Inhibitiory Activity of Lactic Acid Bacteria against Hazardous Microbes

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ABSTRACT : One hundred of lactic cultures were evaluated for their ability to inhibit hazardous microbes, such as *Salmonella enteritidis*, *Salmonella typhimurium*, *Escherichia coli*, *Listeria monocytogenes*, and *Bacillus cereus* by agar well diffusion method. None of them showed inhibitory halo against *S. enteritidis*, while 27 strains showed inhibitory activity against *S. typhimurium*, 6 against *E. coli*, 9 against ampicillin resistant *E. coli*, 31 against *L. monocytogens*, 10 against *B. cereus*. pH of the culture does not explain for the inhibitory activity except against *B. cereus*. A neutralized culture from corn silage showed highest inhibitory activity against *S. typhimurium*, and the size of inhibitory halo was same as 10 ug/mL of ampicillin. The culture was identified to be *Lactobacillus buchneri* on the basis of biochemical characteristics and utilization of substrates. Using the culture as probiotics could be expected to reduce antibiotics for animal feeding. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 10 : 1550-1554)***

Key Words : Lactic Acid Bacteria, Inhibitory Activity

INTRODUCTION

Probiotics are viable bacterial cell preparation or foods containing viable bacterial cultures or components of bacterial cells that have beneficial effects on the health of the host (Delley et al., 1990; Hertel et al., 1991; Ehrmann et al., 1992; Ehrmann et al., 1994; Charteris et al., 1997). Many of these probiotics are lactic acid bacteria (Lee et al., 1999). The pioneering evidence of the competitive exclusion concept of lactic acid bacteria was obtained from poultry chickens by Nurmi and Rantala (1973). The newely hatched birds do not obtain the normal gut flora of the adult, owing to modern management methods. Since normal flora is lacking, the intestines of the birds are easily colonized by pathogens, most often by *Salmonella* or Coliforms. When the chickens were inoculated just after birth with the caecal contents of an adult bird, the frequency of *Salmonella* infections was radically reduced and the number of *Salmonella* needed to colonize the caeca of the birds increased. The specific role of lactic acid bacteria as a probiotic has been extensively discussed by Juven et al. (1991). Lactic acid bacteria produce many kinds of metabolites which might affect the other microbes in the gut. Lactic acid produced both by homolactic and heterolactic strains reduce pH of the luminal contents, which is most obvious in the stomach of neonatal piglets (Cranwell et al., 1976). Moreover, acetic acid and hydrogen peroxide produced by lactic acid bacteria are inhibitory against coliforms, *salmonella*, and clostridia *in vitro* (Nousiainen, 1993).

Lactic acid bacteria play an essential role in the majority of food fermentations, and a wide variety of strains are routinely employed as starter cultures in the manufacture of dairy, meat, vegetable and bakery products. One of the most important contributions of these microorganisms is the extended shelf life in the fermented product by comparison to that of the raw substance. Growth of spoilage and pathogenic bacteria in these foods is inhibited due to competition for nutrients and the presence of starter-derived inhibitors such as lactic acid, hydrogen peroxide and bacteriocins (Ray and Daeschel, 1992). Currently, artificial chemical preservatives are employed to limit the number of microorganisms capable of growing within foods, but increasing consumer awareness of potential health risks associated with some of these substances has led researchers to examine the possibility of using bacteriocins produced by lactic acid bacteria as biopreserativates (Abee, 1995). However, the inhibitory activity of bacteriocins is confined to Gram-positive bacteria and inhibition of Gram-negatives by them has not been demonstrated.

The aim of this study was to screen lactic bacterial cultures having inhibitory activity against hazardous bacteria for the development of animal probitics and food biopreservatives.

MATERIALS AND METHODS

Microorganisms and media

The *Lactobacilli* cultures were obtained from ATCC and KTCC, and isolated from silages and dairy products. Isolation from silages and dairy products was carried out by incubating the samples in MRS agar containing sodium azide for 2 days. Acid producing, Gram positive, and rod cultures were presumed as *Lactobacillus* organisms. Acid production was measured by pH, and shape of the cell was

