Contribution to the Immunomodulatory Characteristics of Probiotic Bacteria

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Abstract

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We monitored the impact of selected probiotic strains on the human immune system. 13 strains of lactic acid bacteria (*Lactobacillus acidophilus*, *Lactobacillus helveticus, Enterococcus faecium*, *Streptococcus thermophilus*, and *Propionibacterium freudenreichii* subsp. *shermanii*), used in the production of functional foods, were tested from the point of view of their ability to stimulate a range of cytokines. The selected cytokines have regulatory properties; they affect the progression of inflammation and the inhibition of inflammatory response, and they play a role in humoral immunity, allergies, and cell-mediated immunity. The tested strains showed a specific interaction with the immune system. It was found that the *P. freudenreichii* strain showed significant levels of IFN gamma cytokine.

Keywords: *Lactobacillus acidophilus*; *Lactobacillus helveticus*; *Enterococcus faecium*; *Streptococcus thermophilus*; *Propionibacterium freudenreichii* subsp. *shermanii*; immunomodulation

Probiotics are microorganisms that exhibit a positive influence on human health by improving their intestinal microbial balance, and that may have a therapeutic effects. Probiotics are often administered through functional fermented foods with the aim to increase their health and nutritional benefits. Probiotics are currently defined as live, orally administered bacteria, that have a positive influence on human health by improving their intestinal microbial balance, and that may have therapeutic effects (Aureli *et al.* 2011).

It is known from historical sources that the technique of the production of food fermented by microorganisms as a therapeutic means was already used by anging Romans. The positive role of certain bacteria was described by Mechnikov. Alfred Nissle isolated bacterium *E. coli,* today called *E. coli* Nissle, from the faeces of healthy soldiers in the First World War, and he also successfully used it for the first time in the therapy of ulcerative colitis (Schultz 2008).

Colonisation of the intestinal mucous membrane after the birth represents a source of microbial stimulus essential for the development of the mucous system. Diversions in this process may precede the development of serious diseases. The influence of artificial colonisation of intestines in the newborns by the non-pathogenic *E. coli* strain O83 and its beneficial effects had been monitored by Lodinová-Žádníková (2002) along with her co-workers at the Institute for the Care of Mother and Child at Prague-Podolí (Czech Republic) since the sixties. The results of her studies were published in many papers. A significant finding of her long-term study is the reduction of the risk of nosocomial infections by peroral colonisation of the probiotic strain of *E. coli* after the birth, and its influence on the frequency of repeated infections and on the reduction of the incidence of allergies after ten and twenty years.

Gueimonde *et al.* (2006) identified a positive effect of *Lactobacillus rhamnosus* GG used by

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mothers on the development of intestinal microflora in the newborns.

Besides some *E. coli* strains (e.g. *E. coli* Nissle), selected yeasts, bifidobacteria, lactobacilli, and other lactic acid bacteria belong to probiotics. The administration of probiotics may positively affect the disorders of the gastrointestinal tract (Verna & Lucak 2010), otorhinolaryngological (Smith *et al.* 2011) and urogenital diseases (Hoesl & Altwein 2005), and the level of cholesterol (Fabian & Elmadfa 2006). Similarly, the beneficial action of probiotics may positively affect not only the diseases of the oral cavity (Meurman *et al.* 2004) but also the diseases that are mediated by TH1 or TH2 clones (Mosmann & Coffman 1989; Kokešová *et al*. 2006). Even many advertisements try to persuade us of the great effects of probiotic bacteria in dairy products. Experimentally, it was proved that the oral administration of *Lactobacillus acidophilus* 92 strain activates the T lymphocytes producing TGF beta and controlling the TH1 and TH2 cytokine response (Torii *et al.* 2007).

The antiallergic effects of *Lactobacillus pentosus* S-PT84 strain were again experimentally exhibited by inducing the regulatory TH lymphocytes producing IL-10. On the contrary, some other strains may negatively affect the activation of regulatory TH lymphocytes (Lammers *et al.* 2003). The therapeutic effects of probiotics should be therefore aimed at influencing those clones of T lymphocytes that were still not yet activated by the disease.

The objective of our study was to monitor the effects of the application of 13 selected strains of lactic acid bacteria on cytokine production, and to find what clones of T lymphocytes are affected, and what therapeutic effects can be expected from the individual strains.

