Influence of *Lactobacillus rhamnosus GG* metabolites on immunological processes *in vitro*

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Abstract

The Lactobacillus species belong to bacteria favourably modulating human immune response, nevertheless they may cause infection in extreme immunodeficiency disorders. Several cases of infections provoked by Lactobacillus strains referred to: short intestine syndrome, prematurity and status after cardiosurgery operation. The aim of this paper was to evaluate the influence of Lactobacillus rhamnosus GG (L.GG) metabolites on certain immunity components in vitro. The bacteria were placed inside alginate microcapsules to prevent host invasion by L.GG.

No growth of L.GG was observed on the external surface of membranes under scanning microscope examination, proving the microcapsules were impenetrable for bacteria. Further the microcapsules containing L.GG were lyophilised and added to a culture of PHA stimulated human lymphocytes obtained from the whole blood. Statistically significant increase of lymphoblasts percentage was noted. While the medium significant increase of IL-5 concentration and only a slight increase of TNF-β concentration were observed.

Key words: Lactobacillus rhamnosus GG, cytokines: IL-5, TNF-β.

Introduction

Lactic acid rods are antagonistic against a number of pathogenic strains of bacteria and fungi, compete for receptors of digestive tract cells surface, tighten the intestine epithelium through the increased release of mucus, stimulate or inhibit the apoptosis of epidermal cells. They also restore the microflora balance in the intestine reducing the share of anaerobes [1-3]. The bacteria also reduce the infection status through the normalization of the intestinal flora, thus decreasing pathological permeability of the mucous membrane and penetration of food allergens [4-6]. It was discovered that CpG dinucleotide can stimulate Th1 lymphocytes and macrophages through TLR receptors leading to a change in the profile of released cytokines from Th2 to Th1 in atopic children, resulting in a lower frequency of atopic skin inflammation and digestive tract disturbances [7-9].

Besides desirable influence, certain side-effects were also recorded. Lactobacilli, together with *Streptococcus mutans*, cause caries [10, 11]. *L.GG* is the exception, because it does not decompose fructose and glucose, and, therefore, may be used in treatment of buccal cavity diseases. It was also reported that in patients with significant immunodeficiency Lactobacilli may cause generalized infection. The reported occurrence of such cases, from which the Lactobacilli were isolated, included bacteriemia after extent surgery, liver abscess, urinary system infection or cerebrospinal meningitis [12-14].

The literature reports the possibility of causing arthritis by parts of peptidoglycan presented on a surface of the synovial membrane. Also the potential of vancomycin resistance transfer from naturally resistant Lactobacilli to enterococci (VRE) is currently being considered.

Materials and Methods

Bacterial cultures

The lyophilisate of *Lactobacillus rhamnosus GG* ATCC 53103, purchased from the London collection of ATCC strains, was used for the study. The lyophilizate of

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L.rhamnosus GG was suspended in liquid MRS (acc. to Man Rogos Sharpe) (Oxoid). The bacteria were cultured for 24 h at 36.6°C until the medium became turbid.

Preparation of microcapsules

The cultured bacteria were immobilized in alginate microcapsules. Those were hydrogel microspheres obtained by precipitation of polymers from sodium alginate solution through calcium cations instillation. The resulting microcapsules were subjected to lyophilisation process.

Examination of lyophilised microcapsules surfaces during storage

One of the stages of the study was a test aimed to identify possible biofilm formation on the surface of capsule membranes. Lyophilised microcapsules containing *L.GG* were suspended in MRS medium and incubated at 36.6°C for 14 days. Microcapsules were sampled every second day and dried at 50°C for 24 h. Dried preparations were vacuum coated with gold and platinum alloy (Au Pt 40). Images of the microcapsules were performed by scanning microscope Joel JSM 6100. Assessment of *L.GG* survival in lyophilised and non-lyophilised microcapsules.

Suspension of the microcapsules in 0.9% NaCl was stored at 4°C. Microcapsules samples were collected every second day, diluted with 1.4% sodium citrate and the contents was then plated onto MRS agar base and incubated at 36.6°C.

