Appl Microbiol Biotechnol (2013) 97:809–817
DOI 10.1007/s00253-012-4241-7

Antioxidant properties of potentially probiotic bacteria: in vitro and in vivo activities

Alberto Amaretti · Mattia di Nunzio · Anna Pompei · Stefano Raimondi · Maddalena Rossi · Alessandra Bordoni

Received: 7 May 2012 / Revised: 11 June 2012 / Accepted: 13 June 2012 / Published online: 12 July 2012

Abstract Thirty-four strains of lactic acid bacteria (seven *Bifidobacterium*, 11 *Lactobacillus*, six *Lactococcus*, and 10 *Streptococcus thermophilus*) were assayed in vitro for antioxidant activity against ascorbic and linolenic acid oxidation (TAA_{AA} and TAA_{LA}), trolox-equivalent antioxidant capacity (TEAC), intracellular glutathione (TGSH), and superoxide dismutase (SOD). Wide dispersion of each of TAA_{AA}, TAA_{LA}, TEAC, TGSH, and SOD occurred within bacterial groups, indicating that antioxidative properties are strain specific. The strains *Bifidobacterium animalis* subsp. *lactis* DSMZ 23032, *Lactobacillus acidophilus* DSMZ 23033, and *Lactobacillus brevis* DSMZ 23034 exhibited among the highest TAA_{AA}, TAA_{LA}, TEAC, and TGSH values within the lactobacilli and bifidobacteria. These strains were used to prepare a potentially antioxidative probiotic formulation, which was administered to rats at the dose of 10^7, 10^8, and 10^9 cfu/day for 18 days. The probiotic strains colonized the colon of the rats during the trial and promoted intestinal saccharolytic metabolism. The analysis of plasma antioxidant activity, reactive oxygen molecules level, and glutathione concentration, revealed that, when administered at doses of at least 10^8 cfu/day, the antioxidant mixture effectively reduced doxorubicin-induced oxidative stress. Probiotic strains which are capable to limit excessive amounts of reactive radicals in vivo may contribute to prevent and control several diseases associated with oxidative stress.

Keywords *Bifidobacterium* · *Lactobacillus* · Probiotic · Antioxidant · In vivo · In vitro

A. Amaretti · S. Raimondi · M. Rossi (✉)
Department of Chemistry,
University of Modena and Reggio Emilia,
via Campi 183,
41100 Modena, Italy
e-mail: rossi.maddalena@unimore.it

M. di Nunzio · A. Bordoni
Department of Food Sciences, University of Bologna,
piazza Goidanich 60,
47023 Cesena, FC, Italy

A. Pompei
Department of Pharmaceutical Sciences, University of Bologna,
via Belmeloro 6,
40100 Bologna, Italy

Introduction

Reactive oxygen metabolites (ROM), generated through normal reactions within the body during respiration in aerobic organisms, can cause damage in proteins, mutations in DNA, oxidation of membrane phospholipids, and modification in low-density lipoproteins. Excessive amount of ROM can result in cellular damage which, in turn, promotes chronic diseases including atherosclerosis, arthritis, diabetes, neurodegenerative diseases, cardiovascular diseases, and cancer (Babbs 1990; Firuzi et al. 2011). To neutralize the oxidant molecules, the human body synthesizes antioxidant enzymes and molecules that, together with the antioxidants contained in food, form the biological antioxidant barrier. However, in certain circumstances, the defense system fails to protect the body against oxidative stress; consequently, the possibility of increasing antioxidant defenses is considered important in the maintenance of human health and disease's prevention (Serafini and Del Rio 2004).

In this direction, a novel pioneering approach is represented by the development of probiotics exerting antioxidant activity and counteracting the oxidative stress in the host. Probiotics are defined as "live microbes which, when administered in adequate amounts, confer a health benefit to the host" (FAO/WHO working group 2001). In particular, besides the long history of consumption of lactic acid

bacteria, probiotic strains belonging to the genera *Lactobacillus* and *Bifidobacterium* have been reported to have a range of health-promoting features (Rossi and Amaretti 2010). Although the molecular mechanisms of probiotics have not been completely elucidated yet, their modulation of the intestinal microbiota, antibacterial substance production, improvement of the epithelial barrier function, and reduction of the intestinal inflammation are already well established. At first, probiotics were consumed to modulate and improve the gut microbiota balance; nowadays, properly selected probiotics strains have developed, in order to respond to specific physiological targets (Pompei et al. 2007a, b), to prevent and treat pathogen-induced diarrhea (Guandalini 2011), or to manage autoimmune and atopic diseases (Finch et al. 2010).

Among probiotics beneficial effects, some authors have reported the protection against oxidative stress and the capability to decrease the risk of accumulation of ROM (Martarelli et al. 2011; Kaizu et al. 1993; Kullisar et al. 2002). Studies have shown that selected strains of lactobacilli and bifidobacteria present antioxidative properties (Shimamura et al. 1992; Lin and Yen 1999; Kullisaar et al. 2002; Wang et al. 2006; Zanoni et al. 2008; Mikelsaar and Zilmer 2009; Uskova and Kravchenko 2009; Spyropoulos et al. 2011) and can be used to prepare probiotic and fermented dairy products that improve total antioxidant status and decrease markers of oxidative stress in healthy people (Naruszewicz et al. 2002; Songisepp et al. 2005; Virtanen et al. 2007).

The antioxidant mechanisms of probiotics could be assigned to ROS scavenging, metal ion chelation, enzyme inhibition, and to the reduction activity and inhibition of ascorbate autoxidation (Lin and Yen 1999; Talwalkar and Kailasapathy 2003). Probiotic metabolic activities may have an antioxidant effect via the scavenging of oxidant compounds or the prevention of their generation in the intestine (Azcárate-Peril et al. 2011).

However, although the ability of probiotic bacteria to exert antioxidant activity has attracted some attention; so far, no rigorous comparative studies of this feature have been published. The present study wants to fill this gap by exploring in vitro the antioxidant properties of 34 lactic acid bacteria, then assessing the effect of the administration of a selected mix of potentially probiotic bacteria in Wistar rats subjected to an exogenous oxidative stress induced by doxorubicin.

