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Research Article

# Characterization and identification of bacterial flora from infected equine hooves

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## Abstract

**Background:** The aim of this study was to characterize and identify the bacterial flora associated with a specific hoof infection in a population of horses in the Rochester NY area.

**Methods:** Samples taken from horses showing symptoms of infection were first grown on a Brain-Heart Infusion (BHI) solid medium. Forty different bacterial colonies were obtained and characterized microscopically and biochemically. The identity of these bacteria was also confirmed by 16S ribosomal DNA sequencing.

**Results:** We have identified normal inhabitants of the skin such as *Staphylococcus epidermidis* as well as bacteria of the genus *Bacillus* commonly found in soil. In addition, bacteria such as *Enterococcus gallinarum* and *Lactobacillus* normally found in the intestinal tract of mammals were recovered.

Interestingly, *Corynebacterium xerosis*, which is known to cause animal diseases was also isolated from infected horses.

**Conclusions:** Our results indicate that the infection affecting the horses is likely the result of the presence of multiple bacterial genera including members of the normal skin and gut flora, as well as soil bacteria.

## Introduction

Hoof infections in horses are a very common problem. The majorities of infections are mild and easily treatable, but in some cases can result in chronic lameness and even death [1]. The hoof is a very critical and essential component of a horse's anatomy. It acts as the primary weight-bearing structure and protects the internal structures of the foot such as the laminae, the short pastern, and coffin bone [2]. The major components of the hoof are the coronary band, wall, sole, white line, and frog. The hoof wall is composed of three keratinized layers known as stratum externum, stratum medium, and stratum internum [3]. The coronary band is the junction point of the hoof with the skin. It is responsible for the growth of the hoof wall. The wall is the hard exterior of the hoof and connects with the underside of the hoof, the sole, at the white line. The frog is wedged-shaped and projects into the sole from the heel of the hoof. The wall and the frog are the weight-bearing surfaces of the hoof and result from the concaveness of the sole.

The hoof substance is made up of a tubular and intertubular horn. The proportion of tubular to intertubular horns varies with respect to location. For instance, the wall of the hoof is predominantly tubular, but the frog is predominantly composed of the intertubular horn. The tubular horn is produced by the hair-like dermal papillae of the coronary band, sole, and frog. The intertubular horn is produced by the hollows that form between the dermal papillae [4]. Damage to the coronary band causes defective growth of the hoof. Damage to the coronary band is usually the result of physical trauma, although abscesses can also be the cause.



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An abscess is defined as a localized accumulation of pus within the hoof [5]. Abscesses are caused by bacteria entering through a wound in the sole and traveling up the white line [5]. Abscesses can also be caused by deep bruises to the sole of the hoof [5]. In either case, bacteria become trapped inside the hoof and pressure builds up which results in a great deal of pain for the horse. The trademark symptom of an abscess is the severe lameness it causes. In some cases, the horse will actually walk on three legs. This unequal weight bearing can cause laminitis which left untreated can lead to founder. The founder is especially damaging to the overall health of the horse because it causes rotation of the coffin bone within the hoof and leads to prolonged lameness [6]. In severe cases, the horse is euthanized.

Abscesses are treated by opening up the pocket of pus [6]. This relieves the built-up pressure and allows the drainage of the bacteria. Additionally, the hoof can be soaked in Epsom salts and wrapped to aid in healing. Abscesses can also damage the coronary band when the bacteria track up the white line and break out at the coronary band [6].

Another common hoof infection is thrush. Studies have shown that as many as 90% of domestic horses suffer from some degree of bacterial/fungal hoof infection related to thrush. Thrush is an infection of the central and lateral sulci (clefts) of the frog of the horse's foot [7]. The name thrush implies a fungal infection, although, thrush in a horse's frog most often involves bacteria. One species of bacterium, *Fusobacterium necrophorum*, is particularly aggressive, invading and destroying the frog, sometimes exposing the deeper sensitive tissues [7,8]. A combination of factors such as soft or damp footing, improper trimming, and a diet rich in sugars and carbohydrates, makes domestic horses susceptible to thrush. Thrush is treated by removing overgrown areas of the frog and sole as well as scrubbing the infected area with povidone-iodine or hydrogen peroxide. Lastly, white line disease is the separation of the hoof wall from the sole which allows bacteria and fungi to invade the resulting space [9]. Horses are more susceptible to white line disease in damp conditions [9]. Severe white line disease can lead to chronic laminitis in horses. Treatment for white line disease involves removing the infected areas of the white line and applying an anti-bacterial such as iodine.