Establishment of cell culture

The purpose of cell culture establishment was to examine the immunomodulating effects of *L.GG* metabolites. Firstly a solution of heparin (Sigma) in the concentration of 300 units per 10 ml in 0.9% NaCl was prepared. The solution was filtered using sterile Millex GP sets and poured into 50 test tubes, 0.5 ml each. 5 ml of venous blood was obtained from 10 adult volunteers at the blood donation center of Szczecin Clinical Hospital. The blood was poured into test tubes containing 150 units of heparin (Sigma) in 0.5 ml of 0.9% NaCl.

Afterwards the media for lymphocytes cultures were prepared. Typical composition of 250 ml medium included:
• 20% of inactivated calf's serum (Biomed, Lublin),
• 80% of RPMI 1640 medium with L-glutamine and NaHCO$_3$ (Biomed, Lublin),
• 5 µg of phytohemagglutinin PHA-P (Sigma),
• 100 µg of streptomycin in 2.5 ml of 0.9% NaCl,
• 100 U of penicillin in 2.5 ml of 0.9% NaCl.

The complete medium of pH 7.2-7.4 was filtered on sterile Millex GP sets. Culture medium was poured into 50 sterile test tubes (5 ml each) adding 0.1 ml of the whole peripheral blood. The test tubes were divided into five groups.

Control group I consisted only of lymphocytes in culture medium.

Group II contained test tubes with 0.005 mg of empty alginate capsules.

Group III included 0.5 ml of supernatant with metabolites from the 24 h culture of *L.rhamnosus GG* in MRS (7 in McFarland scale) added to test tubes.

Group IV comprised of test tubes with 0.5 ml of liquid MRS medium.

Group V consisted of test tubes with 0.005 mg of lyophilized *L.rhamnosus GG*.

The cultures were incubated at 36.6°C for 5 days, slightly shaken daily. After 5 days the test tubes were centrifuged, 2000 rpm, for 5 minutes at 4°C. Supernatants were collected from the cultures, frozen at -20°C and stored for further study to determine cytokines.

Thin smears of cellular sediments were prepared and preparations were stained using Pappenheim method. The percentage of lymphoblasts was determined by using the microscope counting.

Determination of IL-5 and TNF-β concentrations in cell cultures

The concentration of cytokines was determined by ELISA with R&D Systems kits (USA), in cultures of PHA stimulated lymphocytes obtained from the whole blood. The kits included all reagents and polyester plates with 96 wells coated with monoclonal antibodies against IL-5 or TNF-β.

200 µl of supernatant from cell cultures, or standards of 7.8 to 500 pg/ml for IL-5, or from 31.2 to 10 000 pg/ml for TNF-β were pipetted into each well. The standards and the samples were tested in duplicates. The plates were then incubated for 2 h at room temperature. Next, the wells were rinsed three times with automatic washer EL$_x$50 (BIO-TEK). Further polyclonal antibody conjugated with horseradish peroxidase was added to each well. The plates containing the samples were incubated for 2 h at room temperature. The following washing removed unbound antibodies. The solution of the substrate was then added and the plates were incubated for 20 minutes at room temperature. Colour formation reaction was stopped by adding the 'stop' solution. The colour intensity was directly proportional to the amount of IL-5 or TNF-β. The absorbance was read at 450 nm wavelength by multichannel reader ELISA - EL$_x$808 (BIO-TEK). The accuracy of the method was ± 1% ± 0.010 of absorbance within the range of 0-2.500.

Statistical analysis

In order to answer a question whether the difference between the mean values is statistically significant, i.e. it represents the difference within the population rather than random variation, Shapiro-Wilk test was used for the examination of consistency of normal distribution with the distribution of a variable tested in population. The results were statistically analysed with Statistica 7.1 software version (Statsoft).

If the value of probability p falls below a pre-defined test significance level α, then the hypothesis of the con-
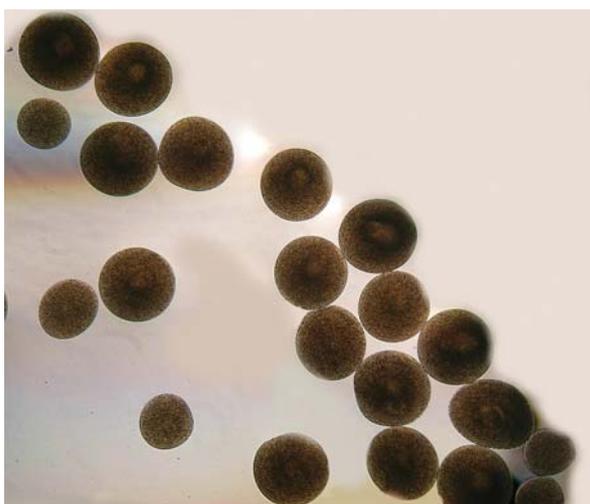
Fig. 1. Image of microcapsules containing the *L.rhamnosus GG* made by Nikon Eclipse TE 2000 – S microscope. Enlargement × 40.

