

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

by

BRENDA LEE DARBY

(Under the Direction of Gary L. Heusner)

ABSTRACT

Four probiotics were isolated to test *in vitro* growth at varying pH levels, in taurocholic acid, inhibition against *S. Typhimurium*, *E. coli* and *C. perfringens* and *in vivo* survival in the GI tract of horses. *B. subtilis* had significantly more growth than *B. licheniformis* at pH 3 ($P<0.001$). In 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 *in vitro* inhibition due to better growth under both acidic and bile environments; inhibition by *B. subtilis* was significantly greater than *L. salivarius* ($P<0.0001$). Both *B. subtilis* and *L. salivarius* survived passage through the GI tract of horses with no significant differences among treatments. *B. subtilis* and *L. salivarius* can be considered as components in a potential equine probiotic to inhibit the growth of enteric bacteria associated with gastrointestinal illness in horses.

INDEX WORDS: Equine, Horses, Probiotics, Inhibition, Lactobacillus, Bacillus

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CHAPTER 1

INTRODUCTION

There are numerous microbial organisms naturally occurring in the environment that, under certain conditions, can become pathogenic when the body's immune system is compromised by illness, surgery or stress. Among these many organisms are *Salmonella*, *Escherichia coli* and Clostridia. *Salmonella* are gram-negative facultative anaerobic bacteria common to the gastrointestinal tract and *S. Typhimurium* is the most pathogenic serotype found in horses (Jones 2004). *E. coli* is another typical inhabitant of the GI tract with some strains known to cause disease in animals or humans (Dunowska, Morley et al. 2006). Thirdly, Clostridia are found in horses of all ages and are among the first bacteria acquired after birth, though they are normally found in low numbers in the gastrointestinal tract (Jones 2004).

Horses shedding salmonellae are a potential source of infection to susceptible horses, as is the environment; for these reasons, salmonellosis is one of the most common nosocomial diseases in horses (Jones 2004). Salmonellosis is a severe and potentially fatal disease that primarily affects horses recovering from abdominal surgery for colic (Ikeda, Hirsh et al. 1986; Traub-Dargatz, Salman et al. 1990). Other risk factors for salmonellosis outbreaks in horses include stress from transport to the veterinary hospital, administration of antibiotics, diarrhea, general anesthesia and major surgery, and nasogastric intubation (Tillotson, Savage et al. 1997; Mainar-Jaime, House et al. 1998; Schott, Ewart et al. 2001). *Salmonella* outbreaks from horses have been reported in several veterinary teaching hospitals, including Universities in California, Georgia, and

Wisconsin and Colorado and Michigan State Universities (Ikeda, Hirsh et al. 1986; Hartmann, Callan et al. 1996; Tillotson, Savage et al. 1997; Schott, Ewart et al. 2001), with estimated costs per outbreak have ranging from \$10,000 to \$428,174 (Kim, Morley et al. 2001). In addition, hospital employees and animal owners are potentially at risk of acquiring the disease, especially if they are immunocompromised or are receiving antimicrobials (Gorbach 1993). According to Ward et al. (Ward, Brady et al.), the teaching hospital at Purdue University was closed for a period of ten weeks due to a strain of *Salmonella* Typhimurium which has been shown to be *in vitro* resistant to a total of thirteen different antibiotics.

E. coli has long been known to cause diarrhea in calves, lambs, pigs and humans and it has been suggested that *E. coli* isolated from diarrheic foals may express some of the same attributes as those found in other neonatal farm animals (Holland, Schmidt et al. 1996). *E. coli* is a naturally occurring component of the gastrointestinal microflora, yet certain pathogenic strains have been associated with diseases such as diarrhea and hemorrhagic colitis (DebRoy and Maddox 2001). Diarrhea associated with acute colitis occurs sporadically in horses of all ages, is highly fatal and treatment is costly due to the massive amounts of intravenous fluids required (McConnico 2004). Attempts have been made to develop treatments such as vaccines or pharmacological agents but have been hindered by the unavailability of acceptable models and the complex equine intestinal pathophysiology (McConnico 2004).

