ISSN 0003-6838, Applied Biochemistry and Microbiology, 2010, Vol. 46, No. 5, pp. 481–486. © Pleiades Publishing, Inc., 2010.
Published in Russian in Prikladnaya Biokhimiya i Mikrobiologiya, 2010, Vol. 46, No. 5, pp. 527–531.

Complete Glutathione System in Probiotic *Lactobacillus fermentum* ME-3[1]

T. Kullisaar[a], E. Songisepp[b], M. Aunapuu[c], K. Kilk[a], A. Arend[c], M. Mikelsaar[c], A. Rehema[a], and M. Zilmer[a]

[a] *Department of Biochemistry of Tartu University, Tartu, 50411 Estonia*
[b] *Bio-Competence Centre of Healthy Dairy Products, Tartu 51014, Estonia*
[c] *Department of Anatomy and Histology of Tartu University Tartu 50411, Estonia*
[d] *Department of Microbiology of Tartu University, Tartu 50411, Estonia*
e-mail: tiiu.kullisaar@ut.ee
Received July 10, 2009

Abstract—There is much information about glutathione (GSH) in eukaryotic cells, but relatively little is known about GSH in prokaryotes. Without GSH and glutathione redox cycle lactic acid bacteria (LAB) cannot protect themselves against reactive oxygen species. Previously we have shown the presence of GSH in *Lactobacillus fermentum* ME-3 (DSM14241). Results of this study show that probiotic *L. fermentum* ME-3 contains both glutathione peroxidase and glutathione reductase. We also present that *L. fermentum* ME-3 can transport GSH from environment and synthesize GSH. This means that it is characterized by a complete glutathione system: synthesis, uptake and redox turnover ability that makes *L. fermentum* ME-3 a perfect protector against oxidative stress. To our best knowledge studies on existence of the complete glutathione system in probiotic LAB strains are still absent and glutathione synthesis in them has not been demonstrated.

DOI: 10.1134/S0003683810050030

Glutathione (*L*-gamma-Glu-*L*-Cys-Gly or GSH) is a major cellular non-enzymatic antioxidant. It eliminates reactive oxygen species (ROS) like lipid and hydrogen peroxides, hydroxyl radical and peroxynitrite mainly via cooperation with selenium-dependent glutathione peroxidase [1]. In addition, GSH is used for detoxification of xenobiotics, for transport and storage of NO, for transport of amino acids into liver and kidney cells, for stabilization of cell membranes, etc. [1]. During the fulfillment of its biofunctions the reduced form of glutathione (GSH) is oxidized to a disulfide (GSSG, oxidized glutathione) by glutathione peroxidase (EC 1.11.1.9, GPx). GSSG is rapidly reduced back to GSH by a concerted action of glutathione reductase (EC 1.6.4.2, GRed) and NADPH in order to maintain the physiological glutathione redox ratio (GSSG/GSH). The latter is known as cellular redox switch that controls/regulates functionality of several principal cellular events and factors (e.g. transcription factors). Thus, glutathione system with its basic components GSH, GSSG, GPx and GRed, has a high impact on viability of cells [2]. In contrast to the extensive information related to glutathione in eukaryotic cells, relatively little is known about glutathione and components of glutathione system in prokaryotes. Studies on systematic determination of all glutathione system components in case of probiotic lactic acid bacteria (LAB) strains are actually absent. Confirmation of the presence of all glutathione system components in LAB gives very valuable information as it shows that a specific LAB strain has especially high oxygen and ROS tolerance under different stress conditions. Tolerance to stress in the digestive system as well as during the production of a functional food is an essential physiological trait for probiotics [3, 4]. A LAB that contains superoxide dismutase (SOD) has protection against superoxide radicals [5–7], but SOD cannot protect against lipid peroxides, direct killing by hydrogen peroxide (H_2O_2) and/or against hydroxyl radical (OH) damage [6]. LAB must contain some other cellular antioxidants which they can synthesize and/or uptake from the environment. A LAB strain with fully operational glutathione system can directly detoxify or eliminate H_2O_2 and lipid peroxyl radicals [8–10] and thus have defense against H_2O_2 accumulation [11, 12]. H_2O_2 detoxification capacity probably defines the degree of oxygen tolerance of LAB [13]. With a more developed network of antioxidative agents a LAB strain has better tolerance against oxidative stress.

