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EFFECT OF HYDROGEN PEROXIDE OF BACTERIAL ORIGIN ON APOPTOSIS AND NECROSIS OF GUT MUCOSA EPITHELIAL CELLS AS A POSSIBLE PATHOMECHANISM OF INFLAMMATORY BOWEL DISEASE AND CANCER

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A series of *in vitro* experiments was arranged to assess effects of different concentrations of H_2O_2 contained in bacterial cultures on apoptosis and necrosis of HT-29 line cells representing human gut epithelium. On the basis of cytofluorimetric assays it was possible to demonstrate that supernatant of the *Lactobacillus* strain producing hydrogen peroxide (*L. delbrueckii* CU/22) was able to induce both apoptosis and necrosis in human epithelial culture cells HT-29. Both effects were more prominent than those visible under influence of supernatant of the non-H_2O_2-producing *Lactobacillus* strain or chemically pure H_2O_2 at the same concentration used as a control. In the light of this study and also other reports on damaging effects of hydrogen peroxide and superoxide radicals of bacterial origin on colonic cells, commensal bacteria of the human gut producing H_2O_2 may be involved in pathomechanisms of IBD by perpetuating the inflammatory reaction and increasing apoptosis and necrosis. There is a promise that probiotic preparations containing *Lactobacillus* bacteria will be successful as adjunct therapy of IBD and it is, therefore, postulated to make a very careful selection of the *Lactobacillus* strains as candidates for probiotics indicated to ameliorate the course of IBD, before starting clinical trials.

Key words: *hydrogen peroxide, apoptosis, gut, epithelial cells, inflammatory bowel disease, cancer, reactive oxygen species*

INTRODUCTION

Probably a very phylogenetically old phenomenon of exporting reactive oxygen species (ROS) out of the bacterial cells to avoid their cell damaging effects was later adopted by phagocyting free living amebas and phagocyting immune cells of the multicellular organisms to destroy invading microorganisms. The phagocyting cells like granulocytes and macrophages are able to produce considerable amounts of superoxide radicals transformed into hydrogen peroxide (H_2O_2) during respiratory burst (1). It is estimated that at *in vitro* conditions population of granulocytes (approximately 10^6 cells) produces around 1 mM of H_2O_2 in 2 hours (2). On the other hand, some species of bacteria belonging to *Streptococcus*, *Enterococcus* and *Lactobacillus* genera residing on the mucus membranes of the human body are also able to produce comparable amounts of H_2O_2 in aerobic conditions. Populations of these bacteria (approximately 1×10^8/ml) can liberate *in vitro* 0.6 to 1.8 mM of H_2O_2 in 24 hours (3). We have been able to demonstrate that bacteria producing increased amounts of H_2O_2 are present in high numbers on the inflamed mucosa of patients with ulcerative colitis (4). This may indicate that H_2O_2 producing bacteria present on mucosal surfaces and especially on gut mucosa may play an active role not only in regulation of human gut microbiota but also interaction with mucosa epithelial cells by initiating their apoptosis or necrosis.

Increased inflammatory cytokine levels and intestinal epithelial cell apoptosis leading to disruption of epithelial integrity are major pathologic factors in inflammatory bowel diseases (IBD). In IBD, cell death can directly or indirectly compromise barrier function, impair effective epithelial restitution, lead to loss of stem cells and regenerative capacity and has relevance in carcinogenesis. The nature of the predominant form of cell death remains uncertain but probably involves both apoptosis and necrosis. Apoptosis is a phenomenon of the programmed cell death and the essential part of the normal cellular phenotype repertoire. In the absence of appropriate survival factors such as short-chain fatty acids, anti-oxidants and surfactants and in the presence of luminal factors like cytokines and ROS in association with inflammation, apoptosis is activated through specific signaling cassettes. The cellular program that triggers apoptosis in epithelial cells may be activated in different ways depending on the initiating factors and cell types (5). Essentially the same factors but acting more rapidly may cause necrosis; a direct cell death with rapid disruption of the cell membrane and liberation of the cytoplasm leading to activation of different mediators of the inflammatory process characteristic for IBD (6). Oxidant-induced necrosis of epithelial cells during IBD may be increased due to iron-mediated Fenton reaction when free H_2O_2 generated near the intestinal epithelial surface can passively diffuse into colonocytes and form hydroxyl radicals (7).

