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Immunomodulation Effect of Metabolites from *Lactobacillus Rhamnosus GG* on Interleukins Release *in Vitro*

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Abstract The aim of the study was to determine *in vitro* whether the metabolites of *Lactobacillus rhamnosus GG* stimulate the release of selected cytokines from lymphocytes. Cell cultures from whole human blood were established. The concentration of TGF-β1, IFN-γ and IL-4 was determined in cultures liquid by ELISA. *Lactobacillus GG* rods were immobilized in alginate microcapsules. To determine the tightness of the capsules their surfaces were observed by electron microscopy. During 14 days the migration of the bacteria to outside of the capsules was not observed. In liquid from cell cultures of lymphocytes the increase of TGF-β1 and IL-4 and the decrease in IFN-γ concentration were observed, influenced by *Lactobacillus GG* metabolites enclosed in lyophilized alginate microcapsules, as compared to control group. The possible stimulants were exopolysaccharide and *Lactobacillus GG* metabolites, i.e. lactic acid, nitrogen oxide and hydrogen peroxide.

Keywords: *microencapsulation, immunomodulation, TGF-β1, IFN-γ, IL-4, probiotics*

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1. Introduction

Numerous studies performed in humans and animals confirm that the onset of allergy symptoms can be affected by the changed composition of gut microbiota. Maturation and development of immunological system depends on microorganisms inhabiting alimentary tract during neonate and infant period. Bacterial flora participates in anatomic, physiological and immunological host development [1]. If, however, any unfavorable factors occur, i.e. microbial infection, antibiotic therapy, stress, also the necessity of caesarean section, then the intestines can be colonized by random microflora leading to the distortion in composition of bacterial flora. As a result the increased permeability of mucous membrane can occur, followed by the unfavorable antigens penetration into the organism. The consequences of prolonged dysbiosis could be lingering inflammation states, recurring infections and allergic diseases.

Lactic acid rods are responsible for the state of eubiosis and affect local and systemic immune response [2]. They take part in maturation and development of immune system linked to mucous membranes, i.e. MALT system. By regulating the composition of gut microflora they indirectly affect the function of immune system, reducing the frequency of inflammations. They normalize the permeability of mucous membranes and in allergic people they reduce the infiltration of food allergens [3,4,5]. They modulate the immunity of the host, among others by the increased release of IgA. They also decrease the inflammatory reaction by reduction of the activity of phagocytes, degradation of food allergens and modulation of cytokines release by Th2 lymphocytes, what leads to milder symptoms of IgE - dependent allergy [6,7,8,9]. Among the lactic acid rods *Lactobacillus GG* is an approved probiotic. Numerous works confirmed the immunomodulating properties of this strain, however there are no data reporting the effect of only their metabolites on immune system. For industrial applications it is easier to standardize and obtain proper concentrations of metabolites than maintain alive constant number of lactic acid bacteria. The experiments were also performed with alginate with immunity stimulating properties, as confirmed by EU patent [10]. The aim of the study was to compare the concentrations of IL-4, TGF-β1 and IFN-γ from the cultures of lymphocytes stimulated by *L.GG* metabolites with *L.GG* immobilized in alginate microcapsules and alginate *in vitro*.

2. Materials and Methods

2.1. Bacterial CULTURES

For the analyses *Lactobacillus GG* ATCC 53103 lyophilisate was used from London ATCC cultures collection. *Lactobacillus GG* lyophilisate was suspended

in cell culture medium /MRS, the breeding ground of bacteria according to De Man Rogosa Sharpe/ (Oxoid CM0359). The bacteria were cultured for 24h at 36.6°C until the medium was turbid.