MATERIAL AND METHODS

To study the effects of probiotics, we used the "buffy coat" from the blood bank which is a fraction of an anticoagulating blood sample after density gradient centrifugation that contains leucocytes and platelets. We diluted the buffy coat 1:1 with X-VIVO 10 (Cambrex, East Rutherford, USA) medium. We applied 15 ml of the Histopaque 1077 (Sigma-Aldrich, St. Louis, USA) solution into a 50 ml tube and carefully overlaid it with the diluted solution of the buffy coat. By phase centrifugation at 600 rpm (200 g) we separated the layer of mononuclear cells that we washed with the x-Vivo medium (one centrifugation at 400 rpm and one at 200 rpm) and diluted it with x-Vivo medium to a concentration of 10^7/ml cells.

Stimulation of cells to produce cytokines. The diluted cells were cultivated with probiotic bacteria, with the medium alone and with pokeweed mitogen (PWM) stimulating T and B cells, respectively.

Pokeweed mitogen – PWM – lectin from *Phytolacca americana* (Sigma-Aldrich, St. Louis, USA), a polyclonal activator, serves as a positive control, diluted with the x-Vivo medium to the concentration of 20 μg/ml.

Selected bacterial strains. In Table 1 are presented the bacteria selected for this research. The strains are deposited in the Culture Collection of Dairy Microorganisms Laktoflora®. These strains are commonly used in the dairy industry for the production of dairy products (Drbohlav 2005).

Preparation of bacterial strains for further processing. *Lbc. acidophilus* strains were selected from the Culture Collection of Dairy Microorganisms Laktoflora® (Prague, Czech Republic) on the basis of their previously tested probiotic properties; these strains were reidentified by biochemical and molecular genetic methods. The selected strains were cultivated in relevant culture media. *Lbc. acidophilus* strains in the collection Laktoflora® were routinely cultivated in MRS broth (Himedia, Mumbai, India). *Streptococcus thermophilus* and *Enterococcus faecium* strains were cultivated in M17 broth (Himedia), and *Propionibacterium*

Table 1. Selected bacterial strains (Collection number Laktoflora®)

Bacterial strains	No.
1. *Lactobacillus acidophilus*	CCDM 79
2. *Lactobacillus acidophilus*	CCDM 109
3. *Lactobacillus acidophilus*	CCDM 149
4. *Lactobacillus acidophilus*	CCDM 151
5. *Lactobacillus acidophilus*	CCDM 152
6. *Enterococcus faecium*	CCDM 922
7. *Lactobacillus acidophilus*	CCDM 193
8. *Lactobacillus acidophilus*	CCDM 217
9. *Lactobacillus acidophilus*	CCDM 406
10. *Lactobacillus acidophilus* reidentified (2009) as *Lactobacillus helveticus*	CCDM 92
11. *Lactobacillus acidophilus*	CCDM 476
12. *Streptococcus thermophilus*	CCDM 144
13. *Propionibacterium*	CCDM 160

Table 2. Cultivation conditions of individual strains

Strain	Cultivation conditions		
	temperature (°C)	time (h)	nutrient medium
Lactobacillus acidophilus	37	24 (in some cases, 48 h)	MRS broth
Streptococcus thermophilus	37	24	M17 broth
Enterococcus faecium	37	24	M17 broth
Propionibacterium freudenreichii subsp. *shermanii*	30	72	propionic medium

freudenreichii subsp. *shermanii* in a special whey medium produced for the cultivation of propionic bacteria (MILCOM a.s., Prague, Czech Republic). The strains routinely passaged in milk (CCDM 92, CCDM 151) were subcultured three times in synthetic media in order to eliminate the impact of protein from cow's milk. The cultivation conditions of individual strains are presented in Table 2.

After the cultivation in the relevant nutrient media, the strains were centrifuged (3000 rpm, 5040 g) for 15 min and subsequently washed in saline solution. Centrifugation and washing in saline solution was repeated three times. Then 3 ml of the X-VIVO (Cambrex) medium was added. In the resulting suspension the counts of microorganisms were determined by the cultivation plate method (Table 3).

The suspension samples were refrigerated and prepared for further tests. In Table 4 is presented the experimental procedure.