A series of *in vitro* experiments was arranged to assess effects of H_2O_2 produced by *Lactobacillus* on apoptosis and necrosis of HT-29 cell line cells representing human gut epithelium.

MATERIALS AND METHODS

Apoptosis as an effect of H_2O_2-producing *Lactobacillus* strain culture on human colon adenocarcinoma cell line HT-29 was assessed using ApoFluor Green Caspase Activity Assay kit (ICN Biomedical Inc.). Chemically pure H_2O_2 and pro-apoptotic staurosporine (Sigma) were used as positive controls while PBS and supernatant of non-H_2O_2-producing *Lactobacillus* strain culture as negative controls. Necrosis of the same cells under influence of either pure H_2O_2 or *Lactobacillus* culture supernatant was also measured after staining the cells with propidium iodide from the same kit.

Bacterial strains: *L. delbrueckii* CU/22 and *L. plantarum* K/12 were incubated in 50 ml of freshly prepared MRS broth (Oxoid) for 24 hours at 37°C in anaerobic conditions. The cultures were than centrifuged at 3000 rpm for 10 min., supernatants discarded and bacterial cells washed with PBS without Ca^{2+} and Mg^{2+} ions (IITD PAN Wrocław) and centrifuged again. Bacteria were transferred to 5 ml of fresh PBS without Ca^{2+} and Mg^{2+} ions and incubated in aerobic atmosphere for 24 hours at 37°C with shaking. The cultures were centrifuged at the same conditions and supernatants filtered through membrane filters with 0.22 μm pore size. Concentration of H_2O_2 in supernatants was measured using appropriate strips (Mercoquant, Peroxide-Test, Merck). *L. delbrueckii* CU/22 culture supernatant contained 0.8 mM of H_2O_2 while this of *L. plantarum* K/12 had no H_2O_2. Chemically pure solutions containing 1.8 and 0.8 mM of hydrogen peroxide (POCH) in PBS without Ca^{2+} and Mg^{2+} ions were prepared immediately before experiments and used as controls.

Tissue culture of the epithelial cell line HT-29 was carried out for 20 days in DMEM medium (IITD PAN Wroclaw) supplemented with 10% of fetal bovine serum (FBS, Sigma) in 6-well vessels (Greiner) in an atmosphere with increased humidity containing 10% CO_2 at 37°C. The medium was exchanged every 48 hours. After reaching monolayer growth phase, the cells were removed by 0.25% trypsin solution (Sigma) treatment for 10 min. The cells were then transferred into 12-well trays (Greiner) to obtain a density of 5×10^5 cells per well and incubated for 24 hours as before and washed with PBS without Ca^{2+} and Mg^{2+} ions before associating the constituents of the experiment as follows. To each well containing the final number of 1×10^6 HT-29 cells, the following samples were added to the final volume of 1 ml:

sample 1: (negative control): DMEM medium + 10% FBS,
sample 2: (negative control): PBS without Ca^{2+} and Mg^{2+} ions,
sample 3: (positive control): PBS without Ca^{2+} and Mg^{2+} ions with 2 μM of staurosporine,
sample 4: (positive control): PBS without Ca^{2+} and Mg^{2+} ions with 0.8 mM of H_2O_2,
sample 5: (positive control): PBS without Ca^{2+} and Mg^{2+} ions with 1.8 mM of H_2O_2,
sample 6: (test mixture): 500 μl of *L. delbrueckii* CU/22 culture supernatant + 500 μl of μl PBS without Ca^{2+} and Mg^{2+} ions,
sample 7: (test mixture): 500 μl of *L. plantarum* K12 culture supernatant + 500 μl of μl PBS without Ca^{2+} and Mg^{2+} ions.

