Food Sci. Biotechnol. Vol. 17, No. 1, pp. 191 ~ 194 (2008)

RESEARCH NOTE

Food Science and Biotechnology

Prophylactic Uses of Probiotics as a Potential Alternative to Antimicrobials in Food Animals

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Abstract The antagonistic activity of probiotic strains (*Bifidobacterium animalis* BB-12, *Bifidobacterium bifidum* A, *Bifidobacterium longum* B6, *Lactobacillus acidophilus* ADH, *Lactobacillus paracasei* ATCC 25598, and *Lactobacillus rhamnosus* GG) against nalidixic acid resistant (NA^R) *Escherichia coli* O157:H7 MF1847, *E. coli* O157:H7 H2439, *E. coli* O157:H7 ATCC 43894, and *E. coli* O157:H7 C7927 was investigated using the agar-overlay, well diffusion, and broth culture tests. *L. paracasei* ATCC 25598 was the most effective probiotic strain in terms of *in vitro* antagonistic activity against NA^R *E. coli* O157:H7, followed by *L. rhamnosus* GG, *B. longum* B6, and *L. acidophilus* ADH. The use of selected probiotic strains could be an effective pre-harvest intervention strategy to reduce the risk of NA^R *E. coli* O157:H7 by maintaining a balanced microflora in animals and might provide many potential benefits *in lieu* of using antimicrobials.

Keywords: probiotic strain, antagonistic activity, *Bifidobacterium*, *Lactobacillus*, nalidixic acid

Introduction

Antimicrobials including cephalosporins, penicillins, sulfonamides, tetracyclines, and quinolones are often administered to animals to improve overall health and prevent occurrences of pathogenic infections (1-3). The use of antimicrobials in food animals is inevitable for disease prevention and growth promotion (4). However, the overuse of antimicrobials has led to an increase in resistant bacteria, which has emerged as a major issue in food animals (5-7). Food animals could be a reservoir of antimicrobial-resistant pathogens, where the pathogen can reproduce and be excreted (4). Antimicrobial resistant bacteria have increasingly been isolated from food animals over the past two decades (7-10). Many isolates were resistant to two or more antimicrobials, which is known as cross-protection (4,8,9). Therefore, recent researches are more focused on developing alternative strategies for minimizing the risk of antimicrobial resistant bacteria.

Because the use of antimicrobials in animal feed is unacceptable, probiotic supplementation as an alternative and holistic approach for improving intestinal microbial balance and reducing foodborne pathogens has recently received much research attention in the animal industry (11,12). Probiotics are known as safe and beneficial live microorganisms, including microflora modulation, competitive exclusion of pathogens, and immune response, of which antagonistic effect against pathogens has become a priority in pre-harvest interventions of food animals (13-16). Probiotic strains can be fed to animals in order to inhibit pathogens in the gastrointestinal (GI) tract (17,18). However, few studies have evaluated the effectiveness of probiotic strains in terms of antagonistic activity against antimicrobial resistant foodborne pathogens. Therefore, the objective of this study was to investigate the *in vitro* antagonistic activity of selected probiotic strains against nalidixic acid resistant (NA^R) *Escherichia coli* O157:H7 strains.

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Received June 26, 2007; accepted September 4, 2007

Materials and Methods

Probiotic strains and culture conditions Strains of *Bifidobacterium animalis* BB-12, *Bifidobacterium bifidum* A, *Bifidobacterium longum* B6, *Lactobacillus acidophilus* ADH, *Lactobacillus paracasei* ATCC 25598, and *Lactobacillus rhamnosus* GG from the Food Microbiology Laboratory, Department of Food Science, University of Missouri Culture Collection (Columbia, MO, USA) were anaerobically grown in de Man, Rogosa, Sharpe (MRS, Difco, BD Diagnostic Systems, Sparks, MD, USA) broth supplemented with 0.05% cysteine-HCl at 37°C for 24 hr. Cultures of probiotic strains were harvested by centrifugation at 4,000×g for 15 min at 5°C.