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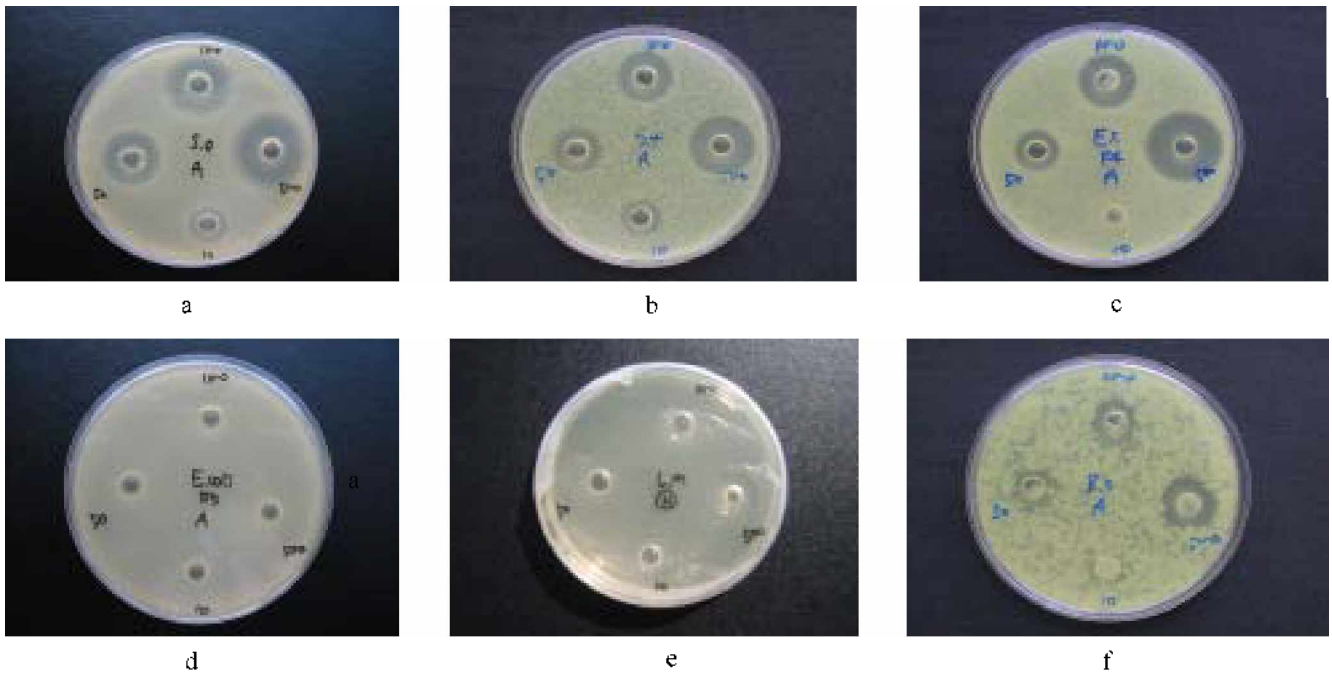

Figure 1. Inhibitory activity of antibiotics against hazardous microbes according to concentration (from 10 to 500 μg/mL).
a: ampicillin against *Salmonella enteritidis* IFO 3313, b: ampicillin against *Salmonella typhimurium* IFO 12529, c: ampicillin against *E. coli* F4, d: ampicillin against *E. coli* F5, e: nisin against *Listeria monocytogene* ATCC 15313, f: ampicillin against *Bacillus cereus* ATCC 11778.