Apoptosis and necrosis assays

Test mixtures and controls were incubated for 24 hours at 37°C as before. The cells were then trypsinized for 5 min, removed, centrifuged at 2000 rpm for 10 min. and resuspended in 300 μl of fresh DMEM medium with 10% of FBS. A fluorescent substrate ApoFluor Green reagent (ICN Biomedicals Inc.) was added to each well in a volume of 5 μl. Cell suspensions with substrate were incubated at 37°C for 1 hour in an atmosphere supplemented with 5% CO_2 and washed several times with HEPES buffer (ICN Biomedicals Inc.) to remove unbound substrate. After this procedure, the cells were suspended in 400 μl of PBS containing 2 μl of propidium iodide solution (250 g/ml). Fluorescence was measured in each sample using flow cytofluorimeter (FACS Calibur, Becton Dickinson) with FL-1 filter (488-525 nm) to measure apoptotic cells and FL-2 filter (488-575 nm) to measure necrotic cells. Each experiment was carried out in duplicate and test results were presented as mean values.

Fenton reaction

Hydroxyl radicals in bacterial supernatants containing H_2O_2 in the presence of ferrous ions were detected by electron spin resonance (ESR) - spin trapping. Bacterial supernatants were prepared as before but always using PBS without not only Ca^{2+}, Mg^{2+} but also Fe^{2+} which was obtained by adding 5 g of Chelex 100 chelator (Sigma) to 1 l of PBS without Ca^{2+} and Mg^{2+} ions and vigorous mixing for 6 hours. Then the buffer was filtered through Whatman 40 paper and sterilized by autoclaving at 121°C for 15 min. Freshly prepared 0.8 mM solution of chemically pure hydrogen peroxide in PBS without Ca^{2+}, Mg^{2+} and Fe^{2+} was a positive control, while the same solution pretreated with catalase (0.1% in PBS without Ca^{2+}, Mg^{2+} and Fe^{2+}) for 10 min. at 37°C served as negative control.

Just before the experiment, a freshly prepared solution of $FeSO_4$ (POCH) in 10 mM phosphate buffer with 0.3 mM EDTA (Sigma) and spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pirroline-*N*-oxide (DMPO, Sigma) were added to bacterial supernatants and controls to obtain final concentrations, 0.2 mM and 100 mM in 1 ml of the total volume of the reaction mixtures, respectively. Spectra were recorded immediately after addition of a spin trap and then in 10, 30 and 60 min. using an X-band (9.2 GHz) ELEXSYS 500 spectrometer (Brucker) with the following parameters: microwave power 100 mW, modulation amplitude 5.0 Gauss, sweep width 200.0 Gauss and time constant 81.92 ms, amplification: 1.42×104 G, conversion time: 163.84 ms. DMPO radical adducts simulation was performed using EPR sim 32 program (8) by making use of constant hyperfine splittings as determined by other authors (9, 10). Each experiment was carried out in duplicate. Appropriate scheme of the reaction mixtures is given in *Table 2*.

Effects of Fenton reaction products on apoptosis and necrosis

Apoptosis and necrosis of HT-29 cells was assayed as described above for bacterial supernatants using HT-29 cells. The reaction mixtures were prepared in parallel also to demonstrate the Fenton reaction. The HT-29 cells were washed with PBS buffer without Ca^{2+}, Mg^{2+} and Fe^{2+} ions and then 1 ml of each mixture was added to consecutive wells according to the following scheme:

sample 1: (negative control): DMEM + 10% FBS
sample 2: (negative control): PBS without Ca^{2+}, Mg^{2+} and Fe^{2+} with 0.2 mM of $FeSO_4$
sample 3: (positive control for apoptosis): PBS without Ca^{2+}, Mg^{2+} and Fe^{2+} with 2 μM of staurosporine
sample 4: (positive control): PBS without Ca^{2+}, Mg^{2+} and Fe^{2+} with 0.8 mM of chemically pure H_2O_2 and 0.2 mM of $FeSO_4$

sample 5: (positive control): PBS without Ca^{2+}, Mg^{2+} and Fe^{2+} with 1.8 mM of chemically pure H_2O_2 and 0.2 mM of $FeSO_4$

sample 6 (negative control): PBS without Ca^{2+}, Mg^{2+} and Fe^{2+} with 0.8 mM of chemically pure H_2O_2 to which 0.1% catalase and 0.2 mM of $FeSO_4$ were consecutively added.

