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Two antioxidative lactobacilli strains as promising probiotics

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Abstract

Two antioxidative strains tentatively identified as *Lactobacillus fermentum*, E-3 and E-18, were isolated from intestinal microflora of a healthy child. Survival time of these strains in the presence of reactive oxygen species (ROS), like hydrogen peroxide, superoxide anions and hydroxyl radicals, was significantly increased compared with a non-antioxidative strain, and also was quite similar to a highly ROS resistant strain of *Salmonella typhimurium*. E-3 and E-8 contain a remarkable level of glutathione, express Mn-SOD, which is important for the prevention of lipid peroxidation, and secrete hydrogen peroxide. Their significant antimicrobial activity combined with antioxidative properties may serve as defensive principles in the intestinal microbial ecosystem and overcome exo- and endogenous oxidative stress.

Keywords: *Lactobacillus fermentum*; Reactive oxygen species; Lipid peroxidation; Glutathione; Superoxide dismutase (SOD)

1. Introduction

The increasing interest in healthy diet is stimulating the innovative development of novel products by the food industry, and intestinal *Lactobacillus* species are particularly in focus (Vaughan et al., 1999). During the past decade, oxidative stress and antioxidative potency have been revealed as the key points in molecular regulation of cellular stress responses (Demple et al., 1999). Oxidative stress occurs when abnormally high levels of reactive oxygen species (ROS) are generated, resulting in DNA, protein and lipid damage. Both eukaryotic and aerobic prokaryotic organisms have developed an overall antioxidative defense system to mitigate the damaging effects of ROS. The important components of the cellular defense system are reduced glutathione (GSH) and antioxidative enzymes like superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT) (Frei et al., 1988; Halliwell et al., 1992; Vervaart and Knight, 1996; Miller and Britigan, 1997).

In some human microbiota, *Lactobacillus*, *Streptococcus* spp., lacking cytochromes and catalase, the flavoproteins (e.g. NADH-oxidase) make oxygen consumption possible, and give rise to an increase of the content of superoxide anions and hydrogen peroxide,

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which are highly toxic to several microorganisms and are considered to have an impact on bacterial antagonism in microbial ecology (Dahiya and Speck,1968; Eschenbach et al., 1989). Concerning *Lactobacillus* spp., it has been concluded that their antimicrobial effect is also expressed via ROS (hydrogen peroxide, superoxide and hydroxyl radicals) which may have a selective influence on the intestinal microbiota, similar to that described for oral streptococci (Garcia-Mendoza et al., 1993).

Lactobacillus spp. are important members of the healthy human microbiota (Mikelsaar et al., 1984; Lidbeck and Nord, 1993; Mikelsaar et al., 1998). Lactic acid bacteria and bifidobacteria are considered to have several beneficial physiological effects, such as antimicrobial activity, enhancing of immune potency and antitumorigenic activities (Fuller, 1991; Salminen et al., 1998). It has been shown that some lactobacilli possess antioxidative activity, and are able to decrease the risk of accumulation of ROS during the ingestion of food (Kaizu et al., 1993; Peuhkuri et al., 1996). Lactic acid bacteria are able to degrade the superoxide anion and hydrogen peroxide (Ahotupa et al., 1996; Korpela et al., 1997). However, the type of superoxide dismutase (SOD) expressed in antioxidative strains has not been assessed. It still remains open if the antioxidative potency of strains is associated with their survival in different environment.

In this study, we report that strains E-3 and E-18, tentatively identified as *Lactobacillus fermentum* with substantial antioxidative activity, express Mn-SOD (manganese superoxide dismutase) and have significantly increased resistance to several ROS, like hydrogen peroxide, superoxide and hydroxyl radicals.

2. Materials and methods

2.1. Strains of microorganisms

Strains of lactobacilli and *Salmonella typhimurium* were used in this study. Lactobacilli (E-3 and E-18) were isolated from the faecal sample of an Estonian child and E-338-1-1 was isolated from another healthy Estonian 1-year-old child (Sepp et al., 1997). Isolation of lactobacilli on de Man–Rogosa–Sharpe agar (MRS agar; CM 361, Oxoid Basingstoke, Hampshire, UK) was followed by the tentative identification to the species level by the API 50 CHL kit and API LAB Plus software, version 4.0 database (bioMerieux,

