ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 3 (9): 603-607, 2004

Inhibition of Growth of *Escherichia coli*, *Salmonella typhimurium*, and *Clostridia perfringens* on Chicken Feed Media by *Lactobacillus salivarius* and *Lactobacillus plantarum*

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Abstract: Two dominant strains of lactobacilli isolated from a botanical probiotic were identified and evaluated to determine their ability to inhibit the *in vitro* growth of *E. coli*, *S. typhimurium*, and *C. perfringens* on a medium that simulated a normal starter and grower diet for broiler chickens. The two strains identified were *Lactobacillus salivarius* and *Lactobacillus plantarum*. In the inhibition assay *in vitro*, both strains of *Lactobacillus* from the probiotic inhibited ($P < 0.001$) growth of *E. coli*, *S. typhimurium*, and *C. perfringens* for both the starter and grower diets when compared to the control diets. Both strains of *Lactobacillus* for both the starter and grower diets produced more ($P < 0.001$) acetic and lactic acid than was found in the control diets. Also, the pH of the media with cultures of *L. plantarum* and *L. salivarius* for both the starter and grower diets was lower ($P < 0.001$) than for the control diets. These results indicate that *L. salivarius* and *L. plantarum* contained in the botanical probiotic can ferment carbohydrates in poultry feed to produce pH levels and concentrations of lactic and acetic acid that inhibit the growth of *E. coli*, *S. typhimurium*, and *C. perfringens*.

Key words: *Escherichia coli*, *Salmonella typhimurium*, *Clostridia perfringens*, *Lactobacillus*, poultry feed

Introduction

Clostridia perfringens, *E. coli* and *Salmonella* sp. may colonize the gastrointestinal tract of chickens. *C. perfringens*, *E. coli* and *Salmonella* are major food borne pathogens associated with processed poultry and may cause severe illness and even death in humans (Tauxe, 1991). The presence of *E. coli* on processed poultry is an indicator of fecal contamination. Antibiotics have been used extensively in animal feed to inhibit the growth of intestinal pathogens. However, the continued feeding of antibiotics at sub-therapeutic levels has created concerns about the extent to which usage increases the possibilities of antibiotic residue, the development of drug-resistant bacteria, and a reduction in the ability to cure bacterial infections in humans (Jensen, 1998). Increased awareness of the potential problems associated with the use of antibiotics has stimulated research efforts to identify alternatives to their use as feed additives. Probiotics (direct-fed microbial) have been suggested as alternatives to the use of antibiotics in food animals. According to Fuller (1989) probiotics are characterized as live microorganisms (e.g., including bacteria, fungi, and yeast) that when ingested by animals have beneficial effects in the prevention and treatment of diseases (Miles and Bootwalla, 1991; Havenaar and Huis in't Veld, 1992). The probiotic bacteria must be able to colonize the gastrointestinal tract, survive the low pH of the stomach and bile acids in the intestines, and compete against other microorganisms in the gastrointestinal tract (Nurrni *et at*., 1983; Chateau *et at*., 1993).

The most commonly used probiotics contain strains of lactic acid producing bacteria (e.g., *Lactobacillus*, *Bifidobacterium* and *Streptococcus*), and of these, lactobacilli are the most studied group (Balevi *et al*., 2001). Lactic acid bacteria have been demonstrated to inhibit the *in vitro* growth of many enteric bacteria, including *Salmonella typhimurium*, *Staphylococcus aureus*, *E. coli*, *Clostridium perfringens*, and *Clostridium difficile*, and have been used in both humans and animals to treat a broad range of gastrointestinal disorders (Silva *et al*., 1987; Hinton *et al*., 1992b; Gibson and Wang, 1994; Meurman *et al*., 1995). Sinha (1986) reported that the major metabolites of lactic acid bacteria, short chain fatty acids (SCFA) and lactic acid are responsible for their antimicrobial activity against *E. coli* in the intestine.