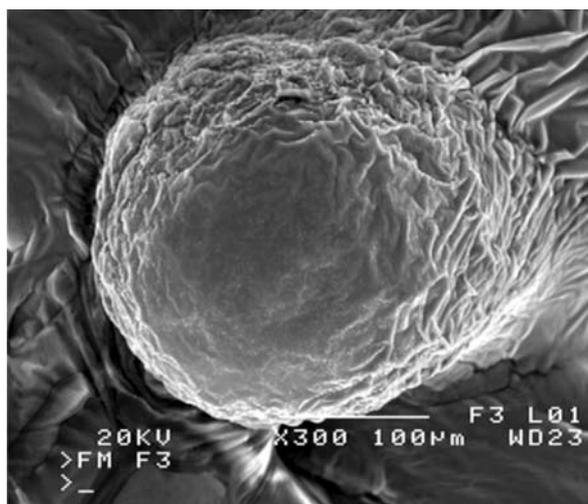**Fig. 2.** Microcapsule image made by a scanning microscope. Enlargment × 300.

sistence with the normal distribution is rejected. The Shapiro-Wilk test is a preferred normality test since it is more powerful comparing to other tests. In case of rejecting the zero hypothesis, in order to show the statistically significant differences in concentration between the groups, non-parametric Wilcoxon match-pairs ranks test is used for results in medical and biological studies [15], which is a non-parametric alternative of t-test for correlated samples.

Results and Discussion

It was observed that in vitro L.GG immobilized in alginate microspheres (Fig. 1) survived longer than those not subjected to the process concerned. The bacteria in liquid cell cultures survived up to 4 days. The immobilized strains suspended in normal saline and stored at 4-6°C survived up to 30 days. Immobilized and lyophilised strains grew on MRS agar base even after 180 days of storage.

The study showed that the *L.rhamnosus GG*, did not form a biofilm outside the capsule membranes (Figs. 2, 3). This fact eliminates the possible threat of bacteria dissemination and infection in immunodeficient patients. Practically, the microcapsules would be easier to remove from infection affected areas within buccal cavity, if necessary. Furthermore, they should protect the bacteria against phagocytes *in vivo*. One of the advantages would be the possibility of obtaining a higher concentration of *L.GG* metabolites in infected tissues.

Enclosing the bacteria in microcapsules (Fig. 1) limited the presentation of *L.GG* antigens to immunological response cells included in cell cultures (*in vitro* tests). Thus the possible stimulants were exopolysaccharide and metabolites, such as nitrogen oxide and hydrogen peroxide, supporting the effect
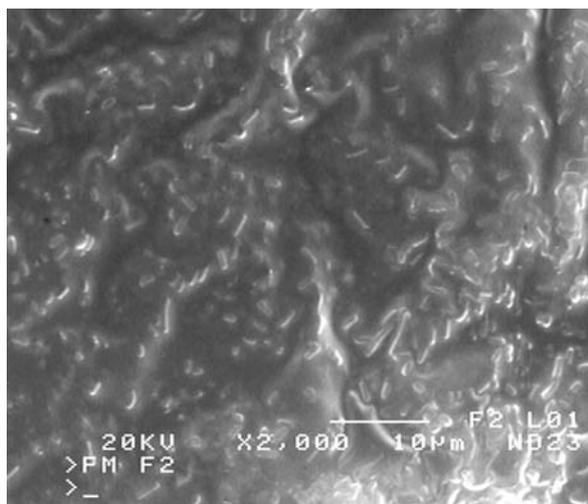

Fig. 3. Microcapsule surface image made by a scanning microscope. Enlargment × 2000.

of lactic acid as well as alginate microcapsules. *L.rhamnosus GG*, as opposed to other LAB, does not produce bacteriocins, hydrogen peroxide not only inhibits the growth of some bacteria [16], but also provokes higher rate of mucus secretion by the intestine epithelium and controls the blood supply and peristalsis. It also activates immune system through stimulation of lymphocytes and cytokins secretion. *L.GG* also produces nitrogen oxide which increases the secretion of stomach mucus, angiectasia and release of certain cytokines, and also inhibits bacterial growth.