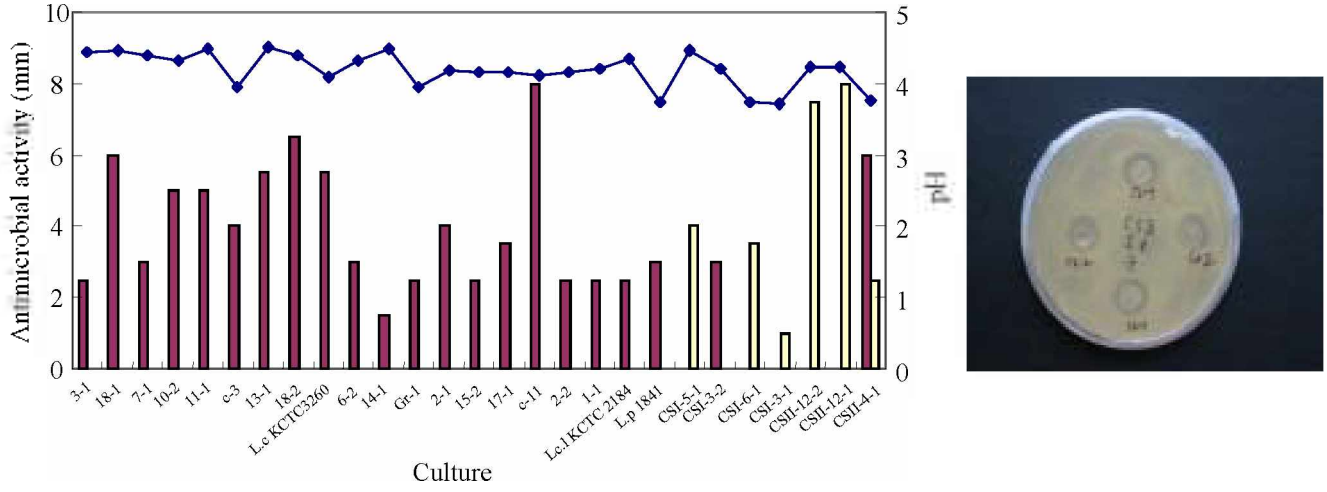

Figure 2. Inhibitory activity of lactic acid bacterial cultures against *Salmonella typhimurium*.
Line: pH of the culture, red stick: antimicrobial activity of lactic cultures, yellow stick: antimicrobial activity of neutralized lactic cultures, photograph: inhibitory halo of the neutralized CSII12-1 and CSII-2 culture against *S. typhimurium*.

examined using light microscopy (×1,500) (Clinigold, USA) after Gram-staining. Identification of *Lactobacillus* species was conducted by API 50CHL kit (bioMerieux, France) and the software (APILAB Plus Ver. 3.3.3; bioMerieux, France). *S. enteritidis* IFO 3313, *S. typhimurium* IFO 12529, *E. coli* F4 and F5, *L. monocytogenes* ATCC 15313, and *B. cereus* ATCC 11778 were maintained by subcultured in tryptic soy broth (TSB) (Difco). *E. coli* F4 and F5 were donated by Lab. of Veterinary Medicine, Seoul National University.

Inhibitory activity of lactic acid bacteria

Agar well diffusion method was modified from that of Tagg and McGIVEN (1971). Petri dishes with metal borer of 8 mm diameter are poured with tryptic soy agar (TSA) and inoculated by testing strain. By removing borer after solidification, the base of each hole was sealed with a drop (0.05 mL) of melted TSA, and then either 100 μl of lactic

culture or neutralized lactic culture filtrate was added to the well. The inoculated plates are incubated at 37°C for 1 day after overnight preincubation at 4°C to allow for diffusion of the inhibiting factors. 10, 50, 100, and 500 μg/mL of ampicillin and nisin (Sigma, USA) were used as control.

RESULTS AND DISCUSSION

The inhibitory activity of ampicillin and nisin against *S. enteritidis* IFO 3313, *S. typhimurium* IFO 12529, *E.coli* F4 and F5, *L. monocytogenes* ATCC 15313, and *B. cereus* ATCC 11778 by agar well diffusion method according to concentration is given in Figure 1. Nisin showed inhibitory activity only against *L. monocytogens*, while ampicillin has inhibitory activity against all of them except *L. monocytognes* and *E. coli* F5. Although *B. cereus* generally shows high resistance to ampicillin, colistin and polymyxin (Bernhard et al., 1981), the type of antibiotic and the concentration affecting vegetative growth of *Bacillus* strains may differ (Russell, 1982; Ochi and Freese, 1983).

Among those hundred strains of lactic acid bacteria, no strain was found to have inhibitory activity against *S. enteritidis*. Human illness caused by infection with *Salmonella enterica* serovar Enteritidis (*S. enteritidis*) increased worldwide beginning as early as the mid-1970s and, by 1990, this serovar displaced *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) as the primary cause of Salmonellosis in the world (Baumler et al., 2000; Anonymous, 2001). It can be assumed that inhibition of *S. enteritidis* by lactic acid bacteria seems have little possibility, but possible in case of *S. typhimurium*. 22 strains of LAB cultures and 6 strains of neutralized LAB cultures showed inhibitory activity against *S. typhimurium* (Figure 2). One strain showed inhibitory activity in both culture and neutralized culture. Among those strains, C-11, CSII-12-1, and CSII-12-2 showed higher inhibitory activity than that of others. C-11 strain isolated from rye silage was