Table 1
Total antioxidative activity (according to TAS and LA-test), hydrogen peroxide content, glutathione redox ratio and activity of superoxide dismutase for *Lactobacillus fermentum* strains E-3, E-18 and E-338-1-1

	Lactobacillus fermentum E-3[a]	*Lactobacillus fermentum* E-18[b]	*Lactobacillus fermentum* E-338-1-1[c]
Intact cells			
LA-test (%)	29 ± 0.7 (n = 5)	21 ± 0.8 (n = 5)	0
TAA (mmol/l)	0.16 ± 0.03 (n = 5)	0.20 ± 0.03 (n = 4)	0
H_2O_2 (μg/ml)	31 ± 26 (n = 3)	34 ± 24 (n = 3)	49 ± 20 (n = 3)
Cell lysate			
LA-test (%)	59 ± 3.8 (n = 5)	51 ± 7.8 (n = 4)	0
H_2O_2 (μg/ml)	229 ± 37 (n = 4)	251 ± 58 (n = 4)	137 ± 25 (n = 3)
TGSH	12.5 ± 4.1	14.3 ± 4.2	5.5 ± 3.0
GSSG (μg/ml)	2.59 ± 2.01	4.98 ± 3.92	5.5 ± 2.4
GSH (μg/ml)	9.95 ± 3.30	9.32 ± 4.70	Traces
GSSG/GSH	0.28 ± 0.17	0.81 ± 0.70[d]	0[e]
SOD (U/mg protein)	0.859 ± 0.309 (n = 3)	0.761 ± 0.014 (n = 3)	n.e.

LA-test—linolenic acid test; TAA—total antioxidative activity; TGSH—total glutathione; GSSG—oxidized glutathione; GSH—reduced glutathione; SOD—superoxide dismutase; n.e.—not estimated; the data are expressed as the mean value ± SED.

[a] E-3—API 50 CHL identification level very good, %ID < 99.6, T index > 0.87.

[b] E-18—API 50 CHL identification level very good, %ID < 99.6, T index>0.87.

[c] E-338-1-1—identification level very good, %ID < 99.6, T index>0.87.

[d] $p < 0.05$ E-18 versus E-3.

[e] Cannot be calculated.

Marcy l'Etoile, France). The three identified *Lactobacillus fermentum* (Table 1) strains were included into the study of antioxidative properties. *S. typhimurium*, *Shigella sonnei*, *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis* were obtained from the culture collection of the National Reference Laboratory of Microbiology, Estonia.

2.2. Assay of resistance of lactobacilli to ROS

2.2.1. Resistance to hydrogen peroxide

Strains E-3, E-18 and E-338-1-1 were grown on MRS agar for 24 h. The overnight cultures of E-3, E-18, E-338-1-1 and *S. typhimurium* were suspended at the level of 10^7 CFU/ml in isotonic saline (sodium chloride, S-9625, Sigma) and incubated with 0.4 mM hydrogen peroxide (30 wt.% solution in water; Sigma-Aldrich Chemie, Steinheim, Germany) at 37 °C. At 30-min time intervals, the removed aliquots were plated onto MRS or Peptone Agar (Unipath Basingstoke, Hampshire, UK) plates and the number of viable cells was estimated by using the semi-quantitative method of Buchmeier et al. (1997). The incubation of the peptone agar plates for cultivation of *S. typhimurium* was performed at 37°C for 24 h, and the incubation of MRS agar for the cultivation of lactobacilli was performed in 10% CO_2 (CO_2 thermostat IG 150, Jouan, France) at 37 °C for 48 h.

2.2.2. Resistance to superoxide anions

Resistance of lactobacilli to superoxide anions induced by paraquat (1,1′-dimethyl-4,4′-bipyridinium, M-2244, Sigma) was tested using the diffusion assay procedure (Bauer et al., 1966). Overnight cultures of *L. fermentum* E-3, E-18 and *S. typhimurium* were harvested and suspended in saline (10^7 CFU/ml). 0.1 ml of particular cell suspension was spread onto MRS agar plate of 0.5% glucose agar plate, respectively, and allowed to dry. Ten microliters of 10 mM paraquat solution in sterile Millipore water was spotted on the paper disk in the center of the agar plate, which was incubated overnight at 37 °C. The zone of growth inhibition (mm) was measured.