Lactobacillus fermentum ME-3 (DSM14241, *L. fermentum* is of human origin [14] and has proven its safety as a probiotic exhibiting both antimicrobial and antioxidative benefits under different in vitro and

[1] The article is published in the original.

in vivo conditions [5, 4, 15–17]. We have previously shown that *L. fermentum* ME-3 contains glutathione [5]. The aim of this study was to investigate: (a) does *L. fermentum* ME-3 have the complete glutathione system necessary for glutathione recycling, and (b) can it synthesize and/or transport glutathione.

MATERIALS AND METHODS

The probiotic strain *L. fermentum* ME-3 was originally isolated from a faecal sample of an Estonian child [14]. Isolation of a Lactobacillus on de Man-Rogosa-Sharpe (MRS agar, CM 361, Oxoid Ltd. Basingstoke, UK) was followed by the identification to the species level by API 50 CHL kit and API LAB Plus software, version 4.0 database (bioMerieux, Marcy l'Etoile, France).

Growth conditions of probiotic *L. fermentum* ME-3 for detection of GSH synthesis and transport. The *L. fermentum* ME-3 strain was grown in MRS broth (CM 359, Oxoid Ltd. Basingstoke, UK) for 24 h under microaerobic conditions at 37°C with or without a GSH synthesis inhibitor *L*-buthionine sulfoximine (BSO, Sigma, USA). Thereafter three passages (the inoculation dose of 100 μl) into 3 ml of sterile (120°C, 15 min) milk (2.5% fat), incubation time 24 h at 37°C, were carried out. The culture grown on BSO-containing MRS was grown in BSO-containing milk at this stage and further. The 24 h old culture was then inoculated into 9 ml of sterile milk (the inoculation dose was 1 ml) as follows: (a) milk containing only the strain *L. fermentum* ME-3 served as the control, (b) milk containing the strain and BSO in final concentration of 6.0 mM, (c) milk containing the strain and 0.3 mM GSH (Sigma, USA), (d) milk containing the strain and 0.6 mM GSH.

The presence of GSH or GSH uptake/synthesis by *L. fermentum* ME-3 was measured at 9 and 24 h from the final inoculation. Bacterial cells were harvested by centrifugation (2500 g/10 min) at 4°C and the pellet was washed twice with MilliQ water. The cell pellet was resuspended in MilliQ water. The density of the suspension was adjusted spectrophotometrically to an OD_{600} of 1.1 (approximately 10^9 bacterial cells ml^{-1}). The cell suspension was used for the measurement of GPx and GRed.

To get lysates 1 ml of cell suspension was added to a vial along with 1 g of glass beads, and cells were broken with a mini bead beater (BioSpec Products Inc., www.biospec.com) for 1 min several times at 4°C. The cell suspension was centrifuged at 4°C 10 000 g for 10 min (Hermle Labortecnik GmbH, Z252 Mk, Germany) and the supernatant was used for the GSH assay. The protein concentration in the supernatant was determined by using the method of Lowry [18].

Reduced and oxidized glutathione. To eliminate proteins from the sample 10% solution of metaphosphoric acid (Sigma, USA) was added to the equal volume of the sample and mixed vigorously. This mixture was allowed to stand at room temperature for 5 min and centrifuged at 3000 g for 5 min. The supernatant was carefully collected and stored at –20°C, if the assay was not performed immediately. We had to derivatize reduced glutathione (GSH) to measure oxidized glutathione (GSSG). This was done by adding 0.1 ml of 1 mM 2-vinylpyridine in ethanol (Sigma-Aldrich, Germany) followed by vortexing and incubation at room temperature for 1 h. To determine the content of GSSG (as well as the content of total glutathione) 0.005 ml of 4.0 M solution of triethanolamine in water was .added to 0.1 ml of derivatized sample, 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 (Sigma, USA) and 0.3 mM NADPH (Sigma, USA). The enzymatic reaction was initiated by addition of 0.1 ml of 1.0 mM 5,5'-dithio-bis-2-nitrobenzoic acid (Sigma, USA) in 0.2 M sodium phosphate buffer (pH 7.5) containing 0.01 M EDTA [19]. The change in optical density at 412 nm was measured after 10 min. 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 nmol/mg protein of sample.