Table 1. Utilization of carbohydrate substrates of the C-11

Glycerol	-	Mannitol	-	D-raffinose	+
Erythritol	-	Sorbitol	-	Amidon	-
D-arabinose	-	α-Methyl-D-mannoside	-	Glycogen	-
L-arabinose	-	α-methyl-D-glucoside	-	Xylitol	-
Ribose	+	N acetyl glucosamin	-	β-gentiobiose	-
D-xylose	-	Amygdalin	-	D-turanose	-
L-xylose	-	Arbutin	-	D-lyxose	-
Adonitol	-	Esculin	-	D-tagatose	-
β-methyl-D-xyloside	-	Salicin	-	D-fucose	-
Galactose	-	Cellobiose	-	L-fucose	-
D-glucose	+	Maltose	+	D-arabitol	-
D-fructose	+	Lactose	-	L-arabitol	-
D-mannose	-	Melibiose	-	Gluconate	+
L-sorbose	-	Saccharose	+	2-keto-gluconate	-
Rhamnose	-	Trehalose	-	5-keto-gluconate	+
Dulcitol	-	Inulin	-		
Inositol	-	Melezitose	-		

Table 2. Utilization of carbohydrate substrates of the CSII12-1(CSII12-2)

Glycerol	-	Mannitol	-	D-raffinose	+
Erythritol	-	Sorbitol	-	Amidon	-
D-arabinose	-	α-methyl-D-mannoside	-	Glycogen	-
L-arabinose	+	α-methyl-D-glucoside	-	Xylitol	-
Ribose	+	N acetyl glucosamin	-	β-gentiobiose	-
D-xylose	+	Amygdalin	-	D-turanose	-
L-xylose	-	Arbutin	-	D-lyxose	-
Adonitol	-	Esculin	-	D-tagatose	-
β-methyl-D-xyloside	-	Salicin	-	D-fucose	-
Galactose	+(-)	Cellobiose	-	L-fucose	-
D-glucose	+	Maltose	+	D-arabitol	-
D-fructose	+	Lactose	+(-)	L-arabitol	-
D-mannose	-	Melibiose	-	Gluconate	+
L-sorbose	-	Saccharose	+	2-keto-gluconate	-
Rhamnose	-	Trehalose	-	5-keto-gluconate	+
Dulcitol	-	Inulin	-		
Inositol	-	Melezitose	-		

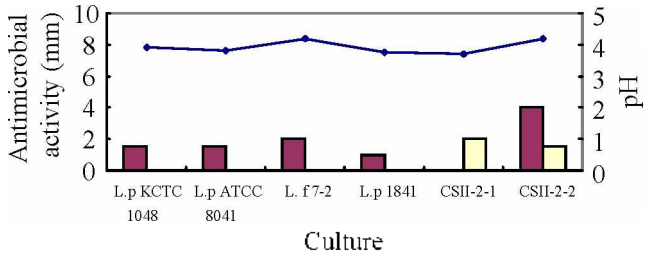

Figure 3. Inhibitory activity of lactic acid bacterial cultures against *Escherichia coli* F4.
Line: pH of the culture, red stick: antimicrobial activity of lactic cultures, yellow stick: antimicrobial activity of neutralized lactic cultures

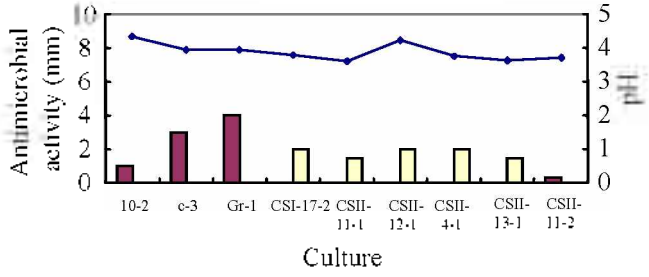

Figure 4. Inhibitory activity of lactic acid bacterial cultures against *Escherichia coli* F5. Line: pH of the culture, red stick: antimicrobial activity of lactic cultures, Yellow stick: antimicrobial activity of neutralized lactic cultures.