2.2.3. Resistance to hydroxyl radicals

Strains E-3, E-18 and E-338-1-1 were grown on MRS agar for 24 h and suspended in isotonic saline at the level of 10^8 CFU/ml. For testing the survival of *L. fermentum* cells and *S. typhimurium* in the environment of ROS, the samples were exposed to hydroxyl radicals which were generated via the Fenton reaction (Barreto et al., 1995). Thus, the hydroxyl radicals generation was performed in the solution which contained 10 mM terephthalic acid (1,4-benzenedicarboxylic acid, T-7774, Sigma) in phosphate buffer (disodium phosphate, S-9763 Sigma), *L. fermentum* and *S. typhimurium* at the level of 10^8 CFU/ml, and 0.01 mM $CuSO_4 \times 5H_2O$ (C-7631 Sigma), whereas the reaction was started by adding 1 mM H_2O_2. At 15-min time intervals, the number of viable cells was estimated as CFU on MRS plates as indicated above. The experiments in the medium of 10 mM terephthalic acid in phosphate buffer or in 1 mM hydrogen peroxide served as the controls.

2.3. Antioxidative indices of L. Fermentum strains E-3 and E-18

Lactobacilli were grown in MRS broth (CM359, Oxoid Basingstoke) overnight and then harvested by centrifugation at 4 °C (1500 p/min) for 10 min, washed with isotonic saline (4 °C) and resuspended in 1.15% KCl (potassium chloride-P4504, Sigma). The density of suspension was adjusted to an OD_{600} of 1.1 (approximately 10^9 bacterial cells ml^{-1}). To get lysates, the cells were disrupted by sonification (B-12 Branson Sonic Power Company, Danbury, CT) 35 vibrations s^{-1} for 10 min in ice-bath and then for 10 min at -18 °C. The suspension was centrifuged at 4 °C 10,000 × *g* for 10 min (Hermle Labortecnik, Z252 Mk, Wehlengen, Germany) and supernatant was passed through Millipore filters (MILLEY-GS, nonpyrogenic, sterile 0.22 μm; Millipore, 67 Molsheim, France) to get cell-free extracts. The protein concentration was determined by using the method of Lowry et al. (1951).

2.4. Total antioxidative activity

The total antioxidative activity (TAA) of the bacteria was assessed by using the linolenic acid test (LA-test). This test evaluates the ability of the sample to inhibit linolenic acid (L-2376, Sigma) oxidation (Starkopf et al., 1995; Pähkla et al., 1998). The standard

of linolenic acid in 96% ethanol (1:100) was diluted in isotonic saline (1:125). To the 0.4 ml linolenic acid, diluted in isotonic saline, was added 0.01% sodium dodecyl sulphate (lauryl sulfate L-5750, Sigma) and the sample (0.045 ml of lactobacilli cell lysates or cells). The incubation was started by adding 0.1 mM $FeSO_4$ (F-7002, Sigma) and the mixture was incubated at 37 °C for 60 min. Then the reaction was interrupted by adding 0.25% butylated hydroxytoluene (B-1378, Sigma), the mixture was treated with 0.5 ml acetate buffer (pH 3.5) consisting of acetic acid glacial and sodium acetate trihydrate (A-6283 and S-8625, respectively, Sigma), and heated with freshly prepared 1% thiobarbituric acid solution (TBA) (T-5500, Sigma) at 80 °C for 40 min. After cooling, the mixture was acidified by adding 0.5 ml cold 5 M HCl, extracted with 1.7 ml cold 1-butanol (BT-105, Sigma) and centrifuged at $3000 \times g$ for 10 min and absorbance of butanol fraction was measured. The TAA of sample was expressed as the inhibition by sample of LA-standard peroxidation as follows: $[1 - (A534 (sample)/A534 (LA as control)] \times 100$. The higher numerical value (%) of TAA indicates the higher TAA of the sample. Peroxidation of LA-standard in the isotonic saline (without samples) served as a control.

The second method for assessing the total antioxidative activity was the TAS-method which uses the commercially available kit (TAS, Randox Laboratories Ardmore, UK). This method is based on the inhibition of the absorbance of the ferrylmyoglobin radicals of 2,2′ -azinobis-ethylbenzothiazoline 6-sulfonate (ABTS+) generated by the activation of metmyoglobin peroxidase with H_2O_2. The suppression of the absorbance of ABTS+ radicals by sample depends on the total antioxidative activity (TAA) of the sample under investigation (Rice-Evans and Miller 1994). The assay procedure was as follows. To the 1 ml chromogen (metmyoglobin) solution was added 0.02 ml of cell lysate in isotonic saline (blank was Millipore water) and standard (6-hydroxy-2,5,7,8-tetramethylchroman), mixed well and initial absorbance was read. Then 0.2 ml of substrate (hydrogen peroxide in stabilized form) was added, mixed, incubated at 37 °C and absorbance was read exactly after 3 min at 600 nm. TAA values are expressed as the trolox units (mmol/l).