The aim of this study was to identify the bacteria causing the weakening of the white line and the foul-smelling odor of the hooves. The horses involved in this research all have a history of abscesses, thrush, and white line disease.

## Methods and materials

### Sample collection

Samples were collected from the infected hooves of four horses. Two were 20-year- old Paint mares, and the other two were approximately 14-year-old Thoroughbred geldings. Horses reside at the same location and are therefore exposed to the same elements. The infected areas were cleaned out using a hoof knife and sterile swabs were used to sample the area. The swabs were placed in sterile tubes containing saline solution and refrigerated until plating. A total of eleven samples were taken which included nine samples from infected regions on the hoof, one control, and one sample from a hoof clipping to establish the general flora of the hooves.

### Bacterial isolation

The eleven samples were plated in duplicate on Brain-Heart infusion (BHI) Agar (BD Biosciences). One set of plates was incubated at 37˚C for 48 hours in aerobic conditions, and one set was incubated in an anaerobic jar (BD BBL) at 37˚C for 48 hours. Following incubation, each plate was investigated and macroscopic observations were recorded. Different colonies were then isolated using the streak plate technique on new BHI agar and incubated at 37˚C overnight in the presence or absence of oxygen. A total of 35 colonies were isolated and further characterized.

The presence of *Fusobacterium necrophorum* was determined by plating each of the eleven samples on a duplicate selective medium [10]. The medium contains (per liter): trypticase, 32g; digested casein, 5.2g; yeast extract, 2.8g; thiotone, 4g; glucose, 1g; MgSO4, 9.6mg; Na2HPO4, 5g; agar, 16.6g. The ingredients were combined, autoclaved, and allowed to cool in a water bath. Phenylethyl alcohol (2.7ml), an egg yolk, 90 ml of 9% saline solution, and 50 ml of 0.8% crystal violet (in sterile water) were aseptically added to the medium.

### Identifying bacteria method 1

The first approach used for the identification of the bacterial population present in the infected hooves was based on the morphology, the Gram stain reaction, as well as the biochemical properties of each isolated bacterium. First, a Gram stain was performed on each bacterium and the results, including the cell morphology and the morphological arrangement, were recorded. Based on these results, several biochemical tests were then performed for further identification.

A. Carbohydrate fermentation: The ability to ferment carbohydrates with the production of acid was determined by inoculating 3 ml of a purple broth base broth (BD Difco) containing bromocresol purple as a pH indicator, as well as the appropriate carbohydrate at a final concentration of 1% with a single colony, and incubating at 37ºC for 48 hours. In the presence of acidic products resulting from fermentation, the broth will turn yellow (pH ≤ 5.2), indicative of a positive result. In the absence of fermentation, the broth will remain purple.

B. The presence of the enzyme catalase was determined by transferring a small number of bacteria onto a glass slide followed by the addition of one drop of 2% hydrogen peroxide. The production of bubbles was indicative of the generation of oxygen from hydrogen peroxide catalyzed by the enzyme catalase.

C. Starch hydrolysis: A starch agar (BD Difco) was inoculated in order to detect the presence of the enzymes amylase and maltase capable of hydrolyzing starch. Following



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incubation at 37ºC for 48 hours, the plate was flooded with Lugol's iodine solution (Thermo Scientific). Starch hydrolysis is visualized by the presence of a clear zone around the bacterial growth within 30s.