LUMINEX – detection of cytokines. After 5-day cell stimulation, the cytokine production was determined in tissue culture media by the multiplex method that allowed us to monitor quantitatively 8 cytokines in one sample.

Working procedure. All reagents and solutions (RD5K calibrator, Flurokine standard, microparticules, Biotin Antibody Cocktail, Streptavidin PE) were prepared. The membranes in the 96-well plate were washed with 100 µl washing solution and the fluid was sucked off with a vacuum pump. The diluted microparticles (50 µl) and standards or samples (50 µl) were added into each well and incubated at room temperature in the horizontal shaker for 3 hours. After the incubation, the plate was washed 3 times with the washing solution followed every time by the subsequent sucking off of the fluid. This was followed by adding 50 µl of the prepared biotin antibody complex to each well, incubating in the horizontal shaker for 1 h, adding 50 µl Streptavidin-PE to each well after incubating and washing, and incubating in the horizontal shaker for 30 minutes. After another incubation and washing, we resuspended the microparticles in 100 µl of the washing solution, incubated the suspension for 2 min, and measured the samples using the LUMINEX 100 Analyzer (LUMINEX Corp., Austin, USA) for up to 90 minutes.

Table 3. Counts of bacteria in suspension

Bacterial strains	CFU/1 ml MRS agar 37°C/72 h anaerobically	CFU/1 ml M17 agar 37°C/48 h aerobically	CFU/1 ml propionic agar 37°C/7 days anaerobically
1. CCDM 79	7.91	–	–
2. CCDM 109	7.67	–	–
3. CCDM 149	8.18	–	–
4. CCDM 151	7.18	–	–
5. CCDM 152	7.68	–	–
6. CCDM 922	–	8.28	–
7. CCDM 193	7.34	–	–
8. CCDM 217	7.32	–	–
9. CCDM 406	7.00	–	–
10. CCDM 92	7.45	–	–
11. CCDM 476	7.96	–	–
12. CCDM 144	–	7.48	–
13. CCDM 160	–	–	7.77

Table 4. Experiment scheme

Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1.6 ml X-VIVO	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
1.8 ml X-VIVO															+
0.2 ml of cells	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.2 ml of bacteria	+	+	+	+	+	+	+	+	+	+	+	+	+		
0.2 ml of PWM														+	

RESULTS AND DISCUSSION

The use of health-supporting bacteria for therapeutic purposes in medicine has a long tradition. The number of clinical studies scientifically examining the prevention, alleviation, or treatment of diseases has been growing recently (Hoesl & Altwein 2005; Fabian & Elmadfa 2006; Verna & Lucak 2010; Smith *et al.* 2011), in order not only to find evidence for health claims for a particular group of "healthy consumers", but also to test the use of pro-and prebiotics in medicine (in the prevention and treatment). The studies are based on the knowledge that the interaction of microflora with the mucosa-associated immune system of the gut has a significant influence on the development and function of the immune system, and hence on the health of the whole organism (Prakash *et al.* 2011). Despite important advances in the research of these interactions, many of them still remain unknown. The aim of the study was to monitor the cytokine response in human mononuclear cells isolated from the peripheral blood cells (PBMC) after the interaction with *Lactobacillus acidophilus*, *Enterococcus faecium*, *Streptococcus thermophilus*, and *Propionibacterium freudenreichii* strains. Cytokines are defined as secreted regulatory proteins, and their most important function is to regulate the immune processes and to ensure homeostasis under normal and pathological conditions.

Most cytokines are pleiotropic in their action which means that they affect different types of cells, often in a cascade, where one cytokine induces the formation of the second one, or where it may be replaced by the second one. Therefore, detailed knowledge of the functions of cytokines and their mutual functional interactions is of great importance.