Growth conditions of *L. fermentum* ME-3 for total antioxidative activity and hydrogen peroxide measurements. For the measurements of total antioxidative activity (TAA) and hydrogen peroxide *L. fermentum* ME-3 was grown in MRS broth for 48 h at 37°C under microaerobic conditions. Bacterial cells were harvested at different time points (3, 6, 9, 18, 24 and 48 h) by centrifugation (2500 g/10 min) at 4°C and the pellet was washed twice with MilliQ water. The cell pellet was resuspended in MilliQ water. The density of the suspension was adjusted spectrophotometrically to an OD_{600} of 1.1 (approximately 10^9 bacterial cells ml^{-1}).

Total antioxidative activity. TAA of *L. fermentum* ME-3 was assessed with the linolenic acid test (LA-test). This test evaluates the ability of a sample to inhibit linolenic acid (Sigma, USA) oxidation [5, 20]. The standard of linolenic acid in 96% ethanol (1:100) was diluted in isotonic saline (1:125). To 0.4 ml linolenic acid, diluted in isotonic saline, 0.01% sodium dodecyl sulphate (lauryl sulphate, Sigma, USA) and the sample (0.045 ml of *Lactobacilli* cells) were added. The incubation was started by adding 75 μM $FeSO_4$ (Sigma, USA) and the mixture was incubated at 37°C for 60 min. Then the reaction was interrupted by adding 0.25% butylated hydroxytoluene (BHT, Sigma, USA), the mixture was treated with 0.5 ml acetate buffer (pH 3.5) consisting of acetic acid glacial and sodium acetate trihydrate (Sigma, USA), and heated

with freshly prepared 1% thiobarbituric acid solution (TBA, Sigma, USA) 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 (Sigma, USA) and centrifuged at 3000g for 10 min. The absorbance of butanol fraction was measured. The TAA of samples was expressed as fraction of peroxidation in LA-standard as follows: [1-(A_{534} (sample)/A_{534} (LA as control)] × 100. The higher numerical value (%). of TAA indicates the higher TAA of sample. Peroxidation of LA-standard in the isotonic saline (without samples) served as a control.

Direct detection of hydrogen peroxide concentration. Direct measurement of hydrogen peroxide concentration was performed with Apollo 4000 free radical analyzer (WPI, Germany) with an ISO-HPO_2 type of electrode (WPI, Germany). Cells frozen at different growth stages were warmed to 37°C and vortexed thoroughly before measurement. The electrodes were let to calibrate in cell suspension until a stable level was reached (about 30 s). Thereafter the signal was registered for two more minutes and average value was taken. Each experimental point was measured in 3 independent parallels. In order to estimate the concentration the signal of unknown samples was compared to a standard curve of known concentrations [21].

The detection of GPx and GRed. The *L. fermentum* ME-3 cell suspensions were fixed with 10% buffered formalin solution on poly-*L*-lysine coated SuperFrost slides (Menzel-Gläser, Germany) (10 min). Slides were washed in PBS (pH 7.2), treated with normal 1.5% goat serum (Gibco, Invitrogen Co., USA) for 20 min at room temperature and then incubated with the first antibody (GPx, diluted 1 : 200, GRed, diluted 1 : 200, both Biogenesis, USA) 1 h at room temperature. Then sections were washed in PBS buffer and incubated in peroxidase blocking solution (0.3% H_2O_2 in methanol) (Merck, Germany). After washing in PBS, slides were incubated with biotinylated secondary antibody (1 : 500, Vector Labs, USA) for 30 min at room temperature. Then after washing in PBS the slides were incubated in VECTASTAIN ABC-AP Reagent (Vector Labs, USA) at room temperature (30 min), incubated in DAB (3,3'-diaminobenzidine) (Vector Labs, USA) for 5 min and counterstained with hemalaun (Fisher Diagnostics, USA). Slides were rinsed in distilled water, dehydrated through ethanol and mounted with DPX (mounting media, a mixture of distyrene, tricresyl phosphate and xylene) (Fluka, Switzerland). The GPx and GRed intensity is expressed in an arbitrary scale ranging from 0 to 3 (0 no staining, 1 weak staining, 2 moderate staining, 3 strong staining). Two independent observers in a blinded fashion performed the evaluation [22].