D. Novobiocin resistance: Resistance to the antibiotic novobiocin was determined by plating onto a Mueller-Hinton II medium (BD Difco), placing a sterile disk containing 30µg novobiocin onto the plate, and measuring the diameter of the zone of growth inhibition around the disk after incubation at 37ºC for 48 hours.

E. Citrate utilization: The ability of a bacterium to use citrate as the sole source of carbon and energy was determined by inoculating a Simmons citrate agar (BD Difco), which contains the pH indicator bromothymol blue, followed by incubation for 48 hours at 37˚C. In the event that citrate is utilized, the production of alkaline end products turns the medium blue, as opposed to remaining green in the absence of any citrate utilization.

F. Gelatin hydrolysis: The presence of the enzyme gelatinase was assessed by inoculating a semi-solid nutrient broth containing 12% gelatin and incubating for 48 hours at 37˚C. Following incubation, the gelatin tube was placed on ice for 30 min. If gelatin has been hydrolyzed, the content of the tube remained liquid.

G. Vogues-Prausker (VP) test: The VP test is used to detect the production of 2,3-butanediol, a neutral end product of glucose metabolism. A buffered peptone broth containing 3% glucose was inoculated with a single colony and incubated at 37ºC for 48 hours. Following incubation, 5 drops of Barritt's reagent A and 5 drops of Baritt's reagent B (Gibson Bioscience) were added and the tube was incubated at room temperature for 15 min. The presence of a pink to cherry coloration indicates a positive result.

H. Hemolysis: The ability of the bacterium to break down red blood cells was determined by plating it onto a blood agar plate and incubating overnight at 37˚C.

I. Endospore formation: To test for endospore formation, 1 drop of an overnight culture of the bacterium was transferred to 3 ml of nutrient broth and heated at 80˚C for 10 minutes. The presence of endospores was assessed by microscopic observations at a total magnification of 1000X. In addition, following heat treatment, the culture was plated onto BHI agar, followed by incubation overnight at 37˚C.

J. Growth on mannitol salt agar: The ability of bacteria from the genus *Staphylococcus* to ferment mannitol with the production of acid was examined by plating it onto a mannitol-salt agar (MSA) medium. The presence in the MSA of 7.5% sodium chloride is selective for bacteria of the genus *Staphylococcus*. In addition, the presence of phenol red as a pH indicator allows the determination of whether the bacteria can ferment mannitol with the production of acid (*S. aureus*) or is unable to ferment mannitol (non-pathogenic *Staphylococcus sp*).

### Identifying bacteria method 2

In order to confirm the results of the bacterial identification, we set up to amplify and sequence the 16S ribosomal DNA of the previously obtained bacterial isolates.

**Genomic DNA preparation:** Isolated colonies were grown in 3ml of BHI Broth overnight at 37ºC. Genomic DNA was obtained using the Wizard® SV Genomic DNA Purification System (Promega) with the following modifications: bacterial cells were centrifuged at 13,000 x g for 2min. The supernatant was removed and the pellet resuspended in 150µl of Wizard SV Lysis Solution and transferred to a mini-column fitted to a collection tube. Following centrifugation at 13,000 x g for 3min, the flow-through was discarded, and the column was washed 3 times with 650µl of column wash solution followed by centrifugation at 13,000 x g for 1 min. The genomic DNA was eluted with 250µl of Nuclease-Free water containing 2µl of RNase A solution for 2min at room temperature and spun at 13,000 x g for two minutes. The Genomic DNA was stored at -20˚C.

**PCR amplification:** Approximately 0.5-2µg of genomic DNA was amplified in a 100-µl reaction using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) and using oligonucleotides fD1(5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3') [11]. PCR conditions consisted of 35 cycles of 95˚C for 2 min, 42˚C for 30 s, and 72˚C for 4min.

**Cloning:** The amplification products were purified using the Silica Bead DNA Gel Extraction Kit (Thermo Scientific), ligated into the pJET1.2/blunt cloning vector (Thermo Scientific), and transformed into *Escherichia coli* DH5α cells. Plasmids were isolated from ampicillin resistant colonies using the GeneJET Plasmid Miniprep Kit (Thermo Scientific), and the 16S rDNA sequence determined using primer pJET-F: (5'-CGACTCACTATAGGGAGAGCGGC-3').

