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Inhibitory Affects of Hoof-Zink®, Copper Sulfate and Zinc Sulfate Solutions on the Growth of *Prevotella melaninogenicus* and *Fusobacterium necrophorum*

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OBJECTIVE

The objective of this study was to determine if different concentrations of Hoof-Zink®, copper sulfate and zinc sulfate solutions have antibacterial activity against *Prevotella melaninogenicus* (formerly *Bacteroides melaninogenicus*) and *Fusobacterium necrophorum,* two bacteria known to cause foot rot in dairy cattle.

TRIAL 1: Methods and Results

Bacterial strains

The bacterial strains used for this study were obtained from the American Type Culture Collection (ATCC).

Prevotella melaninogenicus	ATCC No. 25845
Fusobacterium necrophorum	ATCC No. 25286

Dehydrated bacteria were resuspended in 5 mL of Brain Heart Infusion (BHI) broth and incubated anaerobically for 48 hours at 37ºC. A total of 20 uL of each resuspended bacterial solution was plated on two 5% Trypticase Sheep Blood Agar (BA) plates and incubated anaerobically for 48 hours at 37ºC to ensure that a pure culture was maintained throughout the resuspension process. Six (6) vials of each bacterium were frozen down from the resuspended broth in a 20% glycerol solution (500 uL bacteria: 500 uL 40% glycerol).

One vial from the frozen stock of each bacterial strain was grown in 100 mL of Difco Anaerobe Broth MIC. *P. melaninogenicus* was grown anaerobically for 24 hours at 37ºC and *F. necrophorum* was grown anaerobically for 48 hours at 37ºC. After incubation, one hundred (100) 1 mL-vials of each bacterium were frozen down in a 10% glycerol solution.

Colony forming units/mL (CFU/mL) for each bacterium was determined by thawing 3 vials of each bacteria and making serial 1:10 dilutions out to 10^{-7}. Separate serial dilutions were made for each of the three vials. A total of 100 uL of dilutions 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} were plated in duplicate for each vial on BA plates and incubated

under the appropriate culture conditions for each. The number of colonies growing on each dilution plate was counted and the CFU/mL for each dilution was determined. The average CFU/mL for each bacterium was calculated and used for all further assays.

Bacteria	CFU/mL
P. melaninogenicus	2.38×10^7
F. necrophorum	9.75×10^5

Bacterial inhibition assay

The Hoof-Zink® was received as a solution and diluted with distilled water to achieve concentrations of 5%, 10%, 15%, and 20%. The $ZnSO_4$ (spray-dried powder) and $CuSO_4$ (feed grade crystals) were resuspended in distilled water to achieve 5%, 10%, 15%, and 20% solutions. All solutions were filter-sterilized to ensure sterility and later analyzed at Baltzell Agri Products using atomic absorption spectroscopy. It was later determined after analyzing the solutions that the solutions were not diluted correctly. The dilutions were performed in volumetric flasks using volume to volume dilution for Hoof-Zink® and weight to volume dilutions for $CuSO_4$ and $ZnSO_4$ instead of weight to weight dilutions. The percentages of each solution were determined to be as follows:

	Concentrations
Hoof-Zink®	8.0, 12.9, and 26.6%
$ZnSO_4$	8.3, 12.2, and 16.5%
$CuSO_4$	9.4, 13.5, and 18.0%

After filter-sterilizing the solutions, three replicates of each concentration were incubated with a known concentration of each bacterium on 48-well tissue culture plates.

F. necrophorum: Four plate replicates of the bacterial inhibition assay were completed for *F. necrophorum*. The bacterium was used at a concentration of $1.0e^5$ CFU/mL per well. A total of 500 uL of each mineral concentration was added to the appropriate wells according to the schematic in Figure 1 and 500 uL of the *F. necrophorum* solution was added to all wells except for the negative control and blank wells. Positive control wells containing bacteria and media only were included on each plate. The absorbance was read at 490 nm at 0 hours and after 48 hours of anaerobic incubation. Mineral concentrations with an increase in absorbance when compared to the positive control were plated to determine the CFU/mL.

