

IN VITRO INHIBITION OF GROWTH OF *ESCHERICHIA COLI*, *SALMONELLA* TYPHIMURIUM, AND *CLOSTRIDIA PERFRINGENS* USING PROBIOTICS ISOLATED FROM EQUINE FECES

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ABSTRACT

This study was conducted to compare the *in vitro* inhibition of selected isolates against growth of *Salmonella* Typhimurium, *E. coli*, and *C. perfringens*. Four potential probiotic species were isolated from equine feces to test *in vitro* survivability and growth at varying pH levels and in the presence of bile acid. There was significantly more growth by *B. subtilis* than *B. licheniformis* at pH 3 ($P < 0.001$); at pH 4 and 5 there was growth by *B. subtilis*, *B. licheniformis* and *L. salivarius*; at pH 6 and 7 there was growth by all four bacteria with no significant differences at pH 6. In the presence of bile, growth of *L. agilis* was significantly greater than *B. subtilis*, *B. licheniformis* and *L. salivarius* ($P < 0.0001$). Isolates *B. subtilis* and *L. salivarius* were selected for the *in vitro* inhibition portion due to their better growth under both acidic and bile environments. Inhibition by *B. subtilis* against *S. Typhimurium*, *E. coli*, and *C. perfringens* was significantly greater than *L. salivarius* ($P < 0.0001$). These results suggest *B. subtilis* and *L. salivarius* can be considered as components in a potential equine probiotic that may inhibit the growth of enteric bacteria associated with gastrointestinal illness in horses.

Key Words: Probiotics, Bacilli, Lactobacilli, Equine

INTRODUCTION

Salmonella Typhimurium, *Escherichia coli*, and *Clostridium perfringens* are pathogenic organisms found in horses (Jones, 2004) and these pathogens are known to cause gastrointestinal disease in other animals, including humans (Dunowska, Morley et al., 2006). Due to growing concern over potential pathogenic bacteria such as these, there is increasing interest in developing antimicrobial alternatives as a means of preventing or reducing the prevalence of antibiotic resistant pathogens in horses. Probiotics have been suggested as an alternative to antibiotics for the prevention and treatment of diseases but there is limited evidence on the beneficial effects of probiotics in horses (Parraga, Spier et al., 1997; Kim, Morley et al., 2001), possibly because the probiotic organisms were not host-species specific. Therefore, the objectives of this study were: 1) to isolate and identify specific probiotic species of equine origin with beneficial properties that might be useful in the prevention or treatment of the colonization in the intestinal tract of horses by *Salmonella* Typhimurium, *E. coli*, and *C. perfringens*; 2) to test survivability and growth at varying pH levels and in the presence

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of taurocholic acid; and 3) to compare the *in vitro* inhibition of the selected isolates against growth of *Salmonella* Typhimurium, *E. coli*, and *C. perfringens*.

MATERIALS AND METHODS

Isolation of lactobacillus

Fecal samples were collected during rectal palpation from clinically healthy mature horses, aged 7 to 10 years old. Serial dilutions of fecal material were prepared in 0.1% peptone water and plated on Lactobacillus agar. Plates were incubated anaerobically in a Coy Anaerobic Chamber (Coy Laboratory Products, Inc., Grass Lake, MI, USA) at 37°C for 48h. The streak plate method (Chan et al., 1993) on Lactobacillus agar was used to subculture isolated colonies for purification and the isolates were identified using the MIDI Sherlock Microbial Identification System (MIDI Inc., Newark, DE).

Isolation of bacillus

The same fecal samples were serially diluted, heated at 80°C for 15 minutes, and plated on Tryptic Soy Agar (Difco). Cultures were incubated aerobically at 34°C for 24h. Isolates were streaked on Tryptic Soy Agar for purification and identified as described above.

Determination of growth in acid

Lactobacillus MRS Broth (Difco) 69964 (ingredients in grams/liter; universal peptone 10.0, meat extract 5.0, yeast extract 5.0, D(+)-Glucose 20.0, dipotassium hydrogen phosphate 2.0, diammonium hydrogen citrate 2.0, sodium acetate 5.0, magnesium sulfate 0.1, manganous sulfate 0.05, agar 12.0) and Tryptic Soy Broth (Difco) for lactic bacteria and bacillus, respectively, were adjusted to pH 3.0, 4.0, 5.0, 6.0, and 7.0 by the addition of hydrochloric acid and measuring the pH of the media with a Mettler Toledo MA 235 pH meter. The pH of both broths, prior to adjustment was 7.0 ± 0.2. Isolates were grown in pure culture on Lactobacillus MRS Broth and Tryptic Soy Broth for 48h and 24h, respectively, and 1 ml of this suspension was inoculated into 9 ml of each broth at pH 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0. The size of bacteria populations was determined by plating serial dilutions of each pH broth, incubating for 24h and counting colony-forming-units (CFU).

