Identification and Characterization of Hydrogen Peroxide-generating *Lactobacillus fermentum* CS12-1

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ABSTRACT : Lactic acid bacteria were isolated from silage, which produce high level of hydrogen peroxide in cell culture supernatant. The 16S rDNA sequences of the isolate matched perfectly with that of *Lactobacillus fermentum* (99.9%), examined by a 16S rDNA gene sequence analysis and similarity search using the GenBank database, thus named *L. fermentum* CS12-1. *L. fermentum* CS12-1 showed resistance to low pH and bile acid. The production of hydrogen peroxide by *L. fermentum* CS12-1 was confirmed by catalase treatment and high-performance liquid chromatography. *L. fermentum* CS12-1 accumulated hydrogen peroxide in culture broth as cells grew, and the highest concentration of hydrogen peroxide reached 3.5 mM at the late stationary growth phase. The cell-free supernatant of *L. fermentum* CS12-1 both before and after neutralization inhibited the growth of enterotoxigenic *Escherichia coli* K88 that causes diarrhea in piglets. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 1 : 90-95)***

Key Words : Lactic Acid Bacteria, *Lactobacillus fermentum*, Hydrogen Peroxide, Probiotics

INTRODUCTION

Lactic acid bacteria (LAB) are increasingly important as probiotic organism in the human and animal intestinal tract (Gilliland, 1979; Fuller, 1989; Lee and Salminen, 1995; Jin et al., 1996). Until now, many researchers have extensively studied LAB for their potential use as probiotics. (Reinheimer et al., 1990; Ahn et al., 2002; Bae et al., 2002; Pato, 2003; Surono, 2003).

Probiotic LAB produce many kinds of metabolites which might affect bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella spp.*, etc. (Abee et al., 1995; Bae et al., 2003; Ham et al., 2003). The inhibitory activity against pathogens is mostly due to the metabolites, such as organic acids, hydrogen peroxide (H_2O_2), bacteriocins produced by the probiotic bacteria (Gilliland and Speck, 1977; Fuller, 1989; Oh et al., 2003). The antimicrobial properties of hydrogen peroxide have been recognized for many years (Baldry, 1983; Kullisaar et al., 2002; Annuk et al., 2003), and, currently, hydrogen peroxide is widely used in the food industry for aseptic packaging of fluid foods (Ito et al., 2003). Many reports have demonstrated that hydrogen peroxide produced by LAB inhibits the growth of pathogens, such as *Staphyloccus aureus* (Dahiya and Speck, 1968), *Salmonella typhimurium* (Watson and Schubert, 1969), and *Listeria monocytogenes* (Siragusa and Johnson, 1989), therefore, hydrogen peroxide generating LAB could be beneficial for not only prevention of growth of food borne pathogens but also human and animal health as probiotics.

We have selected from corn silage LAB that produce high levels of hydrogen peroxide in cell cultures, and showed that their cell-free filtrates containing hydrogen peroxide were very effective in inhibiting enterotoxigenic *Escherichia coli* K88 that induces piglet diarrhea.

MATERIALS AND METHODS

Screening for lactic acid bacteria that produce hydrogen peroxide

Various lactic acid bacteria (LAB) were isolated from silage and pig feces. Screening for the hydrogen peroxide (H_2O_2) producer was performed using the agar well diffusion method modified from that of Kim and Rajagopal (2001). The culture supernatants of LAB were prepared by growing the cultures in MRS broth for 24 h at 37°C and removing the cells by centrifugation. The supernatant was stored at -60°C before use. Filter sterilized catalase (Sigma) was added to the supernatants to obtain a final concentration of 2.5 mg/ml and was incubated for 3 h at 37°C to examine the loss of antimicrobial activity against *E. coli* K88 by catalase treatment. Prepoured LB (Luria-Burtani) agar plates were overlaid with 4 ml of soft LB agar inoculated with 0.1 ml of 1:100 dilution of an overnight culture of *E. coli* K88. After allowing the media to harden at room temperature for 20 min, wells of 9 mm diameter were made with a sterile cork borer, and 100 μl of the cell-free culture supernatant was placed into each well. The inoculated plates are incubated at 37°C overnight after preincubation for 2 h at 4°C and were subsequently examined for zones of inhibition.