Clostridiosis is an important cause of acute enterocolitis in horses, most commonly caused by *Clostridium perfringens* and *C. difficile*, though other species such as *C. septicum*, *C. cadaveris*, and *C. sordellii* have also been isolated from horses with

enterocolitis (Jones 2004). The main pathologic features of *C. perfringens* are edema, necrosis and death, regardless of the site of action (Long 2004). *C. perfringens*, specifically, has been associated with 68% of foals with diarrhea that died and another 47 of 54 with progressive enterocolitis that often had a fatal outcome (Jones 2000). The specific role of clostridia in enterocolitis has often been widely debated; arguments range from that of primary cause of hemorrhagic enterocolitis to that of opportunistic growth in an altered intestinal environment (Traub-Dargatz and Jones 1993).

Due to growing concern over pathogenic bacteria such as these, there is increasing interest in antimicrobial alternatives as a means of preventing or reducing the prevalence of antibiotic resistant pathogens in horses. In veterinary medicine, there is increased interest in probiotic therapy as an alternative approach for the prevention and treatment of diseases. By definition, a probiotic is a living microorganism that, when administered orally in adequate levels, provides beneficial effects beyond their nutritional value. However, there is limited evidence on the beneficial effects of probiotic administration in horses. Commercially available probiotics have shown little effect in horses (Parraga, Spier et al. 1997; Kim, Morley et al. 2001), possibly because the probiotic organisms were not host species-specific. Therefore, the objective of this study will be to isolate and identify specific probiotic species of equine origin with beneficial properties that will be useful in the prevention or treatment of *Salmonella* Typhimurium, *Escherichia coli*, and *Clostridium perfringens* in horses.

CHAPTER 2

LITERATURE REVIEW

Salmonella

According to Smith et al. (1978), *Salmonella* infection is a common occurrence among hospitalized horses, sometimes attributed to nosocomial infection. Stress factors are also frequently associated with salmonellosis in horses and can include long distance transportation, antimicrobial treatment, colic surgery, changes in diet, food deprivation, parturition, anesthesia and anthelmintic treatment (House, Mainar-Jaime et al. 1999). According to the review by Reich (2005), salmonellosis is characterized by an acute colitis with profuse watery diarrhea that is often malodorous and varying in color from green to black. Secondary complications can include laminitis, septicemia, renal failure and pneumonia. For susceptible horses, the surrounding environment is their greatest risk. *Salmonella* species are very adaptable to varying conditions and have been recovered from contaminated soil after more than 300 days and from water after 9 months (Reich 2005). While they can be killed by desiccation or exposure to sunlight, *salmonellae* will survive in dried feces for more than two years.

Traub-Dargatz et al. (2000) sampled 972 equine operations in 28 states for prevalence of fecal shedding of *Salmonella* spp. Results from Traub-Dargatz et al. (2000) showed that the overall prevalence of operations with ≥ 1 horse shedding *Salmonella* in the feces was 1.8%, with prevalence being higher in summer than winter and among operations from southern rather than northern states. Other studies have shown shedding rates ranging from 2.8 to 5% with no clinical signs of salmonellosis (Smith, Reina-Guerra et al. 1978; Begg, Johnston et al. 1988). When horses shed

Salmonella without showing any clinical signs of salmonellosis, the organisms can easily spread to other animals, therefore increasing the risk for development of clinical salmonellosis (Ewart, Schott et al. 2001).

In a study by Alinovi et al. (2003) at the Purdue University School of Veterinary Medicine, presenting illness was identified as the primary risk factor for whether a horse will shed *Salmonella*. Alinovi et al. (2003) based their conclusion on the general teaching hospital population rather than only those involved in a salmonellosis outbreak. Also, horses presented for elective reasons (surgery, lameness) and those accompanying sick animals were at reduced risk of shedding *Salmonella* while those that presented with gastrointestinal disorders were at intermediate risk (Alinovi, Ward et al. 2003). In disagreement with some previous studies (Owen, Fullerton et al. 1983; House, Mainar-Jaime et al. 1999), Alinovi et al. (2003) and Traub-Dargatz et al. (1990) both showed that antibiotic use was not associated with the risk of a horse being culture positive for *Salmonella* nor did they find a positive association between nasogastric intubation and culture status.