2.5. Reduced and oxidized glutathione and glutathione redox status

To eliminate the proteins from the sample, 10% solution of metaphosphoric acid (M-5046, Sigma) was added to the equal volume of sample and mixed vigorously. This mixture was allowed to stand at room temperature for 5 min and centrifuged at $3000 \times g$ for 5 min. The supernatant was carefully collected and stored at -20 °C, if the assay was not performed immediately. For assay of oxidized glutathione (GSSG), reduced glutathione (GSH) was derivatized by adding 0.1 ml of 1 mM 2-vinylpyridine in ethanol (13,229-2 Sigma-Aldrich) mixing on a vortex mixer and keeping at room temperature for 1 h. To determine the content of GSSG (as well as the content of total glutathione), to the 0.1 ml of derivatized sample was added 0.005 ml of 4 M solution of triethanolamine in water, mixed immediately and added 0.5 ml of 0.2 M sodium phosphate buffer (pH 7.5) containing 0.01 M EDTA, 0.5 U glutathione reductase (G-4751, Sigma) and 0.3 mM NADPH (N-7505, Sigma). The enzymatic reaction was initiated by the addition of 0.1 ml of 1 mM 5,5′ -dithio-bis-2-nitrobenzoic acid (D-8130, Sigma) in 0.2 M sodium phosphate buffer (pH 7.5) containing 0.01 M EDTA (Griffith, 1980). The change in optical density was measured after 10 min at 412 nm on a spectrophotometer. The glutathione content was quantitated on the basis of a standard curve generated with known amounts of glutathione. The amount of GSH was calculated as the difference between the total glutathione and GSSG (total glutathione − GSSG = GSH). The glutathione content was expressed as microgram per milliliter of sample or as glutathione redox ratio (GSSG/GSH).

2.6. Hydroxyl radicals eliminating ability

The procedure was performed using terephthalic acid (THA) as a chemical dosimeter for hydroxyl radicals (Barreto et al., 1995). To the 2 ml of 10 mM THA solution in a sodium phosphate buffer (pH 7.5) was added 0.1 ml of *L. fermentum* cell lysate (10^9 microbial cells per milliliter of isotonic saline; pure isotonic saline was used as a control). Then, the hydroxyl radicals were generated via the Fenton reaction by adding 0.1 ml $CuSO_4 \times 5\ H_2O$ and 0.1

ml of hydrogen peroxide (the final concentration of the latter was 0.01 mM). The reaction product of THA with hydroxyl radicals (THA-adduct) was measured fluorometrically at 312 nm excitation and 426 nm emission. The data was expressed as the inhibition percent of generation of THA-adduct by sample.

2.7. Superoxide dismutase activity

The activity of superoxide dismutase (SOD) was measured by using commercially available reagents kit (RANSOD, Randox Laboratories, Ardmore, UK). This method employs xanthine and xanthine oxidase and 2-(4-iodophenyl)-3(4-nitrophenol)-5phenyltetrazolium chloride to form a red formazan dye. Inhibition of this reaction by sample indicates its superoxide dismutase activity. To the 0.05 ml of cell-free extract of lactobacilli was added 1.7 ml mixed substrate (RANSOD sample diluent was used as a reagent blank) incubated at 37 °C, and 250 ml xanthine oxidase was added, mixed and the first absorbance read after 30 s and the final absorbance after 3 min. The activity of SOD was expressed as U/mg protein.

2.8. Identification of the type of superoxide dismutase

An isoenzyme of SOD was detected by separating the cell-free extracts (30 μg of protein) on 10% non-denaturing polyacrylamide gels (PAAG, Sigma) and stained for SOD activity with nitroblue tetrazolium (NBT, N-6876, Sigma) (Beauchamp and Fridovich, 1971). To identify the type of SOD, duplicate gels were incubated with 15 mM H_2O_2, washed and treated with bovine catalase (50 U/ml) to remove any residual H_2O_2 before staining for activity with NBT (Chang and Hassan,1997).

