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Effect of *Lactobacillus rhamnosus GG* encapsulation on interleukin 10 release *in vitro*

EDYTA BALEJKO[1], ELŻBIETA KUCHARSKA[1], JERZY BALEJKO[2]

[1]Faculty of Food Sciences and Fisheries, Department of Human Nutrition, West Pomeranian University of Technology in Szczecin, Poland
[2]Department of Food Engineering, West Pomeranian University of Technology in Szczecin, Poland

Abstract

Gram-positive rods of Lactobacillus rhamnosus GG (Lactobacillus GG) contribute to development and maturation of intestinal lymphatic system and, moreover, by increased IL-10 activity, decrease the expression of symptoms at the early stage of alimentary allergy and atopic dermatitis development. The aim of the study was to determine in vitro whether the metabolites of Lactobacillus rhamnosus GG stimulate the release of IL-10 from lymphocytes. The analyses were performed on whole human blood cell cultures using ELISA to determine the concentration of IL-10 in cultures liquid. Lactobacillus GG rods were immobilized in alginate capsules. 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 cell cultures the increase in IL-10 release was determined, influenced by Lactobacillus GG metabolites contained 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. Sodium alginate did not show synergistic activity with Lactobacillus GG metabolites.

Key words: *microencapsulation, immunomodulation, IL-10, probiotics.*

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Introduction

Using functional food products serves as a preventive measure in development of several diseases, including immune system disorders. Currently functional food is very popular due to consumers demand and consciousness. Probiotic bacteria belong to the components which give the food products unique properties. Several studies proved that probiotics can have immunomodulating effect by increasing the synthesis of some antibodies, T and B lymphocytes, macrophages and NK cells in intestinal tract [1]. It was determined that *Lactobacillus GG* can decrease the expression of symptoms of alimentary allergy and atopic dermatitis development by stimulation of Th3 lymphocytes, that are able to release IL-10 and TGF-β [2-4]. This is caused by colonization of the intestines by *Lactobacillus GG* interacting with immune system mainly through cell membrane structures and exopolysaccharide. In available literature there are no data concerning the effect of only *Lactobacillus GG* metabolites on immune system, but there are reports showing the possibility of causing bacteriemia in people with extremely reduced immunity. Probiotic bacteria are considered as safe, but in case of extreme low immunity, as any other 'safe' microorganism, they can become the cause of infection. It should be stressed that the frequency of infections caused by lactic acid bacteria is incomparably lower than infections caused by other Gram-positive and Gram-negative comensal microflora colonizing human organism. Infections caused by *Lactobacillus* strains were reported e.g. after liver transplants, chirurgical surgeries, long-term antibiotic therapies or chemotherapy [5, 6].

Taking the above aspects into consideration it seemed justified to enclose the bacteria in alginate microcapsules. The lack of direct contact with bacteria can, on one hand, protect immunosuppressed consumer from the infection and, on the other hand, can reduce the possibility of bacte-

Correspondence: Edyta Balejko, Faculty of Food Sciences and Fisheries, Department of Human Nutrition, West Pomeranian University of Technology in Szczecin, Poland, e-mail: Edyta.Balejko@zut.edu.pl

rial phagocytosis, hence facilitating longer survival of probiotic bacteria in the organism.

Previous reports on the effect of alginate on immunity, which suggested its immunomodulating activity [7] had significant influence on the materials selected for this study. The aim of this work was to determine the effect of *Lactobacillus GG* metabolites on IL-10 release. Therefore the concentrations of IL-10 from lymphocyte cultures stimulated by alginate, *Lactobacillus GG* metabolites and *Lactobacillus GG* immobilized in alginate microcapsules were compared *in vitro*.

Material and methods

Bacterial cell cultures

For the analyses *Lactobacillus GG* ATCC 53103 lyophilisate was used from London ATCC cultures collection. *Lactobacillus GG* lyophilisate was suspended in MRS broth (according to De Man Rogosa Sharpe) (Oxoid CM0359). The bacteria were cultured for 24 h at 36.6˚C until the medium was turbid.

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 viscous average molecular mass 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 0.22 µm. 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.