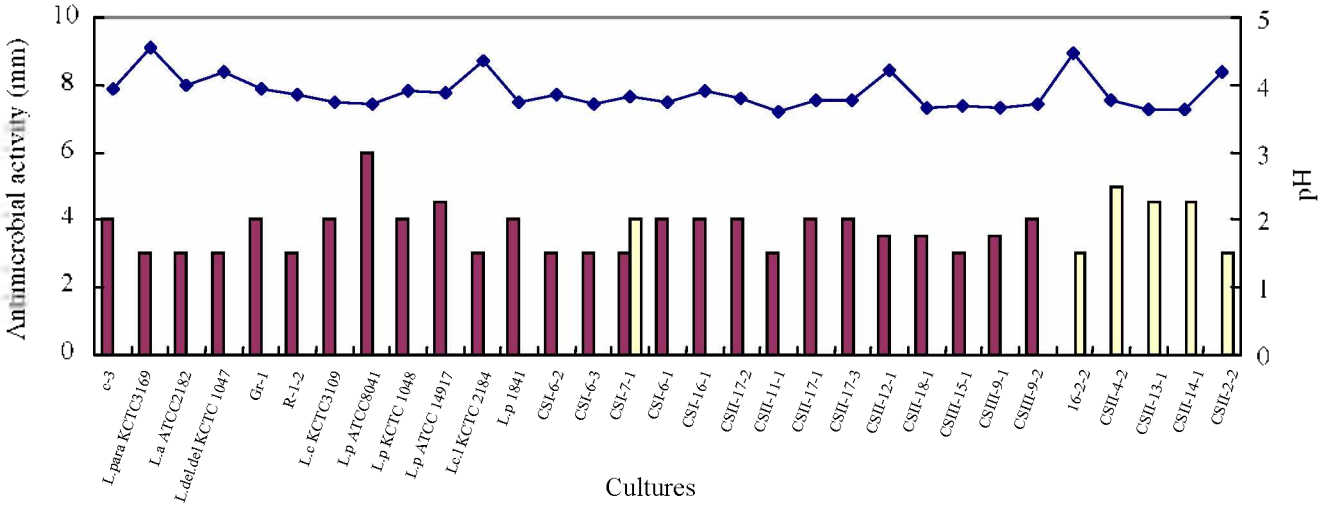

Figure 5. Inhibitory activity of lactic acid bacterial cultures against *Listeria monocytogenes*. Red stick: Antimicrobial activity of lactic cultures, yellow stick: Antimicrobial activity of neutralized lactic cultures.

identified to be *L. buchineri* by API test (Table 1). CSII-12-1 and CSII-12-2 isolated from corn silage were also (Table 2). C-11 and CSII-12-2 could not utilize lactose and galactose. Inhibitory activity of the neutralized cultures might from bacteriocin produced by the LAB because the inhibitory activity of acid would be eliminated and that of hydrogen peroxide would not be made by neutralization. Antibiotic activity of bacteriocin could be affected by pH (Liu and Hansen. 1990). Purification of the bacteriocin is under studying. 5 strains of LAB cultures and 2 strains of neutralized LAB cultures showed inhibitory activity against *E. coli* F4 (Figure 3). One strain showed inhibitory activity in both culture and neutralized culture. But all of them were weak and less than 5 mm. 4 strains of LAB cultures and 5 strains of neutralized LAB cultures showed inhibitory activity against *E. coli* F5 (Figure 4). Because *E. coli* F5 weren't inhibited by ampicillin, further study is required about the inhibiting agent of the neutralized cultures. Especially, neutralized CSII-12-1 culture showed inhibitory activity against *E. coli* F5 as well as *S. typhimurium*. 26 strains of LAB cultures and 6 strains of neutralized LAB cultures showed inhibitory activity against *L. monocytogenes* (Figure 5). It was thought that not many strains showed bacteriocin activity comparing the report of Aroutcheva et al. (2001). Accroding to the report, approximately 80% of the isolated *lactobacilli* from vaginal specimens produced bacteriocin that inhibited growth of 4 strains of *Gardnerella vaginalis*. However, not all strains of *G. vaginalis* could be inhibited by lactobacilli-producing bacteriocin. It was noted that under pH 4 of the cultures could inhibit *Bacillus cereus*, and no neutralized strain could (Figure 6). Role of probiotics in health and diseases is well summarized in 'Handbook of probiotics' (Lee et al., 1999). Orally administered yoghurt bacteria could protect against *S. typhimurium* infection in Male Sprague-Dawley rat. Intraperitoneal administration of *L. casei* strain Shirota could protect against *L. monocytogenes* in male BALB/c mice. It can be inferred from the results, the screened lactic acid bacteria might be useful as a probiotics for protection against hazardous microbes infection with the experimental results in animal model.