2.9. Measurement of H_2O_2 content

Hydrogen peroxide concentration was measured by using the method of Ou and Wolff (1996). Briefly, 0.075 ml of the test sample was mixed with 1.425 ml of FOX 1 (D-sorbitol, S-1876 and xylenol orange, X-0127, Sigma) and ammonium ferrous sulfate hexahydrate (09719 Fluka, Sigma-Aldrich) and incubated at room temperature for 30 min. Flocculated protein and cells were removed by centrifugation and the absorbance was read at 560 nm. The concentration of hydrogen peroxide was found by using standard graph and was expressed as microgram of H_2O_2 per milliliter of the sample.

2.10. Antimicrobial activity

The antimicrobial activity of *L. fermentum* (E-3, E-18 and 338-1-1) against *S. sonnei*, *E. coli*, *S. aureus* and *E. faecalis* was assessed by using a streak line procedure on plates containing MRS medium without triammoniumcitrate and sodiumacetate (pH 7.2). Lactobacilli were cultivated in MRS substrate for 48 h at 37 °C in CO_2 environment and in an anaerobic glove chamber (Sheldon Manufacturing, Shel LAB, USA) with a gas mixture $CO_2/H_2/N_2$:5/5/90% and then inactivated by using chloroform gas for 2 h (Mikelsaar et al., 1987). The test-strains of *E. coli*, *S. sonnei*, *S. aureus* and *E. faecalis* were cultured at 37 °C for 18 h in peptone water (CM9 Unipath Basingstoke, Hampshire, UK) and seeded in duplicate perpendicular to the streak line of lactobacilli. After the incubation of plates for 18 h at 37 °C, the width of the clear zone, extending from the culture line of lactobacilli, was measured (Mikelsaar et al., 1987). The width of the clearing was expressed in millimeters and summarized for each strain tested by four different test-cultures. As a control, the growth of test-strains on modified MRS agar plate was estimated.

2.11. Statistical analysis

For comparison of the numbers of lactobacilli, in ROS resistant assays, the Mann–Whitney rank sum test was used. Calculations were performed using commercially available statistical software packages (Statistics for Windows, StatSoft and GraphPad PRISM Version 2.0). The values are given as mean and standard deviation of the mean. Statistically significant differences between groups were determined with Student's *t*-test. In all analyses, a value of $p < 0.05$ was considered statistically significant.

3. Results

Two strains of *L. fermentum*, E-3 and E-18, had significant antioxidative capacity established by using LA-test and TAS-test (Table 1). We also examined

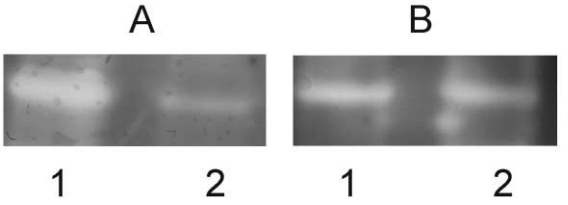

Fig. 1. Effect of hydrogen peroxide (known to inactivate irreversibly Fe-SOD but not inactivate Mn-SOD) on the activity of SOD of *Lactobacillus fermentum* E-3 and E-18 cell free extracts. To identify the type of SOD, duplicate gels were incubated with 15 mM H_2O_2, washed and treated with bovine catalase (50 U/ml) to remove any residual H_2O_2 before staining for activity with NBT (nitroblue tetrazolium). The cell free extracts were prepared and electrophoresed (45 pg of protein/lane) on 10% polyacrylamide gels in duplicate. (A) Without treatment, (B) treatment with 15 mM H_2O_2. *L. fermentum* E-3 (line 1), *L. fermentum* E-18 (line 2).

several parameters of these antioxidative strains (E-3 and E-18) including their survival in the presence of ROS in comparison with the survival of non-antioxidative strain *L. fermentum* E-338-1-1 (Table 1 and Fig. 3) and with a highly ROS resistant strain of *S. typhimurium*.

3.1. Activity of superoxide dismutase and its type

The lysates of E-3 and E-18 were shown to have superoxide dismutase activity (0.859 ± 0.309 and 0.761 ± 0.014 U mg/protein, respectively, Table 1).

