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Synergic Activity of Selenium and Probiotic Bacterium *Enterococcus faecium* M-74 against Selected Mutagens in *Salmonella* Assay

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ABSTRACT. Concentrated extracts of MRS (De Man–Rogosa–Sharpe) media in which probiotic bacterium *Enterococcus faecium* strain M-74 was grown exerted different antimutagenic activity against ofloxacin-, *N*-methyl, *N´*-nitro-*N*-nitrosoguanidine- and sodium 5-nitro-2-furylacrylate-induced mutagenicity in *Salmonella typhimurium* assay depending on the presence (+Se) or absence of disodium selenite pentahydrate (–Se). The antimutagenicity of MRS(+Se) extract was higher than that of MRS(–Se) extract. Selenium enhanced also the antimutagenic effect of both live and killed cells of *E. faecium* M-74, respectively. The live bacteria decreased the mutagenicity of selected substances more than killed cells. Synergic activity of selenium with the bacterium was also manifested.

Selenium is an essential dietary trace element for animals, humans and microorganisms. Foodstuffs of animal and plant origin are the pool of organic forms of selenium (selenoproteins). If the level of Se in soil is very low or Se is absent, it has to be added in inorganic form (selenite, selenate) to the food to avoid Se deficiency which is associated with various chronic diseases including cancer. Several studies have suggested that low serum levels of Se and other micronutrients increase the risk of carcinoma (Chin-Thin *et al.* 2002; Lopez-Saez *et al.* 2003; Shukla *et al.* 2003). On the other hand, higher serum Se levels may be associated with the prevention of several cancers such as esophageal adenocarcinoma (Rudolph *et al.* 2003), prostate cancer (Klein *et al.* 2003; Vogt *et al.* 2003), nasopharyngeal carcinoma (Jian *et al.* 2003) and breast cancer (Ip *et al*. 1994). Generally, Se may be considered as an anticarcinogenic agent that, if present in higher concentrations in the plasma, may have a protective action against human malignancies (Ferenčík and Ebringer 2003; Wojtzak *et al.* 2003).

Probiotics have been defined as "microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host" (Salminen *et al.* 1999). Probiotics are mainly bacteria or yeasts, particularly lactic acid bacteria (LAB; *Lactobacillus* sp., *Bifidobacterium* sp., *Enterococcus* sp.). Consumption of sufficient amounts of selected LAB or other probiotic bacteria, and/or their fermentation products elicits positive health effects in humans and in animals (Hrubý *et al.* 1992; Olasupo 1998; Ashar and Prajapati 2000; Osuntoki *et al*. 2003; Vančíková *et al*. 2003). Tolerance to gastric acidity and bile toxicity (Kos *et al.* 2000; Šušković *et al.* 2000; Annuk *et al.* 2003), antioxidative activity (Kullisaar *et al.* 2002), production of antimicrobial compounds (Ouwehand 1998; Jacobsen *et al.* 1999; Lauková *et al.* 1999; Pantev *et al.* 2002*a*,*b*), ability to modulate immune responses (Kaila *et al.* 1992; Schiffrin *et al.* 1995) and adhesion to gut tissues (Saarela *et al.* 2000) have been shown for selected probiotic LAB.

Enterococci are normal inhabitants of the gut flora of humans (10^5–10^7 CFU per g of stool) and animals. It was reported that *Enterococcus faecium* strain SF68 effectively treats chronic hepatic encephalopathy (Loguercio *et al.* 1995) and acute diarrhea as well as antibiotic-associated diarrhea in humans (Wunderlich *et al.* 1989). The strains SF68 and 18C23 exert inhibitory effects against important enteropathogens (Lewenstein *et al.* 1979; Jin *et al.* 2000). *E. faecium* M-74 was shown to enhance nonspecific immunity *in vitro* and *in vivo* (Ebringer *et al.* 1995; Mikeš *et al.* 1995; Benyacoub *et al.* 2003). Rovenský *et al.* (2002) reported that a combination of methotrexate with *E. faecium* M-74 enriched with organic selenium significantly inhibits markers of both inflammation and arthritis in rats with adjuvant arthritis. It was demonstrated that milk fermented by *E. faecium* M-74 exhibited dose-dependent inhibition of selected mutagens and UV-irradiation in the *Salmonella* and *Euglena* assays (Belicová *et al.* 1999). *E. faecium* M-74 enriched by selenium, and MRS(+Se) extract after the cultivation of this strain possessed higher antimutagenic activity