Materials and methods

Materials

All the chemicals were supplied by Sigma (Stenheim, Germany), unless otherwise stated. Bacterial strains were obtained from ATCC (Manassas, VA, USA), DSMZ (Braunschweig, Germany), and our own collection (formerly the department of Chemistry, University of Modena and Reggio Emilia, Modena, Italy). They were seven strains of the genus *Bifidobacterium*, 11 of the genus *Lactobacillus*, six of the genus *Lactococcus*, and 10 of the species *S. thermophilus* (Table 1). Bifidobacteria and lactobacilli were cultured in Lactobacilli MRS broth (BD Difco, Sparks, MD, USA) containing 0.5 g/L L-cysteine · HCl, and were anaerobically incubated at 37 °C for 24 h. The strains of *S. thermophilus* and *Lactococcus* were cultured aerobically at 42 °C for 24 h in M17 broth (BD Difco).

Preparation of bacterial suspensions and cell-free extracts for antioxidant assays

Bacterial samples were prepared as follows. The biomass from 24-h cultures was harvested by centrifugation (6,000×g for 10 min at 4 °C), washed three times and suspended in a buffer solution, as appropriate for the specific assay. Phosphate-buffered saline (0.15 M NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4) was used in TAA_{AA}, TEAC, and TGSH assays; 11.5 g/L KCl was used in TAA_{LA} assay; 0.1 mM EDTA in 50 mM potassium phosphate buffer (pH 7.8) was used in SOD assay. Cell-free extracts for TEAC, TGSH, and SOD analysis were obtained from cell suspensions containing approximately 3×10^9 cfu/ml, that were subjected ultrasonic disruption (five 1-min strokes at 0 °C, with 5-min intervals) and centrifuged (10,000×*g* for 5 min at 4 °C) to remove the cells debries. Protein concentration of cell-free extracts was quantified according to Lowry et al. (1951). For TGSH analysis, the cell-free extracts were treated with 50 g/L metaphosphoric acid at 4 °C for 15 min to precipitate proteins and remove interference with the assay.

Antioxidant activity in bacterial cells

The antioxidant properties of bacterial samples (intact cells and cell free-extracts) were determined by measuring the inhibition of linolenic acid peroxidation (TAA_{LA}) and ascorbate autoxidation (TAA_{AA}), the TEAC, and the cellular content of total glutathione (TGSH, oxidized plus reduced form), and SOD.

TAA_{LA} was evaluated by measuring the antioxidant activity of cell suspensions against linolenic acid peroxidation and was referred to as the percentage inhibition of color formation (λ=534 nm) arising from thiobarbituric acid assay (Zanoni et al. 2008; Kullisaar et al. 2002). The TAA_{AA} of cell suspensions was referred to as the percentage inhibition against spontaneous ascorbate oxidation, which was evaluated through the measurement of UV absorbance at 265 nm (Mishra and Kovachich 1984; Zanoni et al. 2008).

Table 1 TAA_{LA}, TAA_{AA}, TEAC, TGSH, and SOD of bacterial strains of *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, and *S. thermophilus*

Strains	TAA_{LA} (%)	TAA_{AA} (%)	TEAC (μM)	TGSH (nmol/mg)	SOD (U/mg)
Bifidobacterium					
B. adolescentis DSMZ 18351	15 b	34 a	20 b	0 a	
B. pseudocatenulatum WC 0402	20 a	20	34 a	0 a	
B. pseudocatenulatum WC 0408	5 c	0.3 b	30 a	0 a	
B. breve WC 0421	22 a	12	19 b	5.8	
B. breve WC 0424	8 c	28 a	24 b	0 a	
B. animalis subsp. *lactis* DSMZ 23032	15 b	33 a	63	30.3	
B. longum subsp. *longum* ATCC 15708	2 c	0.4 b	0	0[a]	
Lactobacillus					
L. acidophilus ATCC 4356	25 b	82	54 a	0.2 b	
L. amylovorus DSMZ 20552	15 c	1	25 c	0.1 b	
L. acidophilus WC 0203	25 b	13 c	7 d	0.9 a	
L. acidophilus DSMZ 23033	27 b	20 b	28 c	4.5	
L. brevis DSMZ 23034	34 a	16 b,c	125	0 b	
L. coryniformis ATCC 25600	24 b	38 a	40 b	0 b	
L. helveticus S 40.8	27 b	52	41 b	0 b	
L. plantarum LN 3	28 b	15 c	10 d	1.3 a	
L. plantarum LP 1	33 a	16 b,c	16	2.7	
L. plantarum MB 395	14 c	5	0	0 b	
L. reuteri ATCC 23272	35 a	34 a	51 a	1.9	
Lactococcus					
L. lactis MB 445	33	12 a	56	1.6 a	2.9 b
L. lactis MB 446	26[a]	13 a	86 a	8.0	0.8
L. lactis ssp. *cremoris* ATCC 19257	21 a	35	9 b	26.3	4.1 a
L. lactis ssp. *cremoris* MB 461	2	12 a	87 a	2.4 a	0.2
L. lactis ssp. *diacetilactis* MB 447	23 a	12 a	71	10.5	3.6 a,b
L. lactis ssp. *lactis* ATCC 19435	24 a	25	6 b	4.2	2.8 b
Streptococcus					
S. thermophilus ATCC 19258	34 a	45	15 a	0.1 a	0.5 b
S. thermophilus B 1	10 b	2 c,d	0 b	4.2	0.7 b
S. thermophilus B 2	4 c	0.2 d	0 b	0.1 a	0.2 b
S. thermophilus MB 410	32 a	20 a	17 a	10.3	1.8 a
S. thermophilus MB 417	37 a	19 a,b	39	0.2 a	2 a
S. thermophilus MB 418	35 a	14 b	95	0.1 a	0.7 b
S. thermophilus MB 450	8 b	15 b	0 b	0.3 a	0.2 b
S. thermophilus Z 1	2 c	0.2 d	16 a	0.2 a	0.3 b
S. thermophilus Z 4	6 b,c	4 c	14 a	5.6	0.2 b
S. thermophilus EI-16, MB 426	34 a	26	0 b	19.4	0.4 b

Values are means, $n=3$. TAA_{LA} and TAA_{AA}, SD always <3 %; TEAC, SD always <5 μM; TGSH, SD always <2.5 nmol/mg; SOD, SD always <0.5 U/mg

Within each microbial group, means in a column without a common letter significantly differ, $P<0.05$

To evaluate the TEAC of cell-free extracts, the inhibition of the chemiluminescent reaction between luminol and H_2O_2 was determined and compared to standard Trolox solutions (Girotti et al. 2004; Zanoni et al. 2008).

