A SUSTAINABLE APPROACH TO THE CONTROL OF PATHOGENS: THE FATE OF *STREPTOCOCCI* IN EQUINE COMPOST.

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Abstract

Streptococcus equi subspecies *equi* (*S. equi*), causes the potentially fatal respiratory disease called "strangles" in horses, while the closely related *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*) causes potentially fatal infections in humans. A study was undertaken to determine the survival of these two organisms in equine compost. Compost piles of equine bedding and feed waste were inoculated with 10 x 10^{10} c.f.u. of *S. zooepidemicus* and samples taken at 48, 96, 168 and 336 hours relative to samples placed in the pile at 0 hours. No *Streptococci* were isolated at 48 hours or subsequent time-points. Next, *S. equi* was similarly inoculated into equine compost, with samples taken at 2, 4, 8, 12, 24, 48, 168 and 336 hours later. No *Streptococci* were isolated at any time-point. To rule out killing of *S. equi* by microflora in equine waste, samples of soiled bedding, both autoclaved and un-autoclaved (with water added to match autoclaved moisture) were inoculated with 10 x 10^{10} c.f.u. of *S. zooepidemicus* and *s*ampled at 0, 6, 12, 24, 48, 72, 120, 168 and 264 hours. In autoclaved bedding, *S. zooepidemicus* was isolated from 0 – 120 hours, but replaced by other flora at 264 hours. In un-autoclaved samples, *Streptococci* were not present after 48 hours. A repeated trial with *S. equi* yielded similar results. This data suggest that microbial activity of equine waste bedding may

eliminate streptococci within 24 - 48 hours, indicating that normal microflora may provide sustainable methods for the control of human and animal pathogens.

Keywords: Equine compost, *Streptococcus equi*, *zooepidemicus,* Bioremediation

Introduction

Strangles is a highly contagious respiratory infection of horses, caused by *Streptococcus equi* subspecies *equi* (*S. equi*), a Lancefield group C gram-positive non-motile bacterium. An outbreak of *S. equi* on a farm may last up to 4 to 6 months (Taylor et al., 2006), and outbreaks occur every year in the U.S. Infected horses may continue to shed the organism for over a year after clinical signs resolve, the longest documented shedding being 39 months (Taylor et al., 2006) . Preventing spread of disease is imperative because *S. equi* has a 4-8% mortality rate (Taylor et al., 2006) . While *S. equi* has been seen to persist in the environment on different materials from 63-72 days (Jorm, 1992; Skorobohach et al., 1994), a follow up study showed S. *equi* surviving only 1-3 days (Weese et al., 2009).

Outbreaks cause serious economic hardship, such as loss of riding activities, veterinary expenses, and cost of horse disposal (Weese et al., 2009). Concern exists regarding infectivity of both manure and animals that have died from the illness. In addition, *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*) is an equine commensal closely related to *S. equi* and has been reported to cause serious infection in humans exposed to horses or horse manure (Lee and Dyert, 2004; Pelkonen et al., 2013; Minces et al., 2010; Yi et al., 2014).

In the United States, there has been an increase in organic farming (Greene, 2015), and the use of compost as a soil amendment (Martínez-Blanco et al., 2013). Horse bedding is used as a compost feedstock (Swinker et al., 2009; Romano et al., 2006). A case was reported in which a 79 year old man contracted a fatal infection of *S. zooepidemicus* from the fresh, un-composted manure he spread onto his garden (Lee and Dyert, 2004). Other fatal cases have been reported where people contracted *S. zooepidemicus* following contact with horses and/or horse manure (Pelkonen et al., 2013; Minces et al. 2010).

Effective management of equine waste is needed to prevent spread of strangles. The number of people that come in contact with horses and horse byproducts is high. Infected manure is handled daily and is spread by shovels, wheel barrows, pitch forks, clothing, and shoes (Sweeney et al., 2005; Pelkonen et al., 2013). Many farmers spread fresh manure on their fields (Swinker et al., 2009; Powell et al., 2005). According to the

American College of Veterinary Internal Medicine "manure from infectious animals should be composted in an isolated location" (Sweeney et al., 2005) . However, there is limited research to support whether disease abatement occurs in compost.

Care must be taken when disposing of strangles fatalities to prevent spread of *S. equi* to other horses or farms. Burial is a common disposal option for many horse owners, but can be difficult, especially in winter months, and may be banned in some municipalities (Gwyther et al., 2011). Composting is increasingly recognized as an environmentally sound and economical way to dispose of dead horses (Gwyther et al., 2011). In particular, with ready access to horse manure, composting may be an economically viable and bio-secure option for disposing of strangles cases on horse farms. However, data on the survival of *S. equi* in compost is lacking. Therefore a study was performed to determine if *S. equi* and *S. zooepidemicus* would survive in equine compost, as a step towards improving practices for equine manure handling.

