THE EFFECT OF PHOSPHORYLATED GLUCOSE ON MAJOR PATHOGENS OF BOVINE MASTITIS

An Undergraduate Research Scholars Thesis

by

KENDALL SCHAEFER

Submitted to the Undergraduate Research Scholars program
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Jeffrey Musser

May 2016

Major: Animal Science

TABLE OF CONTENTS

ABSTRACT

The effect of phosphorylated glucose on major pathogens of bovine mastitis

Kendall Schaefer
Department of Animal Science
Texas A&M University

Research Advisor: Dr. Jeffrey Musser
Department of Veterinary Pathobiology

Mastitis is the number one infectious disease on dairies. Treatment and prevention is mainly through good milking hygiene, proper environmental conditions, and antibiotic usage. For organic dairies, mastitis prevention and control can be problematic as there are major restrictions on the use of antibiotics. Phosphorylated glucose, produced by heating (121ºC) a phosphate-buffered solution with added glucose, is bactericidal to *Escherichia coli*. We tested the bactericidal properties of phosphorylated glucose on three major pathogens of mastitis: *E. coli*, *Staphylococcus aureus,* and *Streptococcus agalactiae*. Phosphorylated glucose was bactericidal on the pathogens tested in phosphate-buffered saline. When in the presence of milk, phosphorylated glucose was not bactericidal on *E. coli*, but *Staph. aureus* and *Strep.* agalactiae are still affected. Further studies of phosphorylated glucose on *Mycoplasma,* a pathogen of mastitis that is inherently resistant to β-lactam antibiotics, and on mammary cells are in process. This work will provide organic dairy producers with additional information for the prevention and treatment of mastitis.

ACKNOWLEDGMENTS

I would like to thank Dr. Jeffrey Musser for all of his assistance and encouragement throughout this project. He has instilled a love for microbiology in me, and I could not have completed this project without him.

Rosie Gonzalez has also been of invaluable help in the lab. She has been with me every step of the way, and I greatly appreciate her never-ending support.

NOMENCLATURE

CFU	Colony Forming Units
E. coli	*Escherichia coli*
M. bovis	*Mycoplasma bovis*
MRSA	Methicillin-Resistant *Staphylococcus aureus*
PBS	Phosphate Buffered Saline
Phos 1% Gluc	Heat- and phosphate-catalyzed 1% glucose
Staph. aureus	*Staphylococcus aureus*
Strep. agalactiae	*Streptococcus agalactiae*
TSA	Tryptic soy agar

SECTION I

INTRODUCTION

Bovine mastitis in the dairy industry

Bovine mastitis is primarily caused by a bacterial infection. These pathogens can be differentiated into two categories of pathogens, contagious and environmental pathogens. Contagious pathogens, such as *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma bovis*, are transmitted from cow to cow during the milking procedure and routinely cause chronic subclinical infections (NMC, 2011). Coliform bacteria, *Escherichia coli* being the prototypical bacteria, are environmental pathogens that are acquired from the cow's environment with transmission usually occurring during the period between milkings (NMC, 2010).

Annually in the U.S., the cost of mastitis to the dairy industry is about $1.7-2 billion, representing an 11% loss of total milk production (Jones 2009). This loss is due to reduced milk production, discarded milk, and replacement animals; indirect losses in consumer consumption due to reduced quality, taste, and shelf-life also occur. Proper milking hygiene, good facilities, cow management, and dry-cow antibiotic treatment are essential for the prevention of mastitis, while antibiotic treatments are the main tool in the treatment of mastitis. However, the use of antibiotics is restricted on organic dairies.

Antibiotic use in mastitis treatment and prevention

While antibiotics are heavily restricted on organic dairies, commercial dairies routinely use antibiotics for treatment and prevention of mastitis. In the United States, approximately 90% of

all dairy cows are treated with antibiotics (Sjostrom, 2015). Excluding antibiotics used in medicated feed and water, most antibiotic use is for dry-cow treatment, which is the time period between the cessation of milking and the next lactation. Dry-cow treatment is a common practice for subclinical mastitis control (Østerås, 1999).