Glutathione was analyzed in deproteinized bacterial cell-free extracts using a commercial kit (HT Glutathione, Trevigen, Gaithersburg, MD, USA). The kit exploits the reaction between reduced glutathione and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) giving a colored compound (5-thio-2-nitrobenzoic acid, TNB) measured at 405 nm. The reaction was carried out in presence of glutathione reductase and NADPH to determine total glutathione (TGSH, oxidized plus reduced glutathione).

Finally, to estimate SOD activity of cell-free extracts, the inhibition of epinephrine autoxidation was measured (Misra and Fridovich 1972) and compared with 0 to 10 $U ml^{-1}$ standard solutions of bovine SOD (Sigma-Aldrich). Specific activity was expressed as enzymatic

units per milligram of proteins, one unit being defined as the amount of SOD which inhibits the reduction of nitro blue tetrazolium by 50 % (Beuchamp and Fridovich 1971; Dellomonaco et al. 2007).

In vivo animal trial

The Animal Care Committee of the University of Bologna approved the study (Prot. 20600-X/10). The experiment was carried out with 42 male Wistar rats (90 days old; body weight, 250–300 g), individually housed in cages at 20±2 °C and 60–70 % humidity, with a 12/12 h light/dark cycle. Throughout the trial, rats were fed a standard diet (Mucedola, Milano, Italy), and had free access to water and food. The animals were weighted weekly and food consumption was daily recorded. After a 7-day period of acclimatization, rats were randomly divided into four groups, three of them receiving daily via gastric gavage a 6-ml water suspension containing different concentration of a mixture of the potentially probiotic strains *B. animalis* subsp. *lactis* DSMZ 23032, *Lactobacillus acidophilus* DSMZ 23033, and *L. brevis* DSMZ 23034.

The lyophilized probiotic formulation (Probiotical Ltd, Novara, Italy), which contained equal amounts of the viable counts of each strain and potato maltodextrin as excipient, was reconstituted (10 % *w*/*v*) with water and diluted as appropriate to achieve the following doses. The groups $Pro10^7$ (n=7), $Pro10^8$ (n=14), and $Pro10^9$ (n=7) received 10^7, 10^8, and 10^9 cfu/day, respectively; the fourth group (NS, n=14) received 6 ml of water without any probiotic supplement, as a negative control.

After 18 days of probiotic supplementation, 20 mg doxorubicin (DOXO) per kilogram b.w. were intraperitoneally administered to all $Pro10^7$ and $Pro10^9$ rats, and to seven out of 14 of $Pro10^8$ and NS rats. Besides, the other rats from $Pro10^8$ and NS groups received intraperitoneally a similar volume of physiological solution. Twenty-four hours later, after a fasting period of 3 h, animals anesthetized and sacrificed. Blood was collected using sodium heparin as anticoagulant, plasma was obtained after centrifugation at 2,000×*g* at 4 °C for 15 min, and immediately frozen until analyses.

The concentrations of bifidobacteria, lactobacilli, enterococci, and coliforms in rat feces were monitored at the beginning and after the 18-day probiotic supplementation.

Assays to determine the antioxidant status in vivo

The plasma concentration of ROMs was evaluated spectrophotometrically using the d-ROM test (Diacron, Grosseto, Italy) as reported (Bordoni et al. 2008). The assay is based on the ability of reaction mixture to generate alkoxyl and peroxyl radicals from hydroperoxides by the Fenton's reaction. The reaction produces free radicals that, trapped by an alkylamine, form a colored compound detectable at 505 nm. Results were expressed as milligram of H_2O_2/dl of plasma.

TAA was measured in rat plasma with the method of Re et al. (1999), based on the ability of the antioxidant molecules in the sample to reduce the radical cation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$). TAA was determined in 10 μl plasma by evaluating the decoloration of $ABTS^{+}$, measured as the quenching of the absorbance at 734 nm. Values obtained were compared to the concentration–response curve of the standard Trolox solution and expressed as millimole TEAC.

Plasma reduced GSH concentration was determined as previously described by Di Nunzio et al. (2011) with slight modification. Of plasma, 100 μl were incubated for 30 min in 100 μl reagent buffer (80 mM sodium phosphate, pH 8.0; 2 mM EDTA; 2 % SDS; 250 μM DTNB), and GSH was measured spectrophotometrically by reading at 415 nm the absorbance of TNB. Results were compared to the concentration–response curve of standard GSH solutions and expressed as microgram of GSH/ml plasma.

Analysis of fecal pH and microbiota

The pH of feces was measured with a pH meter, following a 1:10 (*w*/*v*) dilution with distilled water (Pompei et al. 2007a, b). Intestinal bacterial groups were enumerated using specific fluorescence in situ hybridization commercial kits (Microscreen, Groningen, The Netherlands) for the *Lactobacillus* group (*Lactobacillus* 10-ME-H006), the *Bifidobacterium* genus (*Bifidobacterium* 10-ME-H001), the *Escherichia coli* group (*E. coli* 10-ME-H004) and *Enterococcus faecalis* group (*E. faecalis* 10-ME-H015). Depending on the number of fluorescent cells, 30–100 microscopic fields were counted and averaged. All analysis was carried out in triplicate.

Statistical analysis

All values are expressed as means±SD. Differences in TAA_{AA}, TAA_{LA}, TEAC, TGSH, and SOD among the bacterial groups and differences in ROMs, GSH, and TAA among the dietary treatments were evaluated using the one-way ANOVA followed by Tukey's post hoc comparisons. Differences in pH and bacterial concentration in feces were analyzed using two-way ANOVA with repeated measures with diet as the first factor and time as the second factor, followed by Bonferroni post hoc comparisons. Differences were considered significant at $P<0.05$. Calculations were performed using commercially available statistical software packages (Statistic for Windows, StatSoft and graph GraphPad PRISM Version 2.0).

Results

Bacterial antioxidative properties

TAA_{LA} ranged between 2 and 37 % (median, 23 %; mean±SD, 21±11 %). The strains that showed values higher than the 75th percentile (31 %) belonged to *S. thermophilus*, *Lactobacillus* spp., and *Lactococcus lactis* (five, three, and one strains, respectively; Table 1). Due to the wide distribution of TAA_{LA} values, significant differences could not be established among *S. thermophilus* and the other groups (Fig. 1). Conversely, *Lactobacillus* and *Lactococcus* strains presented higher TAA_{LA} than bifidobacteria ($P<0.05$). In particular, the TAA_{LA} of most *Lactobacillus* strains (above their 25th percentile) was higher than the median of all the tested strains (23 %), while the TAA_{LA} of all bifidobacteria was lower.