It is widely known that lactic acid rods affect the local and general immune response. They partake in maturation

Table 1. Percentage of transforming T lymphocytes in cell cultures

Material under study	Average amount of lymphoblasts (%)
Lymphocytes in culture medium + PHA	47.8
Lymphocytes in culture medium + PHA + alginate capsules	55
Lymphocytes in culture medium + PHA + metabolites from 24-hour *L.rhamnosus GG* culture	64
Lymphocytes in culture medium + PHA + liquid MRS	50
Lymphocytes in culture medium + PHA + *L.rhamnosus GG* lyophilisate	58

and growth of mucosa-associated lymphoid tissue (MALT) immune system. By modifying the composition of the intestinal flora, they indirectly influence the immune system functions reducing infections occurrence frequency [17]. They normalize the permeability of mucous membranes and reduce the penetration of food allergens in allergic patients. The bacteria increase host immunity through, among other things, the increased secretion of IgA. They reduce the intensity of inflammatory response by decreasing phagocytes activity, degrading food allergens and modulating Th2 cytokin secretion. They also lower IgE - dependent reaction [18-20]. Lymphocytes T are 'conductors of immunological orchestra', as referred to by Ivan Roitt while discussing their role in immune system. It is due to the release of several pro- and anti-inflammatory cytokines, which affect the remaining cells of the immune response directly involved in the immunity on the basis of feedback control mechanism. Alginate oligomers are extremely bioactive factors, stimulative the secretion of cytokines: TNF-α, IL-1α, IL-β, IL-6, IL-9, IL-12, IL-13 [21, 22].

In this study the influence of metabolites from *L.GG* on the blastic transformation of T lymphocytes in cell cultures was examined. Phytohemagglutinin (PHA) stimulated cultures of lymphocyte *in vitro*.

The conversion of lymphocytes from stationary phase G0 to G1 cell division cycle was observed.

Percentage of lymphoblasts was also calculated (Tab. 1). In comparison to the control group containing lymphocytes in the culture medium with PHA mitogen, the addition of *L.GG* metabolites activated blastic transformation the most. The second stimulant was *L.GG* lyophilisate.

The addition of alginate and liquid MRS medium slightly increased the percentage of lymphoblasts - within statistical error. The non-parametric Wilcoxon match-pairs ranks test, used on results obtained from lymphoblasts cultures, showed statistically significant differences between group V and groups I,II,III,IV, and also between group III and groups I, II, IV, V. There were no statistically significant differences between groups II and IV, and groups I and II.

During the further analyses it seemed interesting to determine the concentration of certain cytokins in culture liquid which were released without the presence of *L.GG* cell wall structures. It is known that live, whole *L.GG* rods may stimulate cellular specific response. In *in vitro* examination, in cultures of mononuclear cells from the peripheral blood, the increase in cytokins released from Th1 lymphocytes and macrophages was observed. They were: TNF-α, IL-1β, IL-6, IL-18 [23, 24]. In another study, *Lactobacillus GG* resulted in a dramatic increase in IgA concentration in response to vaccination against *Salmonella typhi*. Secretory IgA may eliminate pathogenic bacteria thus reducing the intensity of inflammatory processes [25].

Increased concentration of IgA *in vivo* in the faeces of children infected with rotaviruses and given *L.GG* was also observed [26]. *LGG* showed immunostimulating effects on oral mucosa seen as increased allergen specific IgA levels in saliva [27].