The change in absorbance for each mineral concentration was graphed. A lower change in absorbance indicates greater inhibition of bacterial growth (Figure 1). Three wells from each concentration were pooled from each plate replicate. From the Hoof-Zink® and $CuSO_4$ pools, 100 uL was plated on a BA plate to check for bacterial growth. The negative control wells were also pooled and 100 uL was plated. The $ZnSO_4$ and positive control wells were each pooled and serial 1:10 dilutions were made out to 10^{-7}. A total of 100 uL from the undiluted sample and from dilutions 10^{-3} to 10^{-7} were plated on BA plates. All plates were incubated anaerobically for 48 hours prior to counting. The positive control growth was recorded at 1.30×10^7 CFU/ml; however, there was no

growth recovered from any of the mineral salt concentrations tested, despite an increase in absorbance in the $ZnSO_4$ (Table 2).

P. melaninogenicus: The bacterial inhibition assay using *P. melaninogenicus* was designed similarly to that of *F. necrophorum*; however, after the initial stocks were made, all further attempts to grow the bacterium were not successful. New anaerobe broth was made with additional vitamin K and hemin, but those components alone were not adequate to induce bacterial growth. It may be that the bacteria required undefined factors that are present in blood, which is not feasible for measuring the absorbance. For this reason, absorbance data is not available for *P. melaninogenicus*.

To ensure the antibacterial activity of the solutions against both *F. necrophorum* and *P. melaninogenicus*, a zone of inhibition assay were designed using BA plates instead of broth media. This new assay was used with both bacteria to enable comparisons between them.

Zone of inhibition

To determine whether different concentrations of solutions can inhibit the growth of an established culture, a separate blank paper disc was infused with each concentration of mineral salt solution and placed onto BA plates that had been swabbed with established cultures of each bacterium. Plates were incubated anaerobically for 48 hours and the zone (in mm) around each disc with no bacterial growth was measured with a caliper. The assay was repeated three times, and the mean zone of inhibition for each mineral concentration was calculated (Figure 2).

TRIAL 2: Methods and Results

Zone of inhibition

In trial 2, the Hoof-Zink®, $CuSO_4$ and $ZnSO_4$ were diluted with distilled water to 5, 10 and 15% solutions using weight to weight dilutions calculations. To determine whether different concentrations of solutions can inhibit the growth of an established culture, a separate blank paper disc was infused with each concentration of mineral salt solution and placed onto BA plates that had been swabbed with established cultures of each bacterium. Plates were incubated anaerobically for 48 hours and the zone (in mm) around each disc with no bacterial growth was measured with a caliper. The assay was repeated three times, and the mean zone of inhibition for each mineral concentration was calculated (Figure 3).

The zone of inhibition of *P. melaninogenicus* linearly increased as the concentration of the three minerals increased ($P < 0.001$; Table 2). At each of the concentrations, there was a significantly greater zone of inhibition for Hoof-Zink® compared to the other two minerals ($P = 0.01$). The zone of inhibition was not significantly different between $CuSO_4$ and $ZnSO_4$ at each of the concentrations.

The zone of inhibition of *F. necrophorum* linearly increased as the concentration of the all three minerals increased ($P < 0.001$; Table 3). At each of the concentrations, the zone of inhibition was the greater for $CuSO_4$ compared to both Hoof-Zink® and $ZnSO_4$ ($P < 0.01$).

SUMMARY

In these experiments, efforts were made to ensure all the Hoof-Zink®, copper sulfate and zinc sulfate were dissolved into the solutions before the test, which is not always the case in the field when using copper sulfate and zinc sulfate in footbaths. Over the entire experiment, the Hoof-Zink® and copper sulfate mineral solutions showed the greatest antibacterial effect on both *P. melaninogenicus* and *F. necrophorum*. However, when completely dissolved into solution the zinc sulfate mineral solutions showed only slightly less antibacterial activity.