against ofloxacin and acridine orange-induced genotoxicity than *E. faecium* M-74 and MRS extract without selenium in *Euglena* assay (Križková *et al.* 2002).

The aim of this study was to determine and compare (*i*) the antimutagenic activity of the MRS media extracts without disodium selenite pentahydrate (–Se) and MRS media with selenium (+Se) after cultivation of *E. faecium* M-74 against ofloxacin (OFL), *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) and 5-nitro-2-furylacrylate (NFA), (*ii*) the differences in the antimutagenic potential of live and killed cells of the probiotic bacterium *E. faecium* M-74, grown in both MRS(–Se) and MRS(+Se) media using *Salmonella* assay (*Salmonella enterica* subsp. *enterica*, serovar Typhimurium).

MATERIALS AND METHODS

Test strain. Enterococcus faecium M-74 was obtained from *Medipharm* (Hustopeče, Czechia). *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain TA98, TA100 (His^- mutants) and TA102 (with a preserved excision-repair system) were obtained from the *Czech Collection of Microorganisms* (Brno, Czechia).

Chemicals. A fresh solution of OFL was prepared by dissolving it in 0.1 mol/L NaOH and solutions of MNNG and NFA in Me_2SO immediately before use. *Lactobacillus* De Man–Rogosa–Sharpe broth (MRS) was purchased from *HiMedia* (India).

Preparation of concentrated media for antimutagenic tests. The strain M-74 was grown on MRS medium supplemented with disodium selenite pentahydrate (+Se) (16 mg/L) for 17 h at 37 ºC under static conditions. The cells were separated by centrifugation (5000 ***g***, 4 ºC), the supernatant was concentrated in a vacuum evaporator to one-tenth of its original volume at 40 ºC and used for testing its antimutagenicity in *S. typhimurium* assay. The same procedure was used for the non-Se-supplemented MRS medium.

Preparation of probiotic bacterial cells for mutagenic studies. After growth on MRS broth (in the presence or absence of Se; concentration 16 mg/L) for 17 h at 37 °C, the M-74 cells were harvested by centrifugation (5000 ***g***, 4 °C, 15 min; type K24, *Janetzki*, Germany). The cell pellets were washed twice with cold sterile phosphate-buffered saline (PBS), resuspended in the PBS and the absorbance at 600 nm was adjusted to 1.0 in order to standardize the cell concentration to 10–17/pL (*i.e.* $1.1–1.7 \times 10^{10}$ CFU/mL). The standardized bacterial cell suspensions were stored at 4 °C and used within 1 d.

Preparation of killed probiotic bacterial cell suspensions for mutagenic studies. The cell suspensions (with or without Se) with $A_{600} = 1.0$ were heat-treated in test tubes by immersing in a water bath for 15 min at 100 °C. After the treatment, the cells were vortexed for about 5 min to break any coagulum formed during heating and then plated on MRS agar for determination of heat treatment efficacy.

Inhibition of mutagenic activity by live and killed cells of probiotic bacteria. Five-mL aliquots of the probiotic bacterial suspensions (with or without Se) were placed in small sterile bottles. Freshly prepared solutions of each of the mutagens were added to reach final concentration (in mg/L: MNNG 100, NFA 50, OFL 1) and, after incubation, the mutagenic activity was measured in soluble fraction; a control sample was prepared for each mutagen in PBS without probiotic bacteria. Briefly, each suspension of mutagens with or without probiotic bacteria (control) was incubated with shaking for 3 h at 37 °C and centrifuged (5000 ***g***, 4 °C). The supernatants were decanted, filtered through a 0.45-μm filter paper (*Millipore*, Austria) and the filtrates were used for determination of remaining mutagenic activity using the *Salmonella* assay (bacterial cells–mutagen suspension according to Maron and Ames 1983). Before testing, the filtrates were kept at –20 ºC. All experiments were done in triplicate.