Nalidixic acid resistant strains To prepare a stock solution, nalidixic acid was dissolved in 0.1 N NaOH. Four strains of *E. coli* O157:H7 MF1847, *E. coli* O157:H7 H2439, *E. coli* O157:H7 (ATCC 43894), and *E. coli*

O157:H7 C7927 were used as indicator bacteria. In order to screen highly nalidixic acid resistant (NA^R) strains, *E. coli* O157:H7 strains were serially cultivated in trypticase soy broth (TSB) and plated on MacConkey-sorbitol agar containing 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25, or 50 μg/mL of nalidixic acid (Fisher Scientific, Fair Lawn, NJ, USA) at 37°C for 18 hr (19). The strains were resistant to 50 mg/mL of nalidixic acid and remained stable. Their survival, growth characteristics, and biochemical properties were found to be similar to the wild-type counterpart as confirmed by standard culturing and via the use of Micro-ID (Remel, Lenexa, KS, USA). NA^R *E. coli* O157:H7 strains were not further characterized at a molecular level. Cultures of NA^R *E. coli* O157:H7 were centrifuged at 4,000×g for 10 min at 5°C and washed twice in peptone water prior to use.

Agar-overlay test Each probiotic bacterium was plated on MRS agar and incubated anaerobically at 37°C for 24 hr. The population of plated probiotic bacterium was approximately 10^6 CFU/plate. The NA^R *E. coli* O157:H7 was mixed with 12 mL semi-solid agar (10^6 CFU/mL) and poured over the solidified MRS agar plate containing visible probiotic colonies. The overlaid plates were incubated in a GasPak anaerobic system (BBL, Cockeysville, MD, USA) at 37°C for 24-48 hr and observed for growth or inhibition. Negative indicated no growth or hazy colonies on plates after incubation.

Well diffusion assay Trypticase soy agar (20 mL) containing 10^6 CFU/mL of NA^R *E. coli* O157:H7 was poured into a petri dish. Wells (dia. 5 mm) were aseptically made in the agar using a sterile cork borer and filled with 100 mL of supernatant of probiotic culture. The plates were incubated at 37°C for 24-48 hr and observed for clear inhibition zones. The diameter of the inhibition zone around the well was measured using an electronic caliper (The L.S. Starrett Co., Athol, MA, USA).

Antagonistic activity in broth Equal aliquots (10^6 CFU/mL) of probiotic bacterium and NA^R *E. coli* O157:H7 were anaerobically incubated in MRS broth at 37°C for 20 hr. Only indicator *E. coli* O157:H7 bacterial cultures were used as the control. After 20 hr of incubation, the cultures were serially diluted (1 : 10) with 0.1% peptone water, and 0.1 mL of each dilution was plated in duplicate on MacConkey Sorbitol Agar (MSA, Difco, BD Diagnostic Systems) containing 50 mg/mL of nalidixic acid. Inhibition values of probiotic strains against NA^R *E. coli* O157:H7 was calculated by log (N/N_c) (20). N and N_c represent the count of NA^R *E. coli* O157:H7 in the co-incubation and in the control with only indicator bacteria, respectively.

Statistical analysis All experiments were performed in duplicate on 3 replicates. Data from the microbiological studies were analyzed using the general linear model (GLM) and least significant difference (LSD) procedures of SAS. Significant mean differences were compared by Fisher's LSD at $p<0.05$.

Table 1. Antagonistic activity of probiotic strains against NA^R *E. coli* O157:H7 strains tested on MRS agar

Probiotic strain	NA^R *E. coli* O157:H7[1)]			
	MF1847	H2439	43894	C7927
B. animalis BB-12	+	–	+	+
B. bifidum A	+	–	–	+
B. longum B6	–	–	–	–
L. acidophilus ADH	+	–	–	+
L. paracasei ATCC 25598	–	–	–	–
L. rhamnosus GG	–	+	–	–

[1)]+ Indicates visible growth at least once in the 3 replicates; – denotes no growth in any of the replicates.

