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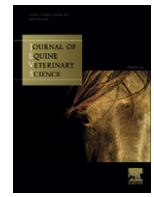

Original Research

Salmonella Antimicrobial Activity of Selected Strains of *Enterolactobacillus* Species Isolated from the Gastrointestinal Tract of the Horse

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A B S T R A C T

The gastric mucosa and the mucosa of the right and left dorsal colon were biopsied in each of the 15 horses, and a total of 45 samples were collected. Mucosal samples were cultured using a *Lactobacillus* enrichment broth. While numerous *Lactobacillus* strains were identified, *Lactobacillus reuteri* was the most common organism identified. Sixteen strains of *Lactobacillus reuteri* were selected for antimicrobial testing. *Salmonella* antimicrobial activity was identified in six out of 16 strains tested. Organisms with *Salmonella* antimicrobial activity were cultured from the stratified squamous epithelium of the stomach and the mucosa of the right and left dorsal colon.

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1. Introduction

Salmonella has been long recognized as a significant cause of equine disease, most commonly an enterocolitis. *Salmonella* is of increased concern in highly dense populations of horses. Outbreaks have occurred at veterinary referral hospitals, breeding farms, racetracks, and other equine sporting events [1].

The primary mode of transmission of *Salmonella* is the fecal oral route. Acidity of the stomach forms the first level of host protection against potential disease. Organisms that survive the acidity of the stomach can cause disease via invasion of intestinal epithelium and the production of exotoxin, endotoxin, and/or cytotoxins. Changes in intestinal contents or composition of nutrients can upregulate *Salmonella* pathogenicity [2]. Numerous host factors play a role in development of disease. Risk factors for the development of disease include antibiotic therapy [3-5], feed restrictions, or dietary changes [4,6]. Foals are at a greater risk of *Salmonella* infection based on exposure, decreased immunocompetency, and lack of a developed normal flora [5]. Stress may also increase susceptibility to infection. Transport and heat stress increase the risk of salmonellosis [4,7,8]. Other risk factors include abdominal surgery, gastrointestinal disease, and colic [5,9-12].

Alterations in intestinal microflora are a frequent precursor to *Salmonella* infection. There is a complex relationship between the host and the normal flora of the host. Competitive exclusion, normal flora preventing the inhabitation by pathogens, is one of the components of this relationship. Recently, the nature of protection has been elucidated as a complex interaction between the microorganism and host and the ability of the microorganism to produce intermediary metabolites to regulate the local environment [13]. *Lactobacillus reuteri* has been intensively evaluated as a unique probiotic species. *Lactobacillus reuteri* is one of the few *Enterolactobacillus* species whose natural ecosystem is the vertebrate gastrointestinal tract [14].

The objective of this study was to identify *Lactobacillus sp.* in defined areas of the gastrointestinal tract and to determine *Salmonella* antimicrobial activity of selected strains.

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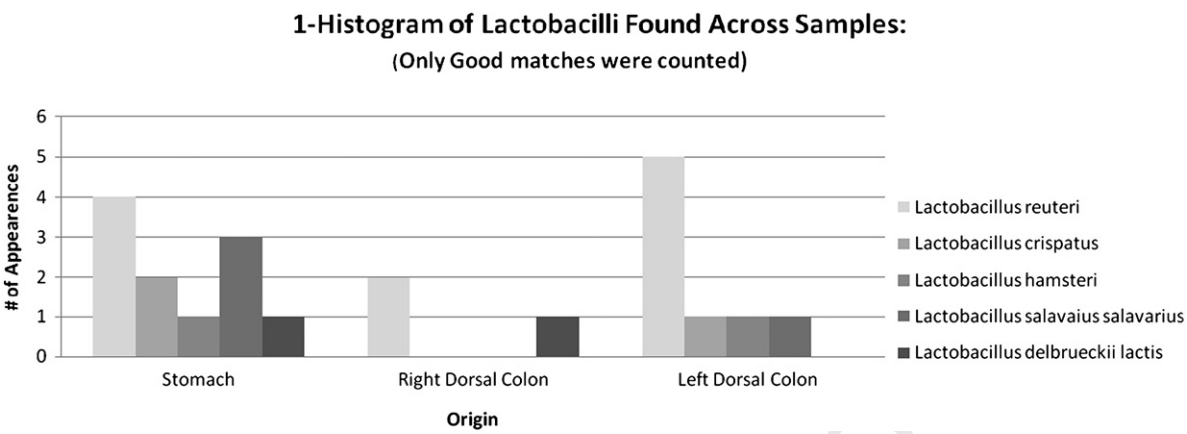

Fig. 1. Histogram of *Lactobacilli* found across samples. Only good matches were counted.