Table 2. Concentration of IL-5 in lymphocyte cultures

Group	Tested samples		Arithmetic mean	Standard deviation
I	lymphocytes	optic density	0.101	0.010
		concentration (pg/mL)	35.685	3.674
II	lymphocytes + empty alginate microcapsules	optic density	0.114	0.013
		concentration (pg/mL)	40.312	4.799
III	lymphocytes + *L.GG* metabolites	optic density	0.149	0.015
		concentration (pg/mL)	52.977	5.232
IV	lymphocytes + MRS	optic density	0.118	0.008
		concentration (pg/mL)	41.725	2.912
V	lymphocytes + *L.GG* lyophilisate	optic density	0.128	0.005
		concentration (pg/mL)	45.223	1.876

The production of antibodies over the infection period directly depends on the amount of released IL-5 and TNF-β, i.e. lymphotoxin α. Enzyme-Linked ImmunoSorbent Assay (ELISA) method was therefore used to determine the concentration of both cytokins in cell cultures *in vitro* and arithmetic means for IL 5 were calculated (Tab. 2).

Using non-parametric Wilcoxon match-pairs ranks test, statistically significant differences in IL-5 concentrations were found between group V and groups I, II, III, IV and between group III and groups I,II,IV and V (Fig. 4). There were no statistically significant differences between the groups II and IV, and groups I and II.

The increased release of IL-5 influenced by *L.GG* metabolites and live lyophilized lactic acid rods (cellular structure) was shown. It is interesting, however, that lyophilized *L.GG* were a weaker stimulant than a liquid with bacteria metabolites added to the culture. The addition of empty alginate capsules also resulted in a slight increase in the concentration of IL-5 in the cultures.

Metabolites and exopolysaccharides from a 2-day *in vitro* culture stimulated IL-5 synthesis to a higher extent than the whole bacteria. It might be concluded that the exopolysaccharide produced by *L.GG* exhibited stronger immunomodulating effect compared to the whole rods, which showed immunogenic effect mainly through cell wall antigens.

The alginate used for microcapsules membranes formation and liquid MRS medium in which *L.GG* was cultured slightly increased IL-5 synthesis as well. The results were statistically insignificant.

The arithmetic means of TNF-β concentrations were also calculated (Tab. 3).

The highest values were obtained in group II – about 10 pg/mL. The lymphocytes in this group were stimulated with empty alginate capsules. Similar results – also about 10 pg/mL, were obtained by adding liquid MRS. In
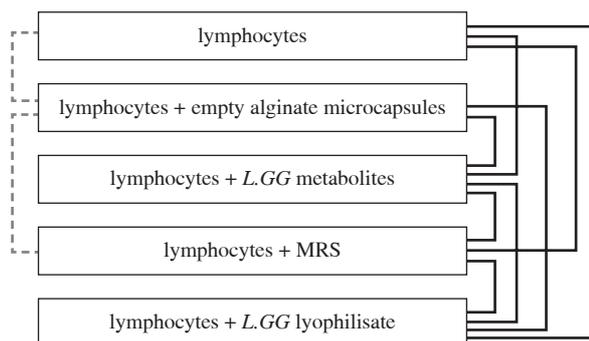

Fig. 4. Probability assessment of occurrence of statistically significant and insignificant differences between IL-5 concentrations in groups under study. Continuous lines represent statistically significant differences, dash lines – lack of statistically significant differences between groups.

lymphocytes cultures where lyophilised *L.GG* were added the observed TNF-β concentration amounted to 9.4 pg/mL. In cultures with *L.GG* metabolites addition the resulting concentration was 9.2 pg/mL, whilst in control culture containing only lymphocytes the concentration obtained reached 9 pg/mL. The non-parametric Wilcoxon match-pairs ranks test showed statistically significant differences in TNF-β concentration between groups I and II, groups I and IV, group II and groups I, III, IV, group III and groups II, IV, group IV and groups I, III. There were no statistically significant differences found between groups I and III, groups I and V, groups II and IV, groups III and V, and groups IV and V (Fig. 5).

In comparison to control group containing lymphocytes in the culture medium, a slightly increased release of TNF-β was determined under the influence of alginate and MRS medium. The addition of *L.GG* metabolites and *L.GG* lyophilised cells did not stimulate TNF-β synthesis.