sample 7: (test mixture): 500 µl of PBS without Ca^{2+}, Mg^{2+} and Fe^{2+} mixed with 500 µl of *L. delbrueckii* culture supernatant containing 0.8 mM of H_2O_2, with 0.2 mM of $FeSO_4$ added.

sample 8: (test mixture): 500 µl of PBS without Ca^{2+}, Mg^{2+} and Fe^{2+} mixed with 500 µl of *L. delbrueckii* culture supernatant containing 0.8 mM of H_2O_2 to which 0.1% catalase and 0.2 mM of $FeSO_4$ were consecutively added.

RESULTS

On the basis of cytofluorimetric assays it was possible to demonstrate that supernatant of the *Lactobacillus* strain producing hydrogen peroxide (*L. delbrueckii* CU/22) was able to induce both apoptosis and necrosis in human epithelial culture cells HT-29. The results were presented as mean percentages of viable, apoptotic and necrotic cells (*Table 1*). Both effects were more prominent than those visible under influence of supernatant of the non-H_2O_2-producing *Lactobacillus* strain (*L. plantarum* K/12) or chemically pure H_2O_2 at the same concentration (0.8 mM) used as positive control.

Our *in vitro* experiments to demonstrate the Fenton reaction and consequently, appearance of hydroxyl radicals, indicated this possibility which took place in the presence of supernatant of the *Lactobacillus* strain producing hydrogen peroxide and

Table 1. Effect of chemically pure hydrogen peroxide and culture supernatants of 2 *Lactobacillus* strains: H_2O_2 producing and non-producing on apoptosis and necrosis of human gut epithelial cells HT-29.

Test mixtures	Percentages of		
	Viable cells	Necrotic cells	Apoptotic cells
DMEM + 10% FBS – negative control	93.8	2.6	3.6
PBS w/o Ca^{+2} and Mg^{+2}–negative control	88.0	6.0	6.0
Staurosporine 2 µM – positive control	41.2	14.7	44.1
Pure H_2O_2 0.8 mM	81.4	8.2	10.4
Pure H_2O_2 1.8 mM	66.1	6.9	27.0
Supernatant *L. delbrueckii* CU/22 containing H_2O_2 0.8mM	74.0	10.0	16.0
Supernatant *L. plantarum K/12*- H_2O_2 containing 0 mM H_2O_2	88.5	6.4	5.1

Table 2. Outline of verifying reactions (*in vitro* conditions) testing for the possibility of Fenton reaction and generation of hydroxyl radicals by culture supernatants of H_2O_2 producing and non-producing Lactobacillus strains. *Table 2* lists the final concentrations of reagents.

Mix-ture no.	**Component I**	**Component II**	**Component III**	**Component IV**	**Fenton reaction product**	**Figure**
1.	0.8mM H_2O_2	0.2mM $FeSO_4$	PBS no ions (Ca^{2+},Mg^{2+}, Fe^{2+})	-	$^{\cdot}OH$ presence confirmed	Fig. 1a
2.	0.8mM H_2O_2	0.2mM $FeSO_4$	PBS no ions (Ca^{2+},Mg^{2+}, Fe^{2+})	Catalase 0.1%	No $^{\cdot}OH$ detection	Fig. 1b
3.	*L. delbrueckii* (CU/22) supernatant with H_2O_2 (0.8mM)	0.2mM $FeSO_4$	PBS no ions (Ca^{2+},Mg^{2+}, Fe^{2+})	-	$^{\cdot}OH$ presence confirmed	Fig. 1c
4	*L. plantarum* (K/12) supernatant w/o H_2O_2 (0mM)	0.2mM $FeSO_4$	PBS no ions (Ca^{2+},Mg^{2+}, Fe^{2+})	-	No $^{\cdot}OH$ detection	Fig. 1d

Table 3. Effect of Fenton reaction products on apoptosis and necrosis of human gut epithelial cells HT-29.