According to the prevailing features, we selected for our study the determination of TNF alpha, IL-6, and IL-8 in the proinflammatory cytokines, of IL-4, IL-6, and IL-10 in cytokines with the inhibitory effect on inflammatory reaction, of IL-4, IL-5, and IL-10 in the cytokines acting in antibody immunity

Table 5. Creation of cytokines after stimulation of mononuclear cells by selected bacteria strains (pg/ml) – $n = 2$

Bacterial strain	IFN gamma	IL-4	IL-17	IL-5	IL-6	IL-8	IL-10	TNF alpha
1. CCDM 79	2.8	0	3.8	1.8	4000.0	1126.6	105.00	3197.00
2. CCDM 109	7.25	0	3.8	1.8	4000.0	1788.0	48.70	3059.00
3. CCDM 149	2.8	0	3.8	1.8	3936.0	1648.0	38.30	2418.00
4. CCDM 151	6.7	0	3.8	1.8	4000.0	2670.0	34.90	3014.00
5. CCDM 152	2.8	0	3.8	1.8	4000.0	3300.0	75.90	3381.00
6. CCDM 922	2.8	2.8	3.8	1.8	352.9	3300.0	0.07	735.90
7. CCDM 193	2.8	0	3.8	1.8	70.0	3300.0	0.07	4.12
8. CCDM 217	68.1	0	3.8	1.8	4000.0	2571.0	207.40	3632.00
9. CCDM 406	2.8	0	3.8	1.8	4000.0	2740.0	12.00	1637.00
10. CCDM 92	77.3	0	3.8	1.8	4000.0	2794.0	204.30	3427.00
11. CCDM 476	2.8	0	3.8	1.8	2655.0	3061.0	7.10	627.60
12. CCDM 144	54.1	0	3.8	1.8	4000.0	3300.0	189.70	3220.00
13. CCDM 160	346.6	0	3.8	1.8	4000.0	3300.0	161.60	3900.00
PWM	16.6	0	3.8	1.8	4000.0	3300.0	58.50	1470.00
Cells and media	11.1	9.7	3.8	1.8	5.3	3109.0	0.97	4.05