Significant differences of TAA_{AA} values could not be established among the groups (Fig. 1). TAA_{AA} ranged between 0 and 82 % (median, 15 %; mean±SD, 20±17 %). Among all the tested strains, the ones presenting TAA_{AA} higher than the 75th percentile (28 %) were *Bifidobacterium breve* WC 0424, *Bifidobacterium animalis* subsp. *lactis* DSMZ 23032, *Bifidobacterium adolescentis* DSMZ 18351, *Lactobacillus reuteri* ATCC 23272, *Lactococcus lactis* ssp. *cremoris* ATCC 19257, *Lactobacillus coryniformis* ATCC 25600, *S. thermophilus* ATCC 19258, *Lactobacillus helveticus* S 40.8, and *Lactobacillus acidophilus* ATCC 4356, in increasing order (Table 1).

TEAC was widely dispersed among the bacterial strains, ranging between 0 and 125 μM (median, 22 μM; mean±SD, 32±32 μM; Table 1). The strains presenting TEAC higher than the 75th percentile (49 μM) were *L. reuteri*

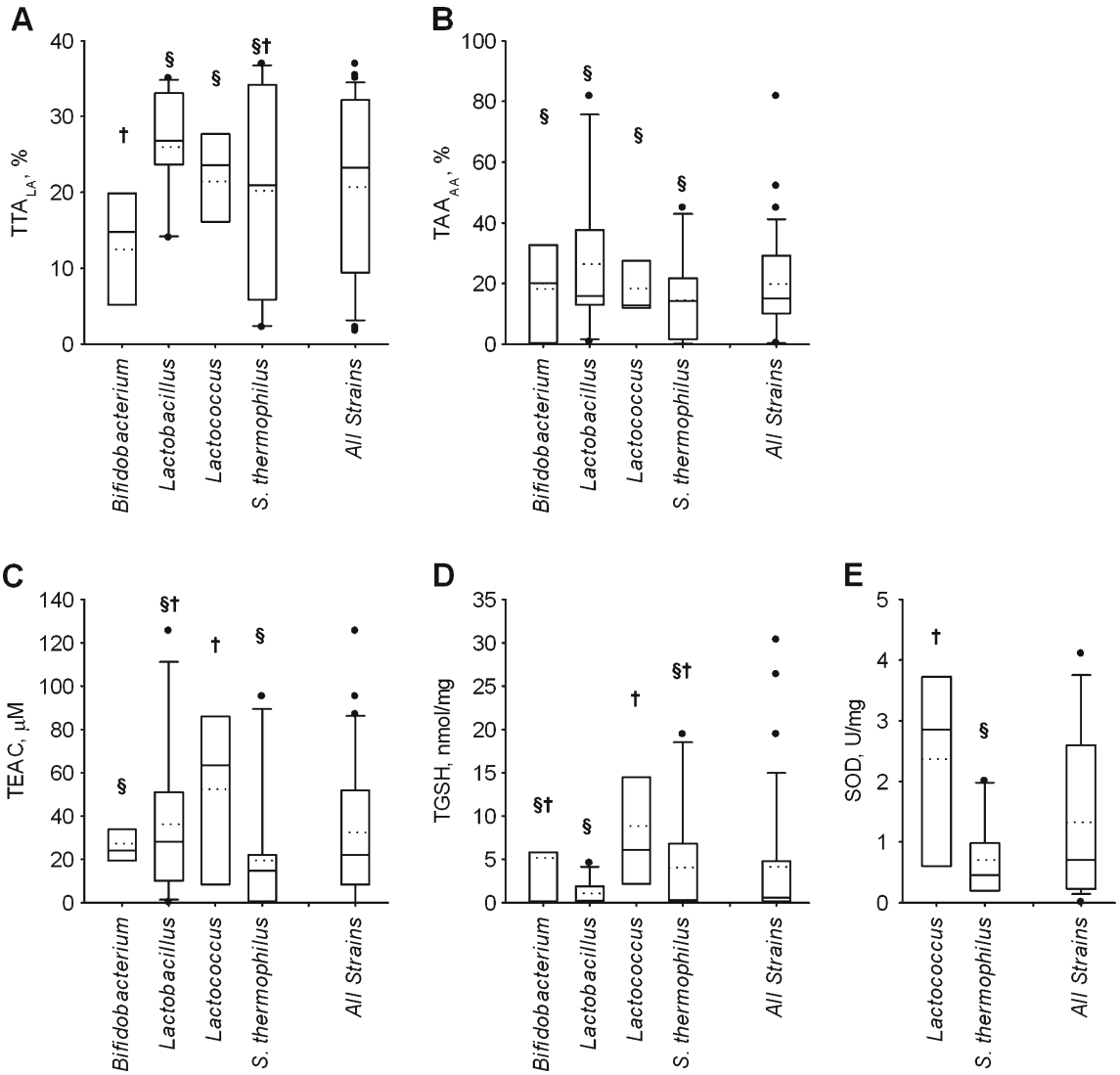

Fig. 1 TAA_{LA} (**a**), TAA_{AA} (**b**), TEAC (**c**), TGSH (**d**), and SOD (**e**) of 34 bacterial strains belonging to *Bifidobacterium* ($n=7$), *Lactobacillus* ($n=11$), *Lactococcus* ($n=6$), and *S. thermophilus* ($n=10$). *Boxes* indicate the median and 25th and 75th percentiles; *whiskers* indicate 10th and 90th percentiles. *Dotted lines* indicate means; means with a common symbol are not significantly different ($P\geq0.05$)

ATCC 23272, *L. acidophilus* ATCC 4356, *L. lactis* MB 445, *B. animalis* subsp. *lactis* DSMZ 23032, *L. lactis* ssp. *diacetilactis* MB 447, *L. lactis* MB 446, *L. lactis* ssp. *cremoris* MB 461, *S. thermophilus* MB 418, and *Lactobacillus brevis* DSMZ 23034, in increasing order (Table 1). On average, TEAC values were higher ($P<0.05$) in *Lactococcus* than in *Bifidobacterium* and *S. thermophilus* strains. In particular, the median of *Lactococcus* group (63 μM) was higher than the 75th percentile of all the strains, while most of *S. thermophilus* were below the median.