Test mixtures	Percentages of		
	Viable cells	Necrotic cells	Apoptotic cells
DMEM + 10% FBS – negative control	93.8	2.6	3.6
PBS w/o Ca^{+2} and Mg^{+2} +$FeSO_4$ – negative control	87.5	6.3	6.2
PBS w/o Ca^{+2} and Mg^{+2} + staurosporine 2 μM – positive control	41.2	14.7	44.1
PBS w/o Ca^{+2} and Mg^{+2} + pure H_2O_2 0.8 mM + $FeSO_4$ – positive control	73.0	5.2	21.8
PBS w/o Ca^{+2} and Mg^{+2} + pure H_2O_2 1.8 mM+ $FeSO_4$ – positive control	50.2	6.2	43.6
PBS w/o Ca^{2+}, Mg^{2+} + $FeSO_4$ + 0.8 mM of chemically pure H_2O_2 + 0.1% catalase	89.0	6.4	4.6
PBS w/o Ca^{2+}, Mg^{2+} + $FeSO_4$ + supernatant *L. delbrueckii* CU/22 containing H_2O_2 0.8mM	68.4	18.8	12.8
PBS w/o Ca^{2+}, Mg^{2+} + $FeSO_4$ + supernatant *L. delbrueckii* CU/22 containing H_2O_2 0.8mM + 0.1% catalase	81.1	13.2	5.7

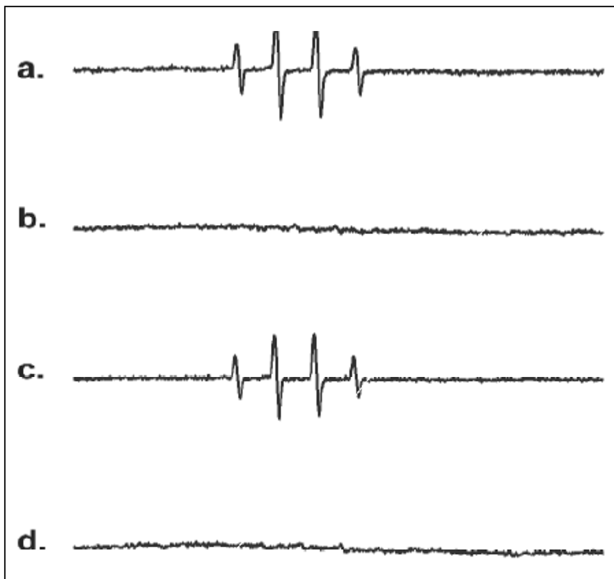

Fig. 1. EPR spectra showing presence or lack of hydroxyl radicals in the tested reaction mixtures: **a** - presence of the radicals in mixture 1 (*Table 2*), **b** - lack of the radicals after catalase adding (mixture 2), **c** - presence of the radicals in mixture 3 containing supernate of H_2O_2 producing *Lactobacillus delbrueckii* CU/22 strain, **d** - lack of the radicals after adding catalase to this mixture 3 in *Table 2*.

iron ions (*Table 2*). The ESR reaction spectra were comparable, although less prominent, to those obtained for chemically pure H_2O_2 at the same concentration as present in the supernatant (*Fig. 1*). Catalase added to the supernatant as well as H_2O_2-negative *Lactobacillus* culture supernatant caused disappearance of visible spectra characteristic for hydroxyl radicals.

Analysis of the effects of Fenton reaction products on apoptosis and necrosis of HT-29 cells revealed that reaction mixtures in which the reaction was initiated by chemically pure H_2O_2 caused more apoptotic than necrotic cells (*Table 3*). On the other hand, the reaction products of supernatant of the *L. delbrueckii* strain producing hydrogen peroxide, containing the same amount of H_2O_2, led to more necrotic than apoptotic cells. This may indicate a presence of some additional compounds in the H_2O_2-containing supernatant which synergistically increase necrotic response in HT-29 cells.