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Table 1. Oligonucleotide primers for amplification of 16S rDNA in lactobacilli (from Pavlova et al., 2002)

Primer*	Sequence (5'-3')	Target site**
F1	AGAGTTTGATCCTGGCTCAG	27
F2	GTGCCAGCAGCCGCGG	530
F3	AAACTCAAAGGAATTGACGG	926
R1	TCTACGCATTCCACCGCTAC	685
R2	GGGTTGCGCTCGTTG	1,100
R3	AAGGAGGTGATCCAGCC	1,525

* F1, F2 and F3: forward primers; R1, R2 and R3: reverse primers.
** According to the numbering of the *Escherichia coli* 16S rDNA.

16S rDNA sequence analysis

16S rDNA sequence analysis of the hydrogen peroxide-producing LAB was performed by the according to the method of Pavlova et al. (2002). To amplify 16S rDNA, universal primers were used corresponding to six conserved regions of the *E. coli* numbering system, as shown in Table 1. The chromosomal DNA of the isolate was isolated using a genomic DNA extraction kit (Qiagen, Germany). Polymerase chain reaction (PCR) was performed in a 50 μl reaction mixture containing primers (50 pmole), template DNA (50 ng), 5 μl of 10×Taq DNA polymerase buffer, 4 μl of 2.5 mM dNTP, and 1 U of Taq DNA polymerase (Takara-Korea Biomedical, Korea). The PCR amplification product was purified using a QIAquick gel extraction kit (Qiagen, Germany), ligated into a pSTBlue-1 vector (Novagen, USA), and transformed into *E. coli* DH5α competent cells. The recombinant plasmids were purified using a DNA purification kit (Promega, USA) and digested with *Eco*RI to confirm the insert. The nucleotide sequence of the insert was determined using a BigDye^{TM}-terminator sequencing kit and ABI PRISM 377 sequencer (Perkin-Elmer, USA), according to the manufacture's instructions. The 16S rDNA sequences were subjected to a similarity search using the GenBank database.

Scanning electron microscopy (SEM)

One typical colony on MRS agar was selected and transferred to filter paper. Then it was fixed with 8% paraformaldehyde and 3% glutaraldehyde in a cacodylate buffer overnight. After washing and dehydration, the sample was coated with gold and observed under a Philips SEM (XL30CP).

Acid tolerance and bile tolerance

Survival of the isolate in MRS broth adjusted between pH 2 to 4 with 1 N HCl was determined. Culture samples were taken up to 4 h of incubation to count surviving cells. Bile tolerance of the isolate was tested in MRS broth containing 0.25 and 0.5% (w/v) bile extract (Sigma) and incubated at 37°C for 24 h. The surviving cells were counted after grown on MRS agar at 37°C.

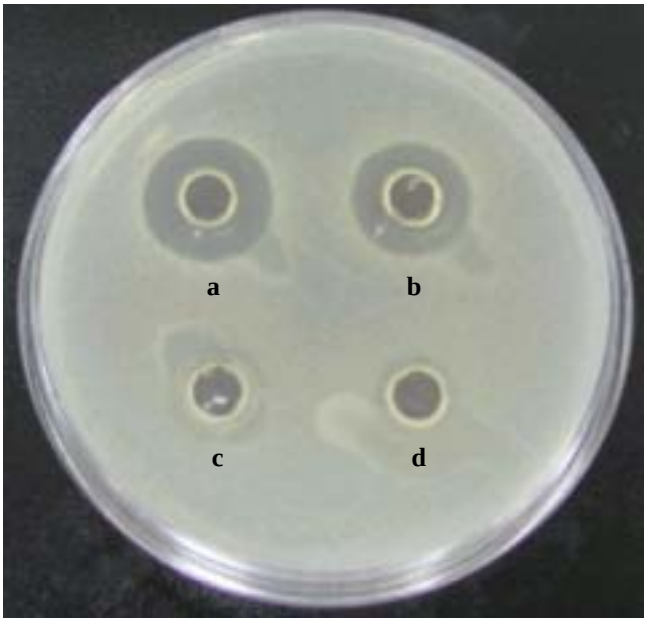

Figure 1. Antimicrobial activity of the cell-free filtrate of the LAB isolate. (a) culture supernatant (b) culture supernatant neutralized (c) culture supernatant after catalase treatment (d) culture supernatant neutralized after catalase treatment.