Results and Discussion

Antimicrobial activity of probiotic strains on agar The inhibitory effects of selected probiotic strains (*B. animalis* BB-12, *B. bifidum* A, *B. longum* B6, *L. acidophilus* ADH, *L. paracasei* ATCC 25598, and *L. rhamnosus* GG) against NA^R *E. coli* O157:H7 strains (MF1847, H2439, 43894, and C7927) were tested by an agar-overlay assay. In the agar-overlay test, plus signs (+) indicate visible growth of the target pathogens at least once in the 3 replicates and negative signs (–) denote no growth in any of the replicates. As shown in Table 1, *B. longum* B6 and *L. paracesei* ATCC 25598 completely inhibited the growth of all NA^R *E. coli* O157:H7 strains, while *B. animalis* BB-12 showed the least antimicrobial effects against NA^R *E. coli* O157:H7 (MF1847, 43894, and C7927). Compared with other probiotic strains, *B. longum* B6 and *L. paracasei* ATCC 25598 showed the highest antagonistic activity against all NA^R *E. coli* O157:H7 strains. No significant difference was observed in the *in vitro* antagonistic activity of selected probiotic strains between wild-type *E. coli* O157:H7 and NA^R *E. coli* O157:H7 (data not shown). This is in agreement with the result in which the antagonistic activity of probiotic strains was more dependent on the species than particular strain (21).

Antimicrobial activity of the culture supernatants of probiotic strains Antimicrobial inhibition zones for probiotics against NA^R *E. coli* O157:H7 strains are shown in Table 2. Compared to the agar-overlay test, a similar inhibitory pattern of probiotic strains against indicator bacteria was observed in the well diffusion assay. In Table 2, the supernatant of *L. paracasei* ATCC 25598 showed the largest inhibition halo against NA^R *E. coli* O157:H7 (H2439; 13.91 mm). *L. paracasei* ATCC 25598 and *L. rhamnosus* GG expressed the highest antagonistic activity against NA^R *E. coli* O157:H7 strains, while *B. animalis* BB-12 and *B. bifidum* A were least effective in inhibiting NA^R *E. coli* O157:H7 strains, showing inhibition zones ranging from 9.23 to 10.83 mm. However, there were no significant differences in antagonistic activity of selected probiotics within NA^R *E. coli* O157:H7 strains. The acidic supernatants (pH <4) of *L. paracasei* ATCC 25598 and *L.*

Table 2. Inhibition zones (mm in diameter) obtained the agar-overlay test for probiotic strains on NA^R *E. coli* O157:H7 strains

Probiotic strain	NA^R *E. coli* O157:H7			
	MF1847	H2439	43894	C7927
B. animalis BB-12	9.23±1.03$^{c1)}$	9.80±0.87^{c}	9.43±0.81^{c}	10.80±1.15^{b}
B. bifidum A	10.17±1.11^{bc}	9.73±0.45^{c}	9.50±0.70^{c}	10.83±1.52^{b}
B. longum B6	11.93±1.10^{ab}	12.10±1.13^{b}	12.13±1.65^{ab}	11.32±1.25^{ab}
L. acidophilus ADH	12.23±1.12^{a}	11.20±0.82^{b}	11.00±0.72^{bc}	11.47±0.84^{ab}
L. paracasei ATCC 25598	12.83±1.07^{a}	13.91±0.66^{a}	11.23±0.25^{b}	12.93±1.62^{ab}
L. rhamnosus GG	13.32±1.04^{a}	11.70±0.46^{b}	13.56±0.55^{a}	13.53±1.15^{a}
$LSD_{0.05}$$^{2)}$	1.784	1.127	1.675	2.295

[1)]Means±SD of 3 replicates with different superscript letters within a column are significantly different at $p<0.05$.
[2)]Least significant difference.