DISCUSSION

The role of the bacterial colon flora in initiating and perpetuating inflammatory bowel disease seems to be well documented, both in humans and in animals (11-13). A few of the mechanisms evoked by bacteria like disruption of the barrier function and sensitization of dendritic cells have been elucidated (14-16) but many others need to be discovered. In this study we arranged a series of experiments to show a possible effect of hydrogen peroxide, one of many substances produced by some bacteria residing in human colon.

The results obtained have confirmed that hydrogen peroxide of bacterial origin exerts apoptosis and necrosis of human colonocytes *in vitro* either directly or by initiating Fenton's reaction. These data not only support hypothesis of Oldenburg (17) on epithelial cells damage caused by Fenton's reaction in IBD but also demonstrate a possibility of Fenton's reaction in the colonic environment colonized by numerous bacterial populations which possess the extracellular ability to produce hydrogen peroxide.

It has been proved before (18, 19) that relatively high amounts of hydrogen peroxide are found in chronically inflamed

tissues. They are produced not only by immune cells but also, as it has been documented before by us (4), by some species of bacteria from *Lactobacillus*, *Streptococcus* and *Enterococcus* genera. Such a double influx of H_2O_2 may more strongly influence various vital processes in the host cells during inflammation. It has been shown that H_2O_2 is able to increase affinity of B cells to antigens due to stimulation of their receptors (20) and to regulate activation of the NF-κB cell system (21). Both mechanisms lead to stimulation of the immune cells to liberate proinflammatory cytokines (22).

It seems however, that one of the most prominent activities of H_2O_2 in chronically inflamed mucosal cells is to regulate their apoptosis. As shown in this study, H_2O_2 in concentration equal to this produced by bacteria contained in gut was able to induce apoptosis of human gut epithelial cells (HT-29). Similar observations have been reported recently by Leung (23) for human stomach cancer cells and Denning and coworkers (24) for gut cells. Such direct activity of H_2O_2 of bacterial origin on gut mucosa cells is even more probable in IBD lesions since they are much deprived of the protective mucus layer. Apoptosis is generally a favorable regulatory mechanism controlling in physiological conditions the removal of aberrant, damaged or infected cells (25, 26). However, in IBD patients protracted apoptosis may cause disruption of the mucosal integrity and impaired tissue healing of the gut mucosa since H_2O_2 and other ROS seem to be unbalanced and continuously activated, being not properly controlled by antioxidative enzymes (18).

These damaging properties to mucosal cells of H_2O_2 produced by some gut bacteria can be potentiated by initiation of the Fenton reaction, the effect which has been shown in the current experiments. Products of the Fenton reaction, hydroxyl radicals, are highly toxic to human cells (25). As it has been demonstrated above, Fenton reaction products induced by culture supernatant of the H_2O_2 producing strain of *Lactobacillus delbrueckii* caused twice more necrotic cells in comparison to the activity of the supernatant alone.

The occurrence of the Fenton reaction in IBD lesions is highly probable because of the presence of both reagents: H_2O_2 produced by both immune cells and bacteria and ferrous ions from extravasated erythrocytes. It has been shown, also by us (4), that numbers of streptococci and lactobacilli, *i.e.* these bacteria which produce H_2O_2 are elevated in IBD lesions. It is of interest that other bacteria, *e.g. Enterococcus* spp. present in human colon microflora are able to produce both H_2O_2 and superoxide that damage colonic epithelial cells (27).

On the other hand, a typical feature of IBD is a chronic loss of blood from colonic and intestinal ulcerations and the presence of free iron ions which are not effectively taken by impaired iron-transferring proteins. Toxic activity of hydroxyl radicals as one of pathomechanisms of IBD has already been discussed (28) since excess of iron amplifies oxidative stress, the inflammatory response and mucosal damage in a rat model of colitis (29, 30). Also, oral iron supplementation used to treat anemia in IBD patients leads to aggravation of the disease clinical symptoms (31) while iron chelators ameliorate oxidative stress and inflammation in colonic biopsies from patients with ulcerative colitis (32).