In conclusion, inhibitory activity of one hundred lactic

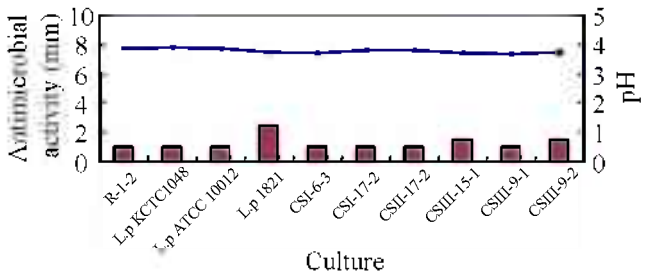

Figure 6. Inhibitory activity of lactic acid bacterial cultures against *Bacillus cereus*. Red stick: Antimicrobial activity of lactic cultures.

acid bacteria strains against hazardous bacteria. *S. enteritidis*, *S. typhimurium*, *E. coli*, *L. monocytogenes*, and *B. cereus* was compared by agar well diffusion method, and *L. buchneri* neutralized culture filtrate from corn silage showed inhibitory activity same as that of 10 μg/ml ampicillin against *S. typhimurium*. Using the culture as probiotics could be expected to reduce antibiotics usage for animal feeding.

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. 28:169-185.

Aroutcheva, A., D. Gariti, M. Simon, S. Shott, J. Faro, J. A. Simoes, A. Gurguis, and S. Faro. 2001. Defense factors of vaginal lactobacilli. Am. J. Obstet. Gynecol. 185:375-379.

Anonymous. 2001. *Salmonella* in Humans: England and Wales (1981-99). Public health Laboratory Service (http://www.phls.co.uk).

Baumler, A. J., B, M. Hargis and R. M. Tsolis. 2000. Tracing the origins of *Salmonella* outbreaks. Science 287:50-52.

Bernhard, K., H. Schrempf and W. Goebel. 1981. Bacteriocin and antibiotic resistance plasmids in *Bacillus cereus* and *Bacillus subtilis*. J. Bacteriology 133:897-903.

Cranwell, P. D., D. E. Noakes and K. J. Hill. 1976. Gastric secretion and fermentation in the suckling pig. Br. J. Nutr. 36(1):71-86.

Charteris, W. P., P. M. Kelly, L. Morelli and J. K. Collins. 1997. Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. Int. J. Food Microbiol. 35:1-27.

Delley, M., B. Mollet and H. Hottinger. 1990. DNA probe for *Lactobacillus delbrueckii*. Appl. Environ. Microbiol. 56(6):1967-1970.

Ehrmann, M., W. Ludwig and K. H. Schleifer. 1992. Species specific oligonucleotide probe for the identification of *Streptococcus thermophilus*. Syst. Appl. Microbiol. 15:453-455.

Ehrmann, M., W. Ludwig and K. H. Schleifer. 1994. Reverse dot blot hybridization: A useful method for the direct identification of lactic acid bacteria in fermented food. FEMS Microbiol. Lett. 117(2):143-150.

Hertel, C., W. Ludwig, M. Obst, R. F. Vogel, W. P. Hammes and K. H. Schleifer. 1991. 23S rRNA-targeted oligonucleotide probes for the rapid identification of meat lactobacilli. Syst. Appl. Microbiol. 14:173-177.

Juven, B., R. J. Meinersmann and N. J. Stern. 1991. Antagonistic effects of *lactobacilli* and *pediococci* to control intestinal colonization of human enterpathogens in live poultry. J. Appl. Bact. 70:95-103.

Lee, Y. K., K. Nomoto, S. Salminen and S. L. Gorbach. 1999. Handbook of probiotics. John Wiley & Sons, Inc. New York, NY.

Liu, W. and J. N. Hansen. 1990. Some chemical and physical properties of nisin, a small-protein antibiotic produced by Lactococcus lactis. Appl. Environ. Microbiol. 56:2551-2558.

Nurmi, E. and M. Rantala. 1973. New aspects of *salmonella* infection in broiler production. Nature 241:210-211.

Nusiainen, J. and J. Setala. 1993. 12. Lactic acid bacteria as animal probiotics In: Lactic acid bacteria. (Ed. Seppo Salminen) Marcel Dekker, Inc. New York.

Ochi, K. and E. Freese. 1983. Effects of antibiotics on sporulation caused by the stringent response in Bacillus subtilis. J. Gen. Microbiol. 129:3709-3720.

Ray, B. and M. A. Daeschel. 1992. Food biopreservatives of microbial origin. CRC Press, Boca Raton, FL.

Russell, A. D. 1982. The destruction of bacterial spores. Academic Press, London.

Tagg, J. R. and A. R. McGIVEN. 1971. Assay system for bacteriocins. Appl. Microbiol. 21(5):943.