TABLES AND FIGURES

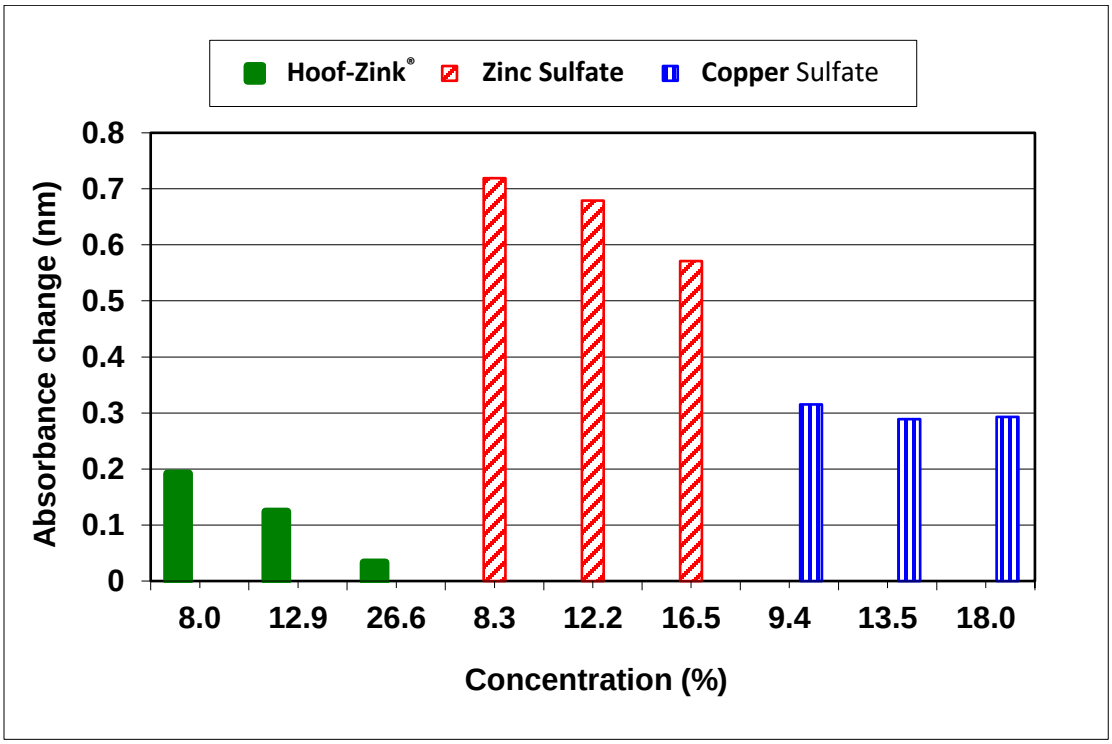

Figure 1. *F. necrophorum* absorbance (490 nm) at each concentration. The positive control remained at 0.5 and negative control remained at 0 absorbance change. A lower change in absorbance indicates higher antibacterial effect (Trial 1).

Table 2. *F. necrophorum* concentration (CFU/mL) plated from wells used in absorbance determination (Trial 1)

	Target Concentration[a]		
Item	10%	15%	20%
Hoof-Zink®	0	0	--
$ZnSO_4$	0	0	0
$CuSO_4$	0	0	0
Positive control	1.30×10^7	1.30×10^7	1.30×10^7
Negative control	0	0	0

[a]Actual concentrations were: 8.0, 12.9, and 26.6% for Hoof-Zink®; 8.3, 12.2, and 16.5% for $ZnSO_4$; and 9.4, 13.5 and 18.0 for $CuSO_4$ due to errors in dilutions.