According to the review by Dargatz and Traub-Dargatz (2004), when bacteria lack certain structures or functions that are targeted by an antimicrobial drug, they are inherently resistant. Bacteria can also acquire resistance through mutation or gene transfer from other bacteria through the uptake of DNA from the environment, acquiring new DNA by phage transfer, or transfer among live cells (Prescott and Baggot 1985). These genes are then retained and activate in the presence of an antimicrobial drug, leading to bacterial resistance to the effects of the antimicrobial (Prescott and Baggot 1985; Martinez and Baquero 2000). In a hospital setting, such as veterinary teaching

facilities, the environment is uniquely capable of housing commensal organisms that can serve as a reservoir for resistant bacteria that are non-pathogenic yet can opportunistically invade a host animal creating a nosocomial infection (Dargatz and Traub-Dargatz 2004).

There have been numerous outbreaks of nosocomial salmonella infection in veterinary hospitals, some resulting in temporary closure of the hospital. Schott et al. (2001) reported that Michigan State University's Large Animal Clinic was closed from Jun 26 to Jul 22, 1996 due to an outbreak of a multiple-drug resistant *S. Typhimurium* in 19 equine patients. In 1999, an outbreak of salmonellosis resulted in the closure of Purdue University teaching hospital for 10 weeks; 33 cases of infection by multi-drug resistant *S. Typhimurium* were detected (Ward, Brady et al. 2005). In 2002, the University of Florida-Veterinary Medical Teaching Hospital (UF-VMTH) conducted a study to estimate the prevalence of *Salmonella* shedding in hospitalized horses (Ernst, Hernandez et al. 2004). The overall prevalence of fecal shedding was 13% with prevalence being higher in foals than in adults. In addition, Ernst et al. (2004) reported that foals presented with gastrointestinal tract (GIT) disease were significantly more likely to be shedding *Salmonella* in their feces than were adults with GIT disease. According to van Duijkeren et al. (2002), 232 strains of *Salmonella* were isolated and tested for antimicrobial susceptibility during the period 1993-2002. van Duijkeren et al. (2002) observed that the predominant serovar was *S. Typhimurium*, with *S. enteritidis* second most prevalent. Phage typing showed that the most common types of *S. Typhimurium* found in horses were pt 506, pt 401, pt 510, pt 506 and pt 508. van Duijkeren et al. (2002) suggests there may be a possible common source since the phage

types found were similar to those found in humans, cattle and pigs during the same period.

Escherichia coli

Information on the association of *E. coli* and diarrhea in horses is limited. While *E. coli* is frequently isolated from the feces of diarrheic foals, it is uncertain whether it is the actual cause of enteric disease among foals (Holland, Schmidt et al. 1996). van Duijkeren et al. (2000) found that horses with diarrhea had different *E. coli* genotypes than those with normal feces but no proof that *E. coli* was the cause of the diarrhea. Holland et al. (1996) found 64 different serotypes among 99 strains of *E. coli* from both healthy and diarrheic foals. With this much diversity, serotyping was useless in distinguishing strains and the enterotoxigenic (ETEC) strains that commonly cause diarrhea among other species did not appear to be involved in the diarrheic foals.

Potentially virulent strains of *E. coli* may be present in the feces but the role of these strains in foal diarrhea requires more clarity (Holland, Schmidt et al. 1996). When challenged with a strain of ETEC *E. coli* from a diarrheic foal, both colostrum deprived and nursing foals failed to develop diarrhea within the 72h observation period (Holland, Grimes et al. 1996). They theorized that this was due to the failure of the ETEC strain to colonize the intestinal epithelium. Ward et al. (1986) isolated *E. coli* from the feces of foals but attachment of the organisms to equine enterocytes was inconclusive in both in vivo and in vitro tests.

According to DebRoy and Maddox (2001), pathogenic strains of *E. coli* can cause considerable losses of neonatal animals and tend to fall into one of four categories: ETEC, enteropathogenic (EPEC), enterohemorrhagic (EHEC) and necrotoxicogenic

(NTEC) based on their virulence properties. Enterotoxigenic strains attach to the brush border membrane of the jejunum and ileum and cause watery yellow, white to grey diarrhea in neonatal animals (DebRoy and Maddox 2001). Enteropathogenic strains of *E. coli* form lesions that destroy the brush border of the small intestine and form pedestal structures so that the bacteria remain in contact with the cells and cause chronic, mucoid diarrhea (DebRoy and Maddox 2001). Enterohemorrhagic *E. coli* colonizes the colon and causes necrosis of the villi; diarrhea is mucoid, sometimes hemorrhagic, and seldom fatal but often recurrent even with treatment, resulting in dehydration and reduced growth (DebRoy and Maddox 2001). Necrotoxigenic strains produce a toxin called cytotoxic necrotizing factor (CNF) and although not much is known about the pathogenesis, CNF-1 occurs in about 15% of equine isolates (DebRoy and Maddox 2001).