## Results

The samples collected from the four horses produced thirty-five colonies on the Brain-Heart infusion plates, which were further isolated and characterized. Interestingly, no growth was observed on either duplicate of the isolation media for *Fusobacterium necrophorum*, indicating that the bacterial infection was not thrush. Gram staining, as well as microscopic observations and biochemical tests, resulted in the identification of 11 different bacterial species. Table 1 shows the results obtained for the 35 bacteria.

In order to establish the general bacterial flora of the hooves, bacteria isolated from a hoof clipping were identified. Using biochemical tests and 16S rDNA sequencing, the following bacterial species were isolated only from the hoof clipping and not the hoof infection:

### *Paenibacillus popilliae*

*P. popilliae* formerly *Bacillus popilliae*, was isolated in one instance. A Gram stain led to the observation of sporulated Gram-positive rods. Further biochemical tests indicated that the isolate was catalase-negative, could produce acid from



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glucose but not mannitol fermentation, could not hydrolyze starch or gelatin, could not utilize citrate as the sole carbon source, was VP negative, and could not grow at 55°C. When the 16S rDNA was obtained, we were able to unequivocally identify the bacterium as *P. popilliae*.

### *Bacillus megaterium*

Microscopic observations of Gram-positive rods arranged in clusters, as well as the presence of spherical structures reminiscent of bacterial endospores, suggested the presence of a bacterium from the genus *Bacillus*. When the isolated colonies were cultured in BHI broth and incubated at 80ºC for 10 min, the presence of endospores could be observed in almost every cell . *B. megaterium* was identified on the basis of its ability to hydrolyze starch and gelatin, ferment glucose and mannitol with the production of acidic products, grow in nutrient broth containing 6.5% NaCl, and use citrate as a sole source of carbon. Unlike *B. subtilis*, this bacterium was unable to produce 2,3-butanediol from glucose fermentation (VP-) or to survive at 55°C. Determination of the 16S rDNA sequence validated the biochemical tests and confirmed the identity of this isolate as *Bacillus* megaterium.

### *Bacillus azotoformans*

Gram staining of this isolate revealed the presence of Gram-positive rods arranged in clusters. In addition, we observed the presence of endospores, especially after the bacteria were cultured in BHI broth and incubated at 80ºC for 10 min. Biochemical tests also led to the identification of this isolate as *B. azotoformans*. In addition, the isolate was catalase-negative, could not hydrolyze starch or gelatin, did not ferment glucose or mannitol, but was able to utilize citrate as a sole carbon source. The isolated bacterium did not grow at 55°C or in the presence of 6.5% NaCl, which is indicative of *B. azotoformans*. This was also confirmed by sequencing of the 16S rDNA.

Several bacterial species were specifically isolated from the hoof infections:

### *Corynebacterium xerosis*

The majority of the samples that we collected showed the presence of Gram-positive club-shaped rods, arranged in clusters. All 18 isolates were catalase-positive, did not form endospores, and could not hydrolyze starch. These characteristics, particularly the club-shaped cell morphology, led us to speculate that we isolated a bacterium belonging to the genus *Corynebacterium*. This was confirmed by the ability of all 18 isolates to produce acid from glucose but not mannitol fermentation, to hydrolyze starch, produce 2,3-butanediol ($VP^+$), but the inability to utilize citrate as the sole carbon source ($cit^-$). Sequencing of the 16S rDNA from representative isolates confirmed their identity as *Corynebacterium xerosis*.

### *Enterococcus gallinarum*

Five different isolates displayed similar microscopic characteristics, as well as identical biochemical properties. Following Gram staining, we observed the presence of Gram-positive cocci arranged in clusters. When a catalase test was performed, we observed a negative reaction for all 5 isolates, indicating that these bacteria did not belong to the genera *Staphylococcus* or *Micrococcus*, but, given their cellular arrangement, were likely members of the genus *Enterococcus*. The ability of the isolates to produce acid from glucose and mannitol fermentation, as well as the absence of gelatin and starch hydrolysis further, suggest their identities as *Enterococcus*. DNA from representative isolates was purified, and the 16S rDNA sequence was determined. This allowed the identification of the isolates as *Enterococcus gallinarum*.