Intracellular glutathione (TGSH, oxidized plus reduced forms) ranged between 0 and 30.3 nmol/mg of proteins. With the exception of *Lactococcus* group and few other bacteria, most of the strains presented low levels, the median and the 75th percentile being 0.6 and 4.4 nmol/mg, respectively. The median of *Lactococcus* group (6.1 nmol/mg) was higher than the 75th percentile of all the tested strains. Most bifidobacteria did not produce detectable amounts of TGSH, whereas *B. animalis* subsp. *lactis* DSMZ 23032 gave 30.3 nmol/mg, the highest value obtained among all the strains. Other strains containing relevant amount of TGSH (≥90th percentile, 10.3 nmol/mg) were *S. thermophilus* MB 410, *L. lactis* ssp. *diacetilactis* MB 447, *S. thermophilus* EI-16, *L. lactis* ssp. *cremoris* ATCC 19257, *B. animalis* subsp. *lactis* DSMZ 23032, in increasing order (Table 1).

SOD activity was analyzed in the strains belonging to *Lactococcus* and *S. thermophilus*, which are expected to bear sodA or sodB genes based on nucleotide sequences database. SOD was found in all the bacteria belonging to these groups, being significantly higher ($P<0.05$) in *Lactococcus* (median, 2.9 U/mg; mean±SD, 2.4±1.6 U/mg) than in *S. thermophilus* strains (median, 0.5 U/mg; mean±SD, 0.7±0.7 U/mg).

According to these results, *B. animalis* subsp. *lactis* DSMZ 23032, *L. acidophilus* DSMZ 23033, and *L. brevis* DSMZ 23034 were selected among the lactobacilli and bifidobacteria which exhibited promising antioxidative properties to prepare the probiotic formulation to be administered to rats.

In vivo effects of probiotic supplementation

In basal condition, the supplementation with the probiotic mixture at the dose of 10^8 cfu/day did not modify plasma ROM concentration (Fig. 2). In NS rats, DOXO treatment caused an oxidative stress, evidenced by the significant increase of plasma ROMs; conversely, no modifications in ROMs concentration were observed in probiotic supplemented rats after DOXO administration. In DOXO treated rats, plasma ROMs concentration was inversely related to the dose of the administered probiotics ($r=0.947$, $P<0.05$). Probiotic administration at the dose of 10^8 cfu/day did not modify plasma TAA in basal condition (Fig. 3). The oxidative stress induced by DOXO treatment caused a significant TAA

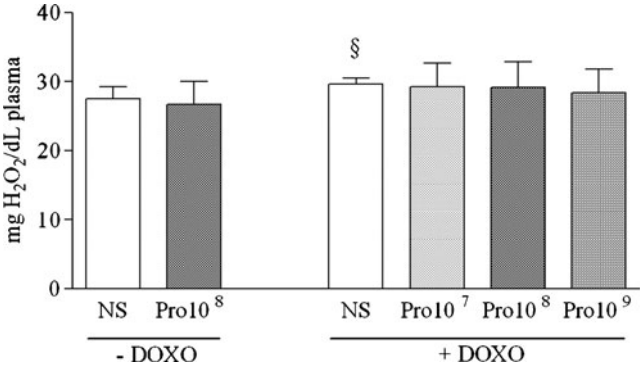

Fig. 2 Plasma ROMS concentration of not supplemented and probiotic supplemented rats, in basal condition and after DOXO administration. Values are means±SD, $n=7$. Statistical analysis was by the one-way ANOVA (ns) with Tukeys as post-test comparing NS rats to other groups (NS vs NS+DOXO; $P<0.05$); the comparison among DOXO-treated rats was by one way ANOVA (ns)

decrease in NS and Pro10^7 rats with respect to NS in basal condition, while no differences were detected in the other probiotic supplemented groups. In DOXO-treated animals, TAA was significantly related to the dose of administered probiotics ($r=0.95$; $P<0.05$). As for the other measured parameters; in basal condition, plasma GSH concentration was not modified by probiotics supplementation (Fig. 4). DOXO administration caused a significant decrease of plasma GSH content in NS rats compared to the corresponding not-stressed counterparts, without affecting GSH level in probiotic supplemented animals. In DOXO-treated rats plasma GSH concentration was significantly related to the dose of administered probiotics ($r=0.948$; $P<0.05$).

Kinetics of microbial groups and pH in feces

The fecal concentration of coliforms, enterococci, lactobacilli, and bifidobacteria was not affected by 18-day administration

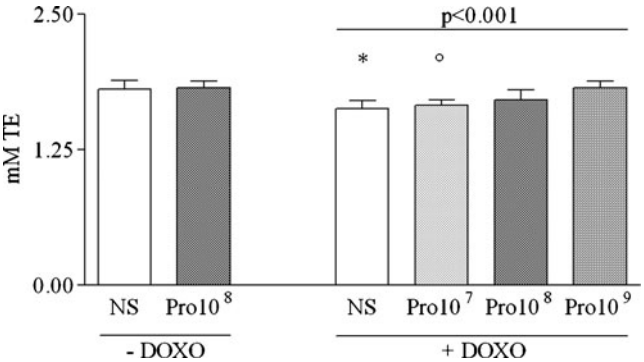

Fig. 3 Plasma total antioxidant activity (TAA) of not supplemented and probiotic supplemented rats, in basal condition and after DOXO administration. Values are means±SD, $n=7$. Statistical analysis was by the one way ANOVA (ns) with Tukeys as post-test comparing NS rats to other groups (NS vs NS+DOXO; $P<0.001$; NS vs Pro10^7+DOXO; $P<0.01$); the comparison among DOXO-treated rats was by one way ANOVA ($P<0.001$)