Methods and Materials:

Experimental Overview

The present study evaluated abatement of *S. zooepidemicus* inoculated into compost piles of 3 different C:N ratios, over a two week period. Dacron bags containing approximately 400g of compost inoculated with *S. zooepidemicus* were placed at 1 and 3 foot depths in three evenly spaced holes on each side of the piles. To provide samples at various time-points, 4 bags were placed at each location, a bag being withdrawn for analysis at 2, 4, 7 and 14 days. The field trial was repeated using *S. equi*, in which only one pile of the highest C:N ratio was used and samples were taken at 2, 4, 8, 12, and 24 hours and then at 2, 4, 7 and 14 days.

To determine the impact of endogenous microflora on survival of *Streptococci* in compost, a second study was performed to determine survival of *S. equi* in sterile versus non-sterile compost feedstock. Because feedstocks were sterilized using steam at 15 p.s.i., non-autoclaved samples were tested at three moisture levels to account for any water up-take by the autoclaved samples.

Composting

Three piles of decreasing C:N ratios were prepared for the *S. zooepidemicus* trial, using compost feedstocks of soiled equine bedding and feed waste at ratios 3:1, 1:1 and 1:4 respectively. Composting was conducted at Highmoor Farm in Monmouth, Maine, USA, which has an isolated composting pad. Compost feedstocks were agitated using a mechanical mixer to ensure homogeneity. Each of the three ratios were piled into an individual windrow 4ft high and 20ft long. Analog and digital thermometers

where placed at 3ft and 1ft depths and pile temperature was recorded daily. For the *S. equi* trial a single pile of a 3:1 ratio of soiled equine bedding to feed waste was used.

Bacterial Growth

Stock cultures of *Streptococci* in this study were propagated in tubes containing 1 mL of fresh heparinized horse blood rotated end over end at 37 °C (Causey et al., 1995). To prepare streptococcal inocula for compost, two flasks containing 125 mL of Todd-Hewitt broth with 10% horse blood were inoculated with 30µL of the streptococcal stock culture and incubated in 20% CO_2 for 12 hours. The flasks were then checked for purity by streaking on blood agar, stored at 4 °C and used within 24 hours.

Inoculation of piles

One heaped tablespoon (approximately 400 g) of compost of each feed-stock ratio was placed into a 5 cm x 10 cm Dacron bag of 50 µm pore size, for a total of 48 bags per feedstock ratio (12 sites per pile x 4 time samplings per site = 48 bags). Each bag contained at least one rayon swab. One mL of the *S. zooepidemicus* culture in Todd Hewitt with 10% horse blood was pipetted directly over the swabs in the compost in the Dacron bag. Each bag was closed with 5ft of non-biodegradable baling twine, labelled to indicate depth, and location in the pile. One bag for each site had two rayon swabs for sampling immediately post inoculation and at 2 days. The remaining 3 bags had one swab for the subsequent time points (4, 7 and 14 days). Compost pile construction, and burial of bags in the pile, occurred 24 hours after inoculating the Dacron bags.

Sample Plating

At each sampling, using the attached twine, one bag was pulled from each site and taken to the laboratory, where the swabs were removed from the bags and put into the matching micro centrifuge tubes, each containing 1 mL of Phosphate-buffered saline (PBS). The swabs were rotated rapidly by hand 10 times in the PBS solution to disperse the *Streptococci*. With a 10 µL-inoculating loop, a sample was taken from each micro centrifuge tube and plated onto a blood agar plate containing 5% sheep blood. When all 12 plates were streaked from each time point, they were incubated at 37 °C for 24h. After 24h, streptococcal colony counts on the plates were assessed according to +++++ = continuous lawn of *Streptococci*, ++++ = partly lawn, partly individual colonies, +++ > 100 individual colonies, 1-99 colonies were counted, and - = no *Streptococci* detected (Causey et al. 1995). Photographs were taken of each plate to record appearance of the plate and to help differentiate endogenous microflora from *Streptococci.* Compost which was

not inoculated with *Streptococci* was sampled in the same way, and plated and documented to show the endogenous background of normal microflora.

Sterilized Feedstock Trials

To determine the impact of endogenous microflora on survival of *Streptococci* in compost, compost feedstocks of varying moisture content were prepared as follows. For the sterilized feedstock, a tablespoon of compost was placed in each of 27 Dacron bags (3 replicates of 9 time periods) and the bags were weighed and autoclaved for 50 minutes at 15 PSI. The bags then were re-weighed and the weight difference before and after autoclaving to determine any uptake of water by compost during the sterilization process. Three non-autoclaved samples of the same feedstock were prepared of various water contents. The first was prepared with no water added, a second with water added to match the weight increase of the autoclaved compost, and a third which was saturated with added water. For each, 1 tablespoon of compost was also placed in each of 27 Dacron bags, giving a total of 27 bags for each stock, and a total of 81 bags overall (27 X 3) of non-sterile compost samples. Moisture content of all 4 stocks was determined by gradual dehydration in a microwave, to the point at which weight reduction no longer occurred as the sample was heated.

