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Short Communication

Bacillus subtilis Strain PB6 Demonstrates Growth Inhibition Toward Equine-Specific Bacterial Pathogens

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ABSTRACT

This study determined the antagonistic activity of a probiotic *Bacillus subtilis* strain PB6 (PB6) toward six bacterial pathogens of equine origin. Antimicrobial activity of PB6 was evaluated using two different in vitro methodologies. A streak line assay resulted in measurable zones of clearing between growth of PB6 and *Clostridium difficile*, *Clostridium perfringens*, *Rhodococcus equi*, and *Streptococcus equi*. A broth micro dilution assay using cell-free supernatant from PB6 culture demonstrated inhibition of *Salmonella* Typhimurium and *R. equi* growth. The results indicate the potential for PB6 to be a beneficial probiotic for use in the equine industry.

1. Introduction

With the widespread overuse of antibiotics for the treatment of animal ailments and the emergence of antibiotic-resistant pathogens [1], direct-fed microbials, or probiotics, are becoming more popular as natural means to treat and prevent animal disease. It is becoming more recognized that changing the microbial balance of the gastrointestinal tract environment through diet or supplementation can have significant health benefits in the animal [2]. Probiotics are defined as microorganisms which when administered provide health benefits to the host through modulation of the microbial balance [3]. Characteristics of beneficial probiotics include the ability to adhere to mucosal tissue/cells and intestinal mucus, competitively exclude and displace enteropathogens, elicit an immune response, and secrete antimicrobial compounds. Previous in vitro and in vivo research conducted using *Bacillus subtilis* PB6 demonstrated antagonistic activity against various human and livestock pathogens, such as *Clostridium* species [4–6].

Probiotics have gained interest as a means to prevent diarrhea in horses and foals; however, to date there have been few published clinical trials showing positive effects of probiotics in equine [7]. Bacteria that are typically associated with enterocolitis and acute diarrhea in horses and neonatal foals are *Salmonella* spp., *Clostridium perfringens*, and *Clostridium difficile* [8]. Salmonellosis is one of the most common cause of diarrhea in horses and the severity of the infection ranges from mild to severe diarrhea and death. *Clostridium* spp. are spore-forming toxin producing gram-positive bacteria that are common inhabitants of the intestines of equine. Antibiotic use, however, can cause a shift in microbial populations leading to the overgrowth and subsequent toxin production of these bacteria [9,10]. Although less common than *Salmonella* and *Clostridium*, both *Escherichia coli* and *Rhodococcus equi* can may also cause diarrhea in foals [11–13]. There is little research on the use of probiotics to prevent infections not associated with the intestinal tract. *Streptococcus equi* is a highly

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contagious bacterium and the causative agent of strangles and bastard strangles in young horses and foals [14]. It was included in the study to evaluate the potential activity, which could lead to investigating alternative administration of probiotics.

The aim of this study was to evaluate *B. subtilis* strain PB6 as a potential probiotic for equine by screening for antagonistic activity against six bacterial pathogens isolated from equine patients.

2. Materials and Methods

2.1. Pathogenic Bacteria

All equine bacterial pathogens were purchased from the Veterinary Diagnostic Laboratory at Iowa State University, Ames, IA. *Clostridium perfringens* #1984 was isolated from the feces of an adult horse presenting with colic and diarrhea. *Clostridium difficile* #42081 was isolated from the feces of a 2-year-old horse with diarrhea and colic. *Salmonella* Typhimurium #06-767 was isolated from the organs of a foal presenting with diarrhea. The foal had failure of passive transfer of colostral immunoglobulin and died from septicemia. *Escherichia coli* # 07-1690 was isolated from the feces of a neonatal (<7 days) foal presenting with diarrhea. No other pathogens were present in the fecal material. *Rhodococcus equi* #45 was isolated from a 1-year-old horse with a history of *R. equi* positive tracheal wash samples, which led to the suspicion that this animal had respiratory abscesses. However, at necropsy, *R. equi* was isolated from an abscess located in the abdomen. *Streptococcus equi* #43006 was isolated from a nasal swab and the mandibular lymph nodes of a 1-year-old horse from a farm with a history of *S. equi* problems.

2.2. Culture Preparation

Each of the pathogen isolates was subcultured into 10 mL brain heart infusion (BHI) broth (Difco, Becton Dickinson, Sparks, MD) and grown aerobically overnight at 37°C ± 2°C, except for *Clostridium* cultures which were grown under anaerobic conditions (GasPak EZ Anaerobic Gas Generating Pouch System, Difco, Becton Dickinson, Sparks, MD). Cultures were concentrated via centrifugation and the pellet was resuspended in a 50/50 mix of BHI and 20% glycerol. The concentrated cultures were aliquoted into 1.5 mL cryovials and frozen at −80°C.