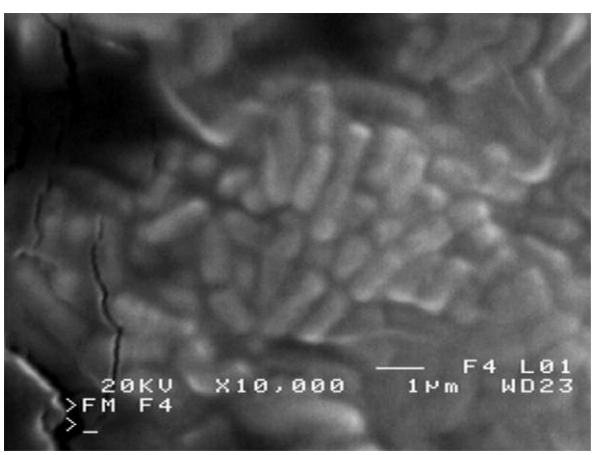

Fig. 1. Microcapsule surface image made by a scanning microscope. Enlargement 10 000×

The factors which had the direct influence on formation of microcapsules of desired spherical shape were: rotation speed of magnetic stirrer – 500 rpm, 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.

Assessment of microcapsules tightness

Lyophilized microcapsules were again suspended in MRS broth. During 14 days samples were collected and preparations were observed by scanning microscope Joel JSM 6100 under a magnification of 10 000× (Fig. 1).

The purpose of cell culture establishment was to examine the immunomodulating effects of *Lactobacillus GG* metabolites. Firstly 5 ml 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 250 ml medium:

- 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. Five ml of 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.

Group I (control): consisted only of lymphocytes in culture medium.

Group II: lymphocytes + the addition of 0.005 mg of empty alginate capsules.

Group III: lymphocytes + 0.5 ml of supernatant with metabolites from the 24 h culture of *Lactobacillus GG* in MRS (7 in McFarland scale).

Group IV: lymphocytes + contained the addition of 0.5 ml liquid MRS medium.

Group V: lymphocytes + 0.005 mg of lyophilized microcapsules containing *Lactobacillus 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.

ELISA analyzes were performed using OptEIA kit (Becton Dickinson and Company USA (cat. No 550613). The kit included polystyrene plate coated with monoclonal antibodies against IL-10 and reagents. 100 µl of serum or standards from 7.8 to 500 pg/ml for IL-10 were added to the wells and plates were incubated for 2 hours at room temperature. Then the wells were washed five times with automatic washer EL×50 (BIO-TEK). Next, monoclonal antibodies against IL-10 coupled with horseradish peroxidase were added and incubated for 1 h at room temperature. After washing each well seven times tetramethylbenzidine was added and samples were incubated for 30 min in the dark. Colour formation reaction was stopped by adding the STOP solution. The absorbance was read at 450 nm wavelength by multichannel reader ELISA-EL_x808 (BIO-TEK). The accuracy of the method was > 2 pg/ml. The analyses were performed in duplicates.

Statistical analysis

Analyses were performed using Statistica 7.1 (Statsoft). To determine the statistically significant differences of concentrations between the analyzed groups, non-parametric Wilcoxon match-pairs ranks test was used [8, 9], which is a non-parametric alternative of *t*-test for correlated samples.

Results and discussion

The concentrations of IL-10 in cell cultures *in vitro* were analyzed using ELISA and the resulting arithmetical means are presented in Table 1. The highest values (188.39 pg/ml) of IL-10 concentration were obtained for Group V, where lymphocytes were stimulated with lyophilized microcapsules containing *Lactobacillus GG*. It was observed that MRS broth (IV Group) also stimulated IL-10 release at the concentration of 62.68 pg/ml. The addition of supernatant culture stimulated the release of cytokine in concentration of 46.33 pg/ml. In Group II, where empty alginate capsules were used, the this value amounted to 23.98 pg/ml, as compared to the control group of non-stimulated lymphocytes culture – 26.58 pg/ml.

Using non-parametric Wilcoxon match-pairs ranks test the statistically significant differences in IL-10 concentrations between the groups V and I, II, II, IV and between group II and IV were determined (Fig. 2, solid lines). The lack of statistically significant differences, however, was observed between the groups I and II (Fig. 2, dashed line).