Scott (2002) showed that there is transfer of genetic material from commensal and pathogenic bacteria in the GI tract because identical resistance genes are present in a variety of bacteria species; this plays a role in the spread of antibiotic resistance.

Dunowska et al. (2006) studied antimicrobial susceptibility of *E. coli* in the feces of horses and found that a combination of both hospitalization and antimicrobial drugs were associated with the prevalence of antibiotic resistance, not just hospitalization alone. Of possible importance was that Dunowska et al. (2006) found that the use of cephalosporins tended to increase multi-drug resistance in non-type specific *E. coli*. Other antimicrobial drugs found to have increased odds of resistance by *E. coli* were streptomycin, sulfonamides and tetracycline. Dunowska et al. (2006) stressed that a possible confounding factor would have been the possible horizontal transfer of resistant bacteria

among hospitalized horses, as well as severity of illness, diet or health history prior to hospital admission.

Clostridia perfringens

Clostridia perfringens is a third commensal/pathogenic organism associated with diarrhea in horses. *Clostridium* species include many aerobic and aerotolerant spore-forming gram-positive rods (Jones 2000). *C. perfringens* is widely distributed in the environment as both vegetative cells and spores (Jones 2000; Feary and Hassel 2006) and is one of the first organisms to colonize the neonatal intestinal tract. With early colonization such as this, the organism can be present for as little as a few hours or as long as months to years before rapid growth and toxin production cause enterocolitis in foals and adult horses (Traub-Dargatz and Jones 1993).

Signs of clostridial enterocolitis in adult horses are seen less regularly or are less commonly diagnosed (Feary and Hassel 2006) but can include death without diarrhea, bloody diarrhea, abdominal pain with or without diarrhea and, occasionally, a reduction in appetite and depression in the early infection stages (Traub-Dargatz and Jones 1993). In foals, there is acute abdominal pain, fever, mild to severe bloody diarrhea, septic and hypovolemic shock and sometimes death before the onset of diarrhea (Feary and Hassel 2006). *C. perfringens* type C in foals has a very high mortality rate, in spite of aggressive medical treatment (East 1998). Clinical signs of clostridial enterocolitis can be confused with those of equine grass sickness or small intestinal strangulation so it is important to confirm a diagnosis (Griffiths 1997). Diagnosis can be made based on isolation of large numbers of *C. perfringens* and identification of the toxin type using PCR assay on either the feces or isolated bacteria (Feary and Hassel 2006).

C. perfringens has four major toxins (α , β , ϵ , and ι) , an enterotoxin (CPE) and three minor toxins associated with it (Songer 1996; Long 2004). Type A *C. perfringens* is the predominant type found in healthy horses as well as foals with diarrhea and foals and adults with enterocolitis, suggesting that other unknown factors can contribute to pathogenic *C. perfringens* (Jones 2000). Type A *C. perfringens* produces α -toxin, which can also be found in other typeable strains, but not with consistent lethal quantities as that of type A (Jones 2000). Other individual cases have been reported to be associated with type B (α -, β - and ϵ -toxins), C (α - and β -toxin), and D (α - and ϵ -toxin) (Songer 1996; Long 2004). Type C is the most commonly found clostridial enteric pathogen found in North America (Songer 1996).

Successful treatment of clostridial enterocolitis involves early and aggressive fluid therapy and broad spectrum antibiotics, with metronidazole being the drug of choice (Jones 2000; Feary and Hassel 2006). With neonates, it is extremely important to identify the disease early, withhold feed and begin immediate partial or total parenteral nutrition (Feary and Hassel 2006). Oral administration of metronidazole may be more effective than intravenous administration by inhibiting small intestinal bacterial populations locally (Feary and Hassel 2006). Acquired resistance to antimicrobials associated with growth promotion and disease prophylaxis have been reported in swine and poultry (Songer 1996) though none has been reported in horses as yet. Various probiotics have been evaluated in laboratory animal models and in vitro tests but data in horses is insufficient (Jones 2000).