Assay for hydrogen peroxide by high-performance liquid chromatography (HPLC) analysis

Reverse phase HPLC based on the method of Barnard and Stinson (1996) was used for analyzing hydrogen peroxide. The culture supernatant incubated for 24 h at 37°C was filtrated before use. 20 μl aliquots, with or without catalase treatment for 3 h at 37°C, were subjected to reverse phase HPLC (Jasco, Japan) with a UV-absorbance detector (UV-1575) on a C_{18} column (4.5 by 250 mm) (Altex Scientific Inc, USA). The mobile phase was aqueous 1 mM HCl at a flow rate of 0.5 ml/min and was monitored for A_{225}.

Hydrogen peroxide production during the growth

To determine the levels of hydrogen peroxide quantitatively along with the growth stage of LAB, the spectrophotometric method was used as previously described (Barnard and Stinson, 1996). Briefly, culture supernatants, aerobically cultivated in cotton-plugged Erlenmeyer flasks with shaking, were mixed with 2 U of horseradish peroxidase (Sigma, USA) and 0.1 μmol of 2, 2'-Azino-Bis-(3-ethylbenz-Thiazoline)-6-Sulfonic acid (Sigma, USA) in a total volume of 1 ml of 0.1 M NaH_2PO_4-Na_2HPO_4 (pH 6.0). The colorimetric reaction was allowed to develop for 2 min, and then the A_{414} was read immediately versus a no-peroxide blank. The concentration of hydrogen peroxide was found by generating a standard curve.

Figure 2. Scanning electron micrograph of the LAB isolate producing hydrogen peroxide. Bar represents 1 μm.

RESULTS

Screening for hydrogen peroxide-producing lactic acid bacteria

To search for hydrogen peroxide-producing LAB, 100 μl of the cell-free filtrate of the isolates with and without catalase treatment was placed into each well of LB (Luria-Bertani) agar plate overlaid with LB soft agar containing *E. coli* K88 as an indicator strain, and incubated at 30°C overnight. The cell-free filtrate of some isolate without catalase treatment (Figure 1a) was inhibitory to *E. coli* K88, while those treated with catalase (Figure 1c) lost almost antimicrobial activity. The inhibition of *E. coli* K88 was not due to acid or low pH, because the neutralized supernatant without catalase treatment (Figure 1b) showed antimicrobial activity while those treated with catalase (Figure 1d) lost antimicrobial activity. A little of residual antimicrobial activity may be due to bacteriocin (data not shown), which should be confirmed by further study. These results indicate that the main inhibitory factor of the isolate against *E. coli* K88 is hydrogen peroxide.

Identification of hydrogen peroxide-producing lactic acid bacteria

The isolate producing hydrogen peroxide was found to be the Gram-positive, non-motile, catalase-negative, and non-spore forming strain (data not shown). Under an electron microscope, the cells were observed to be rod-shaped (Figure 2). The isolate was examined by a 16S rDNA sequence analysis. To amplify 16S rDNA, universal