Therefore, the statistically significant increase of IL-10 release was determined under the influence of lyophilized

Table 1. Concentration of IL-10 in lymphocyte cultures

Group	Tested samples	Concentration of IL-10 [pg/ml]								Average	SD
I	lymphocytes	30.97	26.30	26.99	27.82	26.80	19.74	27.22	26.77	26.58	3.13
II	lymphocytes + empty alginate micropcapsules	22.06	25.82	24.73	21.64	19.44	27.84	26.73	23.59	23.98	2.84
III	lymphocytes + *Lactobacillus GG metabolites*	48.07	32.77	55.56	47.19	42.21	44.54	47.31	52.96	46.33	6.95
IV	lymphocytes + M.R.S.	52.04	81.24	60.46	68.55	56.83	67.1	55.22	59.98	62.68	9.36
V	lymphocytes + *lyophilized microcapsules containing Lactobacillus GG*	193.56	178.23	181.81	184.08	167.88	173.89	218.9	208.78	188.39	17.60

SD – standard deviation

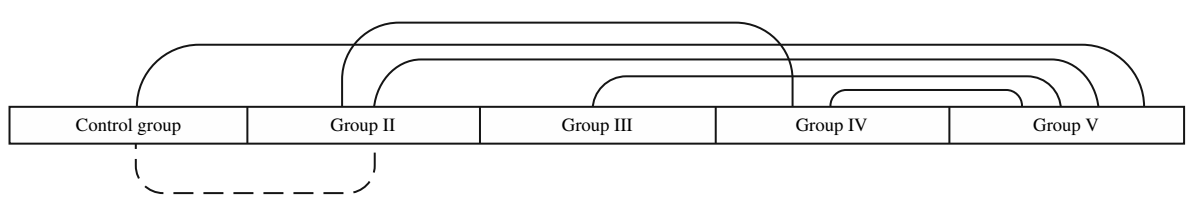

Fig. 2. Statistically significant (solid lines) and insignificant (dashed line) differences between investigated groups

in alginate capsules bacterial cells of *Lactobacillus GG*, as compared to the control and other groups. Enclosing the bacteria in microcapsules limited the presentation of cell membrane antigens (peptidoglycan) of *Lactobacillus GG* to immune response cells. The latter were mainly macrophages and lymphocytes present in cell cultures. Thus the potential stimulants were exopolysaccharide released by bacteria and bacterial metabolites including nitrogen oxide and hydrogen peroxide, supporting the effect of lactic acid [10-13].

It is known that orally admitted *Lactobacillus GG* in people activates Th3 lymphocytes to the synthesis of the cytokines TGF-β and IL-10, which keep the Th1/Th2 balance. Regulating Th3 lymphocytes inhibit pro-inflammatory and allergic response [1, 14]. The studies of Pessi *et al.* and Pochard and Pohjavuori confirmed the favourable influence of this strain on the expression of atopic dermatitis and food allergy symptoms because *Lactobacillus GG* activated IL-10 release [15-17], which in turn inhibited the synthesis of pro-inflammatory cytokines (IL-2 and IL-3). On the other hand it was proved that inappropriate IL-10 concentration can lead to numerous disorders – too high concentration can increase susceptibility to viral infections (e.g. HIV), but too low can intensify inflammatory reactions [18]. Interleukin 10 released in high concentrations exhibits immunomodulating effect to a large extent [19-23]. Moon *et al.* showed significant favourable influence of *Lactobacillus GG* in chronic colitis treatment [24]. The results of many studies confirm synergistic effect of sodium alginate through the increase of T lymphocytes and macrophages proliferation, thus the use of alginate as a stimulant of immune response seemed to be promising in clinical use [25-29].

In our study and with reference to increased IL-10 release *in vitro* the use of microcapsules containing *Lactobacillus GG* can be considered as a components of special food for people with immune disorders. The results justify further studies on the possibilities of the use of *Lactobacillus GG* metabolites *in vivo* as an immunomodulator for localized treatment, bringing the balance between the Th1 and Th2 lymphocytes and safe for humans.

Conclusions

1. In cultures of lymphocytes from whole blood stimulated with metabolites released by *Lactobacillus GG* immobilized in alginate microcapsules a high, statistically significant increase of IL-10 concentration was observed.
2. *Lactobacillus GG* enclosed in alginate capsules did not form biofilm on the outer surface of the membrane during 14 days of observations.
3. No influence of sodium alginate on the concentration of released IL-10 in lymphocyte cultures was observed.

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