Determination of antimutagenicity of concentrated cultivation media and probiotic live and killed cells in the Salmonella *assay.* Quantitative *Salmonella* plate test for evaluation of antimutagenic activity of MRS(+Se) and MRS(–Se) extracts was done according to Maron and Ames (1983). MNNG (concentration of 10 μg per plate), NFA (5 μg per plate) or OFL (0.1 μg per plate) plus MRS(+Se) or MRS(–Se) extracts (0.1, 1, 10 μL per plate) were applied to *S. typhimurium* cells. The number of OFL-, NFA- and MNNG-induced His^+ revertants was counted. Antimutagenic potency (%) on *Salmonella* was calculated:

$$AP = (M - M_{MRS}/M) \times 100$$

where AP is per cent antimutagenic potency, M is the number of mutagen-induced His^+ revertants, and M_{MRS} is the number of mutagen plus MRS(+Se)-induced His^+ revertants.

Statistical analysis. The statistical significance of all the calculated values was determined by paired Student's *t*-test (p_t) and variance analysis ANOVA (*F*-test) (p_A). Values of $p < 0.05$ were considered to be statistically significant; results are means ± SD.

RESULTS AND DISCUSSION

In tests with all three *Salmonella* strains, MRS(+Se) medium possesses at all concentrations used a higher antimutagenic effect than MRS(–Se) medium (Table I). At the highest dose (10 μL) the antimutagenic potency of MRS(+Se) increased from 32 to 44 % in *S. typhimurium* TA98, from 24 to 55 % in *S. typhimurium* TA100, and from 28 to 38 % in *S. typhimurium* TA102. The MRS(–Se) medium decreased the frequency of mutagen-induced revertants in the range of 25–32 %. The results at all concentrations were statistically significant ($p_t < 0.05$; $p_A < 0.01$). Our results were similar to those of Ebringer *et al.* (1996) and Križková *et al*. (1996) in assays with *Euglena gracilis* (the presence of disodium selenite protected chloroplast DNA against the mutagenic effect of ofloxacin). Extracts of milk fermented by *E. faecium* M-74 and *Lactobacillus acidophilus* effectively inhibited the genotoxicity of MNNG in Ames test (Nadathur *et al.* 1995; Belicová *et al.* 1999). It appears that organic acids, butyric and acetic acids in particular, produced by probiotic bacteria contribute to their antimutagenic activity (Lankaputhra and Shah 1998).

Table I. Antimutagenic potency[a] of concentrated MRS(–Se) and MRS(+Se) media after cultivation[b] of *E. faecium* M-74 with NFA, MNNG and OFL

Concentration of media μL per plate	TA98[c]				TA100[d]				TA102[e]			
	–Se		+Se		–Se		+Se		–Se		+Se	
0 (control)	245 ± 65	–	–		1358 ± 756	–	–		567 ± 99	–	–	
0.1	257 ± 22	0	180 ± 15	27	1545 ± 910	0	1169 ± 533	14	604 ± 183	0	494 ± 155	12
1	221 ± 12	10	150 ± 28	39	1401 ± 517	0	848 ± 523	38	504 ± 211	11	461 ± 106	19
10	167 ± 39	32	138 ± 35	44	1038 ± 451	24	609 ± 497	55	410 ± 77	28	350 ± 20	38

[a]Decrease of NFA, MNNG and OFL genotoxicity in tests with *S. typhimurium* TA98, TA100 and TA102 (*left columns* – number of His^+ revertants per plate, means of triplicate plates ± SD for three consecutive experiments; *right columns* – decrease in %); controls (His^+ revertants without mutagen and without probiotic) – *S. typhimurium* TA98 36 ± 11, *S. typhimurium* TA100 181 ± 99, *S. typhimurium* TA102 344 ± 79.
[b]In the absence (–Se) or in the presence (+Se) of selenium (16 mg/L) with [c]NFA (5 μg per plate), [d]MNNG (10), [e]OFL (0.1); for further details *see Materials and Methods*.