Probiotics

Due to growing concern over potential pathogenic bacteria such as *Salmonella*, *E. coli* and *C. perfringens* there is increasing interest in developing antimicrobial alternatives as a means of preventing or reducing the prevalence of antibiotic resistant pathogens. Probiotics have been suggested as just such an alternative. Probiotics are defined as living microorganisms that may beneficially affect the host upon ingestion by improving the balance of the intestinal microflora. To be an effective probiotic, the microorganism(s) must have the ability to: 1) adhere to cells; 2) exclude or reduce pathogenic adherence; 3) persist and multiply; 4) produce acids, hydrogen peroxide and bacteriocins antagonistic to pathogen growth (competitive exclusion of pathogens); 5) be safe, noninvasive, noncarcinogenic and nonpathogenic; and 6) co-aggregate to form normal balanced flora (Kaur, Chopra et al. 2002). A few potential probiotic strains include *Lactobacillus* species, *Bifidobacterium* species and yeasts.

By 1997, farming was the second largest user of antibiotics in Europe and approximately one third were used as feed supplements (Hong, Duc le et al. 2005). With improved biosecurity and farm management practices, the benefits of antibiotics as a feed supplement diminishes (Cartman 2004). As an alternative, probiotic feed supplements have the potential to enhance feed conversion efficiency, rate of weight gain, milk yield, milk quality, egg yield and egg quality as well as increase resistance to infectious diseases (Cartman 2004). Finland has used competitive exclusion products in their poultry feed since 1976 with no safety hazards to man or birds (Schneitz 2005). Denmark banned the use of antibiotics as growth promoters in its swine industry in 2000

(Hong, Duc le et al. 2005) and Europe has banned the use of antibiotics in animal feed since January 2006 (Bernardeau 2006).

Lactobacilli have been used for centuries in fermentation and food processing but their use as a probiotic have only recently been studied (Bernardeau 2006). Lactobacilli are gram-positive non spore-forming rods (Coenye 2003); they are fermentative, aerotolerant or anaerobic, aciduric or acidophilic (Bernardeau 2006). Alakomi et al. (2000) observed that lactic acid, when produced by lactic acid bacteria, functions as a natural antimicrobial by disintegrating the outer membrane of gram-negative bacteria, causing lipopolysaccharide release. While hydrochloric acid also disrupted the outer membrane, lactic acid was observed to be more effective at the same pH than HCl-treated bacteria (Alakomi 2000).

L. salivarius and other lactic bacteria have been successfully used as a component of probiotics. 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 (Nemcova, Laukova et al. 1997). Tsai et al. (2005) isolated two strains of lactobacilli, LAP5 and LF33, from swine and poultry, respectively. Both strains were acid and bile tolerant and were found to inhibit the *in vitro* growth of *E. coli*, *Salmonella* Typhimurium, *Staphylococcus aureus*, and *Bacillus cereus* (Tsai 2005).

Spore forming bacteria have also been studied recently for their probiotic properties. A spore-forming culture can readily be reproduced, has a long shelf life and, in the case of *B. subtilis*, is not virulent. Alexopoulos et al. (2004a, b) reported that BioPlus 2B (containing a 1:1 ratio of *B. subtilis* and *B. licheniformis*), fed at a dose of

400 g/ton of feed equaling 1.28×10^6 viable spores per g of feed, 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. When Kritas and Morrison (2005) compared BioPlus 2B to subtherapeutic doses of antibiotics, they observed similar daily gain, feed intake and feed conversion. Additionally, the cost of feed per pig and per kg of bodyweight were similar with no differences in mortality rate (Kritas 2005). Maruta et al. (1996) observed that *Bacillus subtilis* significantly increased *Bifidobacterium* and *Lactobacillus* significantly decreased *Streptococcus* with the change being more evident in gilts than sows. Additionally, diarrhea in newborn piglets and mortality rate of piglets up to 25 days were reduced (Maruta 1996).