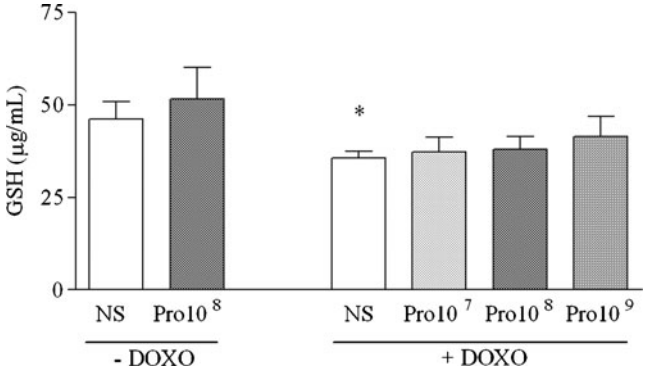

Fig. 4 Plasma GSH concentration of not supplemented and probiotic supplemented rats, in basal condition and after DOXO administration. Values are means±SD, $n=7$. Statistical analysis was by the one way ANOVA (ns) with Tukeys as post-test comparing NS rats to other groups (NS vs NS+DOXO; $P<0.05$); the comparison among DOXO-treated rats was by one-way ANOVA (ns)

of NS diet ($P\geq0.05$; Fig. 5). The administration of probiotic supplements caused both coliforms and enterococci to decrease ($P<0.05$), but differences among Pro10^7, Pro10^8, and Pro10^9 were not statistically significant ($P\geq0.05$). Compared to NS group, lactobacilli, and bifidobacteria remained unchanged ($P\geq0.05$) in Pro10^7 group, but increased by ~1 magnitude order in Pro10^8 group and by two in Pro10^9 group. In this latter group, the mean counts of lactobacilli and bifidobacteria were 7.8 and 7.2 Log_{10} cfu/g, respectively. The pH of rat feces of NS and Pro10^7 groups was not affected by treatment ($P\geq0.05$), whereas it decreased from 6.8 to 6.4 in Pro10^8 and Pro10^9 groups ($P<0.05$).

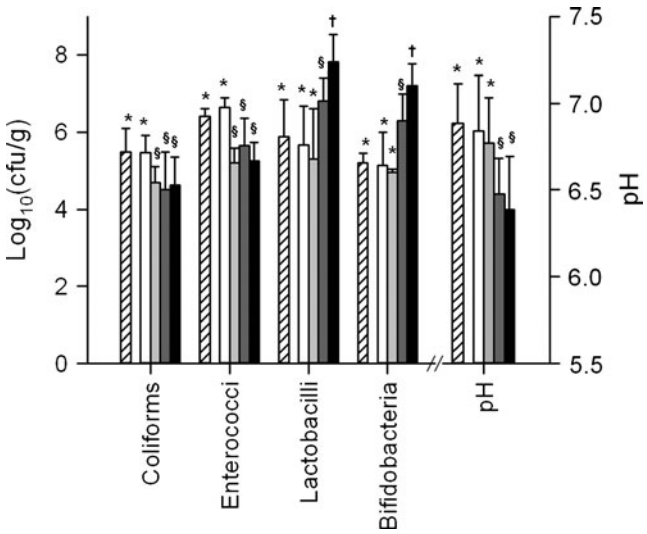

Fig. 5 FISH counts of coliforms, enterococci, lactobacilli, and bifidobacteria and pH of rats feces. *Bars* indicate means±SD at the beginning of the trial (*dashed*) and after an 18-day treatment with NS (*white*), Pro10^7 (*light gray*), Pro10^8 (*dark gray*), and Pro10^9 (*black*). Within each bacterial group and pH, means with a common symbol are not significantly different ($P\geq0.05$)

Discussion

Accumulating evidence suggests that probiotics exert various biological roles through several mechanisms, one of the most debated being the antioxidant activity (Rossi and Amaretti 2010). In fact, among probiotics beneficial effects, the protection against oxidative stress in humans has been reported by several authors (Naruszewicz et al. 2002; Songisepp et al. 2005; Virtanen et al. 2007). In this light, the selection of specific strains and the evidence of their effectiveness, resulting in control of reactive radicals, can be exploited to formulate novel probiotic foods or supplements that can exert a role in the prevention of oxidative stress and related diseases.

This study indicates that the antioxidative properties of probiotic bacteria, measured according to the methods that are commonly used for lactic acid bacteria (Mishra and Kovachich 1984; Lin and Yen 1999; Kullisaar et al. 2002; Zanoni et al. 2008; Mikelsaar and Zilmer 2009), are strain-specific features. Both intact cells and cell-free extracts exhibited antioxidant properties. Within each microbial group, irrespectively by the methods used, wide dispersion of the values of antioxidative parameters was observed, indicating the strain specificity of this trait. For each strain, TAA_{AA}, TAA_{LA}, and TEAC were unrelated to each other, and were not correlated to intracellular TGSH concentration or SOD activity. Such discrepancies may be due to the fact that these methods are based on diverse reactions which may be differently affected by specific molecular mechanisms responsible for defense against oxidative stress.

The screening resulted in a panel of strains exhibiting high values of antioxidative properties. The ones that were included in the probiotic formulation were selected within the genera *Lactobacillus* and *Bifidobacterium*, the most commonly used probiotic bacteria. This approach excluded several promising strains belonging to the genus *Lactococcus* and to the species *S. thermophilus* that presented very high levels of TGSH and SOD activity. Finally, the strains *B. animalis* subsp. *lactis* DSMZ 23032, *L. acidophilus* DSMZ 23033, and *L. brevis* DSMZ 23034 were selected, based also on their technological properties, so that they could be manufactured in adequate amount and incorporated in the lyophilized formula without loosing viability. *B. animalis* subsp. *lactis* DSMZ 23032 and *L. acidophilus* DSMZ 23033 contained the highest amount of TGSH among all the tested strains and lactobacilli, respectively. *L. brevis* DSMZ 23034 presented the highest intracellular TEAC among all the strains and high values TTA_{LA} and TAA_{AA} within the *Lactobacillus* group (≥75th percentile).

The selected probiotic strains acted in concert to counteract the oxidative stress induced in the animal model. During the trial, no differences were detected in animal growth and in food consumption, and all animals appeared in a good state of health. In basal condition, the treatment

with probiotics did not modify the tested markers of oxidative stress; on the contrary, the administration of probiotics protected rats against the DOXO-induced oxidative damage in a dose-related manner. In fact, in not supplemented rats DOXO administration caused an increase of ROM concentration and a decrease in TAA and GSH level, while no modification were detected in animals supplemented with the two higher probiotic concentrations compared to not supplemented, not stressed rats.