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   1 catgcaagtcgaacgcgttggcccaattgattgatggtgcttgcacctgattgattttgg   60
  61 tcgccaacgagtggcggacgggtgagtaacacgtaggtaacctgcccagaagcgggggac  120
 121 aacatttggaaacagatgctaataccgcataacagcgttgttcgcatgaacaacgcttaa  180
 181 aagatggcttctcgctatcacttctggatggacctgcggtgcattagcttgttggtgggg  240
 241 taacggcctaccaaggcgatgatgcatagccgagttgagagactgatcggccacaatggg  300
 301 actgagacacggcccatactcctacgggaggcagcagtagggaatcttccacaatgggcg  360
 361 caagcctgatggagcaacaccgcgtgagtgaagaagggtttcggctcgtaaagctctgtt  420
 421 gttaaagaagaacacgtatgagagtaactgttcatacgttgacggtatttaaccagaaag  480
 481 tcacggctaactacgtgccagcagccgcggtaatacgtaggtggcaagcgttatccggat  540
 541 ttattgggcgtaaagagagtgcaggcggttttctaagtctgatgtgaaagccttcggctt  600
 601 aaccggagaagtgcatcggaaactggataacttgagtgcagaagagggtagtggaactcc  660
 661 atgtgtagcggtggaatgcgtagatatatggaagaacaccagtggcgaaggcggctacct  720
 721 ggtctgcaactgacgctgagactcgaaagcatgggtagcgaacaggattagataccctgg  780
 781 tagtccatgccgtaaacgatgagtgctaggtgttggagggtttccgcccttcagtgccgg  840
 841 agctaacgcattaagcactccgcctggggagtacgaccgcaaggttgaaactcaaaggaa  900
 901 ttgacgggggcccgcacaagcggtggagcatgtggtttaattcgaagctacgcgaagaac  960
 961 cttaccaggtcttgacatcttgcgccaaccctagagatagggcgtttccttcgggaacgc 1020
1021 aatgacaggtggtgcatggtcgtcgtcagctcgtgtcgtgagatgttgggttaagtcccg 1080
1081 caacgagcgcaacccttgttactagttgccagcattaagttgggcactctagtgagactg 1140
1141 ccggtgacaaaccggaggaaggtggggacgacgtcagatcatcatgccccttatgacctg 1200
1201 ggctacacacgtgctacaatggacggtacaacgagtcgcgaactcgcgagggcaagcaaa 1260
1261 tctcttaaaaccgttctcagttcggactgcaggctgcaactcgcctgcacgaagtcggaa 1320
1321 tcgctagtaatcgcggatcagcatgccgcggtgaatacgttcccgggccttgtacacacc 1380
1381 gcccgtcacaccatgagagtttgtaacacccaaagtcggtggggtaacctttaggagcc 1440
1441 agccgcctaaggtgggacagatgattagggtgaagtcgtaacaaggtagccgta      1494
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Figure 3. 16S rDNA sequences of the isolate producing hydrogen peroxide.

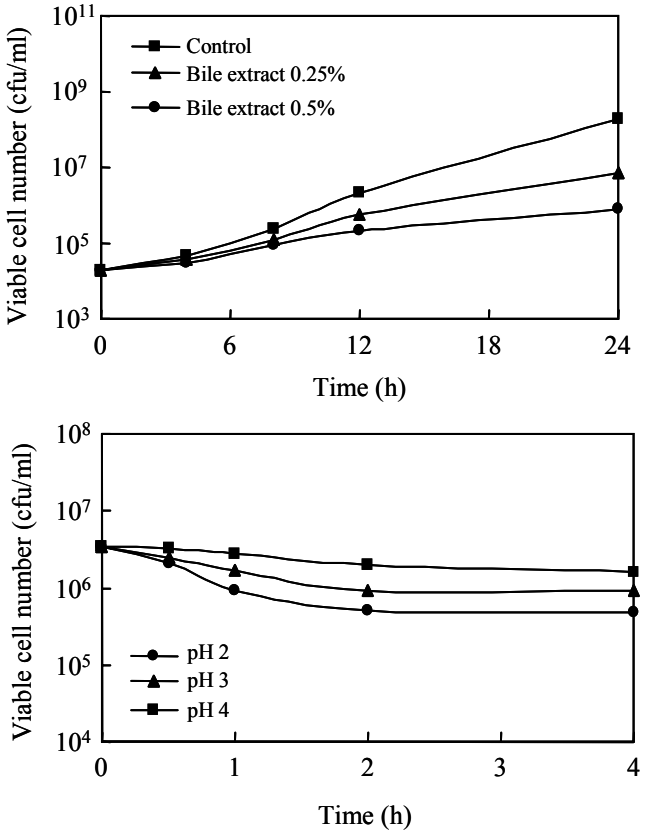

Figure 4. Acid (a) and bile acid (b) tolerance of *L. fermentum* 12-1 in MRS broth.

primers were used corresponding to six conserved regions of the *E. coli* numbering system (Pavlova et al., 2002). It was amplified by PCR and subjected to a similarity search using the GenBank database. The16S rDNA sequences (Figure 3) of the isolate matched perfectly with that of *Lactobacillus fermentum* (99.9%). The utilization of carbohydrates showed the same result (data not shown), therefore, the isolate was named *L. fermentum* CS12-1.

Acid and bile tolerance

The isolate was investigated for acid and bile tolerance. As shown in Figure 4a, *L. fermentum* CS12-1 has high viability in acidic conditions. It survived after incubation for 4 h in MRS broth adjusted to pH 2.0. Bile tolerance of *L. fermentum* CS12-1 was investigated, and the result is shown in Figure 4b. Although the growth rate of *L. fermentum* CS12-1 decreased with increasing concentration of bile extract, it grew well in the presence of 0.5% (w/v) bile in MRS broth. Therefore, it is shown that *L. fermentum* CS12-1 is highly tolerant to acid and bile stress conditions. Acid tolerance and bile tolerance is important characteristics for the survival and growth of the bacteria in the intestinal tract (Gilliland, 1979; Conway et al., 1987).