Our results (Table II) also confirm that antimutagenicity of live cells of strain M-74 is higher than that of killed cells. Cells (live or killed) grown in media with Se showed a higher inhibitory activity than cells grown without Se. The highest inhibitory effect (58 %) was found in *S. typhimurium* TA98 with live M-74 cells grown in the media with Se ($p_t < 0.05$; $p_A < 0.01$).

Table II. Inhibition of NFA, MNNG and OFL genotoxicity in tests with *S. typhimurium* TA98, TA100 and TA102 by live or killed cells of *E. faecium* M-74 in the presence or absence of Se[a]

E. faecium	TA98				TA100				TA102			
	–Se		+Se		–Se		+Se		–Se		+Se	
None (control)[b]	281 ± 69	0	–		1590 ± 554	0	–		742 ± 116	0	–	
Live cells	46 ± 3	23	117 ± 29	58	1389 ± 489	13	671 ± 180	27	607 ± 73	18	336 ± 63	32
Killed cells	280 ± 11	0	120 ± 32	57	1600 ± 473	0	1530 ± 286	4	698 ± 59	6	396 ± 54	28

[a]For experimental conditions and abbreviations *see* footnotes to Table I.
[b]Without live and killed cells (no inhibition of genotoxicity); controls (His^+ revertants without mutagen and without probiotic) – *S. typhimurium* TA98 38 ± 11, *S. typhimurium* TA100 210 ± 23, *S. typhimurium* TA102 389 ± 38.

Lankaputhra and Shah (1998) have also documented that probiotics such as *Lactobacillus acidophilus* and *Bifidobacterium* sp., showed antimutagenic activity; their efficiency in inhibiting the selected mutagens was higher with live cells than with killed ones. Live bacterial cells bound and/or inhibited mutagens permanently whereas killed bacteria released mutagens upon extraction with Me_2SO; our results are in good correlation with the above observations. Moreover, live cells of *E. faecium* M-74 with Se demonstrated a higher antimutagenic potency than those without Se. Orrhage *et al.* (1994) suggested that the efficient and

strong binding of mutagens to microbial cells can be one of the mechanisms of antimutagenicity. Križková *et al.* (2002) reported that MRS media enriched with Se after *E. faecium* M-74 cultivation and the live bacterial cells grown in the presence of Se showed a significantly higher antimutagenic activity in *Euglena gracilis* assays. We demonstrated a similar effect in our testing system.

Foods rich in selenium (*e.g.*, broccoli and garlic) are reported to decrease the incidence of chemically induced colon cancer in a rat model (Lu *et al.* 1996; Finley 2003). Se from high-Se broccoli reduced the incidence of aberrant crypts by more than 50 %. The chemical form of Se in broccoli is similar to that in high-Se garlic. It supports the view that the proliferation of the transformed cells is suppressed by *Se*-methylselenocysteine (Lu *et al.* 1996).

Selenium status in the cells influences their growth and plays an important role in its chemo-preventive effect (Cases *et al.* 2001; Bansal and Kaur 2002). More beneficial Se status was achieved by increasing the Se concentration (organic or inorganic Se compounds), depending on the model used. It was demonstrated that inorganic selenite could be transformed into organic forms *via* binding to proteins, lipids, polysaccharides, and other cell components (Li *et al.* 2003). Hypothetically, cultivation of probiotic bacteria with Se might enhance the level of selenoproteins and thus increase their antimutagenic activity.

Here we demonstrated that the antimutagenic activity of *E. faecium* M-74 was enhanced by addition of disodium selenite into the cultivation medium. We assume that Se-enriched probiotic bacterium *E. faecium* M-74 can be considered as food supplement with beneficial health effects.

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