### *Micrococcus varians*

Two isolates were identified as *Micrococcus varians*. This conclusion was based on the Gram reaction, the cellular morphology, and arrangement (Gram-positive cocci in clusters), but also on the presence of catalase, the production of acid from glucose fermentation, and the inability to ferment mannitol. In addition, this bacterium only grew in the presence of oxygen, and showed susceptibility to novobiocin, two characteristics of the genus *Micrococcus*. The precise identity of the isolates was determined to be *Micrococcus varians* following the sequencing of the 16S rDNA.

### *Psychrobacillus insolitus*

This Gram-positive rod was isolated from 2 different samples. We determined that the bacterium could not hydrolyze starch or gelatin, did not ferment glucose, mannitol, or citrate, was negative for the VP test, but expressed the enzyme catalase. Of particular importance was the observation of endospores that did not deform the cell. In addition, the ability of the isolates to grow in the presence and absence of oxygen points to the genus *Bacillus*. Sequencing of the 16S rDNA further confirmed that the isolates were *Psychrobacillus insolitus*, formerly known as *Bacillus insolitus*.

### *Macrococcus caseolyticus*

Two isolates displayed Gram-positive cocci arranged in clusters. Neither of the bacteria produced yellow colonies on BHI agar, but both were catalase-positive, grew well on MSA agar, fermented glucose but were unable to ferment mannitol. Based on these results and their susceptibility to novobiocin, we speculated that these bacteria were non-pathogenic staphylococci such as *Staphylococcus epidermidis*. However, determination of the 16S rDNA sequence revealed that the isolates were in *Macrococcus caseolyticus*.

### *Bacillus subtilis*

We first observed the presence of white to beige colonies on BHI, combined with microscopic observations of Gram-positive rods arranged in clusters. Most notably, we noticed the presence of spherical structures reminiscent of bacterial endospores. When the isolated colonies were cultured in BHI broth and incubated at 80ºC for 10 min, the presence of endospores could be observed in almost every cell. Biochemical tests led to the identification of the bacteria like *Bacillus subtilis*. The identification was based on its ability to produce acid from both glucose and mannitol fermentation, hydrolyze starch and



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gelatin, produce 2,3-butanediol from glucose fermentation (VP+), utilize citrate as a sole carbon source, and grow in the presence of 6.5% NaCl. In addition, the bacterium was unable to grow at 55°C. The identity of this isolate was confirmed by amplification and sequencing of the 16S rDNA.

### *Lactobacillus sp*

Gram-positive rods were isolated from one sample. The bacterium did not produce catalase, but fermented glucose and hydrolyzed starch. Results from mannitol fermentation, gelatin hydrolysis, citrate utilization, and the VP test were all negative. In addition, the isolate could grow in the presence or absence of oxygen. These results, combined with 16S rDNA sequencing, led us to conclude that the isolate belongs to the genus *Lactobacillus*.

### *Staphylococcus epidermidis*

*S. epidermidis* was isolated from one sample. Gram stain of isolated colonies obtained following growth on BHI medium revealed Gram-positive cocci arranged in clusters, characteristic of the genus *Staphylococcus* and *Micrococcus*. This was confirmed by the ability of those cells to express the enzyme catalase. In addition, as all of the isolated colonies were either white or beige, we concluded that they most likely belong to the genus *Staphylococcus* rather than *Micrococcus*. The colonies grew on MSA but did not turn the medium from red to yellow, indicating the inability to ferment mannitol with the production of acid. At this point, the identity of the bacteria isolated was limited to either *S. epidermidis* or *S. saprophyticus*. However, the fact that the bacterium was susceptible to the antibiotic novobiocin allowed us to conclude that *S. epidermidis* had been isolated. The sequence of the 16S rDNA further confirmed the identity of the isolate to be *S. Epidermidis* (Table 1).