Each Dacron bag represented a time point for sampling and contained at least one rayon swab. Bags were sampled using the same method immediately post inoculation and at 6, 8, 12, 24, 48, 72 hours, and 5, 7, 11 days, yielding a total of ten time points from the nine bags (and two swabs from the first bag). Bags were placed in sealed plastic bags to maintain moisture and were stored at room temperature (21-23 °*C)* during the sampling period. Each sample was plated and read using the plating technique described above.

Results:

In the first study examining the survival of *Streptococci* in compost, *S. zooepidemicus* was below detectable levels within the first 48 hours (Table 1) in all 3 compost piles. A follow - up study with *S. equi* indicated that death of *Streptococci* was occurring while the samples were being stored immediately prior to burial in the compost pile (Table 2). In the second study to determine the role of endogenous microflora in killing *Streptococci,* all *S. zooepidemicus* was eliminated by 24 hours in non-sterile equine waste, whereas in sterilized equine waste *S. zooepidemicus* persisted until 120 hours (Figure 1) at all the moisture contents tested. In a follow up trial with *S. equi,* no bacteria were detected after 36 hours in non-sterilized compost. In the sterilized material, *Streptococci* persisted for 72 hours, but appeared to be

eliminated with the emergence of other flora in the previously sterile feedstock.

Table 1 – Persistence of *Streptococcus zooepidemicus* in 3 compost piles of decreasing C:N ratio.

Compost pile	Streptococcal Growth				
	Post inoculation*	48h**	96h	168h	366h
1	++++	-	-	-	-
2	++++	-	-	-	-
3	++++	-	-	-	-

* Sample taken immediately post inoculation, 24 hours prior to pile construction
** hours after construction of pile
- = no *Streptococci* detected
++++ = partly lawn and partly individual colonies of *Streptococci*

Data recorded at each time point represents summary of 12 replicates for each pile

Table 2 – Persistence of *Streptococcus equi* in a compost pile of the same composition as pile 1 in the previous table

Pile	Streptococcal Growth								
	Post inoculation*	2h**	4h	8h	12h	24h	48h	168h	366h
1	++++	-	-	-	-	-	-	-	-

* Sample taken immediately post inoculation, 24 hours prior to pile construction
** hours after construction of pile
- = no streptococci detected
++++ = partly lawn and partly individual colonies

Data recorded at each time point represents summary of 12 replicates

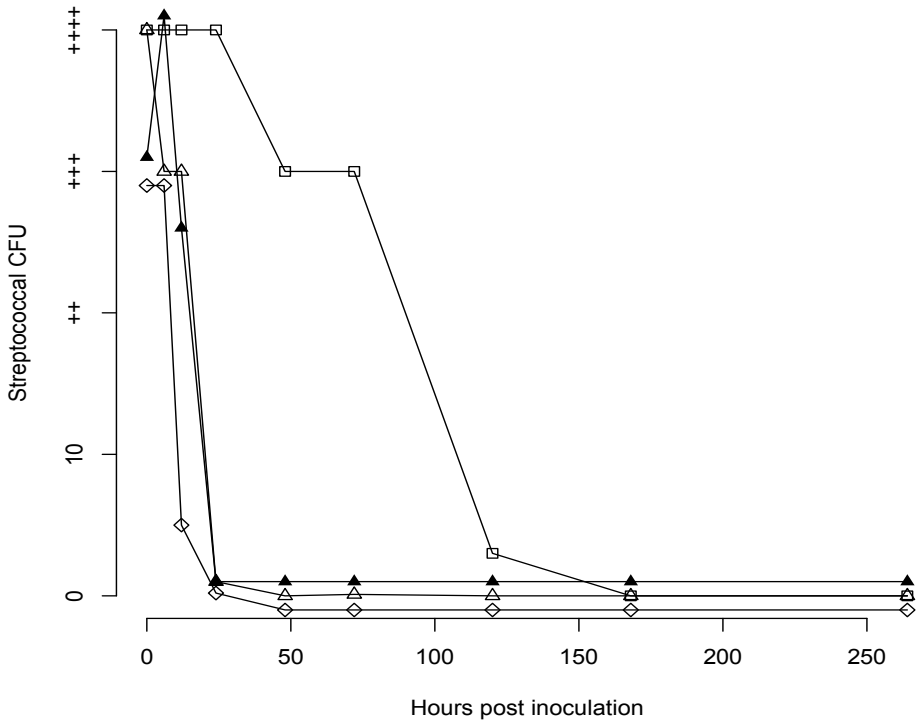

Figure 5: Decline in streptococcal numbers in sterile (box) and non-sterile (triangles and diamond) horse waste.