2.3. Probiotic Organism

The direct-fed microbial product containing *B. subtilis* PB6 (CLOSTAT, Kemin Industries, Inc, Des Moines, IA) was used as the probiotic organism in the inhibition tests.

2.4. Cross-Streak Diffusion Assay

A cross-streak agar diffusion method was used to identify activity of PB6 against equine bacterial isolates. PB6 was inoculated in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) and incubated aerobically at 37°C ± 2°C for approximately 18 hours. The overnight culture was streaked perpendicularly onto prepared tryptic soy agar + 5% sheep blood plates (TSA + SB; Remel, Thermo Fisher Scientific, Lenexa, KS) in triplicate. Plates were incubated aerobically at 37°C ± 2°C for 18 hours. After overnight incubation, the PB6 cultures on the TSA + SB plates were inactivated by placing them in a closed jar with 50 mL chloroform for 2 hours. Each of the six equine isolates was inoculated into BHI broth. Cultures were grown aerobically overnight at 37°C ± 2°C, except for *Clostridium* cultures which were grown under anaerobic conditions. Overnight cultures were streaked perpendicular to the PB6 line at three locations (top, middle, and bottom). Plates were grown overnight as described previously for each particular pathogen. Inhibition was observed as zones of clearing occurring between PB6 and the pathogen, and were measured in millimeters.

2.5. Broth Microdilution Assay

PB6 was inoculated into TSB and incubated aerobically at 37°C ± 2°C for 20–24 hours while shaking at 130 rpm. The culture was centrifuged and the supernatant was filtered through a 0.22 μm in-line syringe filter (cell-free supernatant [CFS]). Equal aliquots of the challenge organism and CFS were dispensed into individual microtiter plate wells. A control consisted of each challenge organism in BHI without CFS addition. Optical density (OD) was read at 620 nm wavelength over 20 hours at 35°C ± 2°C. All results reflect the average OD measurements of four microtiter wells.

3. Results

The results of the cross-streak assay are depicted in Fig. 1 and zone of inhibition measurements are listed in Table 1. There was a measurable inhibitory activity of PB6 against *C. perfringens*, *C. difficile*, *R. equi*, and *S. equi*. No zones of inhibition were observed for *S.* Typhimurium or *E. coli*. The broth microdilution assay (Fig. 2) showed that molecules produced by PB6 were inhibitive to the growth of both *S.* Typhimurium (ST) and *R. equi* (RE). This was indicated by a lower OD in the wells containing the combined pathogen and CFS (•) compared with the pathogen alone (▲). After 20 hours of incubation, no inhibition was observed against *E. coli* (EC) or *S. equi* (SE) as noted by similar ODs in the wells containing the combined pathogen and CFS (•) compared with the pathogen alone (▲).

4. Discussion

B. subtilis strains are known to produce molecules that are inhibitory to many pathogens. These molecules, called bacteriocins, are proteinaceous in nature and stable to high heat, bile salts, and solvents [5,15]. In vivo poultry studies have demonstrated the efficacy of *B. subtilis* PB6 against *Clostridium* spp. and *E. coli* in addition to improving growth performance. In one study, broiler chickens were infected with a pathogenic strain of *E. coli*. After 42 days on trial, birds treated with PB6 had an improved feed:gain ratio, higher weight gain, and reduced mortality as compared with the infected/not treated group [6]. In another study, birds infected with *C. perfringens*, the PB6 supplemented