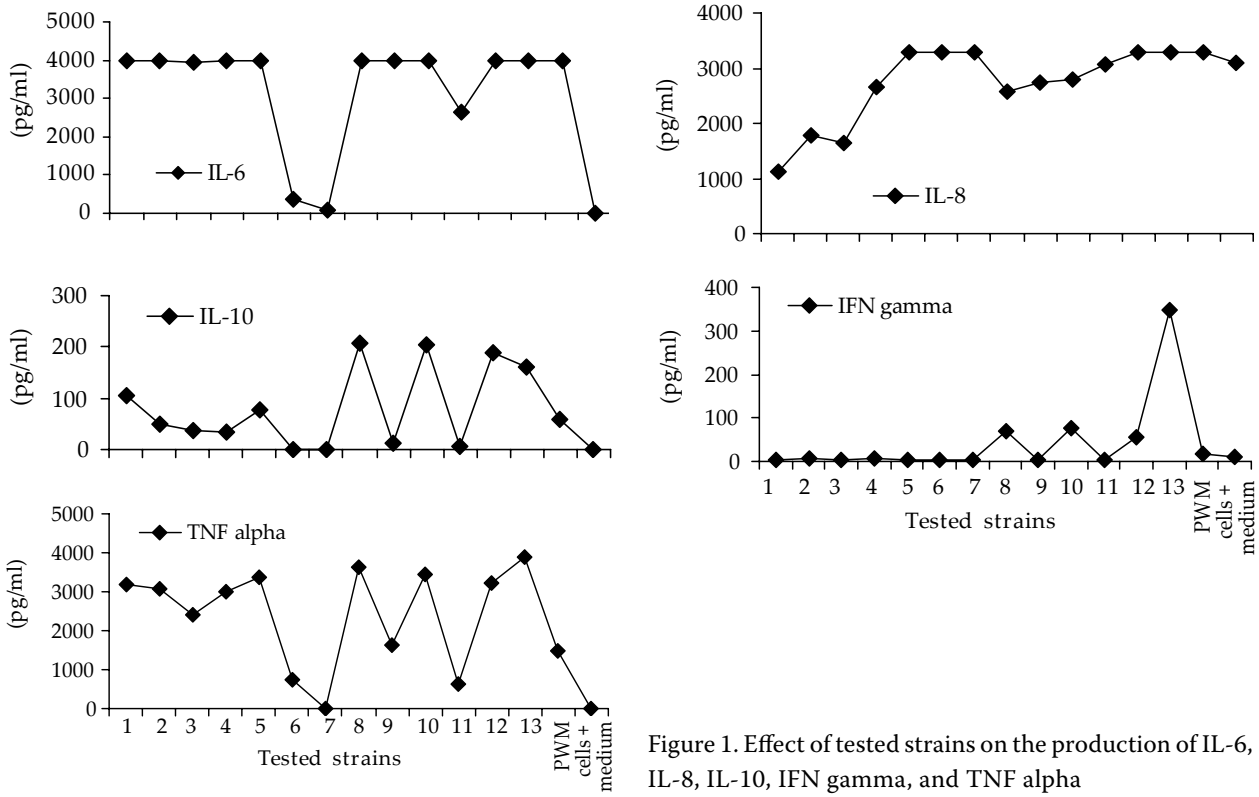

Figure 1. Effect of tested strains on the production of IL-6, IL-8, IL-10, IFN gamma, and TNF alpha

and allergy (TH2 clones of lymphocytes), and of IFN gamma and one of the regulatory cytokines – IL-17 in the cytokines involved in cell-mediated immunity (TH1 clones of lymphocytes).

We compared the effects of the tested bacteria on the production of cytokines with the results of cytokine production after the stimulation with PWM, and solely by the cells with the medium. In comparison with the production of IFN gamma after the cell stimulation with PWM mitogen, or solely by medium and cells, the production increased after the stimulation by *Lactobacillus acidophilus* CCDM 217 and 92, *Streptococcus thermophilus* CCDM 144, and *Propionibacterium freudenreichii* CCDM 160. A higher production of IL-4 was found only after stimulation by *Enterococcus faecium*, but it was lower as compared with the cell stimulation solely by medium and cells. IL-5 and IL-17 production was not affected. In a series of samples, the production of IL-6 was high (4000 pg/ml); a significantly lower production was found after the stimulation by *Enterococcus faecium* CCDM 922, and *Lactobacillus acidophilus* CCDM 193 and CCDM 476. In all media monitored, the production of IL-6 was significantly higher after the cell stimulation by bacteria, as compared with the non-stimulated cultures (i.e. solely cells cultivated in the media). Furthermore, we found the inhibition of IL-8 production after stimulation by *Lactobacillus acidophilus* CCDM 79, 109 and 149 strains, but on the contrary, other strains of bacteria and cells stimulated solely by the medium produced more IL-8. We obtained interesting results when determining IL-10; an increased production of this regulatory cytokine was found after the stimulation of mononuclear cells by *Lactobacillus acidophilus* CCDM 79, 152, 217, and 92, by *Propionibacterium freudenreichii* CCDM 160, and by *Streptococcus thermophilus* CCDM 144 strains. On the contrary, a significant reduction in the production of IL-10 was found after the stimulation by *Enterococcus faecium* CCDM 922, and by *Lactobacillus acidophilus* CCDM 193, 406, and 476. However, as compared with the cells stimulated solely by the medium, the production of IL-10 was higher in all samples. Similar results were obtained when we determined TNF alpha in all samples stimulated by bacteria as compared with the cells stimulated with PWM. *Propionibacterium freudenreichii* CCDM 160, *Streptococcus thermophilus* CCDM 144, and *Lactobacillus acidophilus* CCDM 217, 152, and 92 increased the production of TNF alpha; on the contrary, the inhibition of the production was found after the stimulation by *Lactobacillus acidophilus* CCDM 193, 476, and *Enterococcus faecium* CCDM 922.

CONCLUSION

The knowledge that we acquired after the stimulation of isolated mononuclear cells of *Propionibacterium freudenreichii* CCDM 160 (the strain usually used for the production of Emmentaler cheese types and that which produces vitamin B_{12} and folic acid in dairy products) could be used in clinical research. Lower levels of folic acid and vitamin B_{12} in the serum are often the cause of recurrent aphthous stomatitis, thus it would be interesting to test if the milk products containing this bacterial strain can affect the incidence of the disease. Likewise, the higher levels of homocysteine in the serum of some patients can occur due to reduced levels of B_{12} and folic acid.

It is evident from our experiment that each strain of the tested bacteria interacted with the immune system in a specific way. Our findings stress still more the need for a comprehensive research in this area that could explain in the future the effectiveness or ineffectiveness of probiotic bacteria in the form of nutritional supplements and functional foods in the treatment of specific human diseases or their possible application in disease prevention. Multiplex methods simplifying the testing of cytokine production in the samples could facilitate the targeted deployment of probiotics in clinical practice in the future.

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