In the light of this study and also other reports on damaging effects of hydrogen peroxide and superoxide radicals of bacterial origin on colonic cells, commensal bacteria of the human gut producing H_2O_2 may be involved in pathomechanisms of IBD by perpetuating the inflammatory reaction and increasing apoptosis and necrosis. Different probiotic preparations containing *Lactobacillus* bacteria are now being tested as adjunct therapy of IBD but their clinical effects are rather discrepant (33-35). It is, therefore, postulated to make a very careful selection of the *Lactobacillus* strains as candidates for probiotics indicated to ameliorate course of IBD, before starting clinical trials.

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REFERENCES

1. Clark RA, Nauseef WM. Isolation and functional analysis of neutrophils. *Curr Protoc Immunol* 2001; 7: 7-23.
2. Becker S, Soukup JM, Gallagher JE. Differential particulate air pollution induced oxidant stress in human granulocytes, monocytes and alveolar macrophages. *Toxicol In Vitro* 2002; 16: 209-218.
3. Ocana VS, Pesce de Ruiz Holgado AA, Nader-Macias ME. Selection of vaginal H_2O_2-generating Lactobacillus species for probiotic use. *Curr Microbiol* 1999; 38: 279-284.
4. Strus M, Gosiewski T, Kochan P, Heczko PB. A role of hydrogen peroxide producing commensal bacteria present in colon of children with IBD in perpetuation of the inflammatory process. *J Physiol Pharmacol* 2009; 60(Suppl. 6): 49-54.
5. Zabielski R, Godlewski MM, Guilloteau P. Control of development of gastrointestinal system in neonates. *J Physiol Pharmacol* 2008; 59(Suppl 1): 35-54.
6. Gibson PR. Apoptosis or necrosis-colonic epithelial cell survival. *Novartis Found Symp* 2004; 263: 133-150.
7. Henle ES, Linn S. Formation, prevention and repair of DNA damage by iron/hydrogen peroxide. *J Biol Chem* 1997; 272: 19095-19098.
8. Spalek T, Kruczala K, Sojka Z, Schlick S. Deducing 1D concentration profiles from EPR imaging: a new approach based on the concept of virtual components and optimization with the genetic algorithm. *J Magn Reson* 2007; 189: 139-150.
9. Buettner GR, Moseley PL. EPR spin trapping of free radicals produced by bleomycin and ascorbate. *Free Radic Res Commun* 1993; 19: S89-S93.
10. Zhao BL, Duan SJ, Xin WJ. Lymphocytes can produce respiratory burst and oxygen radicals as polymorphonuclear leukocytes. *Cell Biophys* 1990; 17: 205-211.
11. Sartor RB, Cromartie WJ, Powell DW, Schwab JH. Granulomatous enterocolitis induced in rats by purified bacterial cell wall fragments. *Gastroenterology* 1985; 89: 587-595.
12. Swidsinski A, Weber J, Loening-Baucke V, Hale LP, Lochs H. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *J Clin Microbiol* 2005; 43: 3380-3389.
13. Seksik P, Sokol H, Lepage P, *et al*. Review article: the role of bacteria in onset and perpetuation of inflammatory bowel disease. *Aliment Pharmacol Ther* 2006; 24: 11-18.
14. Pacelli R, Wink DA, Cook JA, *et al*. Nitric oxide potentiates hydrogen peroxide-induced killing of Escherichia coli. *J Exp Med* 1995; 182: 1469-1479.
15. Weersma RK, van Dullemen HM, van der Steege G, Nolte IM, Kleibeuker JH, Dijkstra G. Review article: Inflammatory bowel disease and genetics. *Aliment Pharmacol Ther* 2007; 26: 57-65.
16. Wapenaar MC, Monsuur AJ, van Bodegraven AA, *et al*. Associations with tight junction genes PARD3 and MAGI2 in Dutch patients point to a common barrier defect for coeliac disease and ulcerative colitis. *Gut* 2008; 57: 463-467.
17. Oldenburg B, Koningsberger JC, Van Berge Henegouwen GP, Van Asbeck BS, Marx JJ. Iron and inflammatory bowel disease. *Aliment Pharmacol Ther* 2001; 15: 429-438.