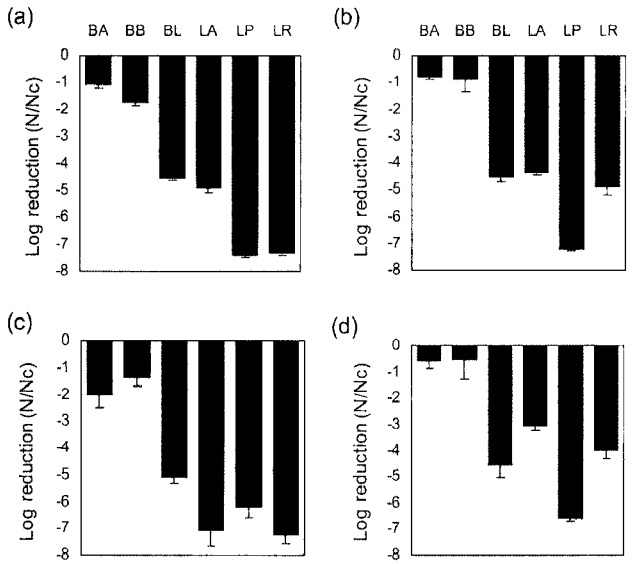

Fig. 1. Inhibitory effect of probiotic strains. BA, *B. animalis* BB-12; BB, *B. bifidum* A; BL, *B. longum* B6; LA, *L. acidophilus* ADH; LP, *L. paracasei* ATCC 25598; LR, *L. rhamnosus* GG against NA^R *E. coli* O157:H7 strains (a, MF1847; b, H2439; c, 43894; d, C7927) in broth.

rhamnosus GG effectively inhibited the growth of NA^R *E. coli* O157:H7 strains (20,22). This result suggests that the antimicrobial activity of probiotic strains is more likely to be related to the production of inhibitory metabolites such as organic acids, hydrogen peroxide, and bacteriocins (20,21).

Antimicrobial activity of probiotic strains in broth The antagonistic activity of probiotic strains in broth was determined as measured by the inhibition of growth of NA^R *E. coli* O157:H7 (Fig. 1). Compared to the control, *L. paracasei* ATCC 25598 significantly reduced NA^R *E. coli* O157:H7 MF1847, H2439, 43894, and C7927 numbers by 7.40, 7.24, 6.21, and 6.62 logs, respectively ($p<0.05$). The growths of *B. animalis* BB-12, *B. bifidum* A, *B. longum* B6, *L. acidophilus* ADH, *L. paracasei* ATCC 25598, and *L. rhamnosus* GG were lowered to pH 4.55, 4.60, 4.13, 3.86, 3.77, and 3.76, respectively. *B. animalis* BB-12 and *B. bifidum* A showed the least antagonistic activity against all NA^R *E. coli* O157:H7 strains (<2 log reduction), which is in agreement with a previous report in which the inhibitory activity of probiotic strains and the pH of the broth were negatively correlated (20,22).

Antagonistic metabolites might be responsible for the antimicrobial activity of probiotic strains against NA^R *E. coli* O157:H7. *L. paracasei* ATCC 25598 and *L. rhamnosus* GG were highly antagonistic probiotic candidates. This result suggests that selected probiotic strains may reduce the risk of antimicrobial resistant *E. coli* O157:H7 contamination of animal foods. Therefore, the use of *L. paracasei* ATCC 25598 and *L. rhamnosus* GG could greatly benefit the animal food industry by providing an alternative, more holistic and potentially effective pre-harvest intervention technique that could contribute to increasing the safety of foods.

Acknowledgments

The authors acknowledge the financial support of the Small and Medium Business Administration, Korea (Regional Industry for the Development of New Technology) and also thank the National Cattlemen's Beef Association (NCBA) for partial financial support and careful review of the article. This study was partially supported by 2007 research grant from Kangwon National University.

References

1. Boothe DH, Arnold JW. Resistance of bacterial isolates from poultry products to therapeutic veterinary antibiotics. J. Food Protect. 66: 94-102 (2003)
2. Johnson JR, Murray AC, Gajewski A, Sullivan M, Snippes P, Kuskowski MA, Smith KE. Isolation and molecular characterization of nalidixic acid-resistant extraintestinal pathogenic *Escherichia coli* from retail chicken products. Antimicrob. Agents Ch. 47: 2161-2168 (2003)
3. Gorbach S. Antimicrobial use in animal feed time to stop. New Engl. J. Med. 345: 1201-1203 (2001)
4. Schroeder CM, Zhao C, DebRoy C, Torcolini J, Zhao S, White DG, Wagner DD, McDermott PF, Walker RD, Meng J. Antimicrobial resistance of *Escherichia coli* O157:H7 isolated from human, cattle, swine, and food. Appl. Environ. Microb. 68: 576-581 (2002)
5. Blanco JE, Blanco M, Mora A, Blanco J. Prevalence of bacterial resistance to quinolones and other antimicrobials among avian *Escherichia coli* isolated from septicemic and healthy chickens in Spain. J. Clin. Microbiol. 35: 2184-2185 (1997)
6. Cohen ML. Changing patterns of infectious disease. Nature 406: 762-767 (2000)
7. Meng J, Zhao S, Doyle MP, Joseph SW. Antibiotic resistance of