Table 3. Concentration of TNF-β in lymphocyte cultures

| Group	Tested samples		Arithmetic mean	Standard deviation
I	lymphocytes	optical density	0.011	0.001
		concentration (pg/mL)	9.096	1.078
II	lymphocytes + empty alginate microcapsules	optical density	0.013	0.001
		concentration (pg/mL)	10.808	0.714
III	lymphocytes + *L.GG* metabolites	optical density	0.011	0.002
		concentration (pg/mL)	9.181	1.847
IV	lymphocytes + MRS	optical density	0.012	0.002
		concentration (pg/mL)	10.585	1.479
V	lymphocytes + *L.GG* lyophilisate	optical density	0.011	0.002
		concentration (pg/mL)	9.351	2.015
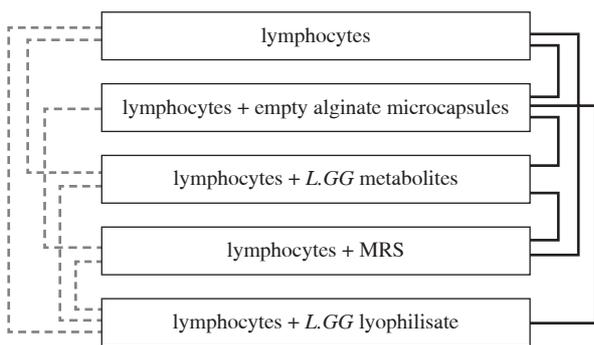
Fig. 5. Probability assessment of occurrence of statistically significant and insignificant differences between TNF-β concentration in groups under study. Continuous lines represent statistically significant differences, dash lines – lack of statistically significant differences between groups.

The results of the study may suggest the lack of stimulating effect on the release of lymphotoxin α (TNF-β) by exopolysaccharide and metabolites of *L.rhamnosus GG in vitro*. TNF-β is a cytokin that induces the maturation of dendritic cells present in solitary follicles and Peyer's patches. Furthermore, it participates in the development of humoral immune response and IgA release [28].

According to the results obtained in this study, i.e. increased IL-5 release in cell cultures by supernatant with *L.GG* metabolites *in vitro*, the use of microcapsules with *L.GG* in allergic patients can be taken into consideration. Eosinophils are main target cells for IL-5. The cytokin stimulates precursor cells proliferation and activation and survival of matured eosinophils. It also stimulates the proliferation of basophils which release histamine. The thesis requires further confirmation by *in vivo* examination and extension of the range of studied cytokines.

The available literature does not contain any publications discussing *in vitro* or *in vivo* effects of strictly bacterial metabolites (excluding the influence of peptidoglycan, teichoic acids and other components of *L.GG* cell walls) on human immune response. The results obtained may form a basis for further research on the possibility of *in vivo* application of *L.GG* metabolites as a constituent of 'safe food' or pharmaceuticals for humans, especially extremely immunodeficient.

Conclusions

Lactobacillus rhamnosus GG immobilized in alginate capsules did not form biofilm outside the membranes.

In cultures of lymphocytes obtained from the whole blood stimulated with PHA the increase in IL-5 concentration and constant level of TNF-β released after adding *L.GG* immobilized in the microcapsules, were observed.

Alginate, the constituent of the microcapsules, demonstrated synergistic immunomodulating effect together with *L.GG* metabolites in increasing the release of cytokines.

L.GG metabolites, together with microcapsule alginate, increased the blastic transformation of PHA stimulated T lymphocytes.