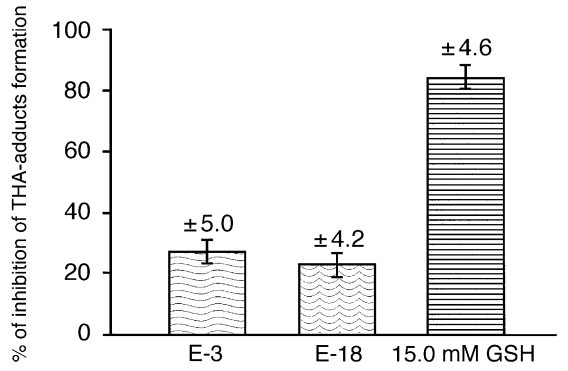

Fig. 2. Influence of *Lactobacillus fermentum* strains E-3, E-18 and 15 mM glutathione on the formation of THA-adduct. The data are expressed as the mean value (± SED) of five separate experiments for E-3 and E-18, respectively, and of four experiments for glutathione. The influence of *L. fermentum* E-338-1-1 on the formation of THA-adduct was about 10–15% (data not shown). (THA = terephthalic acid, GSH = reduced glutathione.)

Concerning the type of SOD, we established (Fig. 1) that a single band on the zymogram revealed the activity of SOD whereas such activity was not inhibited by hydrogen peroxide. The latter information indicates that the antioxidative strains E-3 and E-18 are able to express Mn-SOD (Fig. 1), an antioxidative enzyme.

3.2. Hydroxyl radicals eliminating ability

The lysates of E-3 and E-18 expressed remarkable ability to eliminate hydroxyl radicals as they diminished the formation of THA-adducts for $27 \pm 5\%$ and $23 \pm 4.2\%$, respectively (Fig. 2). Such effects are quite significant as reduced glutathione (at 15 mM), known as a potent scavenger of hydroxyl radicals, decreased hydroxyl radicals production under the same conditions for $84 \pm 4.6\%$ (Fig. 2). In addition, our non-antioxidative strain E-338-1-1 diminished the formation of THA-adducts by 10–15% only (data not shown).

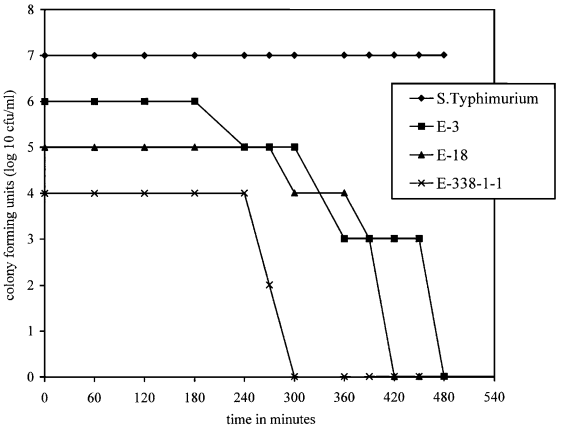

Fig. 3. Survival in the presence of hydrogen peroxide. The overnight cultures of E-3, E-18, E-338-1-1 and *Salmonella typhimurium* were suspended at the level of 10^7 CFU/ml in isotonic saline and incubated with 0.400 mM hydrogen peroxide. At 30-s time intervals, the number of viable cells was estimated by the semi-quantitative method by plating the removed aliquots onto the MRS- and Peptone Agar plates. The incubation of the Peptone Agar plates was performed at 37°C for 24 h, and that of MRS Agar in the 10% CO_2 environment for 48 h. The number of experiments was four. As shown, E-3, E-18 and E-338-1-1 were viable after 450, 390 and 240 min, respectively. In comparison, mutant *S. typhimurium* could survive only for 180 min (Buchmeier et al., 1997) and the wild-type of *S. typhimurium* was resistant to 0.4 mM hydrogen peroxide killing (data not shown).

3.3. Resistance to hydrogen peroxide and hydroxyl radicals

We tested the survival of *Lactobacillus* cells in the presence of 0.4 mM hydrogen peroxide. As shown in Fig. 3, the strains E-3 and E-18 were viable even after 450 and 390 min but the non-antioxidative strain E-338-1-1 was viable only for 240 min. In the environment of 1 mM hydrogen peroxide, the cells of *L. fermentum* E-3 and E-18 were viable for 180 and 150 min, respectively, and E-338-1-1 was viable for 90 min (result not shown).