2.2. Microcapsules Preparation

The cultured bacteria were immobilized in alginate microcapsules obtained by precipitation of polymers from sodium alginate solution through calcium cations instillation. In this study water soluble 1.5% sodium alginate was used obtained from Natura-Sweet Kelco Company (USA), known commercially as Keltone HV, with viscosity average molecular weight M_n-440 000. Prepared 1.5% sodium alginate in physiological salt solution was filtered with sterile Millex GP filtration sets. Filters contained regenerated cellulose membranes with pore size 0.22µm. Alginate solution was combined with previously cultured bacteria. Obtained 0.7% solution (bacteria + alginate) was instilled into 1% $CaCl_2$ in physiological salt solution during 20 minutes. During microcapsule formation $CaCl_2$ in physiological salt solution was mixed with magnetic stirrer. Rapid jellification took place due to the presence of calcium ions. A syringe pump SEP 11S & SEP21S, SEP11 Model (ASCOR S.A.) was used in order to obtain microcapsules of repeatable shapes. Microcapsules were rinsed three times with sterile physiological salt solution to accurately rinse out the solvent and *Lactobacillus GG* cells adhering to the outer surface of the capsules. Obtained microcapsules had spherical shapes.

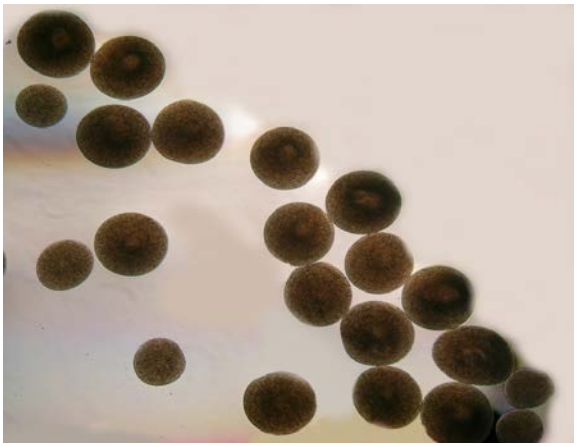

Figure 1. Image of microcapsules containing the *Lactobacillus rhamnosus GG* made by Nikon Eclipse TE 2000 – S. Enlargement x 40.

The factors which had the direct influence on formation of microcapsules of desired spherical shape (Figure 1) were: rotation speed of magnetic stirrer – 500rpm, the distance of needle tip from the surface of $CaCl_2$ solution (in physiological salt solution) – ca. 3 cm, and application of appropriate instillation pressure – 40 ml/h. Obtained microcapsules were subjected to lyophilization.

2.3. Assessment of Microcapsules Tightness

Lyophilized microcapsules were again suspended in Cell culture medium (Figure 2). During 14 days samples were collected and preparations were observed by scanning microscope Joel JSM 6100 under a magnification of 10 000x (Figure 3).

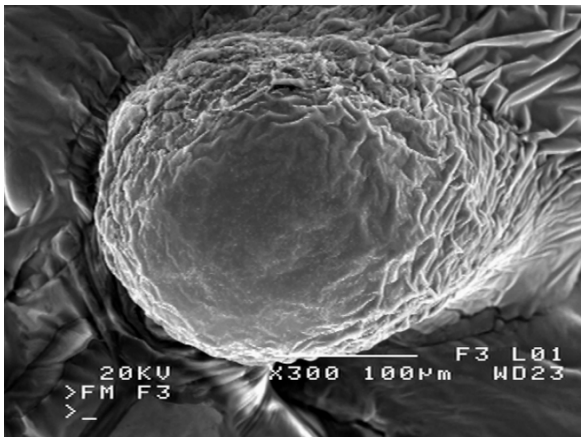

Figure 2. Microcapsule surface image made by a scanning microscope. Enlargement x 300