Most probiotics contain several strains of lactobacilli (*L. acidophilus*, *L. casei* *L. helveticus*, *L. lactis*, *L. salivarius*, and *L. plantarum*) and other lactic acid bacteria, which are found in the native intestinal flora of animals' were natural intestinal strains (Fuller, 1989). Most probiotics may contain single or multiple strains of these bacteria. Results from *in vitro* studies have shown that strains of

L. salivarius have potential for use in probiotics for pigs (Nemcova *et al.*, 1997) and chickens (Garriga *et al.*, 1998). *L. salivarius* offers promising possibilities as a probiotic, specifically because of their ability to inhibit growth of *Salmonella enteritidis* and *E. coli*, their high adhesion efficiency to intestinal mucosal, and their resistance to bile salts and acidic (e.g. pH 3.0) environments (Nemcova *et al.*, 1997; Garriga *et al.*, 1998; Murphy *et al.*, 1999).

Another lactobacillus (*Lactobacillus plantarum*) also shows promise for use as a probiotic. Vescovo *et al.* (1993) reported that *L. plantarum* is found in fermented foods of plant origin and can adhere to human cell lines of intestinal origin (Adlerberth *et al.*, 1996; Ahrne *et al.*, 1998). Recently, *L. plantarum* 299v, isolated from human intestine, has been used as a probiotic in foods for humans with irritable bile syndrome (Nobaek *et al.*, 2000). In a study with rats, Mangell *et al.* (2002) revealed that pretreatment with *L. plantarum* 299v protects against *E. coli*-induced increase in intestinal permeability to [14C] Mannitol.

These findings, although providing support for the potential use of *L. salivarius* and *L. plantarum* as probiotics, were based on the utilizations of single strains of *Lactobacilli* isolated from the gastrointestinal tract of poultry, swine, or humans. There have been no published studies, however, utilizing single or multiple strains of lactobacilli containing *L. salivarius* or *L. plantarum* isolated from fermented plants and used as a probiotic to inhibit pathogenic microorganisms. Therefore, the purpose of this research was to isolate bacteria from a commercial probiotic and test their ability to inhibit the *in vitro* growth of *E. coli* and *Salmonella typhimurium* on a medium that simulated a normal chick diet.

Materials and Methods

Selection of lactic acid and VFA producing bacteria: Serial dilutions of probiotic were prepared and plated on Lactobacilli MRS Agar. Plates were incubated anaerobically in a Coy Anaerobic Chamber (Coy Laboratory Products, Inc. Grass Lake, MI) at 35°C for 48 h. Isolated colonies were transferred to fresh Lactobacillus MRS Agar, incubated at anaerobically at 35°C for 48 h, then stored at 4°C. Isolates were identified using the MIDI Sherlock Microbial Identification System (MIDI Inc., Newark, DE).

Preparation and inoculation of chicken feed broth media: A chicken feed medium was prepared using a corn-soybean based chicken feed obtained from the Poultry Science Department of the University of Georgia, Athens. The feed was composed of 53.32% yellow corn, 37.60% soybean meal (48% protein), 5.46% animal and vegetable fat blend, 1.64% calcium carbonate, 1.22% monocalcium diphosphate, 0.25% broiler vitamin premix, 0.25% sodium chloride, 0.21% methionine hydroxy analog (86% methionine), and 0.05% trace minerals. The chicken feed broth was prepared by blending a 5% (wt/vol) suspension of chicken feed in distilled water at high speed in a Waring blendor for 3 min. The blended suspension was filtered through cheese cloth, dispensed into test tubes, and autoclaved at 121°C for 15 min.

Ten-µ1 inoculating loops (Nunc Inter Med, Roskilde, Den-mark) were used to inoculate test tubes containing 10 ml of the chicken feed broth with the appropriate lactic acid producing bacteria that had been grown in Lactobacillus MRS broth for 24 h. The tubes were incubated anaerobically for 48 h at 37°C. After incubation, the pH of the media was measured with a Corning, Model 450 pH meter (Corning, Inc.; Corning, NY). The concentrations of acetic and propionic acids in the incubated media were determined by gas chromatography (Model 3400; Varian Instrument Group; Sunnyvale, GA) according to the procedures described in Supelco, Inc. (1975) for GC separation of VFA's C2-C5. Lactic acid in the incubated media was determined by gas chromatography of methylated esters according to the procedures described by Holdeman *et al.* (1973).