Statistical analysis. All calculations were performed using commercially available statistical software packages (Statistics for Windows, Stat Soft Inc. and Graph Pad PRISM Version 2.0) and software R, version 1.6.0 for windows (www.r-project.org). The values are given as mean and standard deviation. Statistically significant differences between the groups were determined by using Students's t-test. In all analyses p values <0.05 were considered to be statistically significant. Correlations between the variables were examined using linear regression analysis (software R, version 2.0.1 for Windows).

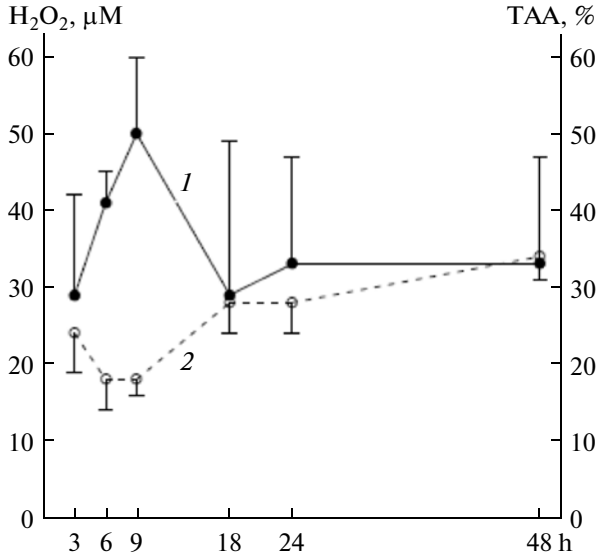

Fig. 1. H_2O_2 production (*1*, *M*) and total antioxidative activity (*2*, TAA, %) in *L. fermentum* ME-3. *L. fermentum* ME-3 grew in MRS broth for 48 h at 37°C in microaerobic conditions. These two parameters had a significant negative correlation ($R = -0.71$, $p < 0.05$, average±SD, $n = 5$).

RESULTS AND DISCUSSION

Hydrogen peroxide content and TAA during the growth of *L. fermentum* ME-3. The H_2O_2 content and TAA were measured at different time-points during *L. fermentum* ME-3 growth. In the exponential growth phase the production of H_2O_2 was found to increase and TAA to decrease initially. In circa 6 h TAA started to increase. In circa 9 h H_2O_2 level started to decrease. When the growth had reached the stationary phase, H_2O_2 concentration remained stable and TAA showed the highest values. We also found a statistically significant negative correlation $r = -0.71$, $p < 0.05$ (Fig. 1) between these two parameters.

GPx and GRed in *L. fermentum* ME-3. To test whether *L. fermentum* ME-3 has the whole glutathione system and therefore the ability to accomplish glutathione redox cycle we tested for the presence of GPx and GRed in *L. fermentum* ME–3 immunohistochemically (Fig. 2). Intensively colored areas (pointed with arrows) in stained *L. fermentum* ME-3