Discussion

In the present study, composting was examined as a method to reduce the spread of an important horse pathogen, *S. equi*. It was shown that the endogenous microflora found in horse manure apparently killed *S. equi* and *S. zooepidemicus*, with important implications for the disposal of manure, bedding and cadavers of infected patients. This study, while limited to horses, nevertheless has some relevance to broader issues of sustainability and disease control.

Composting is a managed aerobic decomposition of decaying plant and animal matter, generating material similar to humus, the organic substrate of soil (Dougherty, 1999). The decomposition occurs through microbial activity which releases heat, causing the temperature of compost to increase. Finished compost has many beneficial uses, such as soil improvement in fields, greenhouses, and gardens, and for water retention in land reclamation (Dougherty, 1999). Compost may also be used for control of plant pathogens and for bio-control of human and animal pathogens in

contaminated waste materials (Nobel and Coventry, 2005; Guan et al. 2004). Composting systems can exist at any scale. They do not cause a significant increase in the emission of greenhouse gases, and produce a beneficial product that contributes to global sustainability. In the present study we focused on the ability of compost to control a specific pathogen of horses, with a view to addressing the possible role of compost in the control of antibiotics and antibiotic resistant bacteria.

Several studies have analyzed bioremediation of antibiotics through composting. Dolliver et al. (2008) examined antibiotic degradation in manure composting of 3 systems: static piles, aerated piles, and in vessels. Specifically, the degradation of chlortetracycline, monensin, tylosin, and sulfamethazine in composted turkey litter were studied. After 35 days it was concluded that chlortetracycline was more than 99% degraded, whereas monensin and tylosin were 54-76% degraded. No degradation of sulfamethazine was detected. In addition, Kim et al. (2012) found that tetracyclines, sulphonamides, and macrolides in composted swine manure compost resulted in all the antibiotics being degraded to within Korea's acceptable daily intake rate in 85 days. Similarly, Ho et al. (2013) showed that doxycycline, trimethoprim, sulfadiazine, norfloxacin, tilmicosin, erythromycin, enrofloxacin, flumequine, fell below detectable levels after 40 days of chicken manure composting. All of these studies conclude that composting can be a valid method for degrading antibiotics, but that further study is required to determine differences between each antibiotic.

There is a risk to human health of antibiotic resistant bacteria entering the food supply through animal manure. Heringa et al. (2010) determined that *E. coli* can be present in compost and that 7% of the isolates were resistant to two or more antibiotics, raising the concern that compost could potentially spread antibiotic resistant *E. coli* into the environment. However, if the process is managed appropriately, with adequate temperature attained, composting could become a widespread practice to remove pathogens in manure, including antibiotic resistant bacteria. Guan et al. (2004) showed that multi-drug resistant *E. coli* could not be detected in chicken manure during composting when temperatures reached 50 ºC or above.

Singh et al. (2012) conducted a study looking at the effect of moisture content and thermal inactivation of *Salmonella spp.* in poultry litter compost. This study used optimum composting conditions and looked at a moisture content of 40 and 50%. In both trials *Salmonella* was abated, however, it took longer in the 40% moisture content with the longest abatement taking 11 days at 50 °C. This study concluded that optimum composting can kill *Salmonella spp.* using moisture content for microbiological activity.

In some jurisdictions in the USA, composting is an acceptable method of disposing of animal carcasses. The compost results in a stable, environmentally-beneficial product that solves many of the problems of large carcass disposal. In 1999, a Northern Right Whale which died on the shore in New Jersey was composted using horse manure. The bones were collected and used in a museum, and the residua the organic material was free from noxious odors and presented no biohazard (Bonhotal et al. 2007). It appears possible that composting may be a sustainable and cost-effective method of animal disposal.

Conclusion:

The balance between the microbiome and pathogens is a delicate one. We have co-evolved with diverse lineages of microorganisms which may help or harm us in our mutual struggle for survival. When exposed to potentially harmful bacteria on door handles we touch, fruit we eat, animals we own, waters in which we swim, we don't automatically contract an illness. The microflora on our skin and mucus membranes is now recognized as a defense against invasion by these pathogens. However, disruption of local flora by antibiotics can disrupt this delicate balance, and promote disease, especially by antibiotic resistant bacteria. In this paper we have shown that the general concept of beneficial microflora may be extended to the control of pathogens in the environment.

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