Inhibition studies: Chicken feed agar medium was made by adding 1.2% Bacto agar (Difco) to the chicken feed broth medium. Ten- µ1 inoculating loops (Nunc Inter Med) were used to make a single streak of the appropriate bacterial cultures across the center of a petri dish containing the agar. These cultures had also been grown for 24 h in Lactobacillus MRS broth, as stated above. The plates were incubated anaerobically for 48 h at 37°C. After incubation, the plates were removed from the anaerobic chamber and 3 ml of the melted feed agar tempered to 50°C was spread over the surface of the plate.

Cultures of *S. typhimurium* and *E. coli* were grown in Trypticase Soy broth (BBL Microbiology Systems, Cockeysville, MD) at 37°C for 18 to 24 h. Each bacterial suspension was diluted to 10^6 CFU/ml, and a sterile spreader was used to spread the bacterial suspension onto the surface of an incubated chicken feed agar plate that had been inoculated with the appropriate isolate(s). The plates were stored at 4°C overnight to allow the inhibitory sub-stances produced by the isolate(s) to diffuse into the agar before the enteropathogens started to grow. Plates were then transferred to a 37°C incubator and incubated for 24 h. After incubation, the width of each enteropathogen's zone of inhibition adjacent to the length of the streak of the isolates was measured.

Statistical analysis: All experiments were repeated three times. Data were analyzed using the GLM procedures of SAS (SAS Inst., Inc., Cary, NC). Treatment means were compared using the PDIFF statement

Table 1: Zones of inhibition of *Escherichia coli*, *Salmonella typhimurium*, and *Clostridia perfringens* on broiler chicken feed media by lactobacillus isolated from probiotic[1]

	Diet									
	Starter			Grower				P-value		
Culture Item	Control	*L. plantarium*	*L. salivarius*	Control	*L. plantarium*	*L. salivarius*	SEM	Culture	Diet	Culture*diet
E. coli	0.00[a]	39.33[b]	31.33[c]	0.00[a]	46.00[d]	32.66[c]	2.00	0.001	0.13	0.25
Salmonella typhimurium	0.00[a]	8.33[b]	9.00[b]	0.00[a]	8.33[b]	8.66[b]	0.66	0.001	0.84	0.96
Clostridium perfringens	0.00[a]	14.33[b]	14.33[b]	0.00[a]	28.33[c]	26.66[c]	1.82	0.001	0.001	0.004

[1]Widths of zones (mm). [a,b,c,d] Means within a row with different superscripts are different (P < 0.05)

Table 2: Concentration of acetic, propionic, and lactic acids and pH of uninoculated chicken feed broth inoculated with bacteria isolated from probiotic

	Diet									
	Starter			Grower				P-value		
Culture Item	Control	*L. plantarium*	*L. salivarius*	Control	*L. plantarium*	*L. salivarius*	SEM	Culture	Diet	Culture*diet
Acetic acid	0.22[a]	7.60[b]	5.85[c]	0.38[a]	11.76[d]	11.96[d]	0.13	0.001	0.001	0.001
Propionic acid	0.02	0.00	0.00	0.00	0.05[b]	0.02	0.02	0.23	0.56	0.14
Lactic acid	0.00[a]	48.25[b]	65.52[c]	0.00[a]	83.74[d]	85.05[d]	1.03	0.001	0.001	0.001
pH	6.21[a]	4.14[b]	4.21[bc]	6.29[a]	4.23[c]	4.13[b]	0.04	0.001	0.002	0.05

[a,b,c,d] Means within a row with different superscripts are different (P < 0.05).

of SAS (SAS Inst., Inc., Cary, NC) when protected by a significant (P < 0.05) treatment effect. Significant differences among treatment means were determined using the F-statistic with results reported as least-square means ± pooled SEM.

Results

Results of the inhibition of *E. coli*, *S. typhimurium*, and *C. perfringens* on chicken feed agar by the 2 lactobacilli isolated from probiotics are listed in Table 1. No zones of inhibition of *E. coli*, *S. typhimurium*, or *C. perfringens* were produced on the uninoculated control plates. Zones of inhibition of *E. coli*, *S. typhimurium*, and *C. perfringens* were produced on the plates inoculated with *L. plantarum* and *L. salivarius* for both the starter and grower diets. Zones of inhibition of *E. coli* on the plates inoculated with *L. salivarius* were lower (P < 0.001) than those for *L. plantarum*. Zones of inhibition of *S. typhimurium* on the plates inoculated with *L. salivarius* were not different (P > 0.05) than those for *L. plantarum*. There was a culture*diet interaction (P < 0.004) for zones of inhibition for *C. perfringens*. Zones of inhibition of *C. perfringens* were produced for both the starter and grower diets but the zones were significantly larger for the grower diet compared to those of the starter diet. Zones of inhibition of *C. perfringens* produced on the plates inoculated with *L. plantarum* and *L. salivarius* were not different (P > 0.05) for the starter or grower diets.