Antimicrobial resistant bacteria have been found on dairies and potentially have a public health significance. *Staph. aureus* isolates were found to be penicillin and multidrug resistant, 9 and 15% respectively, and *E. coli* isolates were found to be tetracycline and multidrug resistant, 15 and 63% respectively (Saini et al., 2012).

As to the issue of MRSA, it is theorized that the use of cloxacillin, which is used extensively as a dry-cow treatment, may increase the prevalence of MRSA (Saini et al., 2012). Presently, few to no MRSA isolates have been reported from mastitic milk (Saini et al., 2012; Vanderhaeghen et al., 2010) and bulk tank milk is not a common source for MRSA isolates (Virgin et al., 2009). Although MRSA has the potential to complicate treatment of mastitis (Vanderhaeghen et al., 2010), in commercially pasteurized milk, it should not be a major health issue.

Heat- and phosphate-catalyzed glucose

Heat- and phosphate-catalyzed glucose is bactericidal against *E. coli* O157:H7 on starvation media and in nutrient broths (Byrd, 1999). A phosphorylated 1% glucose solution reduces colony forming units (CFUs) of *E. coli* O157:H7 by 99.9% and has the potential to be used as an antimicrobial in processed food.

Due to the restrictions on antibiotic use on organic dairies and the increase in antibiotic resistant bacteria on dairy farms, alternative preventative measures and treatments for mastitis are desired. Phosphorylated glucose has shown bactericidal activity in other situations, thus it may be useful in mastitis prevention and treatment. We hypothesize that phosphorylated glucose will have antimicrobial properties against *E. coli*, *Staph. aureus*, and *Strep. agalactiae*.

SECTION II

MATERIALS AND METHODS

Bacteria and reagents

All three pathogens, *E. coli*, *Staph. aureus*, and *Strep. agalactiae*, were obtained from stored archival samples at -80º C. Bacteria were thawed, *Strep. agalactiae* was cultured on blood agar media, and *E. coli* and *Staph. aureus* were cultured on TSA media. The cultures were incubated at 37º C for 24 hours. The bacteria were removed from incubation to be utilized for the project. This process was repeated for each experiment.

Phosphorylated glucose was made by adding glucose[a] to PBS[b] to achieve a final concentration of 1% glucose. The solution was then autoclaved at 121ºC for 15 minutes. This solution, Phos 1% Gluc, was used for all experiments. A fresh solution was made for each experiment.

Pasteurized skim milk[c] was used in all experiments involving the efficacy of Phos 1% Gluc against these bacteria in the presence of milk.

Experimental protocol

Using the 24 hour bacterial cultures, a bacterial suspension in PBS, at a concentration equal to a 0.5 McFarland standard, was made from each bacteria. Two concentrations of bacteria were tested: a 0.5 McFarland and a 1:10 dilution of the 0.5 McFarland. Three milliliters of each bacterial suspension were added to 3 mL of Phos 1% Gluc. These suspensions were incubated at

37ºC and used throughout the experiment, with a fresh bacterial suspension being made in each section.

Bactericidal properties of phosphorylated 1% glucose in PBS

To test the bactericidal properties of Phos 1% Gluc in PBS, a 0.5 McFarland and a 0.05 McFarland standard were made from the 24 hour bacterial cultures. *Streptococcus agalactiae* was cultured on blood agar media and *E. coli* and *Staph. aureus* were cultured on TSA at times t=0, 24, and 48 hours for each concentration of each bacteria. The plates were incubated for 24 hours, the CFU were counted, and the bacteria solutions were incubated throughout.

Time to bactericidal effect in PBS

The experimental protocol was used. The bacteria were streaked at times t = 0, 1, 2, 4, 6, 8, and 24 hours. The plates were incubated for 24 hours.

Bactericidal properties of phosphorylated 1% glucose in skim milk

The experimental protocol was used for the preparation of a 0.5 McFarland standard. For each bacteria, a 1:5 dilution was performed on the 0.5 McFarland standard. One and a half milliliters of the 1:5 dilution were added to 1.5 mL of skim milk, giving a 1:10 dilution in the presence of skim milk. Three different concentrations were made: 1:1, 5:1, and 10:1, Phos 1% Gluc to skim milk solution.