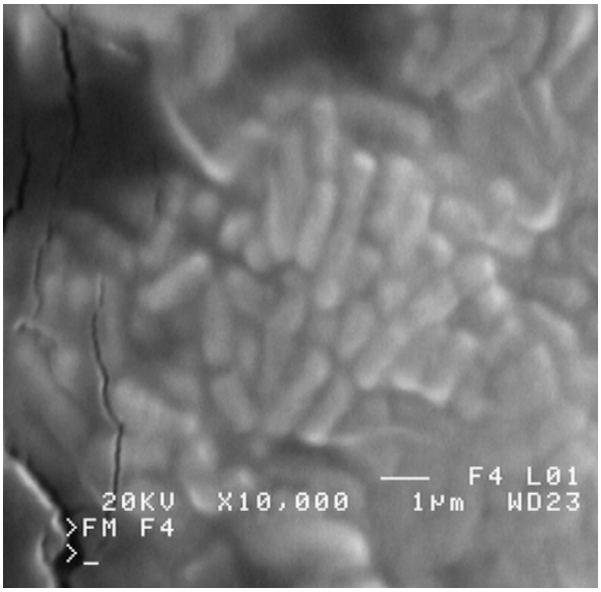

Figure 3. Microcapsule surface image made by a scanning microscope. Enlargement x 10 000

2.4. Cell Cultures

The purpose of cell culture establishment was to examine the immunomodulating effects of *Lactobacillus GG* metabolites. Firstly 5ml of venous blood was obtained from 10 adult volunteers. 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.

Composition of 250ml medium:

- 20% of inactivated calf's serum (Biomed, Lublin)
- 80% of RPMI 1640 medium with L-glutamine and $NaHCO_3$ (Biomed, Lublin)
- 5µg of phytohemagglutinin PHA-P (Sigma)
- 100µg of streptomycin in 2.5ml of 0.9% NaCl
- 100U of penicillin in 2.5ml of 0.9% NaCl

The complete medium of pH 7.2 – 7.4 was filtered on sterile Millex GP sets. 5ml of culture medium was poured into 50 sterile test tubes (5ml each) adding 0.1ml 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 had the addition of 0.005mg of empty alginate capsules.

To **group III** 0.5ml of supernatant with metabolites from the 24h culture of *Lactobacillus* GG in MRS (7 in McFarland scale) was added.

Group IV contained the addition of 0.5ml MRS medium.

To **group V** 0.005mg of lyophilized microcapsules containing *Lactobacillus* GG were added.

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. Supernanants were collected from the cultures to determine the concentration of the cytokines.

2.5. Determination of IL-4, TGF-β1, IFN- γ Concentrations

The analyses of selected cytokines were performed with ELISA using Lucio – Medical Elisa kits, Germany (cat. No REF ELI-6299).

Absorbance was read with EL_x808 (BIO-TEK) at 450nm. The accuracy of the method was > 2pg/ml.

2.6. Statistical Analysis

Analyses were performed using Statistica 10. (Statsoft). To determine the statistically significant differences of concentrations between the analyzed groups, non-parametric Wilcoxon match-pairs ranks test was used [11,12], which is a non-parametric alternative of t-test for correlated samples.

3. Results

To determine the immunomodulating effect of *L.GG* metabolites only the bacteria were fixed inside alginate microcapsules. Even after 14 days of culture the disruption of "packaging" or presence of bacteria on membranes surface were not observed, thus proving that the lactic acid bacteria *Lactobacillus rhamnosus GG* do not form biofilm outside the capsules, so also the administration of "packaging" to ill people should additionally eliminate possible risk of bacteria dissemination and inflammation in immunodeficient patients. Moreover, the microcapsules inside the host organism should protect the bacteria against phagocytes (Figure 3).

The concentration of IL-4, TGF- β1 and IFN- γ was determined by ELISA in cell cultures divided into five groups *in vitro*, and the arithmetical mean values for the obtained results were calculated (Table 1). The highest concentration of IL-4 was observed in group III (1.507 pg/ml), where the lymphocytes were stimulated exclusively by *Lactobacillus GG* metabolites. It was determined that lyophilized microcapsules containing *L.GG* (V group) also increased the release of IL-4 (1.466 pg/ml). The addition of empty alginate capsules to lymphocytes (1.283 pg/ml), as compared to lymphocytes only (1.224 pg/ml), caused almost no activation of IL-4 release, but the addition of cell culture medium, used for culturing of *L.GG*, decreased the release of this cytokine (0.293 pg/ml).

