1% gelatine. Isotypic controls (IgG1-FITC, IgG1-PE and IgG2a-PE), anti-CR1 (CD35-FITC), anti-FcγRI (CD64-FITC), anti-FcγRII (CD32-PE), anti-FcγRIII (CD16-FITC), and anti-FcαR (CD89-PE) were purchased from Immunotech (A Coulter Company, Marseille, France). Anti-CR3 (CD11b-PE) was obtained from Biodesign International (Kennebunk, ME, USA).

Samples

Peripheral, EDTA-anticoagulated (1.5 mg EDTA/mL blood) blood samples were collected by venopuncture prior to and after the one-week challenge. For flow cytometry, leucocytes were isolated by lysing the erythrocytes with ammonium chloride (1.5 mL blood, 8.5 mL of 0.83% ammonium chloride) at room temperature for 15 min. After lysation, leucocytes were centrifuged (400 ***g*** for 10 min) and resuspended in 500 μL ice-cold Ca^{2+}- and Mg^{2+}-free HBSS.

Measurement of receptor expression

The expression of complement receptors (CR1, CR3), receptors for IgG (FcγRI, FcγRII, FcγRIII), and for IgA (FcαR) on neutrophils and on monocytes was assayed by flow cytometry [16].

Leukocytes (3×10^5) were incubated with monoclonal antibodies for 30 min at 4 °C in a volume of 90 μL. The control sample was incubated with isotype-matched monoclonal antibodies directed to irrelevant antigens. After incubation cells were washed with cold Ca^{2+}- and Mg^{2+}-free HBSS.

Flow cytometric analysis was performed with a Coulter EPICS XL (Miami, FL, USA) flow cytometry with an argon ion laser. The laser excitation wavelength was 488 nm. Emitted light was collected through 550 nm and 600 nm dichromic filters and 525 nm and 575 nm bandpass filters. The fluorescence of 5000 cells was measured using logarithmic amplification. A relative measure of receptor expression on neutrophils and on monocytes was obtained by determining the mean log fluorescence intensity (MFI) after the two-colour spectral compensation network adjustment. In assay CV% of the test was on average 6.4% (range: 1.7–7.9%, depending on the receptor measured).

Ethics

Informed consent was obtained from the participants and the study protocol was approved by the local committee on ethical practice.

Statistics

The Wilcoxon signed rank test was used in statistical comparisons. Data are presented as values of mean and standard deviation (SD) and as changes (%) during the challenge.

Results

The receptor expression results are presented in Table 1. These indicate receptor expression changes in percentage values during milk challenge with or without *Lactobacillus GG* in control and milk-hypersensitive subjects. There were no significant differences in the baseline values of the receptor expressions between the groups. In milk-hypersensitive subjects, milk challenge significantly increased the receptor expression while *Lactobacillus GG* down-regulated this increase: (mean [SD] receptor expression prior to vs after the challenge) CR1 in milk challenge 5.9 (0.9) vs 6.9 (1.3), $P = 0.008$ and in milk challenge with *Lactobacillus GG* 7.0 (1.4) vs 6.7 (1.7), $P = 0.38$; CR3 4.1 (1.7) vs 5.6 (2.4), $P = 0.02$ and 4.5 (1.7) vs 4.7 (2.1), $P = 0.74$; and FcαR 9.2 (1.7) vs 10.4 (1.9), $P = 0.008$ and 9.8 (1.3) vs 9.1 (1.5), $P = 0.20$ in monocytes. Similar changes were seen in CR1, FcγRI and FcαR in neutrophils.

A distinct pattern of immunomodulatory response was seen in controls. Milk challenge did not influence receptor expression but milk with *Lactobacillus GG* significantly increased the expression of CR1, CR3, FcγRIII and FcαR in neutrophils but not in monocytes.

Discussion

Results demonstrate that milk increased the expression of phagocytosis receptors (CR1, CR3, FcγRI and IgαR) while *Lactobacillus GG* prevented the increase in milk-hypersensitive subjects. This indicates that the probiotic bacteria can down-regulate the milk-induced immuno-inflammatory response. In the control group *Lactobacillus GG* had an immunostimulatory effect seen as increased receptor expression when consuming milk with *Lactobacillus GG*. Therefore, probiotic bacteria can modulate the

Table 1. Receptor expression changes as percentage values during 1 week milk challenge (after/prior to the challenge × 100%) in control and in milk-hypersensitive subjects with milk only (without LGG) or with milk containing *Lactobacillus GG* (with LGG). No changes during the challenge = 100%. Wilcoxon signed rank test was used in determining *P* values.