The concentration of acetic, propionic, and lactic acids that the isolates produced in the chicken feed broth are presented in Table 2. There was a culture*diet interaction (P < 0.001) observed for the levels of acetic acid. Cultures of *L. plantarum* and *L. salivarius* produced significantly more acetic acid than was found in the controls for both the starter and grower diets, but the concentration was significantly higher for the grower diet than for the starter diet. The concentration of acetic acid produced on the plates inoculated with *L. plantarum* and *L. salivarius* were not significantly different for the grower diet, whereas the concentration of acetic acid produced by *L. plantarum* was significantly higher than for *L. salivarius* for the starter diet. There was no difference (P < 0.23) in the concentration of propionic acid produced by cultures of *L. plantarum* and *L. salivarius* for the starter and grower diets compared to the controls. There was a culture*diet interaction (P<0.001) observed for the levels of lactic acid. Cultures of *L. plantarum* and *L. salivarius* produced significantly more lactic acid than was found in the controls for both the starter and grower diets, but the concentration was significantly higher for the grower diet than for the starter diet. The concentration of lactic acid produced in media inoculated with *L. plantarum* and *L. salivarius* were not significantly different for the grower diet, whereas the concentration of lactic acid produced by *L. salivarius* was

significantly higher than for *L. plantarum* for the starter diet.

There was a culture*diet interaction ($P < 0.05$) observed for the pH of the media. The pH of the media with cultures of *L. plantarum* and *L. salivarius* for both the starter and grower diets was significantly lower than for the controls. However, the pH of the media inoculated with *L. plantarum* and *L. salivarius* were not significantly different for the starter diet, whereas for the grower diet, the pH of the media produced by *L. plantarum* was significantly higher than for *L. salivarius*.

Discussion

The *in vitro* (Hinton *et al.*, 1992a) and *in vivo* (Hinton *et al.*, 1990) inhibition of the growth of enteric bacteria by lactic acid producing bacteria has been correlated with the ability of the lactic acid bacteria to produce inhibitory substances by metabolizing environmental substrates. Some of these inhibitory substances have been identified as short-chained volatile fatty acids (VFA) and organic acids. Lactic acid bacteria ferment carbohydrates to produce high concentrations of lactic acid that decrease the pH of their environment and reduce the growth of other bacteria (Hinton *et al.* 1992a). Although *L. plantarum* and *L. salivarius* produced detectable concentrations of acetic and propionic acids when grown in starter or grower feed, the concentration of these VFA was probably too low to contribute to the inhibition of the growth of *S. typhimurium* and *E. coli* in the present study. Previously, *in vitro* inhibition of *S. typhimurium* and *E. coli* O157:H7 by bacteria isolated from the cecal contents of poultry was detected after the production of approximately 25 µmol/ml of acetic acid and 35 µmol/ml of propionic acid by the cecal isolates (Hinton *et al.*, 1991). In other studies, concentrations of approximately 30 µmol/ml of lactic acid have been shown to inhibit the growth of *S. typhimurium* and *E. coli* O157:H7 (Hinton *et al.*, 1992a). The low pH of the media was probably due to the lactic acid produced in the media by cultures of *L. plantarum* and *L. salivarius*. By producing high concentrations of lactic acid and low pH levels both lactobacilli were able to inhibit the growth of *S. typhimurium* and *E. coli* when grown on poultry feed.

Acknowledgements

The authors wish to acknowledge Kaypi Trade International, Inc. for providing the ProBioFeed7. This study was supported by the Globis Center, University of Georgia. We also thank Brenda Darby, Department of Animal and Dairy Science, University of Georgia, Patsy Mason, Jerrie R. Barnett, Kimberly D. Ingram, and Fredda J. Murray, Agricultural Research Service, United States Department of Agriculture for technical assistance.

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