Table 1. Concentration of IL-4, TGF- β1, IFN- γ in lymphocyte cultures [pg/ml]

Group	Fasted samples	IL-4		TGF-β1		IFN-γ	
		mean value	std. dev.	mean value	std. dev.	mean value	std. dev.
I	Lymphocytes	1,224	0,13	288,428	38,82	0,408	0,04
II	Lymphocytes + empty alginate microcapsules	1,283	0,17	316,556	33,70	0,461	0,07
III	Lymphocytes + *Lactobacillus GG* metabolites	1,507	0,12	404,574	21,10	0,389	0,03
IV	Lymphocytes + M.R.S.	0,293	0,04	263,137	31,51	0,225	0,04
V	Lymphocytes + *Lactobacillus GG* lyophilisate	1,466	0,15	386,085	39,80	0,326	0,02

Using the non-parametric Wilcoxon match-pairs ranks test the statistically significant differences in IL-4 concentrations (Figure 4) were determined between the groups I and II, IV and V and between the groups II, III and IV. Moreover, the differences were also shown between the groups III and IV and V and between IV and V. The lack of statistically significant differences was present between the groups I and III and between II and V. Thus the statistically significant increase in IL-4 release was shown under the influence of *Lactobacillus GG* bacterial cells lyophilized in alginate capsules, alginate and Cell culture medium, as compared to the control group.

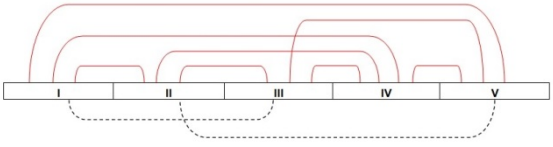

Figure 4. The analysis of the probability of the presence of statistically significant and insignificant differences between determined concentrations of IL-4 in investigated groups. Solid lines represent statistically significant differences, dashed lines – the lack of statistically significant differences between the groups

The highest average concentration of TGF- β1 (Table 1) were obtained in cell cultures stimulated with *L.GG* metabolites in the third group (404.574 pg/ml). Lyophilized microcapsules with *L.GG* (V group) stimulated lymphocytes to the synthesis of TGF- β1 to an average extent (386.085 pg/ml). Alginate microcapsules (II group) only slightly stimulated the lymphocytes (316.556 pg/ml) and Cell culture medium did not cause the increase of TGF- β1 concentration (IV group).

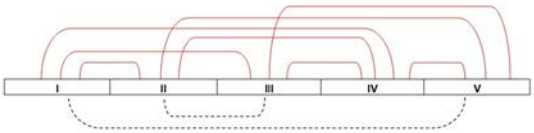

Figure 5. The analysis of the probability of the presence of statistically significant and insignificant differences between determined concentrations of TGF- β1 in investigated groups. Solid lines represent statistically significant differences, dashed lines – the lack of statistically significant differences between the groups

Using the non-parametric Wilcoxon match-pairs ranks test the statistically significant differences in TGF- β1 concentration (Figure 5) were determined between the

groups I and II, III, IV, the groups II, IV and V, and also between the group III and IV, V and the groups IV and V. There were no statistically significant differences between the groups I and V and the groups II and III.

In comparison to the control group of lymphocytes (0.408 UI/ml), the addition of lyophilized *L.GG* (0.326 UI/ml) and the probiotic metabolites only (0.389 UI/ml) caused the decrease of IFN- γ release (Table 1).

Using the non-parametric Wilcoxon match-pairs ranks test the statistically significant differences in IFN- γ concentration (Figure 6) were observed between the groups I, II, IV and V, the groups II, III, IV and V, the groups III, IV and V, and between the groups IV and V. The lack of statistically significant differences was determined between the groups I and III.