2. Materials and Methods

2.1. Animals

Horses used were presented to a commercial slaughter facility. There was no previous knowledge of the age, gender, place of origin, or physical condition of the horses. Fifteen horses were used. Animals were killed with a captive bolt.

2.2. Sample Collection

The gastrointestinal tract was immediately removed by facility employees. While the gastrointestinal tract was being processed, the stomach and the right and left dorsal colon were sampled aseptically. The stomach and the left or right dorsal colon were incised with a sterile disposable scalpel. A piece of the mucosal surface was elevated with sterile disposal forceps and incised with a second disposable scalpel. Samples were placed in sterile vials and labeled. A total of 45 samples were placed on ice and shipped overnight to a laboratory for culture.

2.3. Sample Processing

Within 20 minutes of reception, each sample was checked for weight and aseptically transferred to a laminar flow biological cabinet or platted. Those not transferred were platted by incubating the samples in separate flasks containing Mann, Rugosa, and Sharpe (MRS) media broth for *Lactobacilli* enrichment for 48 to 72 hours at 37°C. An aliquot of MRS medium broth was then platted by having the enrichment serially diluted and aseptically transferred onto previously prepared and dried MRS medium in Petri plates. Observations for Colony Forming Units per 1 mL or 1 g of sample were made after 48 to 72 hours of incubation at 37°C for anaerobic counts.

2.4. Strain Identification

Bacterial strains were streaked onto BiOLOG^{R}Universal Anaerobic Growth agar w/5% Sheep blood (BUA + Blood). All strains were allowed to incubate at 37°C for 24 to 48 hours until sufficient growth for analysis was achieved. Incubation was completed anaerobically using the AnaeroPack System manufactured by Mitsubishi Chemical Co. After substantial growth occurred, sample strains were suspended into sterile saline solution, then the solution was loaded into the appropriate micro-titer plates (BiOLOG^{R} AN). The plates were incubated at 37°C and were examined at 24 hours by using an automated micro-plate reader and compared against version 4.20 of the BiOLOG^{R} AN database to obtain the bacterial identification.

2.5. Strain Selection

The 16 *Lactobacilli* strains obtained in this study were selected on the basis of the phenotypic colony morphologies most closely resembling isolates of this genus. An additional selection criteria used was the ability of the isolate to grow well under the laboratory conditions applied.

2.6. Salmonella Resistance

Selected *Lactobacillus* strains were toothpick-replicated onto MRS medium agar Petri plates and incubated for 72 hours at 37°C under anaerobic conditions. After the incubation period, the *Lactobacillus* species were exposed to chloroform and covered with a *Salmonella* indicator strain, seeded or inoculated into 0.7% w/v trypticase soy agar, and incubated for 24 hours at 30°C. Resistance was observed and measured in millimeters as a clear “halo” or “zone of inhibition” and documented.

3. Results

Numerous *Lactobacillus* strains were identified. Bacterial identification was only considered definitive and reported as *Lactobacillus sp*. if the similarity coefficient was greater than 0.5, a distance coefficient of less than 7.0, and a probability approaching 100%. Results of *Lactobacilli* found in the mucosa of the stomach and the right and left dorsal colon are shown in the histogram (Fig. 1). *Lactobacillus reuteri* was the most common strain isolated. Other strains included: *Lactobacillus crispatus*, *Lactobacillus hamsteri*,

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Table 1
Q10 Strains tested for antimicrobial activity