Blood agar media (*Strep. agalactiae*) and TSA media (*E. coli*, *Staph. aureus*) were streaked at times t=0 hours, t=24 hours, and t=48 hours for each concentration of each bacteria. The plates were incubated for 24 hours and the bacteria solutions were incubated throughout.

Statistical analysis

Descriptive statistics, mean, minimum, and maximum were reported.

SECTION III

RESULTS

Bactericidal properties of phosphorylated 1% glucose in PBS

For all three bacteria, the addition of Phos 1% Gluc to the bacteria reduced the number of CFU, and there was a greater reduction with increased contact time with the Phos 1% Gluc (Table 1). Incubation of the bacterial suspension with Phos 1% Gluc for 24 and 48 hours reduced the CFU greatly; 100% reduction of CFU was seen at 24 hours for all three bacteria, with the exception of *E. coli* at the 0.5 McFarland concentration.

Following the seven days of further incubation of the culture plates with no CFU, no bacteria were observed.

Table 1. Phosphorylated 1% Glucose in PBS

Time exposed [hrs]	***E. coli*** [CFU* (range)]		***Staph. aureus*** [CFU (range)]		***Strep. agalactiae*** [CFU (range)]	
	0.5	0.05	0.5	0.05	0.5	0.05
0	TNTC**	>350	TNTC	TNTC	TNTC	TNTC
24	83 (0, 250)	0	155 (23, 400)	44 (10, 100)	27 (0, 52)	8 (0, 24)
48	58 (0, 175)	0	0	0	0	0

** CFU = colony forming units*

*** TNTC = too numerous to count*

Time to bactericidal effect in PBS

After 8 hours of contact between Phos 1% Gluc and the bacteria, a reduction in CFU was noted, with no CFU observed at 24 hours (Table 2).

Table 2. Time to Bactericidal Effect in PBS

Time exposed [hrs]	***E. coli*** [CFU*]	***Staph. aureus*** [CFU]	***Strep. agalactia*** [CFU]
0	300	325	TNTC**
1	20	TNTC	TNTC
2	TNTC	200	400
4	150	TNTC	400
6	250	TNTC	300
8	70	320	75
24	0	0	0

** CFU = colony forming units*

*** TNTC = too numerous to count*

Bactericidal properties of phosphorylated 1% glucose in skim milk

The addition of skim milk to the suspension reduced the efficacy of the Phos 1% Gluc for all three bacteria (Table 3). The bactericidal effect of Phos 1% Gluc on *E. coli* was negated by the addition of skim milk. However, *Strep. agalactiae* was still sensitive to Phos 1% Gluc in the presence of skim milk. At the 5:1 concentration of Phos 1% Gluc to bacteria, the presence of

CFU was reduced by 85% by 24 hours. The 10:1 concentration was more effective, with 100% reduction of CFU at 48 hours.

Table 3. Phosphorylated 1% Glucose in Skim Milk

Time exposed [hrs]	***E. coli*** [CFU* (range)]		***Staph. aureus*** [CFU (range)]		***Strep. agalactiae*** [CFU (range)]	
	5:1¶	10:1	5:1	10:1	5:1	10:1
0	300 (200, 400)	275 (250, 300)	230 (190, 300)	177 (31, 250)	250 (200, 300)	170 (150, 200)
24	TNTC**	TNTC	230 (120, 300)	129 (67, 200)	33 (11, 70)	5 (1, 12)
48	TNTC	TNTC	317 (250, 400)	27 (18, 39)	10 (0, 19)	0

** CFU = colony forming units*

¶ ratio Phos 1% Gluc to milk

*** TNTC = too numerous to count*

SECTION IV

DISCUSSION

Heat- and phosphate-catalyzed 1% glucose has a bactericidal effect on *E. coli*, *Staph. aureus*, and *Strep. agalactiae*. Following seven days of further incubation of cultures, no bacterial growth was noted, suggesting that the effect is bactericidal and not bacteriostatic. However, the bactericidal effect is time, bacterial concentration, and suspension medium dependent. Bacteria need to be in contact with the Phos 1% Gluc for a minimum of eight hours before the bactericidal effect is noted, but a 100% reduction in CFU was seen following 24 hours of contact time. This factor may play a role in the usefulness of Phos 1% Gluc in mastitis treatment and prevention, as multiple intramammary infusions over a 24 hour period may be required for efficacy.