	Control subjects				Milk-hypersensitive subjects			
	Without LGG		With LGG		Without LGG		With LGG	
	Change (%)	*P*	Change (%)	*P*	Change (%)	*P*	Change (%)	*P*
CR1								
Neutrophils	104	0.72	128	0.004	122	0.02	105	0.74
Monocytes	101	0.86	108	0.15	117	0.008	96	0.38
CR3								
Neutrophils	95	0.86	133	0.05	127	0.06	122	0.15
Monocytes	83	0.14	109	0.14	137	0.02	104	0.74
FcγRI								
Neutrophils	104	0.81	109	0.07	114	0.02	104	0.64
Monocytes	105	0.86	101	0.95	100	0.74	103	0.74
FcγRII								
Neutrophils	106	0.37	110	0.09	107	0.27	97	0.31
Monocytes	99	0.81	106	0.14	98	0.84	105	0.08
FcγRIII								
Neutrophils	104	0.21	108	0.04	100	0.95	104	0.15
Monocytes	114	0.31	91	0.37	177	0.15	117	0.41
IgαR								
Neutrophils	102	0.68	110	0.03	114	0.02	102	0.95
Monocytes	96	0.68	104	0.14	113	0.008	93	0.20

immune response differently in healthy and hypersensitive subjects.

The pathophysiology of milk hypersensitivity is not known precisely. Nevertheless, clinical and laboratory findings in numerous studies indicate that milk hypersensitivity may be mediated through all the four mechanisms characterized by Coombs and Gell [17]. Immediate, IgE-mediated reaction type is best known and studied. However, this may not be the major immunological mechanism for food hypersensitivity in adults [18–20]. In the authors' previous studies, increased phagocyte activity and receptor expression were seen in milk-hypersensitive infants [10] and adults [18]. Therefore, an overactive phagocytic process appears to be at least part of the mechanism in milk hypersensitivity. Both the type II (antibody-dependent cytotoxic) and the type III (immune complex-mediated) reactions can activate phagocytosis. Increased phagocyte activity may cause an inflammation in the intestine as during phagocytosis, phagocytes release lysosomal enzymes and oxidizing agents that may damage the surrounding tissues [21]. This can induce chronic gastrointestinal symptoms, such as abdominal pain and diarrhoea.

How can probiotic bacteria affect milk hypersensitivity? Probiotic bacteria must stay viable throughout the gastrointestinal tract and then colonize the gut or adhere to intestinal mucosa, and, secondly, bacteria must have beneficial effects on the host. *Lactobacillus GG* is an extensively studied strain with the ability to survive in the gastric and bile acids [22]. It can also adhere to intestinal mucosa *in vitro* [23] and *in vivo* [24]. The dose chosen in this study was 2.6×10^8 c.f.u. per day which, as consumed with milk, is adequate for intestinal colonization (M. Saxelin, unpublished data). Probiotic bacteria have been shown to normalize the intestinal permeability and thereby decrease the permeation of antigens in hypersensitive subjects [12]. On the other hand, the bacteria can enhance gut local humoral response, particularly IgA response [4]. Antigen with bound secretory IgA may facilitate the uptake through Peyer's patches and thus sustain the mucosal immune response [25]. In addition to these mechanisms, the enzymes of probiotic bacteria can degrade milk proteins [26,27]. All these mechanisms may lead to improved immune exclusion and immune elimination of antigens, and may modulate immune regulation which is manifested in suppression of phagocyte activity.

Differences in immunomodulatory effects of immunoregulatory cytokines have recently been documented between healthy and hypersensitive subjects [28]. In a similar manner, the immunological effects of microbial stimulation were distinctive in this study's milk-hypersensitive subjects and healthy controls. *Lactobacillus GG* had an immunostimulatory effect on controls, seen as increased receptor expression. Similar results have also been obtained with other probiotic strains. For instance, certain *Lactobacillus acidophilus* and *Bifidobacterium* strains can enhance phagocyte activity both *in vitro* [29] and *in vivo* in mice [30] and in humans [6]. The augmentation of the immune response by probiotic bacteria seems to be similar to that of cholera toxin [4]. Therefore, as adherence of a food antigen with cholera toxin B to enterocytes may enhance the intestinal immune response [31], a similar effect may also occur in adherence of probiotic bacteria to intact enterocytes. Adherent bacteria interact with gut-associated lymphoid tissue and may therefore directly affect leucocytes by stimulating phagocytosis [32]. Probiotic bacteria may also hydrolyse milk proteins, producing bioactive peptides which may trigger gut immune responses [26,27,33]. Alternatively, probiotic bacteria can stimulate cytokine production [7] which can further induce receptor expression. For example, interferon gamma (IFN-γ) can increase the expression of FcγRI and FcγRIII in neutrophils but not alter the expression of FcγRII, CR1 and CR3 [34]. Oral allergen challenge reduces production of IFN-γ by peripheral blood mononuclear cells in hypersensitive subjects [35]. On the other hand, in milk-hypersensitive subjects with abnormal handling of milk antigens the immune response by probiotic bacteria may be different, manifesting as down-regulation of immunoinflammatory response as observed in this study.

In conclusion, specific probiotic lactic acid bacteria strains, such as *Lactobacillus GG*, have an effect on down-regulating the immunoinflammatory response after milk consumption in milk-hypersensitive adults. This may lead to improvement of hypersensitivity reactions [13]. On the other hand the use of probiotic bacteria by healthy subjects appears to stimulate the nonspecific immune response and may therefore give an effective aid in eradication of pathogens. It is important to understand the mechanisms of immune regulation to develop new probiotic functional foods for different target populations.

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