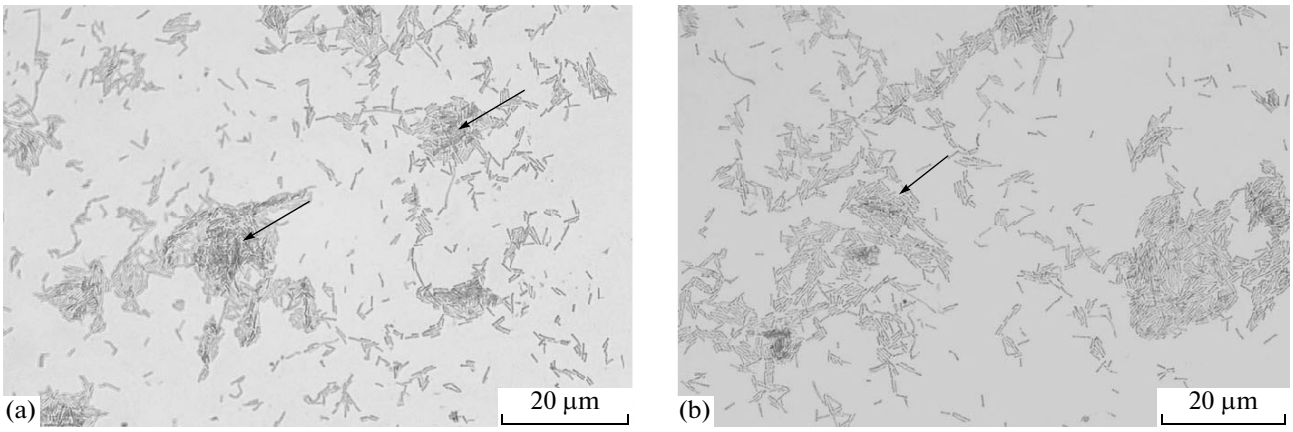

Fig. 2. Glutathione peroxidase (a) and glutathione reductase (b) in *L. fermentum* ME-3 cell suspension. The color intensity is expressed in an arbitrary scale from 0 to 3 (see Material and Method). Two independent observers in a blinded fashion performed the evaluation (the arrow points to GPx and GRed).

cell suspensions demonstrate the existence of both enzymes. The GPx and GRed intensity is expressed on an arbitrary scale ranging from 0 to 3. The staining of control strain preparations were evaluated to be 0 for GPx and GRed. The *L. fermentum* ME-3 slides of cultures 24 and 48 h got scored "2" for both GPx and GRed by two independent blinded observers.

The synthesis and transport of glutathione. To detect whether *L. fermentum* ME-3 is able to synthesize GSH we used y-glutamylcysteine as a precursor and GSH syntethase inhibitor (BSO) at final concentration 6.0 mM. We compared the levels of GSH in *L. fermentum* ME-3 after growth in MRS or milk for 9 or 24 h at the presence or absence of the inhibitor (data not shown). We found that synthesis as well as uptake of GSH was the highest in *L. fermentum* ME-3 grown in milk for 24 h—20.0 ± 10.0 nmol GSH/mg protein and in the case of an inhibitor (BSO) the synthesis decreased about 3 times (6.0 ± 4.0 nmol GSH/mg protein). To determine whether *L. fermentum* ME-3 can uptake GSH we used different GSH concentrations (0.3 and 0.6 mM) in milk. The content of GSH in *L. fermentum* ME-3 was increased 4–6 fold when 0.3–0.6 mM GSH was present in the environment (96.0 ± 32.5 nmol GSH/mg protein in the culture grown on the medium containing 0.3 mM GSH and 120.0 ± 25.4 nmol GSH/mg protein in the culture grown on the medium containing 0.6 mM GSH).

Our results show that probiotic *L. fermentum* ME-3 has all principal components of the glutathione system. As it also contains MnSOD [5] this strain is well protected against both intracellular and environmental ROS. It is important since aerobic growth of LAB is accompanied by formation of H_2O_2, O_2^- and $OH^\bullet$ that can be detrimental to LAB. It has been shown that H_2O_2 accumulation in stationary phase may induce growth defects of LAB. The killing effect of H_2O_2 becomes significant when *L. lactis* cells reach their stationary growth phase and the difference in survivability of cells with and without GSH presence becomes increasingly greater [23]. Continuous production of H_2O_2 in stationary phase is not yet shown but it may be just a function of the greater biomass in that culture [13]. In the exponential growth phase the content of H_2O_2 in *L. fermentum* ME-3 was increasing and TAA (%) was decreasing with a significant negative correlation ($r = 0.71$, $p < 0.05$) (Fig. 1). When *L. fermentum* ME-3 reached stationary growth phase the level of H_2O_2 remained stable and TAA % had the highest values, which means that *L. fermentum* ME-3 has excellent resistance against H_2O_2 killing (Fig. 1). From literature it is known that antioxidative activity in lactobacilli has significant positive correlation with cellular GSH levels [24, 25]. Previously we have shown that *L. fermentum* ME-3 contains GSH [5]. In fact Gram-positive bacteria have been considered not to be able to synthesize GSH until two publications showed that *Streptococcus agalactiae* [26, 27] and *Listeria monocytogenes* [28] can do it. Still little is known about all physiological roles of glutathione in Gram-positive bacteria [29]. The activity of GPx and GRed has been detected in strains of *L. lactis* [23]. GPx can also function as a H_2O_2 receptor and redox transducer [30]. This very short H_2O_2-mediated signal transduction pathway uses GPx in a scavenging action but also as a hydroperoxide receptor and a redox transduction intermediate. Some other LAB, like *Streptococcus thermophilus* and *Enterococcus faecalis*, have GRed activity, which is increased at high O_2 concentration [31]. This means that for optimal protection against oxidative stress both enzymes of glutathione system, GPx and GRed, play an important role. Our study showed that *L. fermentum* ME-3 is capable of glutathione redox-cycling since both necessary enzymes

are present. As we already mentioned above GPx can, additionally, directly detoxify or eliminate hydrogen peroxide and peroxyl radicals. This fact is in good correlation to TAA of *L. fermentum* ME-3 and our previous results [5].