The probiotic strains colonized the colon of the rats during the trial. The marked decrease of fecal pH in the treated groups, which did not occur in the control group, indicates that the probiotic diets promoted the saccharolytic metabolism and the subsequent acidification of feces, due to the fermentation of carbohydrates to short chain fatty acids (Pompei et al. 2008). The decrease of coliforms confirmed the predominance of healthy saccharolytic microbial processes over the harmful proteolytic/putrefactive ones.

Given the colon colonization by administered probiotics, it is conceivable that their overall protective effect could be related to activities taking place at intestinal level, i.e., secretion of enzymes like SOD, metal-chelating activities, promotion of the production of antioxidant biomolecules such as exopolysaccharides showing an in vitro antioxidant and free-radical scavenging activities. Furthermore, the intestinal microflora provides additional enzymatic activities involved in the transformation of dietary compounds, thus increasing the bioavailability of dietary antioxidants (Davis and Milner 2009). Some authors hypothesize that probiotics exert their protective effects against oxidative stress by restoring gut microbiota (Nardone et al. 2010; Forsyth et al. 2009).

Notwithstanding, other mechanisms could also be at the basis of the antioxidant effect, as indicated by the maintenance of GSH level in stressed rats after probiotic supplementation. According to Spyropolous et al. (2011) probiotics may concretely enhance antioxidant defenses in the host, producing and releasing GSH and vitamins which are absorbed and distributed in the organism. Interestingly, probiotics supplementation in rats is able to induce transcription of genes involved in glutathione biosynthesis in the intestinal mucosa (Lutgendorff et al. 2009) and to increase glutathione synthesis in pancreatic cells (Lutgendorff et al. 2008). A similar effectiveness in restoring GSH concentration after an oxidative stress has been already reported (Peran et al. 2007) in rats supplemented with *Lactobacillus casei*, *L. acidophilus*, and *Bifidobacterium lactis*.

Although further studies are needed to elucidate the possible mechanisms involved, the herein reported study confirms the effectiveness of selected probiotic strains in the counteraction of oxidative stress, confirming previous studies in animals and humans (Hathout et al. 2011; Martarelli et al. 2011; Songisepp et al. 2005). This study supports the hypothesis that selected probiotic strains can underlay the enhancement of cellular antioxidant defenses in the host. The screening of physiological traits resulted still a powerful approach to assess the potential of probiotic bacteria for specific functions that can be subsequently verified in animal models. Acting this way, antioxidant probiotic strains can be selected and investigated as promising candidates for the prevention and control of several free radical-related disorders.

Acknowledgments This study was partially supported by Anidral/Probiotical Ltd., Novara, Italy.

References

Azcárate-Peril MA, Sikes M, Bruno-Bárcena JM (2011) The intestinal microbiota, gastrointestinal environment and colorectal cancer: a putative role for probiotics in prevention of colorectal cancer? Am J Physiol Gastrointest Liver Physiol 301:G401–G424. doi:10.1152/ajpgi.00110.2011

Babbs CF (1990) Free radicals and the etiology of colon cancer. Free Radic Biol Med 8:191–200. doi:10.1016/0891-5849(90)90091-V

Beuchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to polyacrylamide gels. Anal Biochem 44:276–28. doi:10.1016/0003-2697(71)90370-8

Bordoni A, Danesi F, Malaguti M, Di Nunzio M, Pasqui F, Maranesi M, Biagi LP (2008) Dietary Selenium for the counteraction of oxidative damage: fortified foods or supplements? Br J Nutr 99:191–197. doi:10.1017/S0007114507793911

Davis CD, Milner JA (2009) Gastrointestinal microflora, food components and colon cancer prevention. J Nutr Biochem 20:743–752. doi:10.1016/j.jnutbio.2009.06.001

Dellomonaco C, Amaretti A, Zanoni S, Pompei A, Matteuzzi D, Rossi M (2007) Fermentative production of superoxide dismutase with *Kluyveromyces marxianus*. J Ind Microbiol Biotechnol 34:27–34. doi:10.1007/s10295-006-0158-4

Di Nunzio M, Valli V, Bordoni A (2011) Pro- and anti-oxidant effects of polyunsaturated fatty acid supplementation in HepG2 cells. Prostaglandins Leukot Essent Fatty Acids 85:121–127. doi:10.1016/j.plefa.2011.07.005

FAO/WHO working group (2001) Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Córdoba, Argentina (October 1–4, 2001)

Finch J, Munhutu MN, Whitaker-Worth DL (2010) Atopic dermatitis and nutrition. Clin Dermatol 28:605–614. doi:10.1016/j.clindermatol.2010.03.032

Firuzi O, Miri R, Tavakkoli M, Saso L (2011) Antioxidant therapy: current status and future prospects. Curr Med Chem 18:3871–3888

Forsyth CB, Farhadi A, Jakate SM, Tang Y, Shaikh M, Keshavarzian A (2009) *Lactobacillus* GG treatment ameliorates alcohol-induced intestinal oxidative stress, gut leakiness, and liver injury in a rat model of alcoholic steatohepatitis. Alcohol 43:163–172. doi:10.1016/j.alcohol.2008.12.009

Girotti S, Ferri E, Fini F, Bolelli L, Sabatini AG, Budini R, Sichertova D (2004) Automated and manual luminescent assay of antioxidant capacity: analytical features by comparison. Talanta 64:665–670. doi:10.1016/j.talanta.2004.03.041

Guandalini S (2011) Probiotics for prevention and treatment of diarrhea. J Clin Gastroenterol 45:S149–S153. doi:10.1097/MCG.0b013e3182257e98

Hathout AS, Mohamed SR, El-Nekeety AA, Hassan NS, Aly SE, Abdel-Wahhab MA (2011) Ability of *Lactobacillus casei* and *Lactobacillus reuteri* to protect against oxidative stress in rats fed aflatoxins-contaminated diet. Toxicon 58:179–186. doi:10.1016/j.toxicon.2011.05.015

Kaizu H, Sasaki M, Nakajima H, Suzuki Y (1993) Effect of antioxidative lactic acid bacteria on rats fed a diet deficient in vitamin E. J Dairy Sci 76:2493–2499. doi:10.3168/jds.S0022-0302(93)77584-0

Kullisar T, Zilmer M, Mikelsaar M, Vihalemm T, Annuk H, Kairanc C, Kilk A (2002) Two antioxidative lactobacilli strains as promising probiotics. Int J Food Microb 72:215–224. doi:10.1016/S0168-1605(01)00674-2