References

1. Grajek W, Sip A (2006): Antagonistic activity of probiotics against pathogenic microorganisms. Zakażenia 6: 49-50, 52-54.
2. Heczko PB, Strus M, Drzewiecki A (2004): Probiotyki a zakażenia. Zakażenia 4: 33-38.
3. Strus M, Kucharska A, Kukla G et al. (2005): The in vitro activity of vaginal Lactobacillus with probiotic properties against Candida. Infect Dis Obstet Gynecol 13: 69-75.
4. Cukrowska B, Ceregra A, Rosiak I (2006): Probiotyki w profilaktyce i leczeniu atopowego zapalenia skóry. Zakażenia 6: 55-60.
5. Cukrowska B, Czarnowska E (2007): Wpływ probiotyków na układ immunologiczny. Zakażenia 1: 59-64.
6. Isolauri E, Rautava S, Kaliomaki M et al. (2002): Role of probiotics in food hypersensitivity. Curr Opin Allergy Clin Immunol 3: 263-271.
7. Ghadimi D, Folster-Holst R, de Vrese M et al. (2008): Effects of probiotic bacteria and their genomic DNA on TH1/TH2-cytokine production by peripheral blood mononuclear cells (PBMCs) of healthy and allergic subjects. Immunobiology 213: 677-692.
8. Klinman DM, Beaucage SL (1996): CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12 and interferon gamma. Proc Natl Acad Sci USA 93: 2879-2883.
9. Ongol MP, Iguchi T, Tanaka M et al. (2008): Potential of selected strains of lactic acid bacteria to induce a Th1 immune profile. Biosci Biotechnol Biochem 72: 2847-2857.
10. Saxelin M (1997): Lactobacillus GG – a human probiotic strain with thorough clinical documentation. Food Rev Int 13: 293-313.
11. Ahola AJ, Yli-Knuuttila H, Suomalainen T et al. (2002): Short-term consumption of probiotic-containing cheese and its effect on dental caries risk factors. Arch Oral Biol 47: 799-804.
12. Szajewska H (2007): Lactobacillus rhamnosus GG w chorobach biegunkowych: przegląd badań z randomizacją. Zakażenia 1: 53-58.
13. Land M, Rouster-Stevens K, Woods C et al. (2005): Lactobacillus sepsis associated with probiotic therapy. Pediatrics 115: 178-181.
14. Kunz A, Noel J, Fairchok M (2004): Two cases of Lactobacillus bacteremia during probiotic treatment of short gut syndrome. J Pediatr Gastroenterol Nutr 38: 457-458.
15. Therrien CW. Decision estimation and Classification. John Wiley and Sons, Inc., 1989.
16. Kochan P (2005): Wybrane schorzenia dróg moczowo-płciowych kobiety i leczenie wg CDC*. Kryteria WHO/FAO** dla probiotyków i ich zastosowanie w ginekologii w świetle najnowszych badań. Ginekol Prakt 87: 11-18.
17. Borruel N (2007): Probiotics and prebiotics in inflammatory bowel disease. Gastroenterol Hepatol 30: 419-425.
18. Merk K, Borelli C, Korting HC (2005): Lactobacilli - bacteria-host interactions with special regard to the urogenital tract. Int J Med Microbiol 295: 9-18.
19. Prescott SL, Wichens K, Westcott L et al. (2008): Supplementation with Lactobacillus rhamnosus or Bifidobacterium lactis probiotics in pregnancy increases cord blood interferon-γ and breast milk transforming growth factor-beta and immunoglobin A detection. Clin Exp Allergy 38: 1606-1614.

20. Seksik P (2007): Probiotics and inflammatory bowel diseases. Cahiers de Nutrition et de Dietetique 42: 2S51-2S59.
21. Yamamoto Y, Kurachi M, Yamaguchi K, Oda T (2007): Induction of multiple cytokine secretion from RAW264.7 cells by alginate oligosaccharides. Biosci Biotechnol Biochem 71: 238-241.
22. Yamamoto Y, Kurachi M, Yamaguchi K, Oda T (2007): Stimulation of multiple cytokine production in mice by alginate oligosaccharides following intraperitoneal administration. Carbohydr Res 342: 1133-1137.
23. Miettinen M, Matikainen S, Voupio-Varkila J, Varkila K (1996): Production of human tumor necrosis factor alpha, interleukin-6 and interleukin-10 is induced by lactic acid bacteria. Infect Immun 64: 5403-5405.
24. Miettinen M, Matikainen S, Voupio-Varkila J et al. (1998): Lactobacilli and Streptococci induce interleukin-12 (IL12), IL-18 and gamma interferon production in human peripheral blood mononuclear cells. Infect Immun 66: 6058-6062.
25. Fang H, Elina T, Heikki A, Seppo S (2000): Modulation of humoral immune response through probiotic intake. FEMS Immunol Med Microbiol 28: 47-52.
26. Guandalini S (2006): Probiotics for Children: Use in Diarrhea. J Clin Gastroenterol 40: 244-248.
27. Piirainen L, Haahtela S, Helin T et al. (2008): Effect of Lactobacillus rhamnosus GG on rBet v1 and rMal d1 specific IgA in the saliva of patients with birch pollen allergy. Ann Allergy Asthma Immunol 100: 338-342.
28. Gołąb J, Jakóbisiak M, Lasek W. Immunologia. Wydawnictwo Naukowe PWN, Warszawa, 2007.