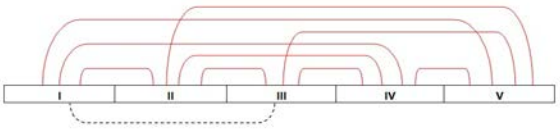

Figure 6. The analysis of the probability of the presence of statistically significant and insignificant differences between determined concentrations of IFN- γ in investigated groups. Solid lines represent statistically significant differences, dashed lines – the lack of statistically significant differences between the groups

4. Discussion

It is known that in healthy human organism there is equilibrium between the group of Th1 cytokines (cell response), Th2 cytokines (humoral immunity) and Th3 cytokines (immune tolerance). Cytokines released by Th1 lymphocytes (IL-2, IL-12, INF-γ, TNF-α, IL-15, IL-17, IL-18) are pro-inflammatory; they facilitate the elimination of viruses and modulate antibacterial and anticancer immunity. Th2 type cytokines (IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, TGF-β) act in an opposite way, i.e. are anti-inflammatory. *Lactobacillus* activates Th3 lymphocytes to the synthesis of TGF-β and IL-10, which in turn maintain the Th1/Th2 balance. Regulating Th3 lymphocytes inhibit pro-inflammatory and pro-allergic response [2,13]. For a long time it has been known that physiological bacterial flora of alimentary tract lowers the activity of Th2 lymphocytes and facilitates Th3-dependent food tolerance [14,15,16,17]. Immuno-modulating properties of probiotics have been shown to reduce the presence of allergic diseases in people [18,19].

In an organism of a person suffering from food allergy, due to the contact with allergenic factor, mast cells release the mediators, which cause inflammatory reactions. Mast cells, stimulated by IgE, release a number of multifunctional cytokines. The balance between Th1 and Th2 is disturbed. Th2 lymphocytes release cytokines necessary for the course of allergic reaction: IL-4, IL-5, Il-10 and IL-13. Interleukin 4 can be regarded as the most crucial for the allergy; it causes the maturation of Th2 lymphocytes by stimulating IgE and eosinophil granulocytes migration to the tissues. Il-5 can take part in chemotaxis and activation of inflammatory cells in site of allergic reaction. The role of cytokines from mast cells *in vivo* is still unknown [20,21].

It should be stressed that there are discrepancies between the studies on using probiotics in clinical trials and *in vitro*. The study of Pessi et al. [22] confirmed, *in vivo*, the favorable effect of *Lactobacillus rhamnosus GG* on the course of atopic dermatitis in food allergy. *L.GG* activated the release of IL-10 cytokine, which inhibits excessive synthesis of some Th1 cytokines: IL-2, IFN-γ, TNF-α and IL-12, and also IL-4 released by Th2. In our previous studies no significant immunomodulating effect of *Lactobacillus GG* metabolites was shown on the concentration of TNF-β in cell cultures from the whole blood. However, the increased IL-10 release was observed in lymphocyte cultures influenced by lyophilized *L.GG* bacterial cells immobilized in capsules, as compared to the control group containing lymphocytes only. There was a difference in immunomodulating effect of microcapsules with *L.GG* and exclusively *L.GG* metabolites [23,24]. In *in vitro* studies performed by Kopp et al. a significant increase in IL-10 and IFN-γ concentration was shown in liquids from cell cultures stimulated with the addition of the whole *L.GG* cells. However, the obtained results did not correlate with the studies *in vivo* performed with the participation of mothers and infants from a high risk group of food allergy [25]. The results of *in vitro* studies performed by Kekkonen et al. show a slight decrease in TNF-α, IL-12, IFN-γ and IL-10 concentration due to *L.GG* [26]. Tests on infants with atopy, allergic on cow milk protein and being given casein degraded by *Lactobacillus GG*, showed the reduction of IL-4 release without the increase of the level of IFN-γ [27]. When comparing the immunostimulating activity of six probiotic strains, whole living cells (*Lactobacillus casei Shirota*, *L. rhamnosus GG*, *L. plaratarum* NCIMB 8826 and *L. reuteri* NCIMB 11951) and bifidobacteria (*Bifidobacterium longum* SP 07/3 and *B. bifidum* MF 20/5) in cultures of mononuclear cells from human peripheral blood, Dong et al. showed increased production of anti-inflammatory cytokines by bifidobacteria, whereas *Lactobacillus* promoted Th1 cytokines. Adding of *L.GG* to cell culture did not result in the increase of IFN-γ concentration. Slightly higher levels of IL-12 were observed by the addition of *L.GG*. Other *Lactobacillus* strains showed stronger stimulating activities. *Bifidobacterium* strains displayed a definite stronger stimulating effect on the production of IL-10, as compared to *Lactobacillus* strains. *L.GG* significantly stimulated the synthesis of IL-1β and TNF-α, and also IL-8, in comparison with the control group containing peripheral blood cells only [28].