18. Kruidenier L, Kuiper I, Lamers CB, Verspaget HW. Intestinal oxidative damage in inflammatory bowel disease: semi-quantification, localization, and association with mucosal antioxidants. *J Pathol* 2003; 201: 28-36.
19. Rezaie A, Parker RD, Abdollahi M. Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause? *Dig Dis Sci* 2007; 52: 2015-2021.
20. Reth M. Oligomeric antigen receptors: a new view on signaling for the selection of lymphocytes. *Trends Immunol* 2001; 22: 356-360.
21. Schreck R, Baeuerle PA. Assessing oxygen radicals as mediators in activation of inducible eukaryotic transcription factor NF-kappa B. *Methods Enzymol* 1994; 234: 151-163.
22. Cao WG, Morin M, Metz C, Maheux R, Akoum A. Stimulation of macrophage migration inhibitory factor expression in endometrial stromal cells by interleukin 1, beta involving the nuclear transcription factor NFkappaB. *Biol Reprod* 2005; 73: 565-570.
23. Leung AM, Redlak MJ, Miller TA. Oxygen radical induced gastric mucosal cell death: apoptosis or necrosis? *Dig Dis Sci* 2008; 53: 2429-2435.
24. Denning TL, Takaishi H, Crowe SE, Boldogh I, Jevnikar A, Ernst PB. Oxidative stress induces the expression of Fas and Fas ligand and apoptosis in murine intestinal epithelial cells. *Free Radic Biol Med* 2002; 33: 1641-1650.
25. Kaczmarek M, Frydrychowicz M, Nowicka A, Kozlowska M, *et al*. Influence of pleural macrophages on proliferative activity and apoptosis regulating proteins of malignant cells. *J Physiol Pharmacol* 2008; 59(Suppl 6): 321-330.
26. Chiozzi V, Mazzini G, Oldani A, *et al*. Relationship between Vac A toxin and ammonia in Helicobacter pylori-induced apoptosis in human gastric epithelial cells. *J Physiol Pharmacol* 2009; 60(3): 23-30.
27. Rutkowski R, Pancewicz SA, Rutkowski K, Rutkowska J. Reactive oxygen and nitrogen species in inflammatory process. *Pol Merkur Lek* 2007; 23: 131-136.
28. Huycke MM, Abrams V, Moore DR. Enterococcus faecalis produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. *Carcinogenesis* 2002; 23: 529-536.
29. Oldenburg B, van Berge Henegouwen GP, Rennick D, Van Asbeck BS, Koningsberger JC. Iron supplementation affects the production of pro-inflammatory cytokines in IL-10 deficient mice. *Eur J Clin Invest* 2000; 30: 505-510.
30. Reifen R, Matas Z, Seidel L, Berkovitch Z, Bujanover Y. Iron supplementation may aggravate inflammatory status of colitis in a rat model. *Dig Dis Sci* 2000; 45: 394-397.
31. Uritski R, Barshack I, Bilski I, Ghebremeskel K, Reifen R. Dietary iron affects inflammatory status in a rat model of colitis. *J Nutr* 2004; 134: 2251-2255.
32. Erichsen K, Ulvik RJ, Nysaeter G, *et al*. Oral ferrous fumarate or intravenous iron sucrose for patients with inflammatory bowel disease. *Scand J Gastroenterol* 2005; 40: 1058-1065.
33. Millar AD, Rampton DS, Blake RD. Effects of iron and iron chelation in vitro on mucosal oxidant activity in ulcerative colitis. *Aliment Pharmacol Ther* 2000; 14: 1163-1168.
34. Rahimi R, Nikfar S, Rahimi F, *et al*. A meta-analysis on the efficacy of probiotics for maintenance of remission and prevention of clinical and endoscopic relapse in Crohn's disease. *Dig Dis Sci* 2008; 53: 2524-2531.
35. Heczko PB, Strus M, Kochan P. Critical evaluation of probiotic activity of lactic acid bacteria and their effects. *J Physiol Pharmacol* 2006; 57: 5-12.
36. Mach T. Clinical usefulness of probiotics in inflammatory bowel diseases. *J Physiol Pharmacol* 2006; 57: 23-33.

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