Escherichia coli O157:H7 and O157:NM isolated from animals, food, and humans. J. Food Protect. 61: 1511-1514 (1998)
8. Golding SS, Matthews KR. Intrinsic mechanism decreases susceptibility of *Escherichia coli* O157:H7 to multiple antibiotics. J. Food Protect. 67: 34-39 (2004)
9. Mathew AG, Saxton AM, Upchurch WG, Chattin SE. Multiple antibiotic resistance patterns of *Escherichia coli* isolated from swine farms. Appl. Environ. Microb. 65: 2770-2772 (1999)
10. Wray C, McLaren IM, Carroll PJ. *Escherichia coli* isolated from farm animals in England and Wales between 1986 and 1991. Vet. Rec. 133: 439-442 (1993)
11. Bach Kundsen KE. Development of antibiotic resistance and options to replace antimicrobials in animal diets. P. Nutr. Soc. 60: 291-299 (2001)
12. Fuller R. Probiotics in man and animals. J. Appl. Bacteriol. 66: 365-378 (1989)
13. Nurmi E, Nuotio L, Schneitz C. The competitive exclusion concept: Development and future. Intl. J. Food Microbiol. 15: 237-240 (1992)
14. Lee N-K, Kim H-W, Chang H-I, Yun C-Y, Kim S-W, Kang C-W, Paik HD. Probiotic properties of *Lactobacillus plantarum* NK181 isolated from *jeotgal*, a Korean fermented food. Food Sci. Biotechnol. 15: 227-231 (2007)
15. Lim S-M, Lee G-J, Park S-M, Ahn D-H, Im D-S. Characterization of *Lactobacillus cellobiosus* D37 isolated from soybean paste as a probiotic with anti-cancer and antimicrobial properties. Food Sci. Biotechnol. 15: 792-798 (2006)
16. Kim J-T, Jung H-Y, Lee N-K, Rhim S-L, Paik H-D. Isolation, identification, and probiotic properties of *Lactobacillus reuteri* HY701 from human feces. Food Sci. Biotechnol. 15: 677-682 (2006)
17. Servin AL, Coconnier M-H. Adhesive of probiotic strains to the intestinal mucosa and interaction with pathogens. Best Pract. Res. Clin. Ga. 17: 741-754 (2003)
18. Servin AL. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol. Rev. 28: 405-440 (2004)
19. Zhao TM, Doyle MP, Harmon BG, Brown CA, Mueller POE, Parks AH. Reduction of carriage of Enterohemorrhagic *Escherichia coli* O157:H7 in cattle by inoculation with probiotic bacteria. J. Clin. Microbiol. 36: 641-647 (1998)
20. Annuk H, Shchepetova J, Kullisaar T, Songisepp E, Zilmer M, Mikelsaar M. Characterization of intestinal lactobacilli as putative probiotic candidates. J. Appl. Microbiol. 94: 403-412 (2003)
21. Lee YK, Lim CY, Teng WL, Ouwehand AC, Tuomola EM, Salminen S. Quantitative approach in the study of adhesion of lactic acid bacteria to intestinal cells and their composition with enterobacteria. Appl. Environ. Microb. 66: 3692-3697 (2000)
22. Hütt P, Shchepetova J, Lõivukene K, Kullisaar T, Mikelsaar M. Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uro-pathogens. J. Appl. Microbiol. 100: 1324-1332 (2006)