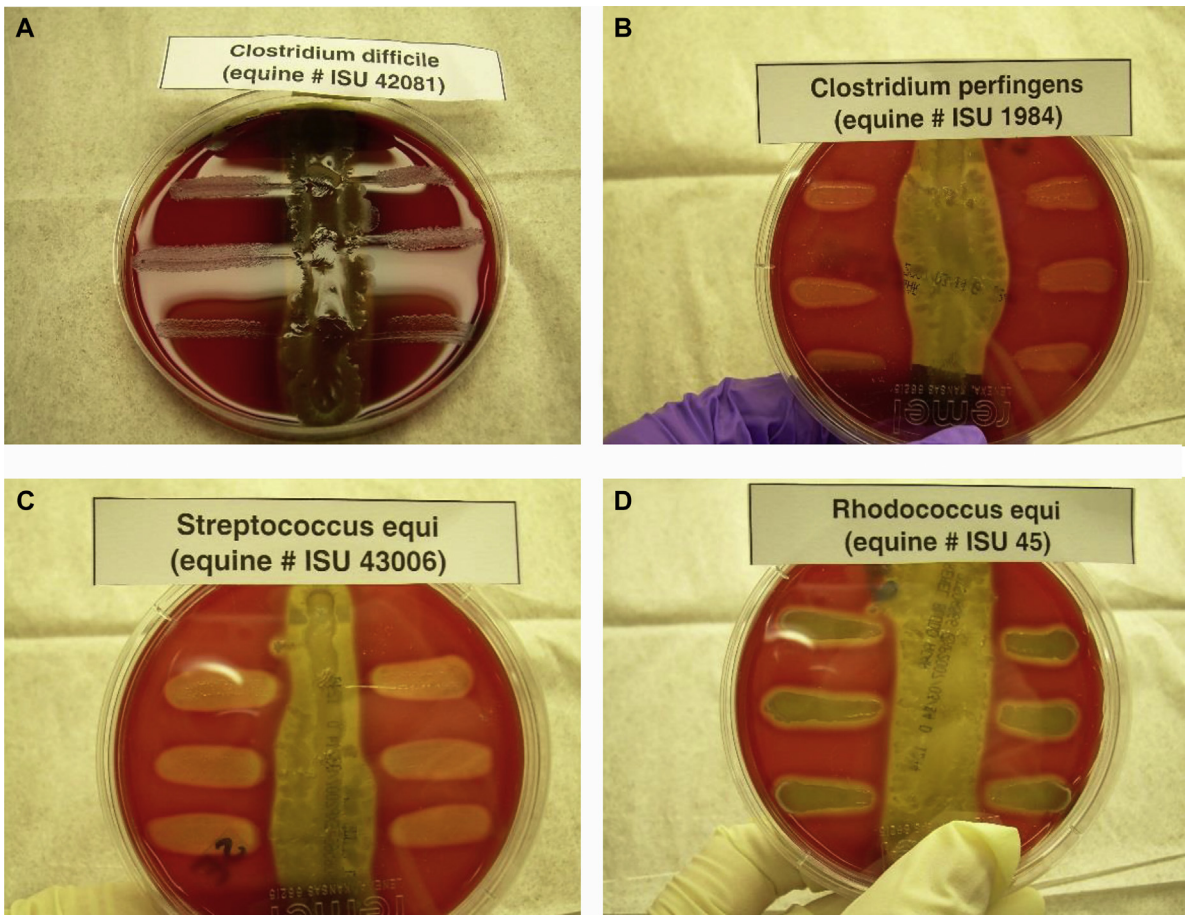

Fig. 1. PB6 inhibited the growth of *Clostridium difficile* (A), *Clostridium perfringens* (B), *Streptococcus equi* (C), and *Rhodococcus equi* (D) as demonstrated with a cross-streak agar diffusion assay. PB6 is the vertical streak with the specific pathogen as the horizontal streak. PB6, *Bacillus subtilis* PB6.

group had an improved feed:gain ratio and reduced *C. perfringens* counts in the intestines. The PB6 group also had an increased villi length to crypt depth ratio indicating improved gut health [16].

Further studies identified immunomodulatory activity in animals treated with PB6. One study with poultry resulted in an enhanced innate immune response as noted by increased bursa weights, decreased *E. coli* populations in the lower intestine, and increased phagocytosis of *E. coli* [17]. In a standardized colitis model, mice treated with PB6 demonstrated an increase in expression of the anti-inflammatory cytokine IL-10 [18]. In a clindamycin-induced *C. difficile*–associated diarrhea model, hamsters treated with PB6 showed reduced weight loss, less diarrhea, and lower mortality than the untreated hamsters. The reduction in mortality on cessation of treatment was similar to that of vancomycin [19]. In an inflammatory bowel disease model, colitis was induced in male rats. The authors theorized that the reduction in inflammation observed in the study could be from the surfactins produced by *B. subtilis* PB6. In different animal models, surfactins have demonstrated anti-inflammatory activity [19]. Therefore, it was of significant interest to identify antagonistic activity of the PB6 strain toward pathogens of equine importance as a first step in identifying its potential use as a probiotic for this species.

Table 1
Mean zones of inhibition (n = 9; ±SEM) measured for each equine isolate in millimeters (mm).

Equine Isolate	Zone of Inhibition, mm
Clostridium perfringens	18.00 ± 0.99
Salmonella Typhimurium	None
Escherichia coli	None
Streptococcus equi	10.89 ± 1.06
Rhodococcus equi	9.11 ± 0.48
Clostridium difficile	13.33 ± 0.65

Abbreviation: SEM, standard error of the mean.