Lin MY, Yen CL (1999) Antioxidative ability of lactic acid bacteria. J Agric Food Chem 47:1460–1466. doi:10.1021/jf981149l

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Lutgendorff F, Trulsson LM, van Minnen LP, Rijkers GT, Timmerman HM, Franzén LE, Gooszen HG, Akkermans LM, Söderholm JD, Sandström PA (2008) Probiotics enhance pancreatic glutathione biosynthesis and reduce oxidative stress in experimental acute pancreatitis. Am J Physiol Gastrointest Liver Physiol 295: G1111–G1121. doi:10.1152/ajpgi.00603.2007

Lutgendorff F, Nijmeijer RM, Sandström PA, Trulsson LM, Magnusson KE, Timmerman HM, van Minnen LP, Rijkers GT, Gooszen HG, Akkermans LM, Söderholm JD (2009) Probiotics prevent intestinal barrier dysfunction in acute pancreatitis in rats via induction of ileal mucosal glutathione biosynthesis. PLoS One 4:e4512. doi:10.1371/journal.pone.0004512

Martarelli D, Verdenelli MC, Scuri S, Cocchioni M, Silvi S, Cecchini C, Pompei P (2011) Effect of a probiotic intake on oxidant and antioxidant parameters in plasma of athletes during intense exercise training. Curr Microbiol 62:1689–1696. doi:10.1007/s00284-011-9915-3

Mikelsaar M, Zilmer M (2009) *Lactobacillus fermentum* ME-3—an antimicrobial and antioxidative probiotic. Microb Ecol Health Dis 21:1–27. doi:10.1080/08910600902815561

Mishra OP, Kovachich GB (1984) Inhibition of the autoxidation of ascorbate and norepinephrine by extracts of *Clostridium butyricum*, *Megasphaera elsdenii* and *Escherichia coli*. Life Sci 35:849–854. doi:10.1016/0024-3205(84)90410-7

Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 247:3170–3175

Nardone G, Compare D, Liguori E, Di Mauro V, Rocco A, Barone M, Napoli A, Lapi D, Iovene MR, Colantuoni A (2010) Protective effects of *Lactobacillus paracasei* F19 in a rat model of oxidative and metabolic hepatic injury. Am J Physiol Gastrointest Liver Physiol 299:G669–G676. doi:10.1152/ajpgi.00188.2010

Naruszewicz M, Johansson ML, Zapolska-Downar D, Bukowska H (2002) Effect of *Lactobacillus plantarum* 299v on cardiovascular disease risk factors in smokers. Am J Clin Nutr 76:1249–1255

Peran L, Camuesco D, Comalada M, Bailon E, Henriksson A, Xaus J, Zarzuelo A, Galvez J (2007) A comparative study of the preventative effects exerted by three probiotics, *Bifidobacterium lactis*, *Lactobacillus casei* and *Lactobacillus acidophilus*, in the TNBS model of rat colitis. J Appl Microbiol 103:836–844. doi:10.1111/j.1365-2672.2007.03302.x

Pompei A, Cordisco L, Amaretti A, Zanoni S, Matteuzzi D, Rossi M (2007a) Folate production by bifidobacteria as a potential probiotic property. Appl Environ Microbiol 73:179–185. doi:10.1128/AEM.01763-06

Pompei A, Cordisco L, Amaretti A, Zanoni S, Raimondi S, Matteuzzi D, Rossi M (2007b) Administration of folate-producing bifidobacteria enhances folate status in Wistar rats. J Nutr 137:2742–2746

Pompei A, Cordisco L, Raimondi S, Amaretti A, Pagnoni UM, Matteuzzi D, Rossi M (2008) In vitro comparison of the prebiotic effects of two inulin-type fructans. Anaerobe 14:280–286

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26:1231–1237. doi:10.1016/S0891-5849(98)00315-3

Rossi M, Amaretti A (2010) Probiotic properties of bifidobacteria. In: Mayo B, van Sinderen D (eds) Bifidobacteria: genomics and molecular aspects. Horizon Scientific Press, UK, pp 97–123. ISBN 978-1-904455-68-4

Serafini M, Del Rio D (2004) Understanding the association between dietary antioxidants, redox status and disease: is the Total Antioxidant Capacity the right tool? Redox Rep 9:145–152. doi:10.1179/135100004225004814

Shimamura S, Abe F, Ishibashi N, Miyakawa H, Yaeshima T, Araya T, Tomita M (1992) Relationship between oxygen sensitivity and oxygen metabolism of *Bifidobacterium* species. J Dairy Sci 75:3296–3306. doi:10.3168/jds.S0022-0302(92)78105-3

Songisepp E, Kals J, Kullisaar T, Mändar R, Hütt P, Zilmer M, Mikelsaar M (2005) Evaluation of the functional efficacy of an antioxidative probiotic in healthy volunteers. Nutr J 4:22. doi:10.1186/1475-2891-4-22

Spyropoulos BG, Misiakos EP, Fotiadis C, Stoidis CN (2011) Antioxidant properties of probiotics and their protective effects in the pathogenesis of radiation-induced enteritis and colitis. Dig Dis Sci 56:285–294. doi:10.1007/s10620-010-1307-1

Talwalkar A, Kailasapathy K (2003) Metabolic and biochemical responses of probiotic bacteria to oxygen. J Dairy Sci 86:2537–2546. doi:10.3168/jds.S0022-0302(03)73848-X

Uskova MA, Kravchenko LV (2009) Antioxidant properties of lactic acid bacteria-probiotic and yogurt strains. Vopr Pitan 78:18–23

Virtanen T, Pihlanto A, Akkanen S, Korhonen H (2007) Development of antioxidant activity in milk whey during fermentation with lactic acid bacteria. J Appl Microbiol 102:106–115. doi:10.1111/j.1365-2672.2006.03072.x

Wang YC, Yu RC, Chou CC (2006) Antioxidative activities of soymilk fermented with lactic acid bacteria and bifidobacteria. Food Microbiol 23:128–135. doi:10.1016/j.fm.2005.01.020

Zanoni S, Pompei A, Cordisco L, Amaretti A, Rossi M, Matteuzzi D (2008) Growth kinetics on oligo- and polysaccharides and promising features of three antioxidative potential probiotic strains. J Appl Microbiol 105:1266–1276. doi:10.1111/j.1365-2672.2008.03860.x