Research of probiotics in horses is limited to date, focusing mostly on lactobacilli; the possible use of bacilli in horses has not yet been reported. 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 (Weese and Rousseau 2005). Kim et al. evaluated the use of Fastrack Equine Gel, containing 1.25 billion CFU lactic acid producing bacteria and 25 million live yeast cells, on fecal *Salmonella* shedding. They failed to detect a difference on *Salmonella* shedding either due to inadequate dose or inadequate treatment duration. Also, they only evaluated the probiotic after the onset of GI illness, not as a prevention (Kim, Morley et al. 2001). Parraga et al. (1997) performed a double blind study with coded syringes whose probiotic contents were to be revealed after data were collected and analyzed but were not mentioned in the paper. Neither probiotic protected against

Salmonellosis, salmonella shedding or postoperative diarrhea; nor was there a decrease in hospitalization time or duration of antibiotic therapy (Parraga, Spier et al. 1997).

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CHAPTER 3

***In vitro* inhibition of growth of *Escherichia coli*, *Salmonella* Typhimurium, and *Clostridia perfringens* using Probiotics¹**

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ABSTRACT

Four potential probiotic species were isolated to test *in vitro* survivability and growth at varying pH levels and in the presence of taurocholic acid and then determine *in vivo* survivability in the GI tract of horses. 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 & 7 there was growth by all 4 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* ($P<0.0001$). Fifteen mares were used to test the gastrointestinal survival of the selected isolates, 5 received a sterile distilled water placebo, 5 received *B. subtilis*, and 5 received *L. salivarius*. Both isolates survived passage through the GI tract of horses but there were no significant differences among treatments. 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.

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 of taurocholic acid; 3) to compare the *in vitro* inhibition of the selected isolates against growth of *S. Typhimurium*, *E. coli* and *C. perfringens* and 4) to determine the survivability of selected isolates in the gastrointestinal tract of horses.

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 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) 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

MRS broth and tryptic soy broth were modified by the addition of taurocholic acid, sodium salt hydrate (Sigma) to a concentration of 0 and 0.3%. 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.

Evaluation of intestinal transit in mature horses

Novobiocin resistant mutants (N^r) of Lactobacilli and Bacilli that showed greatest inhibition of *S. Typhimurium*, *E. coli*, and *C. perfringens in vitro* were selected on Novobiocin sodium salt (Sigma) gradient plates (Chan et al., 1993) with a concentration of 0.2% Novobiocin per liter of agar. Fifteen mares were used to test the gastrointestinal survival of the selected isolates, 5 received a sterile distilled water placebo, 5 received *B. subtilis*, and 5 received *L. salivarius*. A 5 ml oral dose of the N^r lactobacilli and bacilli isolates were administered at 10^8 CFU/ml. Fecal samples were collected at day 0 prior to administering the probiotic and at day 1, 3, 5 and 7 post-administration to assess survivability of the selected isolates. Fecal samples were serially diluted and plated on Lactobacillus and Tryptic Soy agar, respectively, with a concentration of 0.2% Novobiocin per liter of agar.

Statistical Analysis

Each experiment was replicated five times. Data were analyzed using the GLM procedures of SAS [6]. 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.T.*, *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*.

Results of the *in vivo* gastrointestinal survivability of N^r *L. salivarius* and N^r *B. subtilis* are listed in Figure 1. N^r *L. salivarius* and N^r *B. subtilis* were not detected in the feces at day 0 prior to administration because the agar had 0.2% Novobiocin to identify the N^r mutants, which inhibited the growth of the naturally occurring bacteria. Though the selected isolates survived the gastrointestinal tract on days 1 through 7, there were no significant differences among treatments or days.