Determination of growth in bile

Both MRS broth and tryptic soy broth were modified by the addition of taurocholic acid, sodium salt hydrate (Sigma) to a concentration of 0.3% and pH 6.5 ± 0.2. Bacterial growth rate was measured as described above for the determination of growth in acid.

Antimicrobial activity

An agar spot procedure that measured the inhibitory activity of actively growing cells of *L. salivarius* and *B. subtilis* against the test microorganisms, *S. Typhimurium*, *E. coli*, and *C. perfringens*, was done. Tryptic soy agar plates were inoculated with serial dilutions of the test microorganisms in 0.1% peptone water. Afterwards, 5 µl of actively growing cells of *L. salivarius*, at a concentration of 9.80×10^9 CFU, and *B. subtilis*, at a concentration of 1.25×10^9 CFU, were aseptically placed on the indicator lawn and incubated overnight at 37°C. The plates were observed for zones of inhibition, as indicated by a halo around the colony. Widths of the zones were measured in millimeters.

Statistical Analysis

Each experiment was replicated five times. Data were analyzed using the GLM procedures of SAS (SAS 9.1). Significant differences among treatment means were determined using the F-statistic with results reported as least-square means \pm pooled SEM.

RESULTS

Four potential probiotic species, *L. salivarius*, *L. agilis*, *B. subtilis* and *B. licheniformis*, were obtained from the feces of 5 mature Quarter Horse mares. Results of the growth of *L. salivarius*, *L. agilis*, *B. subtilis* and *B. licheniformis* in acid are listed in Table 1. There was growth by *B. subtilis* and *B. licheniformis* at pH level 3, but no growth was observed by *L. salivarius*, *L. agilis*, or in the uninoculated broths (Culture x pH, $P < 0.001$). At pH levels 4 and 5, there was growth by *B. subtilis*, *B. licheniformis* and *L. salivarius*, but no growth was observed by *L. agilis* and the uninoculated broths (Culture x pH, $P < 0.001$). However, at pH 6 and 7 growth was observed by *B. subtilis*, *B. licheniformis*, *L. salivarius*, and *L. agilis*, but no growth was observed by the uninoculated broths (Culture x pH, $P < 0.001$).

Results of the growth of *L. salivarius*, *L. agilis*, *B. subtilis* and *B. licheniformis*, in taurocholic acid, sodium salt hydrate are presented in Table 2. No bacterial growth was observed in the uninoculated broth. Growth of *L. agilis* was 1.46%, 3.23%, and 7.19% higher ($P < 0.001$) than growth of *L. salivarius*, *B. licheniformis*, and *B. subtilis*, respectively.

Results of the *in vitro* inhibition of *S. Typhimurium*, *E. coli*, and *C. perfringens* by *L. salivarius* and *B. subtilis* on tryptic soy agar are listed in Table 3. The isolates *L. salivarius* and *B. subtilis* were chosen for *in vitro* inhibition due to their more desirable growth characteristics under acidic and bile salt conditions. Zones of inhibition on plates inoculated with *B. subtilis* for *S. Typhimurium*, *E. coli*, and *C. perfringens* were 52%, 47% and 82.5% higher, respectively, than for plates inoculated with *L. salivarius*.

Table 1. Growth of *L. salivarius*, *L. agilis*, *B. subtilis* and *B. licheniformis* in acidic broth (Log10 CFU/g).

| pH | Culture | | | | | | SEM | P-value | | |
|----|----------------|-------------------------|--------------------|----------------|----------------------|-------------------|------|---------|-------|-------|
| | Ctrl 1 | <i>B. licheniformis</i> | <i>B. subtilis</i> | Ctrl 2 | <i>L. salivarius</i> | <i>L. agilis</i> | | Culture | pH | *pH |
| 3 | 0 ^a | 4.66 ^b | 6.78 ^c | 0 ^a | 0 ^a | 0 ^a | 0.33 | 0.001 | 0.001 | 0.001 |
| 4 | 0 ^a | 4.84 ^b | 7.75 ^c | 0 ^a | 5.47 ^b | 0 ^a | 0.33 | 0.001 | 0.001 | 0.001 |
| 5 | 0 ^a | 8.35 ^b | 8.97 ^b | 0 ^a | 7.24 ^c | 0 ^a | 0.33 | 0.001 | 0.001 | 0.001 |
| 6 | 0 ^a | 8.61 ^b | 8.52 ^b | 0 ^a | 8.75 ^b | 7.80 ^b | 0.33 | 0.001 | 0.001 | 0.001 |
| 7 | 0 ^a | 8.96 ^b | 8.85 ^b | 0 ^a | 8.73 ^b | 7.02 ^c | 0.33 | 0.001 | 0.001 | 0.001 |

a,b,c Means within a row with different superscripts are different (P < 0.001).