## Discussion

Not all of the bacteria identified came from the infection site of the hoof. As stated in the method and materials, a sample was taken from the untrimmed area of the hoof to establish the general flora of the hoof. The bacteria isolated consisted of *Corynebacterium xerosis*, *Paenibacillus popilliae*, *Bacillus megaterium*, and *Bacillus azotoformans*. Of these four, only *C. xerosis* was also isolated from the infection area of the hoof. Thus, it is unlikely that *P. popilliae*, *B. megaterium*, and *B. azotoformans* are responsible for the infection in the hooves. Most likely these bacteria are part of the normal flora of the hoof and under normal circumstances, they do not cause any issue, although they may take advantage of a weakened and/or infected hoof and begin to over reproduce.

Formerly known as *Streptococcus gallinarum*, *Enterococcus gallinarium* is Gram-positive cocci usually arranged in pairs or clusters [12,13]. This bacterium is found mostly in the intestines of domestic fowls and chickens but is also occasionally found in the human gastrointestinal tract [12,14]. In addition, *E. gallinarum* has also been isolated from human clinical specimens [15-17]. Recently, multi-drug-resistant *E. gallinarum* was isolated from synovial sepsis following heel bulb laceration, full-thickness wound to the carpus, and complete articular open fracture of the tibial crest in horses [18].

**Table 1:** Summary of the biochemical test results used to identify bacteria isolated from the infected hooves.

| # | 18 | 5 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | *C. xerosis* | *E. gallinarus* | *M. varians* | *P. insolitus* | *M. caseolyticus* | *B. megaterium* | *B. subtilis* | *B. azotoformans* | *Lactobacillus* | *P. popilliae* | *S. epidermidis* |
| 55°C | ND | ND | ND | - | ND | - | - | - | ND | - | ND |
| NaC | ND | ND | ND | - | ND | + | + | - | ND | - | + |
| -O2 | + | + | - | + | + | + | + | + | + | + | + |
| O2 | + | + | + | + | + | + | + | + | + | + | + |
| No | ND | ND | s | ND | s | ND | ND | ND | ND | ND | s |
| Spo | ND | ND | ND | + | ND | + | + | + | ND | + | ND |
| Hem | ND | - | ND | ND | - | ND | ND | ND | ND | ND | - |
| VP | + | ND | - | - | + | - | + | - | - | - | + |
| Gel | + | - | - | - | - | + | + | - | - | - | - |
| Cit | - | ND | - | - | - | + | + | + | - | - | - |
| Sta | - | - | - | - | - | + | + | - | + | - | - |
| Glu | + | + | + | - | + | + | + | - | + | + | + |
| Ma | - | + | - | - | - | + | + | - | - | - | - |
| Cat | + | - | + | + | + | + | + | - | - | - | + |
| Morpholog | Rods | Rods | Cocci | Rods | Cocci | Rods | Rods | Rods | Rods | Rods | Cocci |
| Colonial characteristics | Milky, yellow, small | Creamy, white, small | Dull, yellow, medium | Milky, white, large | Milky, white, small | Dry, white, medium | Dry, whire, large | Milky, white, medium | Milky, white, small | Fibrous, white, medium | Milky, white, medium |
| G | + | + | + | + | + | + | + | + | + | + | + |

Abbreviations: G: Gram reaction; Cat: Catalase; Man: Mannitol fermentation; Glu: Glucose fermentation; Star: Starch hydrolysis; Cit: Citrate utilization; Gel: Gelatin hydrolysis; VP: Vogues- Prausker; Hem: Hemolysis, Spo: Presence of endospores; Nov: Novobiocin resistance; $O_2$: Aerobic growth; O-: Anaerobic growth; NaCl: Growth in 6.5% NaCl: 55°C: Growth at 55°C; #: Number of isolates; ND: Not Determined; s: susceptible.