According to Kirjavainen et al., [6] the activity of *Lactobacillus* was different *in vivo* and *in vitro*. In infants group, who were administered *Lactobacillus GG* and *Bifidobacterium*, no changes in TNF-α concentrations in blood was observed *in vivo*, whereas the opposite effect was shown *in vitro*. Majamaa et al., [29] however, showed that *Lactobacillus GG* prevents TNF-α release *in vitro*. Stronger induction of the processes of maturation of dendritic cells, which play an important role in formation of immune reaction, was observed by the addition of *L.GG* in cell cultures *in vitro*, as compared to the activity of *Lactobacillus delbrueckii* [30].

Kekkonen et al. [31] stress that therapeutic effect of probiotics is strain-dependent and favorable activity of a particular strain cannot be attributed to another strain of the same genus. Reports from recent years have shown the possibility to use *Lactobacillus rhamnosus GG* in the prevention and treatment of atopic allergy, mainly atopic

dermatitis [32]. In our previous works a significant effect of *Lactobacillus GG* on the level of IL-5 was determined, in comparison to the control group of lymphocytes in culture medium. There was a significant difference between the activity of *L.GG* metabolites and lyophilized bacteria. Metabolites and exopolysaccharide from a two day culture *in vitro* stimulated IL-5 synthesis to a greater extent that the whole bacteria. It could be stipulated that mainly the exopolysaccharide produced by *L.GG* displayed a stronger immunomodulating activity than the whole bacterial rods which had immunogenic activity through cell wall antigens. Alginate, which was used to form the membranes of the capsules, and Cell culture medium, in which *L.GG* was cultured, also slightly increased the synthesis of IL-5 [23].

In available literature no studies on the effect of exclusively *L.GG* metabolites on immune system were found. Enclosing the bacteria in microcapsules limits the possibility of phagocytosis of bacteria by phagocytes and presenting *L.GG* antigens to immune response cells. Therefore the possible stimulants are exopolysaccharide and metabolites, i.e. lactic acid, nitrogen oxide and hydrogen peroxide [33,34].

The bacteria can survive in the microcapsules for 28 days, if not lyophilized. In lyophilized state they can survive for up to six months *in vitro*. Own studies on increased release of IL-4 and TGF- β1, and reduction of IFN- γ, together with the results of previous own research, i.e. the increase of IL-10 and IL-5 *in vitro*, can suggest the suitability of the use of microcapsules with *Lactobacillus GG* as a component of food for special purposes for people with immune disorders. Taking into consideration the lability of probiotic bacteria and difficulties in clinical use of live cells, the results obtained prove the need for further studies on the possibility of the use of *Lactobacillus GG* metabolites *in vivo*, as an immunomodulator used locally, restoring the balance between Th1 and Th2 lymphocytes and being safe for humans.

5. Conclusion

The significant immunomodulating effect of *Lactobacillus GG* metabolites was shown.

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