It seems that increased accumulation of GSH under aerobic conditions observed in the case of some LAB strains is a regulatory mechanism that participates in protection of the cells against oxidative damage. Experiments show that an increase in the intracellular GSH concentration leads to increased resistance to oxidative stress. Even very low concentrations of extracellulary supplied GSH (1 to 10 μM) lead to the significant protection of *L. lactis* against damage by H_2O_2 [23]. Substantially elevated intracellular amounts of GSH in the antioxidative probiotic *L. fermentum* ME-3 grown in milk at different GSH concentrations indicated that it is capable to uptake GSH from the medium. GSH transport system has been described in several LAB, for example, *L. helveticus, Streptococcus thermophilus, L. lactis* [11]. Glutathione when taken up by *L. lactis* ssp. *cremoris* SK11 increases resistance to H_2O_2 stress and prevents H_2O_2 accumulation [23]. In the contrary, GSH in *L. lactis* strain NZ9000 does not increase resistance to H_2O_2 treatment since *L. lactis* NZ 9000 neither produces nor does transport glutathione. This strain possibly lacks the enzymatic machinery (GPx/GRed) to use glutathione for suppression of oxidative stress [11]. When *L. fermentum* ME-3 was grown in milk aerobically, without any kind of supplementation, it was able to synthesize/accumulate glutathione about 20.0 ± 10.0 nmol GSH/mg protein. When a specific glutathione synthesis inhibitor, BSO, was used, glutathione synthesis decreased about 3 fold. This supports the understanding that glutathione synthesis occurs in *L. fermentum* ME-3.

The high levels of synthesized and accumulated GSH in *S. agalactiae* (304 ± 11 nmol/mg protein) give the bacteria an advantage when dealing with oxidative stress, which suffers due to the lack of catalase and superoxide dismutase. Accumulation of GSH in *L. fermentum* ME-3 is not so potent. Like *S. agalactiae L. fermentum* ME-3 does not contain catalase, but it contains an antioxidative enzyme Mn-SOD and a complete glutathione system (that gives the ability of glutathione recycling). Hence, there is no need to synthesize/accumulate very high levels of GSH [5, 26].

According to our best knowledge this is the first time to establish the existence in probiotic lactobacilli the whole glutathione system: synthesis, transport and redox cycling. This, together with Mn-SOD and GSH [5], underlies impresssive antioxidative activity of *L. fermentum* ME-3 and makes it a perfect natural protector against oxidative stress.