Table1. Growth of *L. salivarius*, *L. agilis*, *B. subtilis* and *B. licheniformis* in acidic broth (Log10 CFU/ml)

pH	Culture							P-value		
	Ctrl 1	<i>B. licheniformis</i>	<i>B. subtilis</i>	Ctrl 2	<i>L. salivarius</i>	<i>L. agilis</i>	SEM	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 ^c	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/ml)

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

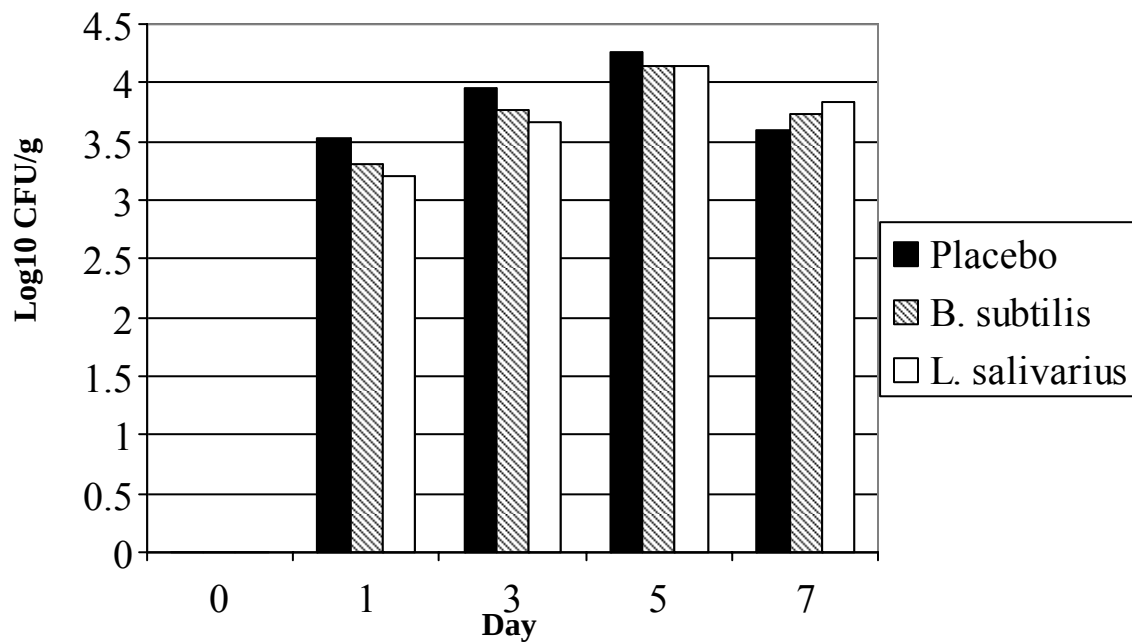
Item	Culture				SEM	P-Value
	Ctrl 1	<i>B. subtilis</i>	Ctrl 2	<i>L. salivarius</i>		
<i>S. typhimurium</i>	0 ^a	12.43 ^b	0 ^a	5.97 ^c	0.095	<0.0001
<i>E. coli</i>	0 ^a	11.37 ^b	0 ^a	6.00 ^c	0.119	<0.0001
<i>C. perfringens</i>	0 ^a	37.43 ^b	0 ^a	6.53 ^c	0.344	<0.0001

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

Figure 1. In vivo survivability of N^r mutations of *B. subtilis* and *L. salivarius* (Log10 CFU/g)



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 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 (Weese, 2005). Several commercial probiotics have been tested in the past but did not demonstrate a significant improvement in gastrointestinal illness in horses (Parraga, Spier 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.

When comparing the equine gastrointestinal survival of the Novobiocin resistant mutants to a placebo, there were no significant differences, which was unexpected. It is possible that this was due to an inadequate dose or inadequate treatment duration or even contamination of the syringes used or hands delivering the probiotic. It is also possible that there was some cross contamination between treatments because all horses were in

the same pasture, sharing hay, feed and water. Coprophagy among the mares, social grooming might have been sources of cross contamination among treatments, as well.

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 survival. 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 a horse 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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CHAPTER 4

CONCLUSIONS

The results of this study indicate that *B. subtilis* and *L. salivarius* isolates recovered from equine feces inhibited the growth of *S. Typhimurium*, *E. coli*, and *C. perfringens in vitro* and inhibition by *B. subtilis* was significantly greater than that of *L. salivarius*. Though there has been no indication of efficacy *in vivo* as yet, the *in vitro* inhibitory effects of *B. subtilis* and *L. salivarius* indicate the need for further evaluation into differing dose levels in the treatment and/or prevention of enteric disease in horses. *B. subtilis* and *L. salivarius* can be considered as components in a potential equine probiotic to inhibit the growth of enteric bacteria associated with gastrointestinal illness in horses.