Hydrogen peroxide assay by HPLC

To confirm the hydrogen peroxide production by *L.*

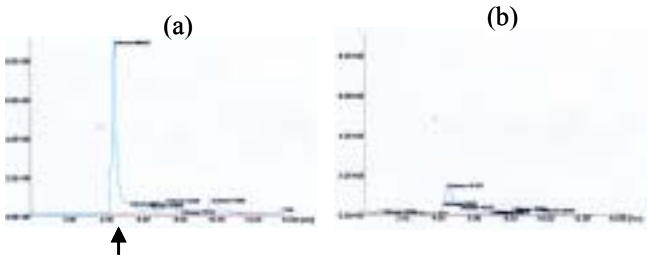

Figure 5. Reverse-phase HPLC profiles of the cell-free filtrate of *L. fermentum* CS12-1 containing hydrogen peroxide. (a) hydrogen peroxide peak before catalase treatment (b) hydrogen peroxide peak after catalase treatment. The elution time of pure hydrogen peroxide as standard indicated by arrow in (a).

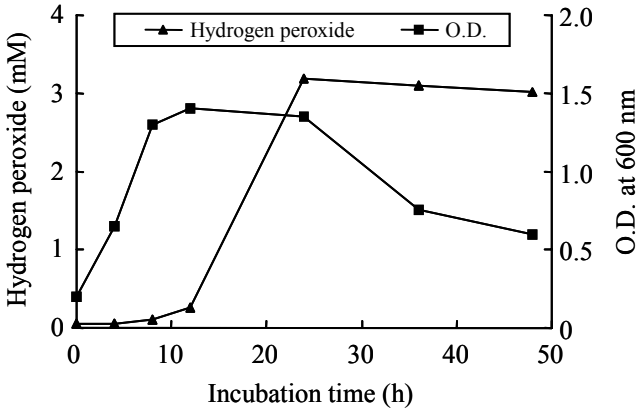

Figure 6. The production of hydrogen peroxide by *L. fermentum* CS12-1 along with cell growth. The values were the means of triplicate experiments.

fermentum CS12-1, the culture supernatant incubated in MRS broth for 24 h at 37°C and was subjected to reverse-phase HPLC in 1 mM HCl. Peak identification was based on the retention time, with hydrogen peroxide as the standard. As seen in Figure 5a, hydrogen peroxide had a retention time of 4 min and 30 sec. When the culture supernatant was incubated with catalase, most of the corresponding peak disappeared (Figure 5b). This observation strongly suggests that *L. fermentum* CS12-1 produces hydrogen peroxide, which inhibits enterotoxigenic *E. coli* K88.

Hydrogen peroxide production along with growth

We investigated the accumulation of hydrogen peroxide in aerated cultures as the cells grew. As shown in Figure 6, *L. fermentum* CS12-1 accumulated hydrogen peroxide as the cells grew, and the highest concentration of hydrogen peroxide in the culture medium was 3.5 mM, which was reached at the late stationary growth phase. A correlation was found between hydrogen peroxide production and antimicrobial activity against *E. coli* K88. Meanwhile, there was no detectable level of hydrogen peroxide in non-agitated growth (data not shown).

DISCUSSION

In this study, we have found the hydrogen peroxide-producing lactic acid bacteria and identified it as *L. fermentum* CS12-1. Production of hydrogen peroxide by LAB has been documented previously. Sakamoto and Komagata (1996) reported that *L. delbrueckii* subsp. *delbrueckii* NRIC1053 accumulated 4.9 mM of hydrogen peroxide in aerobic cultures. The levels of H_2O_2 in *L. crispatus* supernatant increased during its growth and reached its maximum level during the early stationary phase (3.29 mmol H_2O_2 L^{-1}) under aerated conditions (Ocana et al., 1999). Inhibition of pathogenic microorganisms by hydrogen peroxide or hydrogen peroxide-generating LAB has been demonstrated by many authors. Collins and Aramaki (1980) reported that some strains of *L. acidophilus* from dairy origins were able to inhibit *Pseudomonas* species by producing 1.18 to 1.62 mM of hydrogen peroxide in agitated cultures. Venkitanarayanan et al. (2002) reported that hydrogen peroxide with lactic acid effectively killed foodborne pathogens such as *E. coli* O157:H7, *Salmonella enteritidis*, and *Listeria monocytogenes*. Currently, hydrogen peroxide is widely used in the food industry for the asceptic packaging of fluid food (Ito et al., 2003). In addition, Hawes et al. (1996) found that vaginal colonization with hydrogen peroxide-producing lactobacilli was associated with a decrease in the occurrence of bacterial vaginosis.