Both the live culture and CFS preparation of PB6 exhibited inhibitory activity toward selected equine pathogens. The broth microdilution assay was conducted under aerobic conditions; therefore, the two *Clostridium* species were not tested in that assay. In both assays, PB6 markedly inhibited the growth of *R. equi*. Using the cross-streak assay, PB6 additionally inhibited *S. equi*, *C. perfringens*, and *C. difficile* although no inhibition was found against *E. coli* or *S.* Typhimurium. These results were consistent with previous

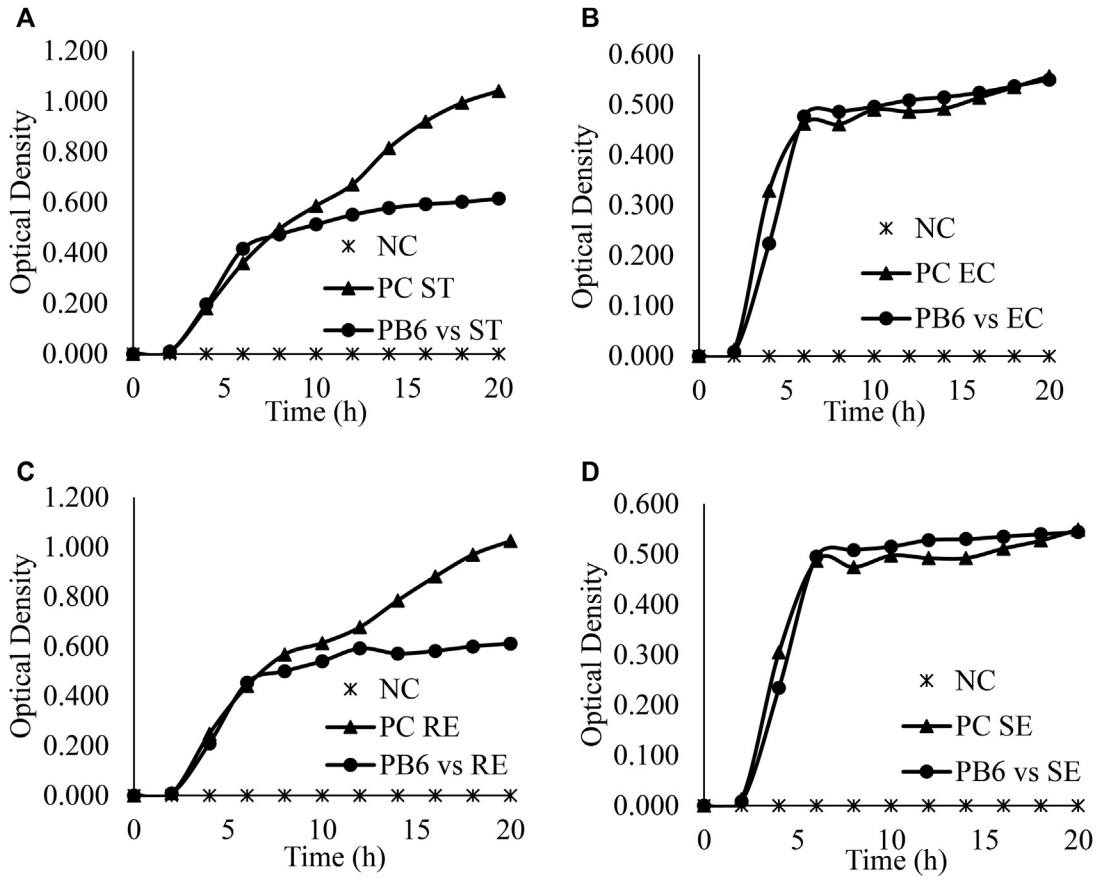

Fig. 2. The inhibitory activity of PB6 toward ST (A), EC (B), RE (C), and SE (D) as determined in a broth microtiter assay. EC, *Escherichia coli*; NC, negative control (broth without bacteria); PB6, *Bacillus subtilis* PB6; PC, positive control (broth with bacteria, without PB6 supernatant); RE, *Rhodococcus equi*; SE, *Streptococcus equi*; ST, *Salmonella* Typhimurium.

findings by other researchers in a similar agar diffusion assay [5]. In the cross-streak assay, zones of β-hemolysis were observed surrounding *B. subtilis* PB6 (Fig. 1) on blood agar plates, further indication of the presence of inhibitory molecules. The correlation between β-hemolysis and antimicrobial activity of secretory lipopeptides has been described in the literature for various *B. subtilis* species [20]. When the broth microdilution assay was used, PB6 was found to be inhibitory against *S.* Typhimurium.

It is not uncommon for in vitro assays to produce conflicting results and is further support for using multiple methodologies when evaluating biological treatments. Production of antimicrobial molecules occurs at different growth phases and has been shown to decrease once growth has entered early stationary phase [21,22]. This may be a possible explanation for some of the differences found between the two assays.

In conclusion, the data collectively indicate potential for *B. subtilis* strain PB6 to have probiotic application in equine species. Although *Bacillus* spp. do not colonize the equine gut, they can reside in that environment. Although the transit time through the equine small intestines is quick (1–3 hours), time through the hind gut can be as long as 2 days [23]. This should be sufficient time for spore germination and production of the antimicrobial molecules; however, it is recognized that in vivo efficacy trials will be needed to confirm any beneficial effect in horses.

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