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*Corynebacterium xerosis* is known as a commensal bacterium of the human skin although it has been associated with diseases such as skin and eye infections, and endocarditis, especially in immunocompromised individuals [19]. Another species, *Corynebacterium pseudotuberculosis*, has been associated with external abscesses in horses [20,21]. A type of infection known as "pigeon fever" is also caused by *C. pseudotuberculosis* in horses. Although it is mainly responsible for external abscesses on the chest, *C. pseudotuberculosis* can also result in the formation of internal abscesses known as ulcerative lymphangitis, which is characterized by the infection of limbs and compromises the lymphatic system [22]. Two other isolates were identified as Micrococcus varians. *M. varians* is a normal inhabitant of the skin of a wide variety of animals, including horses [23]. It has also been shown to cause infection in mice [24]. Although micrococci have been associated with invasive disease in immunocompromised humans and animals, the vast majority of cases are related to *M. luteus*.

Members of the genus *Macrococcus* have been isolated from humans and animals. *M. caseolyticus* was originally isolated from lamb [25]. In addition, two species, *M. equipercius* and *M. carouselicus* have been isolated from the skin of ponies and horses, but have not been associated with any infections [26]. It remains possible that these two species may have taken advantage of the un-going infection and multiplied. Another species, *Macrococcus canis*, previously identified as *M. caseolyticus*, has also been isolated from skin infection sites in dogs [27]. Only two isolates were identified as *Psychrobacillus insolitus* (formerly *Bacillus insolitus*). This psychrophilic bacterium is typically found in soil and has been associated with bread wheat roots, where it promotes growth [28]. To our knowledge, *P. insolitus* has not been associated with any disease in animals. This suggests that the presence of *P. insolitus* may be the result of soil that remained stuck to the hooves even after washing.

*Bacillus subtilis* is a common soil bacterium, but is also present in the gastrointestinal tract of many animals, including horses [29]. The bacterium is considered to be non-pathogenic. Similarly, several members of the genus *Lactobacillus* are present in the gut of many animals including horses. Of these, *Lactobacillus* equigenerosi, *Lactobacillus* equi, and *Lactobacillus* hayakitensis have only been isolated from the horse gut [30,31]. We believe that the isolation of *B. subtilis* and *Lactobacillus* may be attributed to the presence of fecal material in the stables and that the bacterium does not play a role in the hoof infection.

*Staphylococcus epidermidis* is a normal inhabitant of the skin and mucous membranes of many animals. Typically, *S. epidermidis* is non-pathogenic in healthy animals but has been shown to cause opportunistic infections in immunocompromised humans and animals [32]. In addition, antibiotics use has led to the emergence of antibiotic-resistant strains of *S. epidermidis*. Specifically, the emergence of antibiotic-resistant staphylococci in livestock has been reported. It has been suggested that those bacteria serve as reservoirs for the evolution and spread of resistance genes [33,34].

A previous study aimed at characterizing bacteria present in biofilms formed in chronic wounds on the front and rear limbs in horses revealed the presence of several bacterial species, the majority being *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. Other bacteria isolated included *Staphylococcus aureus*, *Serratia marcescens*, *Enterococcus faecalis*, *Providencia rettgeri*, *E. coli*, *Streptococcus dysgalactiae*, *Bacillus* sp., and *Enterococcus* sp [35]. Although our study focused on infected hooves, we have identified some of the same bacteria, namely *Staphylococcus epidermidis*, as well as members of the *Enterococcus*, *Bacillus*, and *Enterococcus* genera. To our knowledge, the horses have not suffered any trauma, although it is likely that minor wounds or cuts contributed to the colonization and proliferation of bacteria within the hoof. Several studies have associated the presence of microbial biofilms following lower limb injuries [36]. The presence of biofilms following traumatic injuries has been shown to delay healing [36].

## Conclusion

Several bacteria were isolated and identified from the infected hooves. Interestingly, these bacteria are all part of either the normal flora of the hoof, the gut, or the soil. This suggests that, although, they may not be pathogenic and under normal circumstances, they may take advantage of a weakened and/or infected area to proliferate and cause disease.

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