REFERENCES

1. Zilmer, M., Soomets, U., Rehema, A., and Langel, A., *Drug Design Reviews*, 2005, vol. 2, pp. 121–127.
2. Valencia, E., Marin, A., and Hardy, G., *Glutathione—Nutritional and Pharmacological Viewpoints*, Part IV: *Nutrition*, 2001, vol. 17, pp. 783–784.
3. Ross, R.P., Desmond, C., Fitzgerald, G.F., and Stanton, C., *J. Appl. Microbiol.*, 2005, vol. 98, pp. 1410–1417.
4. Annuk, H., Shchepetova, J., Kullisaar, T., Songisepp, E., Zilmer, M., and Mikelsaar, M., *J. Appl. Microbiol.*, 2003, vol. 94, pp. 403–412.
5. Kullisaar, T., Zilmer, M., Mikelsaar, M., Vihalemm, T., Annuk, H., Kairane C., and Kilk, A., *Int J. Food Microbiol.*, 2002, vol. 72, pp. 215–224.
6. Bruno-Barcena, J.M., Andrus, J.M., Libby, S.L., Klaenhammer, T.R., and Hassan, H.M., *Appl. Environ. Microbiol.*, 2004, vol. 70, pp. 4702–4710.
7. Bruno-Barcena, J.M., Azcarate-Peril, M.A., Klaenhammer, T.R., and Hassan, H.M., *FEMS Microbiol. Lett.*, 2005, vol. 246, pp. 91–101.
8. Fernandes, L. and Steele, L. *J. Diary Sci.*, 1993, vol. 76, pp. 1233–1241.
9. Stecchini, M.L., Del Torre, M., and Munari, M., *Int. J. Food Microbiol.*, 2001, vol. 64, pp. 183–188.
10. Miyoshi, A., Rochat, T., Gratadoux, J.-J., Le Loir, Y., Costa Oliviera, S., Langella, P., and Azevedo, V., *Genet. Mol. Res.*, 2003, vol. 2, pp. 348–359.
11. Li, Y., Hugenholtz, J., Sybesma, W., Abee, T., and Molenaar, D., *Appl. Microbiol. Biotechnol.*, 2005, vol. 67, pp. 83–90.
12. Marty-Teysset, C., de la Torre, F., and Garel, J.-R., *Appl. Environ. Microbiol.,* 2000, vol. 66, pp. 262–267.
13. Hung, J., Cooper, D., J. Turner, M.S., and Giffard, P.M., *FEMS Microbiol. Lett.*, 2003, vol. 227, pp. 93–99.
14. Sepp, E., Julge, K., Vasar, M., Naaber, P., Björksten, B., and Mikelsaar, M., *Acta Paediatr.*, 1997, vol. 86, pp. 956–961.
15. Truusalu, K., Naaber, P., Kullisaar, T., Tamm, H., Mikelsaar, R.H., Zilmer, K., Rehema, A., Zilmer, M., and Mikelsaar, M., *Microbial Ecol. Health Disease*, 2004, vol. 16, pp. 180–187.
16. Songisepp, E., Kals, J., Kullisaar, T., Mändar, R., Hütt, P., Zilmer, M., and Mikelsaar, M., *Nutr. J.*, 2005, vol. 20, pp. 4–22.
17. Järvenpää, S., Tahvonen, R.L., Ouwehand, A.C., Sandell, M., Järvenpää, M., and Salminen, S., *J. Dairy Sci.*, 2007, vol. 90, pp. 3171–3177.
18. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J., *J. Biol. Chem.*, 1951, vol. 193, pp. 265–275.
19. Griffith, O.W., *Anal. Biochem.*, 1980, vol. 106, pp. 207–212.
20. Pähkla, R., Zilmer, M., Kullisaar, T., and Rägo L., *J. Pineal Res.*, 1998, vol. 24, pp. 96–101.
21. Liu, X. and Zweier, J.L., *Free Radic. Biol Med.,* 2001, no. 31, pp. 894–901.
22. Aunapuu, M. and Arend, A., *VASA*, 2005, vol. 34, pp. 170–175.

23. Li, Y., Hugenholtz, J., Abee, T., and Molenaar, D., *Appl. Environ. Microbiol.*, 2003, vol. 69, pp. 5739–5745.

24. Yoon, Y.H. and Byun, J.R., *Asian-Australian J. Anim. Sci.,* 2004, vol. 17, pp. 1582–1585.

25. Peran, L., Camuesco, D., Comalada, M., Bailon, E., Henriksson, A., Xaus, J., Zarzuelo, A., and Galvez, J., *J. Appl. Microbiol.*, 2007, vol. 103, pp. 836–844.

26. Janowiak, B.E. and Griffith, O.W., *J. Biol. Chem.*, 2005, vol. 280, pp. 11829–11839.

27. Vergauwen, D., De Vos, D., and Van Beeumen, J., *J. Biol. Chem.* 2005, vol. 8, pp. 662–667.

28. Gopal, S., Borovok, I., Ofer, A., Yanku, M., Cohen, G., and Kreft, J., *J. Bactiriol.*, 2005, vol. 187, pp. 3839–3847.

29. Fu, R.-Y., Bongers, R.S., van Swam, I.I., Chen, J., Molenaar, D., Kleerebezem, M., Hugenholtz, J., and Li, Y., *Metabolic Eng.*, 2006, vol. 8, pp. 662–671.

30. Delaunay, A., Pflieger, D., Barrault, M.B., and Vinh, J., *Cell*, 2002, vol. 111, pp. 471–481.

31. Patel, M.P., Marcinkeviciene, J., and Blanchard, J.S., *FEMS Microbiol. Lett.*, 1998, vol. 166, pp. 155–163.