The bactericidal effect of hydrogen peroxide has been attributed to its strong oxidizing effect on the bacterial cell and to the destruction of basic molecular structures of cell protein (Lindgren and Dobrogosz, 1990). Up to now, it is uncertain how *L. fermentum* CS12-1 generates hydrogen peroxide, though, in the cell lysate of *L. fermentum* CS12-1 (data not shown), we have detected pyruvate oxidase activity which catalyzes the oxidative decarboxylation of pyruvate for yielding hydrogen peroxide (Sedewitz et al., 1984). Further study to elucidate the mechanism for the generation of hydrogen peroxide by this microorganism remains. In addition, studies on the *in vivo* antagonistic effect of *L. fermentum* CS12-1 against intestinal pathogenic microorganisms should be investigated.

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REFERENCES

Abee, T., L. Krockel and C. Hill. 1995. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. Int. J. Food Microbiol. 28(2):169-185.

Ahn, Y. T., K. L. Lim, J. C. Ryu, D. K. Kang, J. S. Ham, Y. H. Jang and H. U. Kim. 2002. Characterization of *Lactobacillus acidophilus* isolated from piglets and chicken. Asian-Aust. J. Anim. Sci. 15(10):1790-1797.

Annuk, H. J., J. Shchepetova, T. Kullisaar, E. Songisepp, M. Zilmer and M. Mikelsaar. 2003. Characterization of intestinal lactobacilli as putative probiotic candidates. J. Appl. Microbiol. 94(3):403-412.

Bae, H. C., M. S. Nam and J. Y. Lee. 2002. Probiotic characterization of acid- and bile-tolerant *Lactobacillus salivarius* subsp. *salivarius* from Korean faeces. Asian-Aust. J. Anim. Sci. 15(12):1798-1807.

Bae, J.-S., J.-R. Byun and Y.-H Yoon. 2003. *In vivo* antagonistic effect of *Lactobacillus helveticus* CU631 against *Salmonella enteritidis* KU101 infection. Asian-Aust. J. Anim. Sci. 16(3):430-434.

Baldry, M. G. 1983. The bactericidal, fungicidal and sporicidal properties of hydrogen peroxide and peracetic acid. J. Appl Bacteriol. 54(3):417-423.

Barnard, J. P. and M. W. Stinson. 1996. The alpha-hemolysin of *Streptococcus gordonii* is hydrogen peroxide. Infection and Immunity. 64(9):3853-3857.

Collins, E. B. and K. Aramaki. 1980. Production of hydrogen peroxide by *Lactobacillus acidophilus*. J. Dairy Sci. 63(3):353-357.

Conway, P. L., S. L Gorbach and B. R. Goldin. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci. 70(1):1-12.

Dahiya, R. S. and M. L. Speck. 1968. Hydrogen peroxide formation by lactobacilli and its effect on *Staphylococcus aureus*. J. Dairy Sci. 51(10):1568-1572.

Fuller, R. 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66:365-378.

Gilliland, S. E. and M. L. Speck. 1977. Antagonistic action of *Lactobacillus acidophilus* toward intestinal and foodborne pathogens in associative cultures. J. Food Prot. 40:820-823.

Gilliland, S. E. 1979. Beneficial interrelationships between certain microorganisms and humans; candidate microorganisms for use as dietary adjuncts. J. Food Prot. 42:164-167.

Ham, J. S., H. S. Kim, K. H. Hong, J. G. Kim, S. G. Geong, H. S. Chae, J. N. Ahn, D. K. Kang and H. U. Kim. 2003. Inhibitory activity of lactic acid bacteria against hazardous microbes. Asian-Aust. J. Anim. Sci. 16(10):1550-1554.