Position on Plate	Corresponding Horse Number, Location and Strain Number	Identification of *Lactobacillus*	Antimicrobial Activity	Zone of Inhibition (Millimeters)
1	1 S #2	*Lactobacillus reuteri*	Positive	10 mm
2	1 CL #1	*Lactobacillus reuteri*	Positive	12 mm
3	2 CR #2	*Lactobacillus kefiri*	Positive	11 mm
4	3 CL #2	*Lactobacillus crispatus*	Positive	7 mm
5	4 CL #1	*Lactobacillus reuteri*	Positive	5 mm
6	5 S #3	*Lactobacillus hamsteri*	Faint	Uncertain
7	7 CR #2	*Lactobacillus fermentum*	Negative	None
8	8 S #1	*Lactobacillus salivarius* subs. salivarius	Faint	Uncertain
9	9 CL #1	*Lactobacillus murinus*/paracasei subs. tolerans	Faint	Uncertain
10	10 S #2	*Lactobacillus gasseri*	Negative	None
11	11 CL #2	*Lactobacillus salivarius* subs. *salivarius*	Negative	None
12	12 CR #3	*Lactobacillus salivarius* subs. *salicinius*	Negative	None
13	10 CL #1	*Lactobacillus murinus*/paracasei ss tolerans	Negative	None
14	13 CR #2	*Lactobacillus salivarius* subs. *salivarius*	Negative	None
15	15 S #3	*Lactobacillus reuteri*	Positive	9 mm
16	15 S #1	*Lactobacillus salivarius* subs. *salivarius*	Negative	None

Lactobacillus salivarius subs. *Salivarius*, and *Lactobacillus delbrueckii* subs. *Lactis.*

Sixteen strains were selected for testing of antimicrobial activity based on maintenance of viability and growth under laboratory conditions. Positive antimicrobial activity was identified in six of the 16 strains tested (Table 1). Strains other than *reuteri* that inhibited *Salmonella* were *keferi*, *crispatus*, and *salivarius* (Table 1). All four strains of *Lactobacillus reuteri* tested for antimicrobial activity were positive, with one of the strains being isolated from the stomach (Table 1).

4. Discussion

Six different species of *Lactobacillus* were identified in the stomach or colon of the horse. *Lactobacillus reuteri* was the most commonly identified species. This study identifies *Salmonella* antimicrobial activity in commensal *Lactobacillus* organisms in the horse. Bacterial organisms with antimicrobial activity have been previously identified in the horse [15] by Weese et al, who identified a *Lactobacillus pentosus* in feces of a horse which had antimicrobial activity against *Salmonella spp* and *Escherichia coli*, moderate Q7 inhibitory activity against *Salmonella zooepidemicus* and *Clostridium difficile*, and mild inhibitory activity against *Clostridium perfringens*. *Lactobacillus reuteri* was the most commonly definitively identified organism in this study. *Lactobacillus reuteri* is one of the few *Lactobacillus spp* whose natural ecosystem is the vertebrate gastrointestinal tract [14]. *Lactobacillus reuteri* is indigenous in many animal and human gastrointestinal tracts [16], whereas *Lactobacillus reuteri* and *Lactobacillus gasseri* are the predominant indigenous *Lactobacillus sp* in human infants and adults [17]. *Lactobacillus reuteri* is unique among the *Enterolactobacillus* in its ability to convert glycerol into a potent cell growth inhibitor. This substance called reuterin inhibits the growth of gram-positive and gram-negative bacteria as well as yeast fungi and protozoa [18]. When the biologic Q8 activity of reuterin was tested using an MIC system, it was Q9 found that 2 to 5 U/mL of reuterin inhibited all bacteria tested except lactic acid bacteria, which required four- to fivefold higher concentrations.

Clinically, host-specific strains of *Lactobacillus reuteri* have been used successfully to prevent, treat, or ameliorate gastrointestinal infections such as those caused by *Salmonella typhimurium*, Cryptosporidium parvum, and Candida albicans in chickens, mice, and turkeys. Probiotics can be defined as live commensal microbes administered orally in adequate amounts which are able to confer health effects on the host by improving its intestinal balance. Probiotics have not been well-evaluated in the horse. An equine-origin *Lactobacillus*, *Lactobacillus pentosus*, did not prevent diarrhea in foals. Oral administration of the organism was actually associated with the development of diarrhea [19].

Colonization of the stratified squamous epithelium of the horse by *Lactobacilli* has been previously reported [20]. To the author's knowledge, this report is the first to identify antimicrobial activity in commensal organisms of the equine stomach. This suggests that the stomach of the horse not only protects against invading pathogens through the harsh acidic environment, but also through specific antimicrobial activity. Alterations in the microbial population of the stomach may contribute to the success of invading pathogens. Results suggest that as in other species, *Lactobacillus reuteri* is a major contributor to the normal flora of the gastrointestinal tract of the horse. The inhibition of *Salmonella* emphasizes the significance of *Lactobacillus reuteri's* role in the health of the equine gastrointestinal tract.

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