In the presence of the hydroxyl radicals, the antioxidative lactobacilli strains survived for 34 min, the non-antioxidative strain of lactobacilli (E-338-1-1) survived for 15 min and a highly ROS resistant *S. typhimurium* survived for 56 min (result not shown).

3.4. Resistance to superoxide anions

Superoxide anions were generated by using paraquat. Paraquat is readily reduced to stable, but oxygen-sensitive, cation radicals. The paraquat radicals react with O_2 at the rate of 7.7×10^8 mol^{-1} s^{-1} at 25 °C, and by this, generate superoxide radicals (Buchmeier et al., 1997). Superoxide-dependent growth inhibition was not established in the case of *L. fermentum* E-3, E-18, E338-1-1 and *S. typhimurium* (data not shown). It means that they are resistant to superoxide anions.

3.5. Glutathione content and glutathione redox ratio (GSSG/GSH)

The levels of reduced glutathione (GSH) were 9.95 ± 3.3 and 9.32 ± 4.7 μg/ml for E-3 and E-18, respectively, but the content of GSH in *L. fermentum* E-338-1-1 was not detectable (Table 1). At the same time, the content of oxidized glutathione (GSSG), as well as the glutathione redox ratio for these strains were different (Table 1).

3.6. Content of hydrogen peroxide

The hydrogen peroxide amount in whole cell suspensions was 31, 34 and 49 and in the cell lysates 229, 251 and 137 μg/ml for E-3, E-18, and E-338-1-1, respectively (Table 1).

3.7. Antimicrobial activity of lactobacilli

We found that all three *L. fermentum* strains had a remarkable antagonistic activity against *E. coli*, *S. aureus*, *E. faecalis* and *S. sonnei* (data not shown). The antimicrobial activity of the above-mentioned strains estimated by the streak line procedure was similar to each other and independent of growth conditions: microaerophilic and anaerobic environment. Also, no significant difference between the growth inhibition zone values of G-negative and G-positive bacteria was found.

4. Discussion

Two antioxidative strains of *L. fermentum* were isolated from the intestinal microflora of a healthy child. In this study, we examined the resistance of highly antioxidative strains of *L. fermentum*, named as E-3 and E-18, to the different unhealthy milieu of ROS. We compared the survival of E-3, E-18 both with the non-antioxidative strain of *L. fermentum* E-338-1-1 and with *S. typhimurium*.

Our major findings were as follows: the strains E-3 and E-18 (compared with the non-antioxidative strain E-338-1-1) survived longer in 0.4 mM hydrogen peroxide milieu, possessed the ability to multiply in a medium containing abundant superoxide radicals, and had increased resistance to hydroxyl radicals. The latter phenomenon was proportional to the concentration of hydrogen peroxide (i.e. the higher concentration of hydrogen peroxide the lower the survival). Although the resistance of the antioxidative strains was not comparable with the resistance of *S. typhimurium* (the latter was resistant to 0.4 mM hydrogen peroxide killing even after 48 h and resistant to 1.0 mM hydrogen peroxide after 24 h, data not shown), the antioxidative strains have significantly increased resistance to harsh media compared with the non-antioxidative strain.

In the presence of highly damaging hydroxyl radicals, our antioxidative lactobacilli survived for 34 min. It is a quite impressive fact considering the data that a highly ROS resistant *S. typhimurium* was able to survive in the presence of hydroxyl radicals only two times longer than our antioxidative lactobacilli strains. It is known that the *S. typhimurium* gene

Sly A is responsible both for virulence and survival in a highly oxidative environment of macrophages (Buchmeier et al., 1997). Regarding the latter, it is interesting to underline that E-3 and E-18 could survive in a similar environment twice as long as *S. typhimurium* SlyA negative mutant if we compare our data and literature data (Buchmeier et al., 1997).

Evidently, the high total antioxidative activity (TAA) of our antioxidative strains is one of the reasons for their increased resistance to ROS. Kaizu et al. (1993) found several other strains of different species of lactobacilli with a relatively high antioxidative ability but at a remarkably lower iron concentration compared with our experiments. Lin and Chang (2000) also found that some intestinal lactic acid bacteria, inhibiting linoleic acid oxidation, revealed significant antioxidative activity (in the case of *L. acidophilus* ATCC 4356 an antioxidative capacity was 28–45%). Our lactobacilli strains are microaerophiles and grow both in the anaerobic and aerobic CO_2 enriched milieu. The aerobic growth conditions are very important as one of the purposes was to establish the mechanisms by which our lactobacilli possess ROS eliminating effects. If there is more oxygen in a medium, there will be more oxygen free radicals generated and the antioxidative activity of the lactobacilli can be evaluated more precisely.