Additionally, the presence of skim milk affected the ability of Phos 1% Gluc to reduce bacterial numbers. Heat- and phosphate-catalyzed 1% glucose had no effect on *E. coli* when milk was added*;* in fact, CFU tended to increase. The mechanism for this is unknown, but it is possible that *E. coli* is able to use a different substrate present in the milk in the place of the Phos 1% Gluc. The presence of milk inhibited the effect of Phos 1% Gluc on *Staph, aureus* and *Strep. agalactiae* (Table 3). When the ratio of Phos 1% Gluc to milk was increased, the bactericidal effect was also increased. At a 10:1 (Phos 1% Gluc: milk) ratio, *Strep. agalactiae* was least affected by the presence of milk, as CFU were greatly reduced to 95 and 100% of original numbers at 24 and 48 hours, respectively. This reduction was comparable to the reduction observed when milk was not present (Table 1). This finding is noteworthy as *Strep. agalactiae* is a contagious, obligate bacteria of the mammary gland, thus eradication from a herd is achievable

with proper antibiotic and dry-cow treatment. Due to antibiotic usage restrictions on organic dairies, Phos 1% Gluc may be a useful treatment in the management and eradication of *Strep. agalactiae* on these dairies.

Further research is needed before clinical trials on the effect of Phos 1% Gluc in mastitis prevention and treatment can be done. These studies should include: repeated time trials on bactericidal effect, cytotoxicity of Phos 1% Gluc on mammalian cells, and the bactericidal effect of Phos 1% Gluc on *M. bovis.*

REFERENCES

Byrd, J. J., A. M. Cheville, J. L. Bose, and C. W. Kasper. 1999. Lethality of a heat- and phosphate-catalyzed glucose by-product to *Escherichia coli* O157:H7 and partial protection conferred by the *rpoS* regulon. Appl. Environ. Microbiol. 65:2396-2401.

Jones, G. M. and T. L. Bailey. 2009. Understanding the Basics of Mastitis. Virginia Cooperative Extension. Publication 404-233.

NMC. 2011. A Practical Look at Contagious Mastitis. Accessed Aug. 12, 2015. https://nmconline.org/contmast.htm.

NMC. 2010. A Practical Look at Environmental Mastitis. Accessed Aug. 12, 2015. https://nmconline.org/environmental.htm.

Østerås, O., V. L. Edge, and S. W. Martin. 1999. Determinants of success or failure in the elimination of major mastitis pathogens in selective dry cow therapy. J. Dairy Sci. 82:1221-1231.

Saini, V., J.T. McClure, D. Léger, J.P. Keefe, D. T. Scholl, D. W. Morck, and H.W. Barkema. 2012. Antimicrobial resistance profiles of common mastitis pathogens on Canadian dairy farms. J. Dairy Sci. 95:4319-4332.

Sjostrom, Lucas. 2015. NAHMS Finds Milk Pathogens, Learns about Dry-off Norms in Dairy 2014 Study. Dairy Herd Management. Accessed Nov. 16, 2015. http://www.dairyherd.com/news/nahms-finds-milk-pathogens-learns-about-dry-norms-dairy-2014-study

Vanderhaeghen, W., T. Cerpentier, C. Adriaensen, J. Vicca, K. Hermans, and P. Butaye. 2010. Methicillin-resistant Staphylococcus aureus (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. Vet. Microbial. 144:166-171.

Virgin, J. E., T. M. Van Slyke, J. E. Lombard, and R.N. Zadoks. 2009. Short communication: Methicillin-resistant Staphylococcus aureus detection in US bulk tank milk. J. Dairy Sci. 92:4988-4991.

APPENDIX A

[a] Sigma-Aldrich, St. Louis, MO

[b] Sigma-Aldrich, St. Louis, MO

[c] purchased from HEB, College Station, TX