Hawes, S. E., S. L. Hillier, J. Benedetti, C. E. Stevens, L. A. Koutsky, P. Wolner-Hanssen and K. K. Holmes. 1996. Hydrogen peroxide-producing lactobacilli and acquisition of vaginal infections. J. Infect. Dis. 174(5):1058-1063.

Ito, A., Y. Sato, S. Kudo, S. Sato, H. Nakajima and T. Toba. 2003. The screening of hydrogen peroxide-producing lactic acid bacteria and their application to inactivating psychrotrophic food-borne pathogen. Current Microbiol. 47:231-236.

Jin, L. Z., Y. W. Ho, M. A. Ali, N. Abdullah and S. Jalaludin. 1996. Effect of adherent *Lactobacillus spp.* on *in vitro* adherence of salmonellae to the intestinal epithelial cells of chicken. J. Appl. Bacteriol. 81:201-206.

Kim, J.-W. and S. N. Rajagospal. 2001. Antibacterial activities of *Lactobacillus crispatus* ATCC 33820 and *Lactobacillus gasseri* ATCC 33323. J. Microbiol. 39(2):146-148.

Kullisaar, T., M. Zilmer, M. Mikelsaar, T. Vihalemm, H. Annuk, C. Kairane and A. Kilk. 2002. Two antioxidative lactobacilli

strains as promising probiotics. Int. J. Food Microbiol. 72(3):215-224.

Lee, Y. K. and S. Salminen. 1995. The coming of age of probiotics. Trends Food Sci. Tech. 6:241-245.

Lindgren, S. E. and W. J. Dobrogosz. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev. 7(1-2):149-163.

Ocana, V. S., A. P. Holgado and M. E. Nader-Macias. 1999. Selection of vaginal H_2O_2-generating *Lactobacillus* species for probiotic use. Current Microbiol. 38:279-284.

Oh, S. J., M. H. Kim, J. J. Churey and R. W. Worobo. 2003. Purification and characterization of an antilisterial bacteriocin produced by *Leuconostoc* sp. W65. J. Microbiol. Biotechnol. 13(5):680-686.

Pato, U. 2003. Bile and acid tolerance of lactic acid bacteria isolated from dadhi and their antimutagenicity against mutagenic heated tauco. Asian-Aust. J. Anim. Sci. 16(11):1680-1685.

Pavlova, S. I., A. O. Kilic, S. S. Kilic, J.-S. So, M. E. Nader-Macias, J. A. Simoes and L. Tao. 2002. Genetic diversity of vaginal lactobacilli from women in different countries based on 16S rRNA gene sequences. J. Appl. Microbiol. 92:451-459.

Piard, J. C. and M. Desmazeau. 1991. Inhibiting factors produced by lactic acid bacteria. Oxygen metabolites and catabolism end products. Le Lait. 71:525-541.

Reinheimer, J. A., M. R. Demkow and M. C. Candioti. 1990. Inhibition of coliform bacteria by lactic cultures. Aust. J. Dairy Technol. 45:5-9.

Sakamoto, M. and K. Momagata. 1996. Aerobic growth and activities of NADH oxidase and NADH peroxidase in lactic acid bacteria. J. Ferment. Bioeng. 82(3):210-216.

Sedewitz, B., K. H. Schleifer and F. Gotz. 1984. Purification and biochemical chracterization of pyruvate oxidase from *Lactobacillus plantarum*. J. Bacteriol. 160(1):273-278.

Siragusa, G. R. and M. G. Johnson. 1989. Inhibition of *Listeria monocytogenes* growth by the lactoperoxidase-thiocyanate-H_2O_2 antimicrobial system. Appl. Environ. Microbiol. 55(11):2802-2805.

Surono, I. S. 2003. *In vitro* probiotic perperties of indigenous dadhi lactic acid bacteria. Asian-Aust. J. Anim. Sci. 16(5):726-731.

Venkitanarayanan, K. S., C.-M. Lin, H. Bailey and M. P. Doyle. 2002. Inactivation of *Escherichia coli* O157:H7, *Salmonella enteritidis* and *Listeria monocytogenes* on on apples, oranges, and tomatoes by lactic acid with hydrogen peroxide. J. Food Protection. 65(1):100-105.

Watson, J. A. and J. Schubert. 1969. Action of hydrogen peroxide on growth inhibition of *Salmonella typhimurium*. J. Gen. Microbiol. 57(1):25-34.