Our data indicate that E-3 and E-18 might have some potent mechanisms to eliminate/diminish the effects of ROS. Ahotupa et al. (1996) have shown that *Lactobacillus* GG (*L. rhamnosus*) inhibits lipid peroxidation in vitro due to iron chelation and superoxide anion scavenging ability. Our strains E-3 and E-18 possess remarkable TAA but lack the ability to bind iron (data not shown). The role of SOD is based on the understanding that one of the mechanisms of ROS inactivation in microorganisms involves the expression of SOD. SOD eliminates direct toxicity of superoxide anions and prevents the superoxide anions mediated reduction of iron followed by hydroxyl radicals generation. It is shown that Mn-SOD becomes the predominant form (overcomes the Fe-SOD) when the cells of *E. coli* are exposed to oxidative stress (Hassan and Fridovich, 1978; Touati, 1989; Miller and Britigan, 1997). Our data (including the lack of inhibitory effect of hydrogen peroxide) indicate that E-3 and E-18 express only Mn-SOD. Evidently, this enzyme is located in the cytoplasm as its activity was detectable only in cell lysates.

However, the expression of SOD only cannot guarantee the antioxidative ability of strains E-3 and E-18. The involvement of catalase is excluded as lactic acid bacteria are not able to synthesize the catalase due to the lack of heme. At the same time, it is known that hydrogen peroxide is an important component of the oxidative burst of host macrophages. It will react with iron or copper ions to generate the toxic hydroxyl radicals via the Fenton reaction (Buchmeier et al., 1997). Our study is the first to show that the hydroxyl radical eliminating ability of E-3 and E-18 lysates is comparable to the same effect of GSH (Fig. 2), which is known as an important cellular scavenger of hydroxyl radicals. The possible role of GSH is supported by the fact that E-338-1-1, which has only traces of GSH, possessed about half the hydroxyl radical scavenging ability in comparison with E-3 and E-18. However, the existence of other ROS scavenging thiol-compounds in lactobacilli is not excluded. Such understanding is supported both by data that thioglycollate, cysteine, dithiotreitol, etc., stabilize the multiplication of *L. delbrueckii* due to their low redox potential (Rees and Pirt, 1979) and by data that the glutathione redox status of E-3 and E-18 was different but their TAA was similar (Table 1).

Recently, it has been suggested that among several antimicrobial compounds different lactic acid bacteria can also produce hydrogen peroxide (Owehand,1998). The protective role of vaginal lactobacilli against bacterial vaginosis has been attributed to hydrogen peroxide together with various other antimicrobials (Coconnier et al., 1997). However, the bactericidal activity, based only on hydrogen peroxide, is questionable (Nagy et al., 1991; Fontaine et al., 1996). The secreted hydrogen peroxide concentration of *L. fermentum* E-3 and E-18 cells was relatively low (31–34 μg/ml). The findings are in accordance with Fontaine et al. (1996), who found that the *L. fermentum* cells from the vagina have practically the same hydrogen peroxide concentration. This information is quite consistent with the metabolic activity of lactobacilli. In the presence of oxygen, lactic acid bacteria are able to generate more hydrogen peroxide via the action of flavoprotein-containing oxidases, NADH oxidases and SOD than in anaerobic conditions (Miller and Britigan, 1997; Kemble et al., 1997). The compara-

tively low released amounts of hydrogen peroxide could not be responsible for the good antimicrobial activity of heterofermentative lactobacilli strains. For example, E-338-1-1 released more hydrogen peroxide (Table 1) but its antimicrobial activity was similar to other *L. fermentum* strains. It means that some additional mechanisms, such as the production of lysozyme, ethanol, succinic acid, or other compounds characteristic of *L. fermentum* strains may be involved in antimicrobial activity.

Thus, the significant antioxidative activity is the basis for the increased resistance of some lactobacilli strains to toxic oxidative compounds and helps some isolates of *Lactobacillus* spp. to serve as defensive components in intestinal microbial ecosystem. Such antioxidative strains, with desirable properties, may be a promising material for both applied microbiology and scientific food industry, considering the fact that human microbiota have to be tolerant to endogenous and exogenous oxidative stress.

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