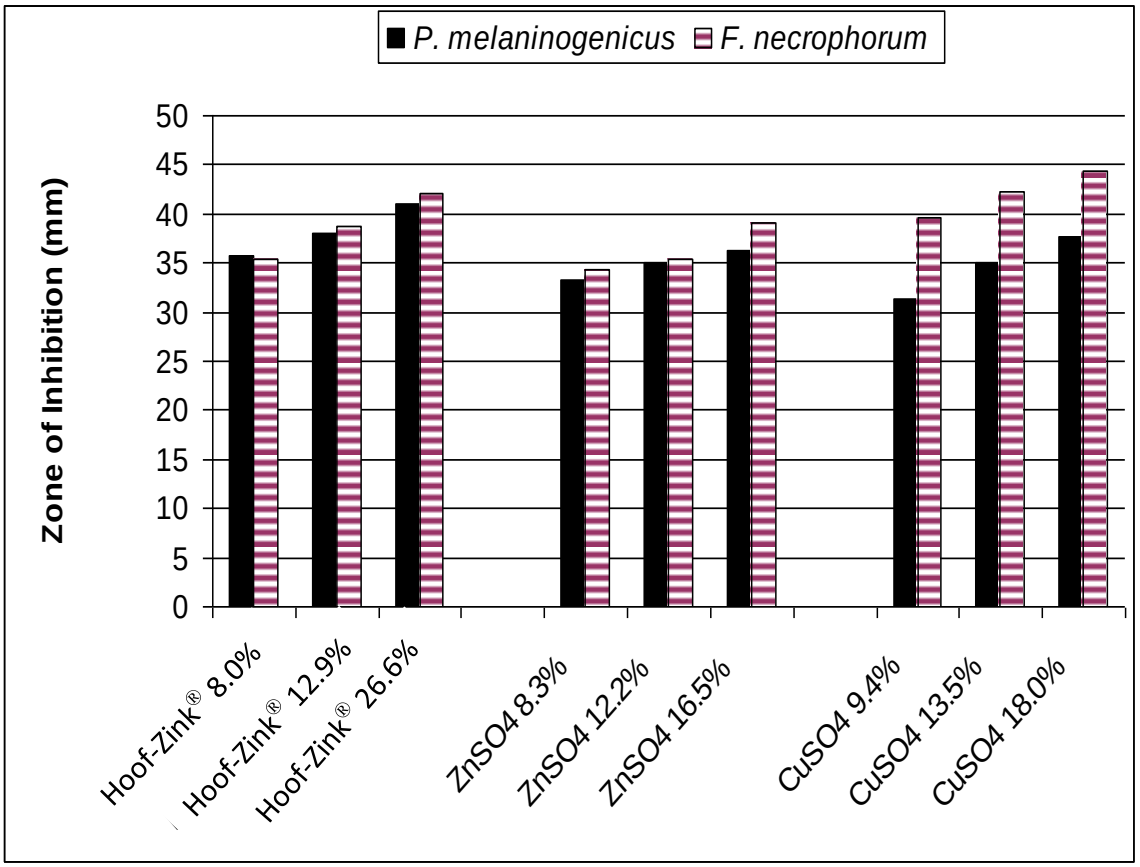

Figure 2. Mean zone of inhibition of each bacteria (mm) at each solution concentration. Higher zones indicate greater antibacterial effect (Trial 1).

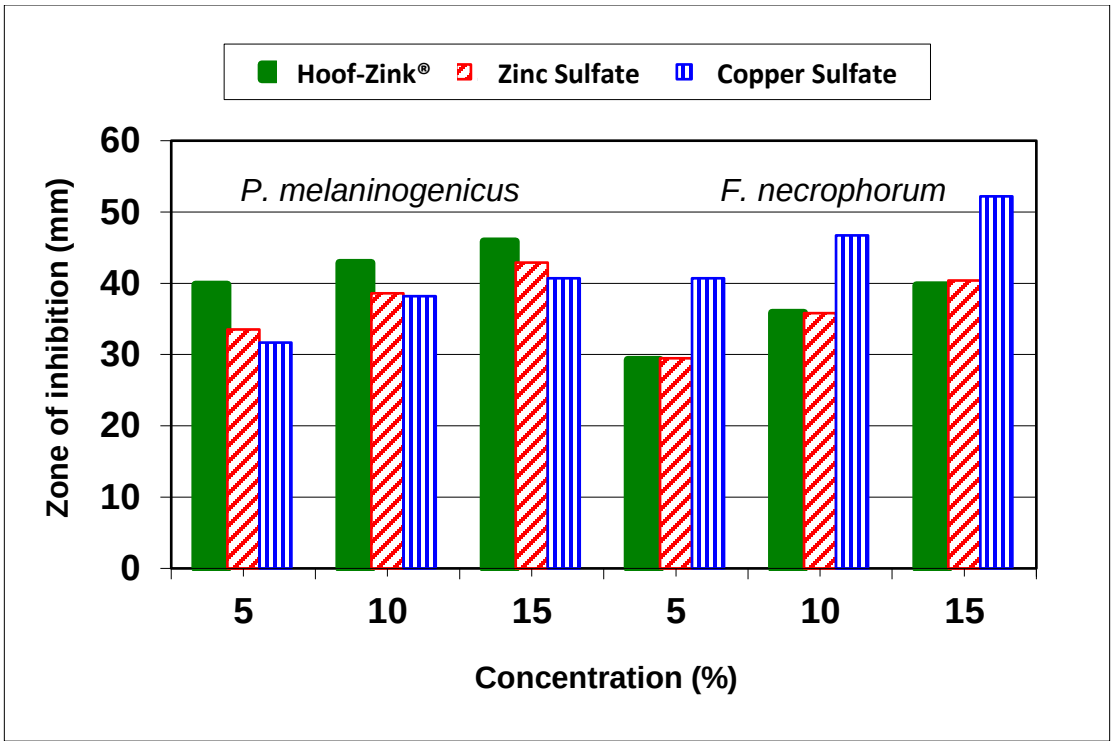

Figure 3. Mean zone of inhibition for each bacteria (mm) at each solution concentration. Higher zones indicate greater antibacterial effect (Trial 2).