Ctrl 1 = Control 1 uninoculated plates, Ctrl 2 = Control 2 uninoculated plates

Table 2. Growth of *L. salivarius*, *L. agilis*, *B. subtilis* and *B. licheniformis* in 0.3% taurocholic acid, sodium salt hydrate (Log10 CFU/g)(pH 6.5 ± 0.2).

| Item | Culture | | | | | SEM | P-Value | |
|----------------|----------------|-------------------------|--------------------|----------------|----------------------|-------------------|---------|------------------|
| | Ctrl 1 | <i>B. licheniformis</i> | <i>B. subtilis</i> | Ctrl 2 | <i>L. salivarius</i> | | | <i>L. agilis</i> |
| 0.3% bile salt | 0 ^a | 9.28 ^b | 8.90 ^c | 0 ^a | 9.45 ^d | 9.59 ^e | 0.02 | 0.0001 |

a,b,c,d,e Means within a row with different superscripts are different (P<0.0001).

Ctrl 1 = Control 1 uninoculated plates, Ctrl 2 = Control 2 uninoculated plates

Table 3. Zone¹ of inhibition of *S. Typhimurium*, *E. coli*, and *C. perfringens* on tryptic soy agar by potential probiotics (pH 6.5 ± 0.2).

| Item | Culture | | | | SEM | P-Value |
|-----------------------|----------------|--------------------|----------------|----------------------|-------|---------|
| | Ctrl 1 | <i>B. subtilis</i> | Ctrl 2 | <i>L. salivarius</i> | | |
| <i>S. typhimurium</i> | 0 ^a | 12.43 ^b | 0 ^a | 5.97 ^c | 0.095 | <0.0001 |
| <i>E. coli</i> | 0 ^a | 11.37 ^b | 0 ^a | 6.00 ^c | 0.119 | <0.0001 |
| <i>C. perfringens</i> | 0 ^a | 37.43 ^b | 0 ^a | 6.53 ^c | 0.344 | <0.0001 |

¹ Width of zones (mm)

a,b,c Means within a row with different superscripts are different (P<0.001)

Ctrl 1 = Control 1 uninoculated plates, Ctrl 2 = Control 2 uninoculated plates

DISCUSSION

This study identified one lactic bacterium and one bacillus isolated from equine feces that grew well in both acid and bile environments. These findings are promising due to the stomach pH ranges between 1 to 2 and 6 to 7, depending on where in the density layer contents are present (Merritt, 2003). The pH of the small intestine ranges from 6 to 7 and the pH of the colon is approximately 6.3 (Argenzio, 1974). They suggest that the isolates have the potential to survive transit through the gastrointestinal tract of horses. *B. subtilis* was shown to inhibit growth of *S. Typhimurium*, *E. coli*, and *C. perfringens in vitro*. The possible use of bacilli as a probiotic in horses has not been reported, but similar findings have been seen in pigs. Alexopoulos et al. (2004a, b) reported that BioPlus 2B (containing a 1:1 ratio of *B. subtilis* and *B. licheniformis*) reduced the mortality as well as the morbidity of pigs associated with *E. coli* diarrhea, as well as increased feed consumption and body weight of sows.

The *L. salivarius* isolate was also shown to inhibit the growth of *S. Typhimurium*, *E. coli*, and *C. perfringens in vitro*. Other lactic bacteria have been used in horses; however they have not been shown to be very effective. For example, Weese et al. (2004) showed that *L. pentosus* WE7 inhibited growth of *Salmonellae*, *C. difficile* and *C. perfringens in vitro* but it didn't prove efficacious *in vivo*. Instead of preventing neonatal diarrhea, probiotic treated foals were more likely to develop diarrhea as well as other clinical abnormalities (2005). Several commercial probiotics, Probiocin, Quick Fix and Fastrack, have been tested in the past but did not demonstrate a significant improvement in gastrointestinal illness in horses (Parraga, Spiere et al., 1997; Kim, Morley et al., 2001). *L. salivarius* and other lactic bacteria, however, have been successfully used as a component of probiotics in swine. According to Nemcova et al. (1997), *L. salivarius* as well as several other lactic bacteria inhibited the growth of *E. coli* 08:K88 ab H9 and *E. coli* 0101:K99 *in vitro* and may be used for possible probiotics in pigs.

CONCLUSION

The findings from this study showed that *B. subtilis* and *L. salivarius* isolates recovered from equine feces inhibited the growth of *S. Typhimurium*, *E. coli*, and *C. perfringens in vitro*. Therefore, *B. subtilis* and *L. salivarius* can be considered as components in a potential equine probiotic that may inhibit the growth of enteric bacteria associated with horses. Further evaluation of these organisms *in vivo* at different dose levels with different treatments in separate pastures is warranted to expand understanding of gastrointestinal transit. Also, it would be fascinating to assess the *in vitro* inhibition of these organisms in a pathogenic environment, such as the gastrointestinal contents taken from horses diagnosed with salmonellosis, *E. coli* diarrhea, or Clostridiosis. The issue of subtherapeutic use for prevention of disease versus therapeutic levels for treatment of disease in both adults and foals needs to be addressed, as well.

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